In vivo and *in vitro* studies of immunodeficiency in Ataxia-telangiectasia

by

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Abstract

Ataxia-telangiectasia (A-T) is a rare neurodegenerative disorder caused by mutations in the *ATM* gene which has a central role in the cellular response to DNA double strand breaks, cell cycle checkpoint control and initiation of the intrinsic pathway of apoptosis. Ataxia-telangiectasia is classified as an immunodeficient disorder with patients commonly showing lymphopenia and abnormalities in immunoglobulin production. They also have a high incidence of leukaemia and lymphoma at young ages. I used multicolour flow cytometry and immunological assays to characterise lymphocyte subsets in a group of 18 A-T patients and analysed the sensitivity of A-T lymphoblastoid cell lines to CD95-mediated apoptosis. I also investigated the potential role for ATM in immune surveillance via DNA damage-induced upregulation of NKG2D ligands. My results confirm a deficiency in naive T and B cells as well as high expression of the death receptor CD95 on all lymphocyte subsets excluding NK cells which together may explain the lymphopaenia in A-T. Analysis of the sensitivity of A-T LCLs to CD95-mediated apoptosis showed increased sensitivity of these cells to apoptosis but there was no evidence for a role of ATM in regulating either CD95 or cFLIP expression. Consistent with this was the increased sensitivity to CD95-mediated apoptosis of T cell prolymphocytic leukaemia (T-PLL) cells. The cause of the tumour is primary loss of ATM activity (either germline loss similar to A-T LCLs or somatic loss) allowing chromosome translocations with malignant potential, as a result of a defect in immune system gene rearrangements. An immediate consequence for A-T patients is an immunodeficiency that is not progressive, but may be described as 'congenitally aged'. Immunodeficiency *per se* is not the cause of cancer in A-T but both immunodeficiency and cancer are consequences of the same basic ATM defect affecting the lymphoid system. There was no evidence of a role for ATM in NKG2D ligand upregulation following DNA damage.

For the virtual student, who may not have worn a lab coat in over thirty years but is still a scientist at heart.

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Firstly I would like to thank my supervisors Paul Moss and Malcolm Taylor for their valuable advice, reviewing of this thesis and the many meetings. I am also grateful to everyone who helped me in the lab or gave me reagents, without them this project would not have been possible. In particular Venkat Srinivasan sequenced the mutations in the A-T PBMCs, Phil Byrd made the panel of isogenic LCLs, Malcolm Taylor made the A-T LCLs, Tanya Stankovic gave me the B-CLL samples, Jade Harris coordinated the selection of control PBMC samples, James Last taught me western blots, Andy McLarnon taught me how to use the LSRII and Oliver Goodyear taught me immunological assays and tissue culture techniques including the 'black art' of T cell cloning. Thanks also to Karen Piper for her advice, Annette Pachnio for proof reading and all my friends in cancer studies for the chats, coffee breaks, cinema trips, meals out and nights in the pub all of which made my PhD a more enjoyable experience. I would also like to thank my family, my parents for their emotional and financial support, Hannah for proof reading and Graeme for making me laugh. Finally thank you to Andy for solving my IT crises, reading sections of my thesis, helping me print, keeping me calm and generally looking after me throughout my PhD.

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Abbreviations

A-EJ	–	alternative end joining
AICD	–	activation-induced cell death
ALPS	–	autoimmune lymphoproliferative syndrome
A-T	–	Ataxia-telangiectasia
ATM	–	*Ataxia-telangiectasia mutated*
B-CLL	–	B cell chronic lymphocytic leukaemia
cFLIP	–	cellular FLICE-like inhibitory protein
DISC	–	death inducing signalling complex
FADD	–	Fas-associated via death domain
FasL	–	Fas ligand
HR	–	homologous recombination
LCL	–	lymphoblastoid cell line
MIC	–	MHC class 1 chain-related
NHEJ	–	non-homologous end joining
NKG2D	–	natural killer group 2, member D
PBMC	–	peripheral blood mononuclear cell
PI	–	propidium iodide
ROS	–	reactive oxygen species
TCR	–	T cell receptor
TEMRA	–	T cell effector memory CD45RA+
T-PLL	–	T cell prolymphocytic leukaemia
ULBP	–	UL-16 binding protein
XIAP	–	X-linked inhibitor of apoptosis

Chapter 1: Introduction.

1:1: Ataxia-telangiectasia, the *ATM* gene and ATM protein kinase.

1:1:1: Ataxia-telangiectasia.

Ataxia-telangiectasia is an autosomal recessive, severe neurodegenerative disorder caused by mutations in the *Ataxia-telangiectasia mutated* (*ATM*) gene which has an important role in repair of damaged DNA and apoptosis. Characteristic features include progressive cerebellar degeneration, early onset ataxia (unsteady gait), occulocutaneous telangiectasia (dilated superficial blood vessels), high levels of serum α-fetoprotein (Taylor et al., 1996) and radiation sensitivity (Taylor et al., 1975). A-T patients have an increased incidence of cancer, particularly lymphoid tumours (Morrell et al., 1986;Olsen et al., 2001) and a variable immunodeficiency, most commonly lymphopenia and immunoglobulin deficiencies (Nowak-Wegrzyn et al., 2004).

The disorder is relatively rare with an incidence in the UK of around one in three hundred thousand live births (Woods et al., 1990). It was first reported by Syllaba and Henner in 1926 (Syllaba and Henner, 1926) and recognised as a separate disorder in 1957 (Boder and Sedgwick, 1957). Ataxia-telangiectasia is clinically heterogeneous; the classic form presents in infancy and shows steady progression whereas milder forms may present later and proceed at a slower rate (McConville et al., 1996). Patients have a reduced life span (median survival of around 19-25 years (Crawford et al., 2006) and usually die of cancer or respiratory tract infections (Nowak-Wegrzyn et al., 2004).

1:1:2: The *ATM* gene.

The *ATM* gene was first identified and cloned by Savitsky in 1995 (Savitsky et al., 1995). It is located on chromosome 11q22-q23 and spans approximately 160 kb of genomic DNA. This

encodes a 13 kb transcript of 66 exons with no evidence of different splice forms (Uziel et al., 1996).

Over four hundred unique mutations in ATM have been described (Leiden Open Variation database). The classic form of A-T results from two truncating mutations which lead to complete loss of ATM protein and therefore total loss of ATM kinase activity. Milder forms of A-T result from missense mutations which may allow expression of mutant protein with some residual kinase activity or leaky splice site mutations which may allow some normal ATM protein to be expressed (Staples et al., 2008;Taylor and Byrd, 2005).

1:1:3: ATM protein kinase.

The *ATM* gene encodes a serine/threonine protein kinase (ATM) which is a member of the phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK) family. ATM is a 350kDa protein which contains 3056 amino acids (reviewed in (Shiloh, 2003)). Like other members of its family it has a FAT domain, FATC domain and a kinase domain. A substrate binding site located near the N-terminus is important for binding of several substrates including p53, NBS1 and BRCA1 (Fernandes et al., 2005). Deletion of this site inactivates the protein (Fernandes et al., 2005). ATM activation occurs through autophosphorylation of the protein, this occurs on at least three sites, Ser367, Ser1983 and Ser1981. Acetylation of ATM at Lys3016 is also required for its activation, this is carried out by the acetyltransferase TIP60 (Sun et al., 2005;Sun et al., 2007) (Fig 1:1:3). ATM kinase is predominantly located in the nucleus although a small amount (~10-20%) is reportedly found in the cytoplasm (Lakin et al., 1996;Watters et al., 1997). The majority of cytoplasmic ATM is located in peroxisomes and endosomes, but it may also be present as a soluble protein (reviewed in (Lavin, 2008)).

Fig 1:1:3: Schematic representation of ATM.

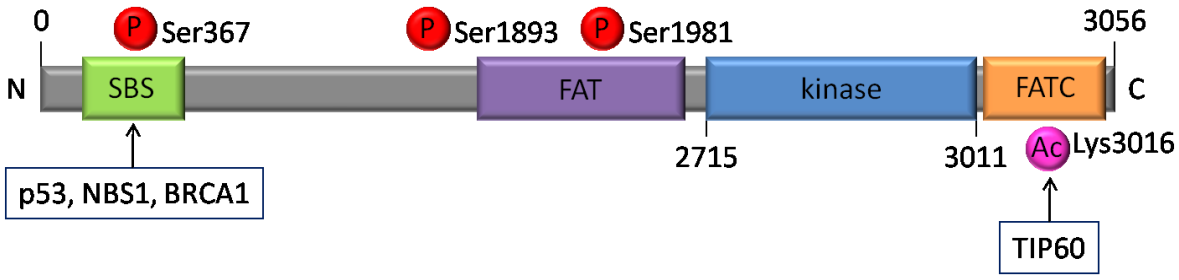


Fig 1:1:3: Schematic of the structure of ATM showing the locations of the SBS, FAT, kinase and FATC domains and the autophosphorylation (P) and acetylation sites (Ac) important in activation of the kinase. The protein is 3056 amino acids long and the kinase domain encompasses amino acids 2715 to 3011. The binding sites of important ATM substrates (p53, NBS1 and BRCA1) and the acetyltransferase TIP60 are also shown. (Modified from (Lavin et al., 2005;Lavin, 2008)).

1:2: ATM regulates the cellular response to DNA double strand breaks.

1:2:1: ATM is activated in response to DNA double strand breaks.

ATM is activated in response to DNA double strand breaks and initiates cell cycle arrest and either DNA repair or apoptosis (reviewed in (Lobrich and Jeggo, 2005b)). The related protein ATR (ATM and Rad3-related protein) has a similar role in responding to DNA single strand breaks.

DNA double strand breaks occur naturally due to collapse of replication forks, physical stress during mitosis and genome rearrangements such as V(D)J recombination, class switching and meiosis. They are also caused by DNA damaging agents such as ionising radiation and certain chemicals (including some chemotherapy drugs). Damage to DNA can result in cell death or genetic alterations including deletions, translocations and chromosome fusions. Therefore an appropriate cellular response to DNA damage is essential for maintenance of genome stability and cancer prevention.

The first stage in the response to a DNA double strand break is the rapid localisation of DNA damage repair and recognition proteins to the site of the break. The proteins Mre11, Rad50 and Nbs1 form the MRN complex which is held at the site of the break by an adaptor protein MDC1 (mediator of DNA-damage checkpoint protein-1). This complex tethers the broken ends of the double strand break. ATM is quickly recruited to the site of damage and initially localises to DNA regions either side of the break before associating with the MRN complex through binding to Nbs1.

Under normal conditions ATM exists as an inactive dimer, however it dissociates into active monomers following DNA damage (Bakkenist and Kastan, 2003). ATM is thought to be

partially activated in response to the relaxation of chromatin adjacent to the double strand break. This partial activation allows the kinase to phosphorylate p53 and possibly other substrates. However, localisation to the break itself through binding to the MRN complex is required for monomerisation and full activation of ATM (reviewed in (Lavin, 2008)) (Fig 1:2:1). Both autophosphorylation of ATM at Ser1981 (Bakkenist & Kastan, 2003;Berkovich et al., 2007) and acetylation at Lys3016 (Sun et al., 2007) are essential for monomerisation and activation of the protein. Autophosphorylation at Ser1981 is also required for interaction with MDC1 which stabilises ATM at the site of the DNA double strand break (So et al., 2009). The function of ATM autophosphorylation at Ser367 and Ser1893 is not known, however this is also essential for ATM activation (Kozlov et al., 2006).

Fig 1:2:1: ATM is activated in response to DNA double strand breaks.

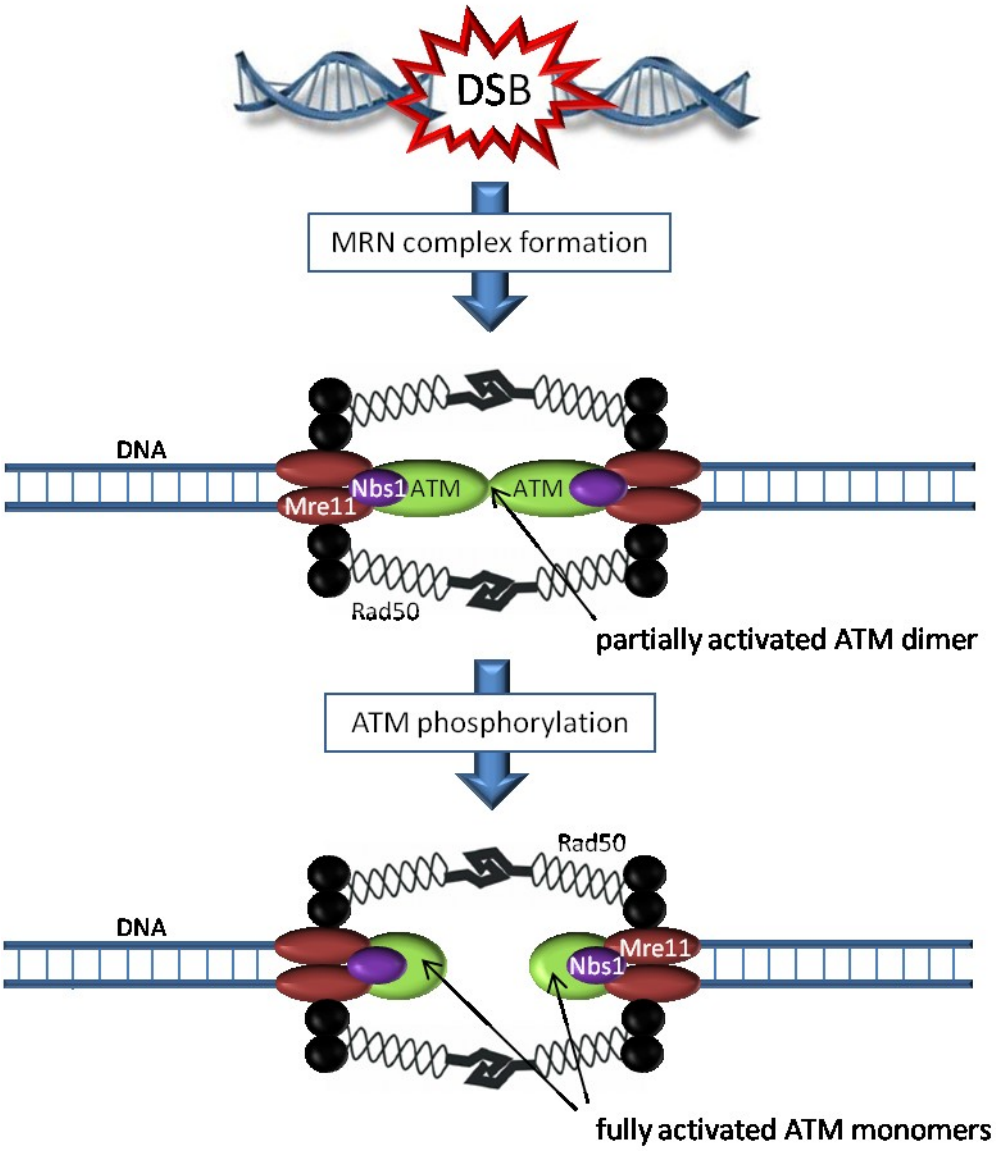


Fig 1:2:1: ATM is activated in response to DNA double strand breaks. Inactive ATM dimers localise to break sites and are partially activated in response to relaxation of chromatin. They then associate with the MRN complex (Mre11, Rad50 and Nbs1) which forms at the site of damage. This initiates phosphorylation of ATM at Ser1981 and acetylation at Lys3016 leading to monomerisation and full activation. Autophosphorylation of ATM on at least two other sites (Ser367 and Ser1893) is also required for activation. (Modified from (Czornak et al., 2008)).

1:2:2: ATM activates cell cycle checkpoints in response to DNA double strand breaks.

Activation of ATM initiates the phosphorylation and activation of many downstream substrates with roles in DNA repair, cell checkpoint activation and transcription. The tumour suppressor p53, is stabilised by phosphorylation and in turn upregulates p21 which is responsible for activation of the G1/S cell cycle control checkpoint (reviewed in (Roos and Kaina, 2006)). ATM also has a role in activation of the S-phase cell cycle checkpoint through phosphorylation of the checkpoint kinase CHK2 (Buscemi et al., 2001;Matsuoka et al., 1998;Matsuoka et al., 2000) and the G2/M checkpoint through phosphorylation of artemis (Zhang et al., 2004). Activation of these checkpoints in response to DNA damage in a normal cell prevents the cell from proceeding through the cell cycle before its DNA has been repaired.

1:2:3: ATM activates pathways of DNA double strand break repair.

MRN complex formation and ATM activation can initiate both the non-homologous end joining (NHEJ) (reviewed in (Lobrich and Jeggo, 2005a)) and homologous recombination (HR) (Morrison et al., 2000) pathways of DNA repair (Fig 1:2:3).

NHEJ is an error prone process which joins together DNA ends without reliance on sequence homology. Therefore it can lead to loss of nucleotides and chromosomal translocations or fusions. It occurs during the G1, G0 and M phases of the cell cycle when sister chromatids are not available. Following MRN complex formation and ATM activation the KU heterodimer (Ku70/80) binds to the broken ends of DNA. A complex of DNA PKcs (DNA dependent protein catalytic subunits) is then formed which tethers the broken ends. Any

single stranded overhang is filled in by nucleases and polymerases and the broken ends are then ligated by the LigIV/XRCC4 complex (reviewed in (van Gent and van der Burg, 2007)).

During the S and G2 phases of the cell cycle sister chromatids are available so DNA repair through homologous recombination is possible. This process is much more accurate than NHEJ as the sister chromatid is used as a template for repair of the broken strand. MRN complex formation and ATM activation occurs in the same way as for NHEJ, however the process of repair is very different. Firstly the broken ends of the DNA are processed by nucleases to create single stranded DNA which is coated with replication protein A (RPA). RPA is then replaced with a Rad51 recombinase to form a nucleoprotein filament. This interacts with Rad52, Rad54, BRCA1 and BRCA2, and invades the sister chromatid allowing the synthesis of complementary strands by DNA polymerases (reviewed in (Li and Heyer, 2008)).

In the absence of ATM DNA double strand break repair may sometimes be possible through the poorly understood process of alternative end joining (A-EJ). This does not require ATM but is inefficient and error prone, commonly resulting in deletions, insertions, microhomology regions and chromosomal translocations associated with lymphoid malignancies (reviewed in (Kotnis et al., 2009)). Evidence of A-EJ during class switch recombination in B cells from A-T patients has been reported (Pan et al., 2002).

Failure of the conventional NHEJ and HR DNA damage repair pathways in A-T patients may contribute to their chromosomal instability leading to chromosome translocations in immune system genes, oncogenesis and high incidence of leukaemia and lymphoma (Taylor et al., 1996). In normal individuals if DNA damage is severe and repair is not possible the cell undergoes apoptosis.

Fig 1:2:3: DNA repair by non-homologous end joining and homologous recombination.

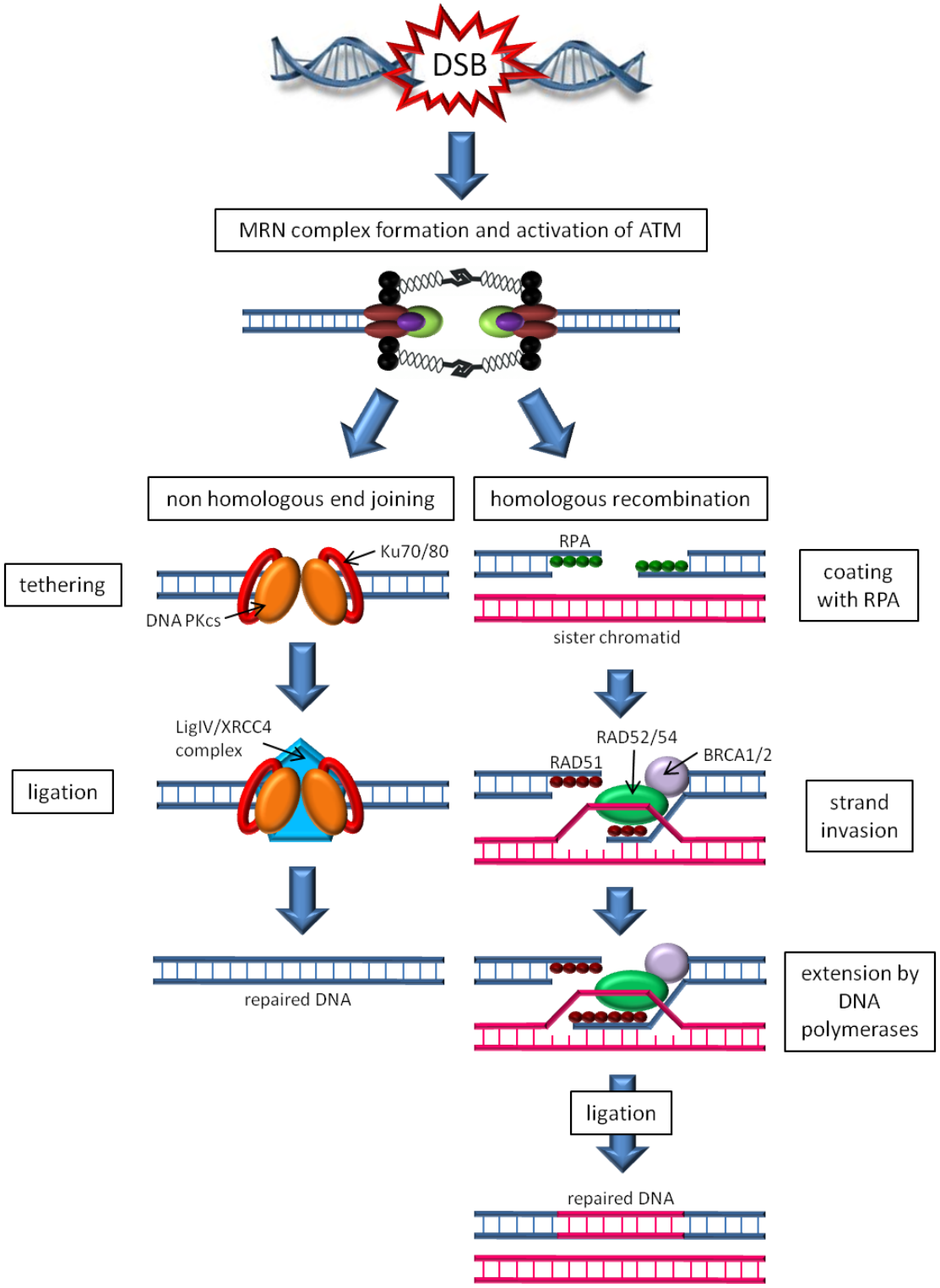


Fig 1:2:3: There are two mechanisms of DNA double strand break repair which can be initiated by MRN complex formation and activation of ATM. Non-homologous end joining begins with tethering of the broken ends by Ku70/80 and DNA PKcs. Missing nucleotides are then filled in by nucleases and the broken ends rejoined by the LigIV/XRCC4 complex. In homologous recombination the ends of the DNA are first processed to produce single stranded DNA which is coated with RPA. This is replaced by Rad51 which together with Rad52, Rad54, BRCA1 and BRCA2 carries out strand invasion of the sister chromatid, providing a template for DNA polymerases to fill in the missing nucleotides. The broken ends of the damaged DNA are then rejoined by ligation. (Modified from (Czornak et al., 2008)).

1:2:4: ATM activates the intrinsic apoptotic pathway in response to severe DNA damage and may also be involved in other apoptotic pathways.

Apoptosis is a process of programmed cell death. This is very important in development, response to cellular stresses and infection and homeostatic control of tissue growth and the immune system. There are two different types of apoptotic processes, intrinsic which does not require external stimuli and extrinsic, or death receptor induced apoptosis (Fig 1:2:4). ATM is involved in the intrinsic apoptotic response to severe DNA damage (Roos & Kaina, 2006;Vit et al., 2000).

The intrinsic process of apoptosis is initiated in response to cellular stresses such as heat shock, UV radiation and severe DNA damage. Similar to cell cycle arrest the apoptotic response to DNA damage relies on the activation of p53 by ATM. When damage is not extensive only a small amount of p53 sufficient for cell cycle arrest is activated. However, in response to a large number of double strand breaks p53 accumulates and activates pro-apoptotic genes such as *Bax* (BCL2-associated X protein), *PUMA* (p53 upregulated modulator of apoptosis) and the FAS receptor (CD95) (reviewed in (Roos & Kaina, 2006)). Bax along with the related protein Bak stimulates permeabilisation of the mitochondrial membranes leading to the release of cytochrome c from the mitochondria. Cytochrome c forms a complex, the apoptosome, with Apaf-1 and the protease caspase-9 (reviewed in (Pradelli et al., 2010)). Caspase-9 is activated and initiates a cascade of activation of caspases (cysteine-dependent aspartate-specific proteases) leading to activation of the executioner caspases (caspase-3, caspase-6 and caspase-7) which initiate the chromatin condensation, DNA fragmentation, nuclear disassembly and plasma membrane blebbing characteristic of apoptosis. In this way DNA damage induced apoptosis helps to ensure that potentially dangerous cells harbouring damaged DNA are not retained in the body.

Extrinsic apoptotic pathways are initiated in response to external cellular stimuli. There are two main types, death receptor mediated apoptosis and the perforin/granzyme pathway. Death receptors include CD95 (Fas) which binds to its cognate ligand; Fas ligand (FasL). Death receptors or ligands may be expressed on the surface of a cell in response to stimuli such as cytokines, cell activation and viral infection. Although the role of ATM in intrinsic apoptotic processes has been investigated (Vit et al., 2000) little is known about the potential role of ATM in extrinsic apoptotic pathways. Recent conflicting reports have suggested that ATM may be involved in regulation of CD95-mediated apoptosis (Ivanov et al., 2009;Stagni et al., 2008). It is possible that abnormalities in extrinsic apoptotic pathways due to *ATM* mutation could contribute to the development of the lymphopenia and/or the lymphoid tumours that are common in A-T patients.

Binding of a death receptor expressed on a potentially apoptotic cell to its ligand (e.g CD95 to FasL) initiates a caspase cascade leading to apoptosis of the cell. Unlike the intrinsic process this does not involve formation of the apoptosome or activation of caspase-9. Instead activation of the initiator caspases, caspase-8 and caspase-10 leads to executioner caspase activation and apoptosis.

A role for ATM is perhaps least likely in the perforin/granzyme pathway of apoptosis. This is the main mechanism by which cytotoxic T cells kill their target cells. The pore forming protein perforin and the serine proteases granzymes are stored within secretory granules of cytotoxic lymphocytes (T cells and NK cells). In response to the formation of an immune synapse between the cytotoxic lymphocyte and a virus infected or transformed target cell the granules are exocytosed into the synapse and can release their contents onto the target cell membrane. Perforin facilitates the entry of granzymes into the target cell cytoplasm although exactly how this is achieved is not yet resolved (reviewed in (Hoves et al., 2010)).

Granzymes are highly proapoptotic and cleave many cytoplasmic proteins. They are able to cleave caspases either directly or indirectly leading to effector caspase activation and apoptosis (reviewed in (Cullen and Martin, 2008)).

Interestingly ATM is cleaved and inactivated by caspase 3 during apoptosis but this does not affect its ability to bind DNA. The presence of the inactive ATM protein may prevent DNA repair and DNA damage signalling in response to the DNA double strand breaks generated during apoptosis (Smith et al., 1999).

Fig 1:2:4: Mechanisms of apoptosis.

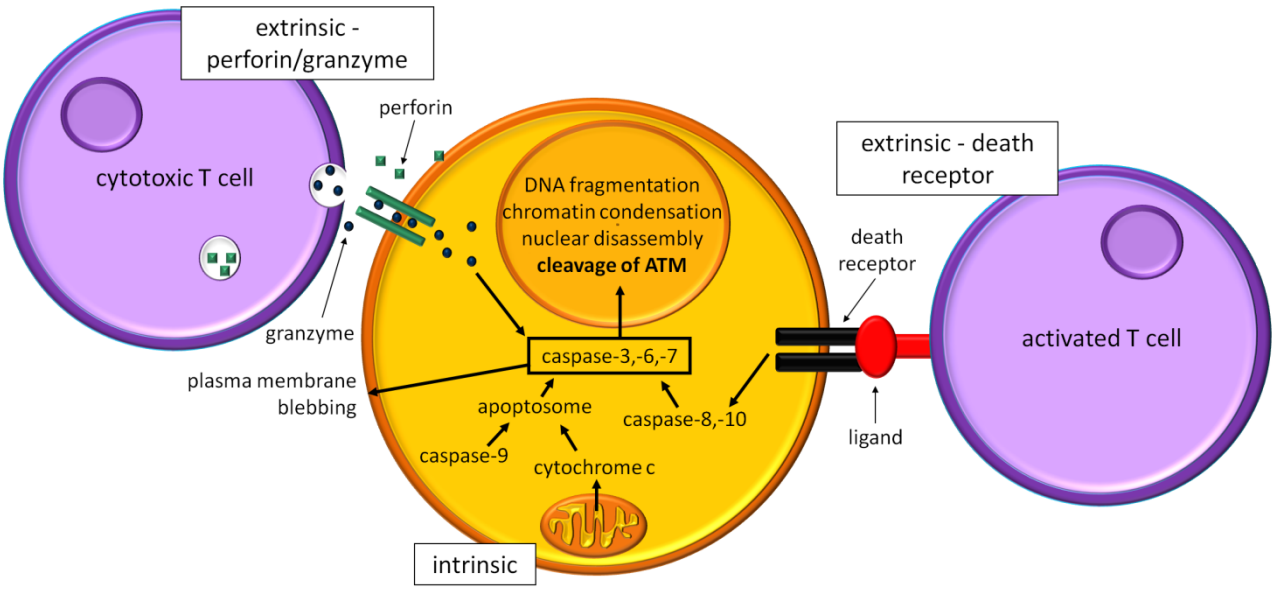


Fig 1:2:4: Extrinsic and intrinsic pathways of apoptosis. The intrinsic pathway of apoptosis is initiated by the release of cytochrome c from the mitochondria which forms a complex with caspase-9 known as the apoptosome. Formation of the complex activates caspase-9 which initiates the caspase cascade leading to activation of effector caspases (caspase-3,-6 and 7) and apoptosis. Extrinsic apoptotic pathways are initiated by external stimuli. In the death receptor pathway binding of a ligand to its death receptor (or vice versa) expressed on the target cell initiates the caspase cascade. Apoptosis can also be initiated by cytotoxic T cells that release cytotoxic granules containing perforin and granzyme. These form a pore in the target cell membrane and like the other pathways they activate the effector caspases leading to apoptosis.

1:3: The immune system in Ataxia-telangiectasia.

1:3:1: Ataxia-telangiectasia patients have immune system abnormalities.

ATM has an important role in lymphocyte development as it responds to the breaks in DNA that occur during differentiation of B and T cells (Lumsden et al., 2004), particularly during V(D)J recombination (Bredemeyer et al., 2006) and class switching. Evidence for the important role of ATM in the immune system comes from the immune system deficiencies seen in A-T patients as well as their high incidence of lymphoid tumours. Interestingly unlike neurological defects the immunodeficiency in A-T is rarely progressive (Nowak-Wegrzyn et al., 2004). The degree of deficiency is highly variable and not all patients are affected (Staples et al., 2008). In general patients who have no ATM kinase activity show a more severe immunological phenotype than those who do retain some ATM activity (Staples et al., 2008).

The principle features of immunodeficiency in A-T are:

1. Lymphopenia of both T and B cells. More than 70% of A-T patients may be lymphopenic (Nowak-Wegrzyn et al., 2004). Naive T and B cell deficiencies are especially pronounced leading to a predominance of memory cells. The naive cell deficiency may be a consequence of thymic hypoplasia and the very low thymic output seen in A-T patients (Micheli et al., 2003).

2. Defects in humoral immunity. Abnormal serum immunoglobulin levels especially deficiencies in the class switched immunoglobulins IgA and/or IgG2 are common and may be the result of a failure of class switching (Reina-San-Martin et al., 2004;Waldmann et al., 1983). A-T patients may also show impaired antibody responses and gammopathy (Sadighi Akha et al., 1999).

Despite these deficiencies systemic bacterial, severe viral and opportunistic infections are rare in A-T patients (Nowak-Wegrzyn et al., 2004) possibly due to their intact T cell responses (Pashankar et al., 2006). However, A-T patients do commonly suffer from recurrent bacterial sinopulmonary infections (McGrath-Morrow et al., 2010) and the incidence of lower respiratory tract infections increases with age. This may be related to neurological decline leading to increased difficulties in chewing and swallowing, increased pulmonary aspiration and poor nutrition (Lefton-Greif et al., 2000;Nowak-Wegrzyn et al., 2004). Response to vaccination is variable with some patients showing normal and some reduced responses (Nowak-Wegrzyn et al., 2004).

1:3:2: ATM has an important role in T cell development.

Haematopoietic stem cells arise in the bone marrow and have the capacity to self renew and to differentiate into multipotent progenitors (MPPs). MPPs are unable to self renew but can differentiate into lymphoid primed multipotent progenitors (LMPPs). These cells express some lymphoid specific genes and have the potential to differentiate into both T and B cells. They may also continue to differentiate in the bone marrow to produce additional progenitor cells. It is possible that these progenitors are further restricted to the B or T cell lineage although this is not yet clear. Some or all of the progenitor cells have the ability to mobilise from the bone marrow, circulate in the blood and enter the thymus (reviewed in (Zlotoff et al., 2008)) where they undergo differentiation into naive T cells.

In normal individuals the thymus is largest (proportional to body size) just before birth. Although it increases in weight to a maximum during puberty the size of its functional compartments, the medulla and cortex, decreases from one year of age onwards. The thymus undergoes a process of involution defined as a decrease in size, weight and activity with age (reviewed in (Appay et al., 2010)). Therefore, the output of naive T cells from the

thymus is greatest in young children and dramatically decreased in the elderly. However, A-T patients may show thymic hypoplasia and their thymic output is very low even in infancy (Micheli et al., 2003).

As they develop in the thymus T cells undergo a process of V(D)J recombination which rearranges their DNA to create functional antigen receptor genes. This process also occurs in developing B cells. Different gene segments which are initially separate from one another are brought together by deletion of intervening DNA sequences. In T cells the joining of different gene segments allows the generation of a huge diversity of antigen receptor specificity. ATM has an important function in the joining together of DNA double strand breaks between gene segments by non-homologous end joining (NHEJ) (Bredemeyer et al., 2006;Corneo et al., 2007) and is also important in maintaining expression of the T cell receptor during recombination. Therefore the process of V(D)J recombination is defective in A-T patients and increased accumulation of unrepaired coding ends during antigen receptor gene assembly has been reported in ATM deficient lymphocytes (Bredemeyer et al., 2006).

However, V(D)J recombination is not completely abrogated in A-T patients as joining together of gene segments can occur in the absence of functional ATM through alternative end joining (reviewed in (Kotnis et al., 2009)). This is an inefficient process which occurs only when conventional NHEJ fails and often produces large deletions (Corneo et al., 2007). Failure of conventional V(D)J recombination leading to an accumulation of unrepaired coding ends (Bredemeyer et al., 2006) may contribute to the increased number of chromosomal translocations involving immune system genes in A-T patients' lymphocytes (Matei et al., 2006).

The most immature subset of thymocyte precursors are the double negative (DN) cells which reside in the cortex of the thymus and lack expression of the T cell receptor (TCR), CD4 and

CD8. At this pro-T cell stage the cells become committed to either the αβ or γδ T cell lineage. In normal individuals the majority (>90%) will give rise to αβ T cells. However, A-T patients have an increased proportion of γδ T cells. This may be due to a recombinational defect and the inability to repair damaged DNA (Carbonari et al., 1990). In thymocytes committed to the αβ lineage RAG-1 and RAG-2 proteins are expressed at the double negative stage and the process of V(D)J recombination of the TCR β chain gene begins with joining of D and J gene segments.

During the next stage of development, the pre-T cell stage, rearrangements between V and DJ regions of the β chain genes occur and the pre-T cell receptor is expressed. Signals from this pre-TCR stimulates proliferation of the pre-T cells and recombination of the α chain of the T cell receptor. It also prevents further rearrangements of the β chain and stimulates the transition to the next stage of development, the double positive stage. Double positive thymocytes express both CD4 and CD8 and undergo rearrangement of α chain genes. The α chain locus lacks D segments so rearrangement involves joining of V and J segments. Following rearrangement of the α chain, double positive thymocytes express TCR αβ heterodimers and migrate from the cortex of the thymus to the medulla.

Double positive cells expressing complete TCR complexes are able to respond to antigen and undergo positive and negative selection processes. The receptors encounter self-peptides displayed on self-MHC molecules in the thymus. Thymocytes which have TCRs which bind with low avidity to self-peptide:self-MHC complexes are stimulated to survive (positive selection) whereas those that bind with high avidity are deleted (negative selection). Thymocytes which do not recognise self MHC also die through apoptosis. These processes ensures that mature T cells which leave the thymus are self-MHC restricted and tolerant to self antigens.

Following positive and negative selection the surviving thymocytes mature into single positive CD4+ve or CD8+ve T cells. CD4+ve T cells acquire the ability to produce cytokines in response to antigen stimulation and to express effector molecules required for interactions with B cells and macrophages whereas CD8+ve T cells become specialised for their cytotoxic function. The mature single positive T cells leave the thymus and migrate through the bloodstream to populate peripheral lymphoid tissues.

Development and differentiation of γδ T cells is similar to that of αβ T cells. Rearrangement of the γ and δ TCR chains occurs through rearrangement and recombination of V, D and J gene segments. γδ T cells do not express CD4 or CD8 and the selection processes that they undergo in the thymus are less well understood than those of αβ T cells. The first stages of maturation take place in the thymic cortex, the cells then migrate to the medulla and continue to mature before leaving the thymus and entering the circulation.

On exit from the thymus T cells are described as naive as they have not yet encountered antigen. These cells express the CD45RA isoform of protein tyrosine phosphatase receptor type C. They circulate between the bloodstream and secondary lymphoid tissues such as lymph nodes, Peyer's patches and the spleen (Weninger et al., 2001). If a naive T cell encounters its cognate antigen presented by mature dendritic cells in the secondary lymphoid tissue it becomes a lymphoblast, proliferates rapidly and acquires effector functions. These effector cells migrate throughout the body and clear infected cells.

When the foreign antigen is no longer present the effector T cell population undergoes a contraction phase during which approximately 95% of cells apoptose. The remaining antigen specific T cells form the memory T cell population. There are three main subsets of memory T cells, central memory (CD45RA-), effector memory (CD45RA-) and TEMRA (T cell effector memory CD45RA+) cells. Central and effector memory T cells are functionally distinct

subsets which can be identified by the differential expression of the homing receptors CD62L (L selectin) and the chemokine receptor CCR7. These receptors have an important role in controlling entry and exit from different lymphoid and non-lymphoid tissues so their expression determines the sites to which the memory T cells can localise.

Central memory T cells express both CD62L and CCR7 and are able to enter lymph nodes spleen and blood. They do not have immediate effector function but are able to stimulate dendritic cells, help B cells and differentiate into CCR7-ve effector cells in response to secondary antigenic stimulation, thereby providing a secondary line of attack. Effector memory T cells do not express CD62L or CCR7. They localise to peripheral non-lymphoid tissues such as lung, liver and intestine as well as the spleen and blood and have immediate effector function allowing them to rapidly respond to infection in peripheral tissues by mediating inflammation and cytotoxicity (Sallusto et al., 1999).

The third subset of memory T cells, TEMRA cells is the only subset which expresses CD45RA. They do not express CCR7 and were initially considered to be terminally differentiated or resting effector memory T cells as they do not express markers of activation, have shortened telomeres, a low proliferative capacity and an increased susceptibility to undergo apoptosis in the absence of costimulation (reviewed in (Sallusto et al., 2004)). However, they also have a high cytotoxic capacity and more recently it has been suggested that they may represent a subset of highly functional long-lived effector memory T cells which do not require proliferation to maintain their population (Cantisan et al., 2010). They accumulate during chronic viral infections including cytomegalovirus (CMV) (Khan et al., 2002), Epstein-Barr virus (EBV) (Dunne et al., 2002) and human immunodeficiency virus (HIV) (Meyer-Olson et al., 2010) and may be involved in the preservation of lifelong immunity to chronic infectious agents.

1:3:3: ATM has an important role in B cell development.

Primary B cell development begins in the bone marrow. Like T cells, B cells develop from progenitor cells derived from haematopoietic stem cells. The most immature B-cell lineage committed cells are known as pro-B cells, they do not produce immunoglobulin but do express the B cell marker CD19. Pro-B cells express RAG genes and undergo V(D)J recombination of the immunoglobulin heavy chain gene.

The next stage in development is the pre-B cell. These cells are the first B cell lineage cells to synthesise immunoglobulin (cytoplasmic μ heavy chain) and express the pre-B cell receptor which stimulates continued maturation and proliferation. It also has a role in allelic exclusion, ensuring that the B cell is specific for only one antigen by providing a signal which prevents rearrangement of the heavy chain genes on the other chromosome. The immunoglobulin light chain is rearranged (V(D)J recombination) and associates with the μ chain to produce complete IgM, the cell is now an immature B cell.

As discussed earlier conventional V(D)J recombination requires ATM, therefore the process is impaired in ATM deficient pre-B cells (Bredemeyer et al., 2008). This may contribute to the increased incidence of chromosome translocations in immune system genes which may lead to the development of B cell tumours in A-T patients.

Like T cells immature B cells undergo a process of positive and negative selection. Those that express functional immunoglobulin receive survival signals (positive selection). Unlike mature B cells, immature B cells do not proliferate and differentiate in response to antigen. Those that express high affinity receptors for self antigens and encounter these antigens in the bone marrow either undergo apoptotic death, fail to mature or undergo an editing

process in which they change specificity by rearrangement of the light chain (negative selection).

Immature B cells leave the bone marrow and complete their maturation in lymphoid organs. Naive mature B cells express both membrane IgM and IgD. They are functionally competent but will die within a few weeks if they do not encounter antigen. On encountering antigen they undergo proliferation to give a clone of antigen specific cells and differentiate into immunoglobulin secreting effector cells and memory cells. Responses to protein antigens require CD4+ve T cell help, whereas responses to non-protein antigens (polysaccharides and lipids) do not.

Effector B cells may undergo heavy chain isotype class switching which results in production of antibodies of different isotypes and effector function but the same antigen specificity as the original IgD and IgM antibodies. Similarly to V(D)J recombination the process of class switch recombination involves the generation and repair of DNA double strand breaks and requires ATM (Lumsden et al., 2004). The process involves intrachromosomal recombination between tandemly repeated switch region sequences located upstream of each immunoglobulin heavy chain constant region gene and excision of intervening DNA sequences. Repair of the DNA double strand breaks generated during class switch recombination predominantly occurs through non-homologous end joining and requires ATM. However, when ATM function is impaired the alternative end joining pathway may be utilised (Kotnis et al., 2009). Failure of the ATM-dependent joining process may account for the low serum antibody levels of A-T patients (Reina-San-Martin et al., 2004).

Effector B cells also undergo a process of affinity maturation which increases the affinity of antibodies for a particular protein. The process is known as somatic hypermutation and involves somatic mutation of immunoglobulin genes and selective survival of the B cells

which produce the highest affinity antibodies. Somatic hypermutation involves the formation of DNA single strand breaks or nicks so requires ATR rather than ATM (Pan-Hammarstrom et al., 2006), therefore the process occurs normally in A-T patients (Pan-Hammarstrom et al., 2003).

1:4: Other effects of *ATM* mutations.

1:4:1: *ATM* mutations result in a defective response to oxidative stress.

Elevated levels of oxidative stress leading to an increase in oxidative damage to lipids and DNA has been reported in A-T patients (Reichenbach et al., 2002). The cause of the increased oxidative stress is unclear, ATM may regulate or modify the activity of antioxidant proteins, it may sense and respond to increased ROS levels directly, or alternatively oxidative stress may result from the effect of unrepaired DNA double strand breaks on cell metabolism (reviewed in (Barzilai et al., 2002)). In addition to increasing oxidative stress *ATM* mutations also result in a defective response to reactive oxygen species (ROS) (Barlow et al., 1999;Ito et al., 2004;Rotman and Shiloh, 1997).

Neurons are particularly sensitive to oxidative damage and oxidative stress has been implicated in the neurodegeneration of A-T patients. A study by Kamsler et al. (2001) using Atm-deficient mice investigated the prevalence of oxidative stress in the central nervous system. They showed that the area that was most severely affected was the cerebellum (Kamsler et al., 2001). This area contains Purkinje and granule neurons, the degeneration of which causes the characteristic progressive ataxia of A-T patients (Boder, 1985). More recent studies using ATM-/- mouse models of A-T have shown that oxidative stress in the absence of ATM leads to reduced survival of Purkinje neurons (Chen et al., 2003), defective

proliferation and increased apoptosis of neural stem cells (Kim and Wong, 2009a) and defective proliferation of astrocytes (Kim and Wong, 2009b).

Although high levels of ROS cause damage to cellular components resulting in cell death, at low levels they have a role in signalling and are generated under normal physiological conditions as by-products of metabolic processes or apoptotic signals and in the cytotoxic response to pathogens. Interestingly they are also generated in response to T cell activation. At low levels ROS amplify signals from the T cell receptor whilst at high levels they are important in initiation of activation-induced cell death (AICD) through activation of the FasL promoter (Devadas et al., 2002;Hildeman et al., 1999). The inability of A-T patients' cells to respond to the generation of ROS in response to T cell activation may contribute to their immunodeficiency.

Bagley et al. showed that mature T cells obtained from ATM deficient mice underwent apoptosis rather than proliferation in response to stimulation through the T cell receptor (Bagley et al., 2007). This also occurred when mature T cells from wildtype mice were stimulated through the T cell receptor in the presence of ATM inhibitors. However, when both ATM deficient and ATM inhibited T cells were stimulated in the presence of the antioxidant *N*-acetyl cysteine (NAC) cell death was prevented and normal levels of proliferation occurred. This suggests that ATM has an important role in T cell activation by regulating the response to reactive oxygen species produced in response to stimulation through the TCR. In the absence of ATM T cells may be unable to proliferate normally and gain effector function in response to antigenic stimulation and this could contribute to the immunodeficiency of A-T patients. However, it seems unlikely that the proliferation defect is as severe in A-T patients as that described in ATM deficient mice as A-T patients are not

generally highly susceptible to opportunistic infections other than those of the respiratory tract and intact T cell responses have been reported (Pashankar et al., 2006).

1:4:2: *ATM* mutations predispose to cancer.

ATM mutations result in the inability to repair double strand breaks, defective V(D)J recombination (Bredemeyer et al., 2006) and aberrant chromosomal translocations in immune system genes (Matei et al., 2006). These, together with the failure to repair DNA damage and remove damaged cells results in a high degree of genetic instability, predisposing A-T patients to the development of cancer (Boultwood, 2001;Gumy-Pause et al., 2003;Taylor et al., 1996).

The incidence of lymphoid tumours in A-T patients is particularly high (Tran et al., 2008), fifteen percent of patients in the UK die of leukaemia or lymphoma as children (reviewed in (Taylor & Byrd, 2005)). There are clear age differences in the types of tumours to which A-T patients are predisposed. A-T children show a high frequency of T cell acute lymphocytic leukaemia and lymphoma whereas young adults with A-T frequently develop T cell prolymphocytic leukaemia (T-PLL) (Taylor et al., 1996). A-T patients may also develop B cell tumours, Hodgkins and non-Hodgkins lymphoma (Olsen et al., 2001;Taylor et al., 1996). Non-lymphoid tumours seen in A-T patients include brain tumours and some carcinomas (Taylor & Byrd, 2005).

Chromosomal translocations in A-T patients typically result from defective repair of the DNA double strand breaks generated in immune system genes during V(D)J recombination and immunoglobulin class switch recombination. As cell cycle checkpoints are defective in A-T broken ends persist and accumulate (Bredemeyer et al., 2006) so translocations are more likely to occur. If such a translocation involves a proto-oncogene it may result in fusion of

the proto-oncogene to a locus that encodes an antigen receptor. The new oncogene will then become activated in response to immunoglobulin or T cell receptor regulatory elements leading to increased expression of the oncogene. Alternatively the fusion of two unrelated genes may lead to the production of new fusion proteins with oncogenic properties. These translocations are the first step in tumourigenesis, however in general further genetic changes are required to provide a proliferative advantage and allow the tumour to escape homeostatic control of cell number.

Interestingly ATM may have a role in signalling the presence of potentially malignant cells to the immune system through the DNA damage-induced upregulation of natural killer group 2, member D (NKG2D) ligands (Gasser et al., 2005). These ligands are recognised by cytotoxic NK cells, NKT cells and T cells which express the NKG2D receptor. Binding of the receptor to the ligand initiates degranulation of the cytotoxic cell which releases perforin and granzme leading to apoptosis of the target cell. Failure to upregulate NKG2D ligands in response to DNA damage in A-T patients due to their *ATM* mutations may allow cells harbouring damaged DNA to escape this process of immunosurveillance. This could contribute to the high incidence of cancer in A-T patients.

ATM mutations are also a frequent characteristic of tumours in non-A-T individuals. They are found in approximately 40% of sporadic B cell chronic lymphocytic leukaemia (B-CLL) (Stankovic et al., 1999) and the majority of T-PLL tumours (Matutes et al., 1991;Nowak et al., 2009;Yamaguchi et al., 2003;Yuille et al., 1998). However, whilst T-PLL is relatively frequent in A-T patients, perhaps because loss of ATM function is important in initiation of this tumour type (Stankovic et al., 2002;Stoppa-Lyonnet et al., 1998), the incidence of B-CLL is low possibly because *ATM* mutation is important in progression of this tumour type.

Approximately 0.5% of the UK population are carriers of mutations in the *ATM* gene. There is evidence that female carriers have an increased risk of developing breast cancer (Geoffroy-Perez et al., 2002;Olsen et al., 2001;Swift et al., 1991). However, as carriers of the *ATM* gene do not show increased sensitivity to ionising radiation the tumours can be treated with radiotherapy without adverse reactions.

1:5: Ataxia-telangiectasia-related disorders.

There are several Ataxia-telangiectasia-related disorders which result from mutations in different components of the DNA damage repair pathway. These disorders have similar clinical phenoypes to A-T but differ in their severity (Table 1:5).

1:5:1: Ataxia-telangiectasia-like disorder.

Ataxia-telangiectasia-like disorder (ATLD) results from mutations in the *Mre11* gene (Stewart et al., 1999) which encodes MRE11, an important component of the MRN complex. It is clinically very similar to A-T and is difficult to distinguish neurologically. However ATLD patients do not show telangiectasia (dilated blood vessels) or raised serum α-fetoprotein levels, onset of ataxia is later and neurological degeneration is slower (reviewed in (Taylor et al., 2004)). ATLD patients have normal levels of total IgG, IgA and IgM although there may be reduced levels of specific functional antibodies (reviewed in (Taylor et al., 2004). The disorder is very rare, and accounts for only a small proportion of patients diagnosed with A-T (reviewed in (Taylor & Byrd, 2005)).

1:5:2: Nijmegen breakage syndrome.

Mutations in the *NBS1* gene result in Nijmegen breakage syndrome (NBS). The gene encodes NBS, another component of the MRN complex (reviewed in (Ball and Xiao, 2005)). This disorder differs from both A-T and ATLD in that patients suffer from microcephaly rather

than neurodegeneration. Patients have a bird like face, short stature, immunodeficiency, chromosomal instability and are predisposed to cancer, particularly B cell lymphomas (Tauchi et al., 2002). The frequency of immunodeficiency and malignancy and the percentage of chromosome rearrangements are higher in NBS than in A-T (Weemaes et al., 1994). Similar to A-T and ATLD patients, NBS patients are radiosensitive (reviewed in (Taylor & Byrd, 2005)).

1:5:3: NBS-like disorder.

Mutations in *RAD50*, the gene which encodes the final component protein of the MRN complex, have been implicated in hereditary susceptibility to breast and ovarian cancers (Heikkinen et al., 2003). However, a genetic disorder resulting from mutations in this gene has only recently been identified (Waltes et al., 2009). Waltes et al. found compound heterozygous mutations in the *RAD50* gene resulting in production of low levels of unstable Rad50 protein in a patient previously misdiagnosed with NBS due to her bird-like features, short stature, microcephaly, mental retardation, cellular radiosensitivity and chromosomal instability. However, unlike NBS patients she was not immunodeficient, had normal immunoglobulin levels, and at time of publication had not developed a lymphoid malignancy (at the age of 23). The patient's cells failed to form DNA damage-induced MRN foci and radiation induced activation of ATM and downstream signalling was impaired as was activation of the G1/S cell cycle checkpoint. The disorder was classified as an NBS-like disorder (NBSLD) (Waltes et al., 2009).

1:5:4: RIDDLE syndrome.

RIDDLE syndrome (radiosensitivity, immunodeficiency, dysmorphic features and learning difficulties) is an extremely rare DNA repair deficiency disorder resulting from mutations in

the E3 ubiquitin ligase, RNF168, which may have a role in recruitment of the DNA repair protein 53BP1 to sites of DNA damage (Stewart et al., 2009). The disorder has so far only been identified in one patient and is characterised by an inability to produce IgG, dysmorphic features, learning difficulties, stunted growth and a slight ataxic gait (Stewart et al., 2007;Stewart et al., 2009).

1:5:5: Ataxia occulomotor apraxia 1 and 2.

Ataxia occulomotor apraxia 1 and 2 (AOA1 and AOA2) also have similarities to A-T. AOA1 results from mutations in the *APTX1* gene which encodes aprataxin, a member of the HIT domain superfamily of nucleotide hydrolases/transferases which may have a role in DNA damage repair (Moreira et al., 2001). AOA1 has similar neurological features to A-T including early onset ataxia, oculomotor apraxia (difficulties with eye movements) and cerebellar atrophy. However AOA1 patients differ from A-T patients in that they do not show immune deficiency, chromosomal instability or radiation sensitivity (Moreira et al., 2001).

AOA2 has similar clinical features to AOA1 but the onset is later; 10-22 years compared to 2-15 years for AOA1 (Moreira et al., 2004). Patients show progressive cerebellar ataxia with peripheral neuropathy, cerebellar atrophy, occasional oculomotor apraxia and elevated serum α-feto-protein levels (Anheim et al., 2009). AOA2 is caused by mutations in the *SETX* gene which encodes sentaxin, a protein which is thought to have DNA/RNA helicase activities and a role in DNA double strand break repair (Moreira et al., 2004). Patients do not have increased radiation sensitivity or susceptibility to cancer (Anheim et al., 2009).

1:5:6: Spinocerebellar apraxia with axonal neuropathy.

Spinocerebellar apraxia with axonal neuropathy (SCAN1) is caused by mutation of the *TDP1* gene which encodes the DNA repair protein tyrosyl DNA phosphodiesterase 1 (Tdp1) (Takashima et al., 2002). Tdp1 is important in repair of DNA single strand breaks resulting from abortive topoisomerase 1 activity, ionising radiation and oxidative stress (el-Khamisy et al., 2005;el-Khamisy et al., 2007). Onset of SCAN1 occurs in late childhood. The disease affects large, terminally differentiated, non-dividing neuronal cells and causes progressive cerebellar ataxia and dysarthria (speech disorder) (Takashima et al., 2002). Lymphoblastoid cell lines derived from SCAN1 patients may show slight radiosensitivity (Zhou et al., 2005).

The highly debilitating clinical phenotypes of Ataxia-telangiectasia and related disorders which result from mutations in components of the DNA damage repair machinery illustrate the importance of effective DNA damage repair in the normal functioning of the immune and nervous systems, maintenance of genomic stability and cancer prevention.

Table 1:5: Ataxia-telangiectasia and related disorders.

	Ataxia-telangiectasia	Ataxia-telangiectasia-like disorder	Nijmegen breakage syndrome	Nijmegen breakage syndrome-like disorder	RIDDLE syndrome	Ataxia occulomotor apraxia 1	Ataxia occulomotor apraxia 2	spinocerebellar apraxia with axonal neuropathy
mutated gene	*ATM*	*Mre11*	*NBS1*	*RAD50*	*RNF168*	*APTX1*	*SETX*	*TDP1*
ataxia	+	+	-	-	+	+	+	+
telangiectasia	+	-	-	-	-	-	-	-
neurodegeneration	+	+	+	-	-	+	+	+
microcephaly	-	-	+	+	-	-	-	-
IR sensitivity	+	+	+	+	+	-	-	+ (slight)
cancer predisposition	+	unknown	+	unknown	unknown	-	-	-
cell cycle checkpoint defects	+	+	+	+	+	-	-	-
chromosome instability	+	+	+	+	-	-	-	-
immunodeficiency	+	-	+	-	+	-	-	-
Ig abnormalities	+	+	+	-	+	-	-	-
dysarthria	+	+	-	-	-	+	+	+
raised serum α-fetoprotein	+	-	-	-	-	-	+	-

Table 1:5: Comparison of the typical characteristics of Ataxia-telangiectasia and related disorders.

Characteristics common to individual disorders are indicated by +ve symbols and yellow cells, -ve symbols and blue cells indicate that the disorder does not typically show the characteristic. The cancer predisposition of patients with ATLD, NBLD and RIDDLE syndrome has not been determined due to the very small number of patients with these disorders. However, there is as yet no evidence of increased cancer risk in these disorders.

1:6: Current and future treatment strategies for Ataxia-telangiectasia.

At present there is no curative treatment for Ataxia-telangiectasia. Therefore current treatment strategies aim to slow the progress of the neurodegeneration and treat tumours with minimal side effects. Immunisations to reduce the risk of sinopulmonary infection and immunoglobulin replacement therapy are commonly administered (reviewed in (Davies, 2009;Lavin et al., 2007)).

1:6:1: Antioxidants.

Possible future therapies include the use of antioxidants to reduce oxidative stress in A-T patients' cells. Studies using ATM deficient mice have suggested that treatment with antioxidants such as N-acetyl-L-cysteine (NAC) may suppress genome rearrangements and DNA deletions, reduce the incidence of lymphoma, increase lifespan and improve neuromotor function possibly by reducing oxidative damage to purkinje cells (Gueven et al., 2006;Reliene et al., 2008;Reliene and Schiestl, 2006). It is unclear if clinical trials analysing the effect of antioxidant treatment on A-T patients are currently underway (Reliene and Schiestl, 2007). However, the data collected using mouse models suggests that the treatment would be advantageous.

1:6:2: Steroids.

Research is also being carried out to investigate the potential of steroid treatment for A-T. There is both anecdotal and clinical evidence that steroids have beneficial effects on A-T patients (Broccoletti et al., 2008;Buoni et al., 2006;Russo et al., 2009). Recent trials have shown that betamethasone can reduce the neurological symptoms although this was not sustained when treatment was discontinued (Broccoletti et al., 2008). Interestingly Broccoletti et al. (2008) also reported an increase in lymphocyte cell count in A-T patients

during bethamethosone treatment. It is possible that the beneficial effects of the drug may be due to an antioxidant mechanism (Russo et al., 2009). Unfortunately steroid treatment has many deleterious side effects, these include immunosuppression which leads to increased frequency of infections and could be very dangerous for already immunodeficient A-T patients.

1:6:3: Mutation-targeted therapies.

Perhaps the most promising area of research into treatment of A-T and other primary immunodeficiency disorders is that of mutation-targeted therapy. These treatment strategies aim to correct mutations at the DNA level before their effects become systemic. Therapies must be tailored to individual mutation type but include chemical suppression of premature termination codons (nonsense mutations) and correction of splicing mutations using antisense oligonucleotides to redirect the splicing machinery to an alternative splice site. Strategies designed to correct frameshift, missense and in-frame mutations are also being developed (reviewed in (Hu and Gatti, 2008)). Mutation-targeted therapy is a relatively new area of research; however it could potentially be highly beneficial for treatment of A-T and related disorders.

1:7: Aims & Objectives.

The aim of this project was to investigate the potential role of ATM in the extrinsic apoptotic processes of CD95-mediated and NKG2D ligand-induced apoptosis and therefore gain a fuller understanding of the cause of the immunodeficiency and cancer predisposition of A-T patients.

Recent studies have suggested a role for ATM in regulation of CD95-mediated apoptosis, however results from different research groups are conflicting. Evidence of both increased

sensitivity and increased resistance to CD95-mediated apoptosis as a result of ATM protein kinase deficiency have been reported. I aimed to clarify the potential role of ATM in the process and also investigate how abnormalities in CD95-mediated apoptosis resulting from *ATM* mutations may affect the immune system in A-T.

The hypothesis of a role for ATM in immune surveillance through the DNA damage induced upregulation of NKG2D ligands was also a new idea and evidence for the concept had only been shown in fibroblasts. As A-T patients have a high incidence of leukaemia and lymphoma I aimed to investigate the potential contributory role of a defect in immune surveillance through NKG2D ligand upregulation on lymphocytes, to the development of these tumours.

Chapter 2: Materials and Methods.

2:1: Collection and preparation of blood samples.

2:1:1: Donors.

Blood samples were obtained from healthy lab donors, A-T patients attending the Nottingham A-T clinic and surplus diagnostic bloods from the Regional Genetics Laboratory, Birmingham Women's Hospital with appropriate ethical approval. Blood samples were collected into heparinised syringes.

2:1:2: Isolation of PBMCs.

Isolation of peripheral blood mononuclear cells (PBMCs) was carried out as soon as possible after sample collection. Blood samples were diluted 1:1 with RPMI 1640 (Sigma-Aldrich, Poole, United Kingdom), layered onto lymphoprep (Nycomed, Sweden) and centrifuged at 1800rpm for 20 minutes with the brake off. The top layer of plasma was collected if required, frozen and stored at -20°C. The buffy layer was then removed into a new sterile tube using a Pasteur pipette and washed twice in RPMI 1640 at 1500rpm and 1200rpm for 10 minutes with the brake on before being resuspended in RPMI media (RPMI 1640 supplemented with 10% foetal calf serum, 2mM L-glutamine (Invitrogen, Auckland, New Zealand) and 1% penicillin-streptomycin solution (Sigma)). Cells were counted using a haemocytometer prior to use in assays or freezing.

2:1:3: Freezing and storage of PBMCs.

PBMCs that were not used immediately in assays were resuspended in freezing media (90% FCS, 10% DMSO) placed in a "Mr Frosty" (Nalgene, Rochester, New York, USA and transferred to a -80°C freezer. Frozen samples were then stored in liquid nitrogen.

2:2: Cell culture.

2:2:1: Lymphoblastoid cell lines.

Lymphoblastoid cell lines were cultured at 37°C, 5% CO_2 in RPMI media. Transfected LCLs were cultured in the same media with the addition of 200μg/ml hygromycin B (Invitrogen). LCL cultures were fed every 3 to 4 days by splitting 1 in 2 and topping up with fresh media.

2:2:2: Fibroblasts.

Human and mouse (NIH3T3) fibroblasts were cultured in horizontal flasks at 37°C, 5% CO_2 in DMEM media (Dulbecco's Modified Eagle medium (Sigma) supplemented with 10% fetal calf serum, 2mM L-glutamine (Invitrogen) and 1% penicillin-streptomycin solution (Sigma)). As fibroblasts are adherent cells detachment was necessary prior to use in assays and for splitting confluent cultures. The media was removed and the cells washed with PBS. 0.05% trypsin-EDTA (Invitrogen) was then added to detach the cells. After a few minutes incubation at 37°C and gentle tapping the cells lifted off the surface of the flask and could be transferred to a sterile tube, washed, counted and reseeded in fresh media or used in assays.

2:2:3: HeLa, K562 and LB23 SARC.

HeLa cells were cultured in DMEM media. As they are adherent cells trypsinisation (as described in section 2:2:2) was necessary for splitting of confluent cultures. The non-adherent cell lines K562 and LB23 SARC were cultured in RPMI media and fed as for LCLs (section 2:2:1).

2:2:4: T cell cloning.

The IFN-γ secretion assay (Miltenyi Biotech, Bergisch Gladback, Germany) was used to detect and isolate viable IFN-γ secreting T cells which could then be cloned by limiting dilution. This method of cloning is considerably more efficient than limiting dilution alone.

Preparation of cloning mix.

Three buffy coats were separated using lymphoprep (as described in section 2:1:2) and individually resuspended in 20ml of RPMI media. They were stimulated overnight by the addition of 1µl/ml PHA, harvested, washed 3 times in RPM1 1640 (Sigma), pooled and resuspended in RPMI media. $20x10^6$ cells of an LCL autologous to the donor whose cells were to be cloned (HLA A2) was also harvested and washed in RPMI. The buffy coats and LCL were irradiated (40Gy) and two separate T cell cloning feeder mixes prepared (one for cloning CD4+ and one for cloning CD8+ T cells). Each mix consisted of $150x10^6$ buffy and $10x10^6$ LCL cells resuspended in 150ml of RPMI media supplemented with 15µl of IL-2.

IFN-γ capture.

A lab donor with the appropriate HLA type (A2) was bled (50ml) and PBMCs isolated using lymphoprep (Nycomed). The PBMCs were resuspended in T cell cloning media (RPMI 1640 supplemented with 10% human serum and 2mM L-glutamine) at a concentration of $10x10^6$cells/ml and plated out into 2 wells (1ml per well). Each well was stimulated for 3 hours with 10µg/ml CMV peptide (VLE for CD8+ T cell cloning, DYSN for CD4+ T cell cloning). This step induces secretion of IFN-γ by VLE- or DYSN-peptide specific T cells. PBMC samples were also stimulated with either DMSO or Staphylococcal Enterotoxin B (100ng/ml) (Sigma) to provide negative and positive controls respectively.

Following stimulation PBMCs were transferred to 15ml tubes, washed in cold MACs buffer (PBS pH 7.2 containing 0.5% bovine serum albumin and 2mM EDTA) and resuspended in 80µl of cold T cell cloning media. 20µl of bi-specific IFN-γ Catch reagent (Miltenyi Biotech) per 10^7 cells was added and the cells incubated for 5 minutes on ice. They were then diluted in warm T cell cloning media to give a concentration of 0.5-1x10^6 cells/ml and left under continuous rotation at 37°C for 45 minutes. Cells were washed twice in cold MACS buffer and resuspended in 80µl of the buffer. 20µl of IFN-γ detection antibody conjugated to PE (Miltenyi Biotech) was added and the cells incubated on ice for 10 minutes. They were washed again in cold MACS buffer, resuspended in 80µl and magnetically labelled by incubation for 15 minutes at 4°C with 20µl of anti-PE magnetic beads (Miltenyi Biotech). Cells were washed and resuspended in 500µl of cold MACS buffer. 50µl of each sample was removed, stained with CD8-PCy5 (Beckman Coulter, High Wycombe, UK) and analysed on an XL Beckman Coulter flow cytometer to analyse the frequency of IFN-γ secreting cells prior to enrichment. Enrichment was performed by magnetic separation using the separation program 'Posseld' on an auto-MACS (Miltenyi Biotech). A small sample of the positively selected cells was also stained with CD8-PCy5 and analysed by flow cytometry to allow the efficiency of enrichment to be determined.

Cloning by limiting dilution.

The positively selected VLE- or DYSN-stimulated T cells were counted, resuspended in T cell cloning media, seeded into cloning mix and plated out in 96 well plates (100µl per well) as follows – 3 plates 30 T cells/well (9000 T cells in 30ml cloning mix), 6 plates 3 T cells/well (1800 T cells in 60ml cloning mix), 6 plates 0.3 T cells/well (180 T cells in 60ml cloning mix) and one plate 0 T cells/well (10ml cloning mix). This gave 30 plates in total (15 plates of VLE-stimulated T cells and 15 of DYSN-stimulated T cells). Plates were wrapped in foil and

incubated at 37°C in 5% CO2 for 2 to 3 weeks. Growing clones were then expanded to 2ml with cloning mix and incubated for a further week. They were tested for specificity by IFN-γ ELISA (as described in section 2:6:2).

2:3: Flow cytometry.

2:3:1: Phenotyping of lymphocyte subsets.

Phenotyping of lymphocyte subsets (T cells, B cells, NK cells, NKT cells, CD4+ve T cells, CD8+ve T cells, CD4+ve and CD8+ve naive, central memory, effector memory and TEMRA T cell subsets, naive and memory B cells and CD56bright and CD56dim NK cells) and analysis of CD95, FasL and CD127 (IL-7Rα) expression was carried out using an 11 colour panel consisting of 10 conjugated antibodies: CD3-Amcyan (BD biosciences, San Jose, California, USA), CD19-eflour 450 (eBiosciences, San Diego, California, USA), CD56-PCy7 (BD biosciences), CD4-APC (BD biosciences), CD8-Q dot 655 (Invitrogen) CD45 RA-AF700 (Biolegend, San Diego, California, USA), CD27-APC AF780 (eBiosciences), CD95-PE (Dako, Glostrup, Denmark), FasL-FITC (Abcam, Cambridge UK) and CD127-PerCP-Cy5.5 (BD biosciences) and the dead cell exclusion die propidium iodide (PI) (Sigma).

Frozen PBMC samples were thawed in a water bath at 37°C for a few minutes, slowly resuspended then washed in warm RPMI 1640 (Sigma) with 10% foetal calf serum. They were washed again in cold MACs buffer prior to staining with an antibody cocktail (conjugated antibodies without PI) for 20 minutes on ice and protected from light. They were then washed in MACs buffer as before, resuspended in 400µl MACs buffer and transferred to FACs tubes. Propidium iodide (Sigma) was added to each tube immediately before analysis on a LSRll flow cytometer (BD biosciences). Data was analysed using DIVA flow cytometry software (BD biosciences).

2:3:2: Analysis of CD95 expression and CD95-mediated apoptosis.

CD95 expression and induction of apoptosis was assessed by flow cytometry on a XL Beckman Coulter flow cytometer. For induction of CD95-mediated apoptosis cells were incubated with 500ng/mL of anti-Fas IgM monoclonal antibody (Tran et al., 2008) (CH11: Upstate, Millipore, Temcula, California, USA) for 15h or as indicated. Apoptosis was quantified using Annexin V-FITC (BD Pharmingen, San Diego, California, USA) and propidium iodide (Sigma) according to the manufacturer's instructions (early apoptosis - AV+ve, PI-ve; late apoptosis - AV+ve, PI+ve). Specific apoptosis was determined as follows: (% of apoptotic cells with CH11/% of apoptotic cells without CH11).

CD95 expression was analysed using a CD95-PE conjugated antibody (DX2: Dako). Mean fluorescence intensity of viable cells (Annexin V-ve, PI-ve) was used to compare the level of CD95 expression. Flow cytometry data was analysed using Flowjo software (Tree Star Inc, Ashland, Oregon USA).

2:3:3: Analysis of perforin/granzyme cytotoxic T cell killing using CFSE.

A flow cytometry assay was developed to analyse perforin/granzyme killing of LCL targets using T cell clones. The clones were tested by chromium release with perforin/granzyme blocking to confirm their mechanism of cytotoxicity (as described in section 2.7.2).

LCL target cells with the appropriate HLA type to allow recognition by the clones (A2 for VLE and DYSN clones) were labelled with 10µM CFSE (Invitrogen) for 10 minutes at 37°C. The reaction was stopped by adding an equal volume of FCS and incubating for 2 minutes at room temperature. The cells were resuspended in RPMI media at a concentration of $5x10^4$cells/ml and plated out in 96 well plates, 100µl per well. T cell clones were counted, resuspended at a concentration of $1x10^4$cells/ml and 100µl per well added to the CFSE

labelled LCL target cells. 100µl per well of RPMI media was added to LCL only control plates. The assay plates were incubated at 37°C for 5 hours.

Wells were harvested, the cells transferred to FACs tubes, washed in cold MACs buffer and resuspended in 300µl. 10µl of counting beads (Caltag Laboratories, Burlingame, California, USA) was added to each tube prior to flow cytometry analysis. This allowed the absolute number of cells to be determined using the formula: absolute count = (number of cells counted/total number of beads counted) x number of beads per µl. As LCLs were labelled with CFSE but florescence is lost on lysis the viable LCL target cells could be distinguished from the T cell clones. The percentage survival of the LCLs was calculated using the formula: (absolute number of CFSE+ve cells after incubation with T cell clones/ absolute number of CFSE+ve cells in the no T cell clone control) x 100/1.

2:3:4: Analysis of NKG2D ligand expression on LCLs, fibroblasts and B cells.

NKG2D ligand expression on LCLs, fibroblasts and B cells was analysed by flow cytometry using an XL Beckman Coulter flow cytometer (Beckman Coulter). For analysis of NKG2D ligand expression on LCLs or fibroblasts cells were harvested, washed in cold MACs buffer and transferred to FACs tubes. $0.5x10^6$ cells per test were stained for 30min on ice with single NKG2D antibodies (MICA-PE, MICB, ULBP-1, ULBP-2-PE, ULBP-3 (R&D systems, Minneapolis, Minnesota, USA)) or isotype control antibodies (IgG2b-PE, IgG2b, IgG2a (BD Pharmingen)). Cells were washed and those stained with congugated antibodies were resuspended in 400µl of cold MACs buffer. Those stained with uncongugated antibodies were blocked with 100µl goat serum for 10 minutes prior to staining with goat anti-mouse-PE secondary antibody (Dako) for 20 minutes on ice. They were then also washed and

resuspended as before. Propidium iodide (Sigma) was added to tubes immediately prior to analysis.

For analysis of MICA expression on B cells staining was carried out as for LCLs but PBMC samples were dual stained with either MICA-PE (R&D systems) and the B cell antibody CD20-FITC (Dako) or the isotype control IgG2b-PE (BD Pharmingen) and CD20-FITC (Dako).

Flow cytometry data was analysed using Flowjo software (Tree Star Inc) and NKG2D ligand expression calculated by subtracting any background staining in the appropriate isotype control from the percentage or MFI of NKG2D+ve cells.

2:4: Western blots.

2:4:1: Analysis of ATM activation, cFLIP expression and ATM activation.

Cell extracts were washed in cold PBS and protein extracted by sonication in UTB buffer (8 M urea, 150 mM β-mercaptoethanol, 50 mM Tris HCl, pH7.5). Protein concentrations of extracts were determined using the Bradford protein assay (Bio-rad, Hercules, California, USA). 30µg of protein extract was separated by electrophoresis on an 8% Tris-Bicine gel and blotted onto nitrocellulose membrane. Following a 2 hour incubation in blocking solution (5% milk in TBST) blots were probed overnight with primary antibodies; total ATM (11G12 mouse monoclonal raised against amino acids 992-1144), phospho ATM Ser1981 (R&D systems), total SMC1 (Bethel Laboratories, Montgomery, Texas, USA), phospho SMC1 Ser966 (Bethel laboratories), total NBS1 (Abcam), phospho NBS1 Ser343 (Bethel laboratories), cFLIP L/S (NF6; Alexis Biochemicals, Lausanne, Switzerland), caspase 8 (5F7; MBL, Watertown, MA, USA), LMP1 (CS1-4) (Rowe et al., 1987), β-actin (A5316, Sigma), Rad50 (Sigma). After washing in TBST they were incubated with the appropriate mouse or rabbit secondary antibodies (Dako). Proteins were visualised using the enhanced chemiluminescence (ECL)

technique (Amersham, Little Chalfont, Buckinghamshire, United Kingdom). Protein bands were quantified by densitometry (Quantity One software).

2:4:2: siRNA knockdown of cFLIP.

$2.5x10^5$ HeLa cells per 6cm dish were plated out in RPMI media and left to become approximately 30-50% confluent overnight.

The next day 1ml warm OPTIMEM (Invitrogen) and 20µl sterile oligofectamine (Invitrogen) were added to RNAse free tubes (Ambion, Austin, Texas, USA). 36µl of 20µM stock of human C-FLAR RNA (ON-Target plusSMARTpool L-003772-00-0005, Human CFLAR (Thermo Scientific Dharmacon, Lafayette, CO, USA)) or 7.2µl of 100µM stock of control RNA (Thermo Scientific Dharmacon) was added and the solution mixed by pipetting then left for 30min to allow RNA and lipids to complex together. The media was removed from the HeLa cells and the cells rinsed with OPTIMEM. 1ml fresh OPTIMEM was then added to each 6cm dish. The RNA was dropped onto the cells and evenly distributed across the dish by gentle rocking.

Following another overnight incubation 2ml of DMEM media (Sigma) with 20% foetal calf serum (no antibiotics) was added to each 6cm dish to give a final concentration of 10% foetal calf serum. Cells were again incubated overnight. The next morning cells were confluent so were detached using 0.05% trypsin-EDTA (Invitrogen) and plated out in 10cm dishes. They were cultured overnight and then harvested for western blotting.

2:5: Activation and inhibition of ATM activity.

2:5:1: Activation of ATM.

Irradiation was used to create double strand breaks in DNA and therefore induce activation of ATM. Cells were irradiated with 5Gy or 10Gy of γ-rays (Cs^{137}) and incubated at 37°C for 30

minutes to allow sufficient time for ATM to be activated prior to use in assays.

2:5:2: Inhibition of ATM.

To inhibit ATM activity, cells were incubated with 10µM ATM inhibitor KU-55933 (KuDOS, Cambridge, United Kingdom) for the times as indicated.

2:6: ELISAS

2:6:1: Measurement of plasma cytokine concentrations.

Analysis of IL-7, IL-15 and IL-21 concentration in plasma samples was carried out in triplicate using commercial kits (Human IL-7 Quantikine HS ELISA kit (R&D systems), Human IL-15 ELISA kit (RayBiotech, North Metro-Atlanta, Georgia, USA), Human IL-21 R ELISA ready-SET-Go! (eBioscience)) according to the manufacturer's instructions.

2:6:2: Interferon gamma ELISA assay.

An IFN-γ ELISA assay (Endogen, Rockford, Il, USA) was used to test the ability of DYSN- and VLE-specific T cell clones to recognise peptide loaded target cells. Irradiated (40Gy) LCLs were used as targets, they were counted, harvested, washed in RPMI 1640 (Sigma) and resuspended in 1ml RPMI 1640. The sample was then split into two 15ml tubes, the cells pelleted and the supernatant poured off. The relevant CMV peptide (DYSN or VLE) was added to one tube (10µg/ml) and an equivalent dilution of DMSO to the other. Cells were incubated for 1 hour at 37°C and the tubes flicked every 15 minutes to optimise peptide loading. They were then washed in RPMI, resuspended in RPMI media at a concentration of $1x10^6$cells/ml and plated out in 96 well plates, 50µl per well to give $5x10^4$cells/well.

T cell clones were counted, washed twice in RPMI 1640 (Sigma) and resuspended in RPMI media at a concentration of $2x10^5$cells/ml. They were added to the LCL targets 50µl per well

to give $1x10^4$T cells/well. The culture plate was incubated overnight then spun down and the supernatant harvested and tested for IFN-γ production by ELISA.

A Maxisorp plate (Nunc, Hatfield, UK) was coated with 50µl per well anti-human IFNγ Ab (Endogen) diluted to a concentration of 0.75µg/ml in coating buffer (0.1M Na_2HPO_4 adjusted to pH 9 with 0.1M NaH_2PO_4). The plate was sealed and left overnight at 4°C. The antibody was then flicked off, the plate blotted with tissue and 200µl of blocking buffer (filtered 1%BSA/PBS with 0.05% Tween-20 (Sigma)) added to each well. It was incubated for 2 hours at room temperature and control standard dilutions of IFN-γ (Endogen) in RPMI media ranging from 2000pg/ml to 31.25pg/ml were prepared.

The Maxisorp plate was washed 3 times with PBS/Tween and assay supernatants and control standards immediately added (50µl/well). The plate was sealed and left at room temperature for 2-4 hours. It was then washed 4 times with PBS/Tween before addition of 50µl/well biotinylated anti-IFN-γ Ab (Endogen) diluted in blocking buffer (2µg/ml). The plate was sealed and incubated at room temperature for 1 hour. It was washed 4 times with PBS/Tween and 50µl of Extra-avidin Peroxidase (Sigma) diluted 1/1000 in blocking buffer added to each well. The plate was sealed and incubated for 30 minutes at room temperature. It was washed a final 8 times with PBS/Tween and 100µl of TMB substrate (TebuBio, Peterborough, UK) added to each well. The plate was incubated for 20 minutes in the dark and then the reaction stopped by the addition of 100µl/well of 0.5M sulphuric acid. The plate was read on an absorbance plate reader at 450nm.

2:7: Chromium release.

2:7:1: CH11 Killing assays.

To test the sensitivity of LCLs to CD95-mediated apoptosis by chromium release LCL targets were labelled with 100µCi of $Na_2{}^{51}CrO_4$ (Amersham-Pharmacia Biotech, UK) for 1 hour, then washed twice, counted and plated out in triplicate to give 2500 cells per well in 100µl of RPMI media. An additional 100µl of RPMI media supplemented with CH11 (Upstate) (to give a final concentration of 10-1000ng/ml) was then added to appropriate wells. To measure spontaneous and maximum lysis for each LCL 100µl of either RPMI media without CH11 (for spontaneous lysis) or 1% SDS (for maximum lysis) was added to appropriate wells. Plates were incubated for 15 hours at 37°C, 5% CO_2 then spun at 1000rpm for 2 minutes to pellet the cells. 100µl of supernatant was harvested from each well and release of radioactivity (lysis) determined using a topcounter (Hewitt Packard). The percentage of CH11-induced lysis was calculated using the equation: 100 x (CH11 release - spontaneous release)/(maximum release - spontaneous release).

2:7:2: Cytotoxicity assays.

Chromium release assays to measure target cell killing by T cell clones were carried out as described in section 2:7:1, however rather than adding 100µl of CH11 supplemented RPMI media to wells containing autologous LCL target cells, 100µl of RPMI media containing resuspended T cell clones was added to give varying effector:target ratios. Plates were incubated for either 6 hours (for perforin killing assays) or 15 hours (for CD95-killing assays) prior to harvest of supernatant.

2:7:3: CD95 and perforin blocking assays.

CD95 and perforin blocking assays were used to determine the mechanism of cytotoxicity of different T cell clones. These assays were variations on the chromium release assay described in section 2:7:2. However to block the CD95-mediated pathway of apoptosis LCL target cells were plated out 2500 cells/well in 90µl of RPMI media, 10µl of 2µg/ml ZB4 (Immunotech, Marseille, France) was added to each well and the plates incubated for 1 hour at room temperature prior to addition of T cell clones. ZB4 blocked and unblocked LCLs incubated with 500ng/ml CH11 (Upstate) were used as positive and negative controls for inhibition of CD95-mediated apoptosis. Perforin blocking was carried out by incubating T cell clones with concanamycin A (Sigma) before incubation with LCL targets. T cells were plated out in 80µl/well of RPMI media and 20µl of 2µg/ml concanamycin A (Sigma) added to each well. They were then incubated at 37°C for 2 hours and LCL targets added.

The percentage of target cell lysis in the presence and absence of blocking was compared. If blocking significantly reduced the percentage of lysis compared to unblocked controls it was concluded that the T cells primarily killed their targets through the blocked pathway. However, if blocking had a no significant effect on the percentage of target cell lysis compared to unblocked controls it was concluded that blocked pathway was not the primary mechanism of cytotoxicity of the T cell clone.

2:8: Statistical methods.

Data was analysed using Graphpad PRISM software (GraphPad Software, INC, San Diego California, USA). The significance of differences between populations of data were analysed using students t test (Mann-Whitney) or one way anova (non-parametric) and Dunn's

multiple comparison test with a level of significance of $p \leq 0.05$. The Spearman test (non-parametric) was used for analysis of correlations.

Chapter 3: Phenotyping of A-T and normal PBMCs.

3:1: Introduction.

The majority of A-T patients are lymphopenic due to reduced numbers of both T and B cells (Nowak-Wegrzyn et al., 2004). They have reduced proportions of naive T cells and naive B cells and increased proportions of memory T cells, memory B cells and NK cells compared to normal healthy individuals (Giovannetti et al., 2002). The cause of the T cell lymphopenia in A-T patients is thought to be reduced output from the thymus, although increased spontaneous apoptosis of naive cells has been suggested as a contributing factor (Schubert et al., 2000). Interestingly elderly individuals also have reduced numbers of naive cells and an increase in both spontaneous and CD95-mediated apoptosis of lymphocytes as well as increased CD95 expression on T cells has been associated with aging of the immune system (Aggarwal and Gupta, 1998;Gupta and Gollapudi, 2008;Potestio et al., 1999). It is possible that a similar sensitivity to CD95-mediated apoptosis in A-T patients could contribute to their lymphopenic phenotype.

In terms of lymphocyte numbers the immune system in A-T is very similar to that of lymphopenic patients such as HIV patients and bone marrow recipients. The cytokine IL-7 is important in regulation of T cell homeostasis and proliferation in response in lymphopenia. It is primarily produced by the stromal cells of the thymus and bone marrow and provides a survival signal by binding to the IL-7 receptor complex on naive and memory T cells. Under normal conditions production of the cytokine is relatively stable and T cell responses are regulated through modulation of the IL-7 receptor α chain (IL7-Rα or CD127) which dimerises with the γ chain to form the IL-7 receptor complex. Naive T cells downregulate CD127 after contact with IL-7 and upregulate it in the absence of the cytokine, this works to conserve IL-7 and control proliferation (Park et al., 2004). However a decrease in T cell number leads to an increase in the amount of IL-7 available per T cell. This provides an

enhanced survival signal and, if the signal is strong enough, lymphopenia-induced proliferation of naive cells (reviewed in (Overwijk and Schluns, 2009)).

Conversely increased availability of the cytokine IL-7 as a response to lymphopenia may also contribute to the lymphopenia as a result of upregulation of CD95 on T cells and consequent sensitivity to CD95-mediated apoptosis. Peripheral T cell depletion associated with increased serum IL-7 concentration and consequent CD95 upregulation in lymphopenic individuals has been reported in HIV patients (Rethi et al., 2008) and bone marrow transplant recipients (Brugnoni et al., 1999). A-T patients are also likely to show increased IL-7 production in response to their lymphopenia and may consequently upregulate CD95 on their T cells leading to both increased sensitivity to CD95-mediated apoptosis and spontaneous apoptosis. Significantly higher CD95 expression on A-T T cells compared to normal controls has previously been described although IL-7 was not investigated (Giovannetti et al., 2002;Schubert et al., 2000). An increased level of spontaneous apoptosis has also been shown in A-T lymphocytes compared to normal controls (Duchaud et al., 1996).

Rethi et al. suggested that the high IL-7 levels associated with lymphopenic conditions may increase the sensitivity of naive T cells to CD95-mediated apoptosis, whilst, in combination with TCR signalling, inducing proliferation of T cells recognising low-affinity antigens through increasing CD95-mediated co-stimulatory signals (Rethi et al., 2008). If this is the case increased sensitivity of A-T patients' cells to CD95-mediated apoptosis could contribute to both their naive T cell deficiency (by increasing CD95-mediated and spontaneous apoptosis) and the predominance of activated memory cells (Schubert et al., 2000) (by inducing their proliferation).

The cytokines IL-15 and IL-21 also have a role in lymphocyte homeostasis and proliferation. IL-15 is thought to be mainly produced by monocytes and macrophages and is particularly important for survival and proliferation of memory CD8+ T cells, NKT cells and NK cells (reviewed in (Overwijk & Schluns, 2009)). IL-21 is produced by CD4+ T cells and NKT cells in response to T cell receptor activation. It has a role in regulating differentiation and proliferation of CD8+ T cells, B cells and NK cells (reviewed in (Sondergaard and Skak, 2009)). Like IL-7, abnormal levels of IL-15 and IL-21 could have a role in shaping the immune system phenotype of A-T patients.

In order to investigate the possible association of CD95 expression and IL-7, IL-15 and IL-21 availability with lymphopenia in A-T patients, PBMC and plasma samples were collected from patients and age matched controls with an age range of 0 to 30 years. An 11 colour antibody panel was developed in order to distinguish different cell subsets and also investigate expression of CD95, Fas ligand (FasL) and the IL-7 receptor α chain (CD127). Plasma concentrations of the cytokines IL-7, IL-15 and IL-21 were measured by ELISA and correlation analysis carried out to investigate any effect of IL-7 concentration on expression of CD95, FasL or CD127. The effect of aging on the proportions of different cell types and CD95, FasL and CD127 expression in normal and A-T individuals was also analysed.

The results of my study were consistent with the reported lymphopenia and naive T and B cell deficiency of A-T patients. As well as reduced naive and increased memory cells A-T patients had significantly higher percentages of NK and NKT cells than normal controls. Overall my findings suggested a congenitally aged immune system phenotype in A-T patients. Samples from A-T patients with no ATM activity showed significantly higher expression of CD95 on all cell types excluding NK cells. This suggests that A-T patients' cells may be more sensitive to CD95-mediated apoptosis than normal cells. There was no

significant difference in IL-7 or IL-15 plasma concentrations between A-T patients and normal controls.

3:2: PBMC sample collection, cell counts and correlations with age.

3:2:1: PBMC sample collection, selection and age matching.

Blood samples were collected from 18 A-T patients with known ATM mutations (Table 3:2:1A), and 33 normal controls (Table 3:2:1B). The age range of the A-T patients at time of bleed was 1 to 28 years. They were divided into two groups according to the presence or absence of ATM kinase activity (previously determined by western blot). The majority of the A-T patients (15) had no ATM activity. The age range of these patients was 1 year 7 months to 28 years 8 months and the mean age was 9 years 4 months (112.1 months). As only three A-T patients had some ATM activity the age range of this group was considerably smaller; 12 years 10 months to 19 years 11 months. The mean age of 16 years 3.7 months (195.7 months) was also older than that of the A-T patients with no ATM activity (Fig 3:2:1A&B).

The majority of the normal control samples (N PBMC1-30) were obtained from the Regional Genetics Laboratory, Birmingham Women's Hospital. These anonymous samples were the surplus from diagnostic bloods obtained from patients aged 1 to 14 years; details of each sample are given in Table 3:2:1B. In order to increase the age range of controls blood samples from 3 healthy lab donors aged 22, 26 and 30 years were also collected (N PBMC31-33). This gave an age range of 1 to 30 years. Blood samples were separated by lymphoprep and matched PBMC and plasma samples collected.

The use of surplus bloods as normal controls is not ideal as although samples from patients with non-haematological disorders were requested it could not be guaranteed that all would have healthy immune systems. However as control samples were required to age match the A-T patient cohort the use of surplus bloods was preferable to bleeding healthy children. The lab donor blood samples were a useful control for checking the 'normality' of the

diagnostic surplus samples. Two samples were omitted from the analysis as they appeared to be abnormal; N PBMC20 had an inverted CD4/CD8 T cell ratio (1:2 - 29% CD4+, 59.3% CD8+) and N PBMC29 had approximately equal proportions of CD4+ (39%) and CD8+ T cells (42.8%), both also had unusually low percentages of naive T cells for their age. The rest of the controls had normal immune system phenotypes like that of the lab donor samples (Appendix 1). This included three samples intended for testing for DiGeorge syndrome, a rare genetic disorder which affects the thymus. However as their T cell phenotype was normal it is unlikely that they had the syndrome.

Omission of the two abnormal samples from the normal controls gave a final age range (1 year 3 months to 30 years 10 months) and mean age (8 years 6.4 months or 102.4 months) very similar to that of the A-T patients with no ATM activity (Fig 3:2:1A&B). Both groups contained samples from the 1 to 30 year age range with similar mean and median ages so were suitably age matched for the study.

Table 3:2:1A: A-T blood samples.

	age		sex	residual ATM protein		*ATM* mutations	
	years & months	months		amount expressed	activity	mutation 1	mutation 2
AT PBMC1	28y 8m	344	M	0%	no	c.2639-?_2838+?del; p.(Gly880fs)	c.8206_8207delAA; p.(Asn2736fs)
AT PBMC2	12y 10m	154	F	5%	yes	c.5763-1050A>G; p.(Pro1922fs)	c.1563_1564delAG; p.(Glu522fs)
AT PBMC3	19y 11m	239	F	5%	yes	c.590G>A; p(Gly197Glu)	homozygous
AT PBMC4	10y 4m	124	M	0%	no	c.5623C>T; p.(Arg1857X)	c.8305insA; p.(Trp2769X)
AT PBMC5	16y 2m	194	F	5%	yes	c.8480T>G; p.(Phe2827Cys)	c.1563_1564delAG; p.(Glu522fs)
AT PBMC6	14y 11m	179	M	0%	no	c.6916delAG; p.2306fs	c.4850T>C; p.(Leu1617Pro)
AT PBMC7	12y 1m	145	M	0%	no	c.5515C>T; p.(Gln1839X)	IVS16-1G>C
AT PBMC8	7y 4m	88	F	0%	no	c.7013T>C; p.(Leu2338Pro)	c.6056A>G; p.(Tyr2019Cys)
AT PBMC9	2y 7m	31	M	0%	no	c.216_217delAG; p.(Glu73fs)	c.8300T>C; p.(Leu2767Pro)
AT PBMC10	4y 8m	56	M	0%	no	c.1402_1403delAA; p.(Lys468fs)	homozygous
AT PBMC11	2y 11m	35	F	trace	no	c.7638_7646del9; p.(Arg2547_Ser2549del)	c.5290_5290delC; p.(Leu1764fs)
AT PBMC12	16y 1m	193	F	trace	no	c.2T>C; p.(Met1Thr)	c.9139C>T; p.(Arg3047X)
AT PBMC13	10y 0m	120	F	10%	no	c.2932T>C; p.(Ser978Pro)	c.8395-8404del10
AT PBMC14	7y 5m	89	F	5%	no	c.8520G>C; p.(Leu2840Phe)	homozygous
AT PBMC15	3y 11m	47	F	5%	no	c.7638_7646del9; p.(Arg2547_Ser2549del)	c.5825C>T; p.(Ala1942Val)
AT PBMC16	1y 7m	19	M	5%	no	c.7638_7646del9; p.(Arg2547_Ser2549del)	c.5825C>T; p.(Ala1942Val)
AT PBMC17	6y 6m	78	M	100%	no	c.1441/2delT	c.9022C>T; p(Arg3008Cys)
AT PBMC18	11y 1m	133	F	100%	no	c.1441/2delT	c.9022C>T; p(Arg3008Cys)

Table 3:2:1A: A-T blood samples.

Age (at time of bleed), ATM protein expression (as % of normal level), ATM activity (measured by western blot) and ATM mutations of A-T patients included in the study. A-T patients with no ATM activity are shown in red; those with some ATM activity are shown in orange.

Table 3:2:1B: Normal control blood samples.

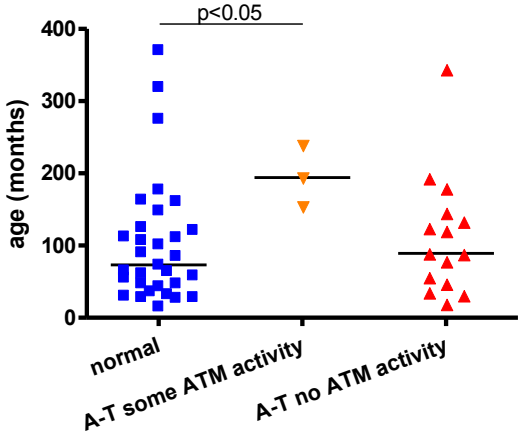
	age		sex	reason for diagnostic testing
	years & months	months		
N PBMC1	2y 8m	32	M	developmental delay, dysmorphic
N PBMC2	1y 3m	15	M	moderate developmental delay
N PBMC3	5y 4m	64	F	developmental delay, possible fragile X syndrome
N PBMC4	3y 7m	43	F	short stature, possible Turner syndrome
N PBMC5	3y 11m	47	M	failure to thrive, dysmorphic
N PBMC6	2y 3m	27	M	unilateral retinoblastoma
N PBMC7	7y 1m	85	F	precocious puberty
N PBMC8	3y 11m	47	M	possible fragile X syndrome
N PBMC9	2y 4m	28	M	speech and language delay, possible DiGeorge syndrome
N PBMC10	10y 1m	121	F	Cleft palate, heart defects, possible DiGeorge syndrome
N PBMC11	5y 5m	65	F	possible Turner syndrome
N PBMC12	7y 6m	90	F	short stature, dysmorphic, for microarray analysis
N PBMC13	2y 6m	30	F	developmental delay, autism, possible fragile X syndrome
N PBMC14	9y 3m	111	F	learning difficulties, microcephaly
N PBMC15	10y 5m	125	F	short stature, possible Turner syndrome
N PBMC16	13y 5m	161	F	arrested puberty
N PBMC17	8y 11m	107	F	language delay
N PBMC18	9y 4m	112	F	short stature
N PBMC19	8y 5m	101	M	learning difficulties, dysmorphism, possible fragile X
~~N PBMC20~~	11y 9m	141	M	tall stature, coordination problems
N PBMC21	4y 10m	58	M	cleft palate, cardiac abnormality
N PBMC22	12y 4m	148	F	short stature
N PBMC23	13y 7m	163	M	developmental delay, dysmorphic, hypotonic
N PBMC24	2y 4m	28	F	overweight, excessive growth
N PBMC25	5y 1m	61	M	communication difficulties, possible ASD
N PBMC26	3y 0m	36	M	developmental delay
N PBMC27	14y 9m	177	F	developmental delay, brachydactyly
N PBMC28	4y 7m	55	M	learning difficulties
~~N PBMC29~~	9y 2m	110	F	possible Turner syndrome
N PBMC30	6y 1m	73	M	mental retardation, possible DiGeorge or fragile X
N PBMC31	30y 10m	370	M	none - healthy lab donor
N PBMC32	26y 7m	319	F	none - healthy lab donor
N PBMC33	22y 11m	275	M	none - healthy lab donor

Table 3:2:1B: Control blood samples.

Age (at time of bleed), sex and reason for diagnostic testing of control samples. N PBMC20 and N PBMC29 were omitted from analysis as lymphocyte phenotyping suggested immune system abnormalities.

Fig 3:2:1: A-T patients and normal controls were age matched.

A.



B.

	normal	A-T some ATM activity	A-T no ATM activity
median age (months)	73	194	89
mean age (months)	102.4	195.7	112.1

Fig 3:2:2: Age distribution of A-T patients and normal controls.

A. There was no significant difference in age (at time of bleed) of A-T patients with no ATM activity and normal controls (line at median).

B. Mean and median ages (at time of bleed) of normal controls, A-T patients with some ATM activity, and A-T patients with no ATM activity.

3:2:2: PBMC cell counts and correlations with age.

Consistent with the reported lymphopenia of A-T patients, samples from individuals with no ATM activity had a significantly lower cell count than the normal controls ($p<0.001$) (Fig 3:2:2A). The A-T patients with some ATM activity also had a lower cell count than normal controls although this was not statistically significant, perhaps because of the small sample size. There was no correlation between cell count and age in any of the groups (Fig 3:2:2B).

Fig 3:2:2: A-T patients were lymphopenic and there was no significant correlation between age and cell count over the 1-30 year age range.

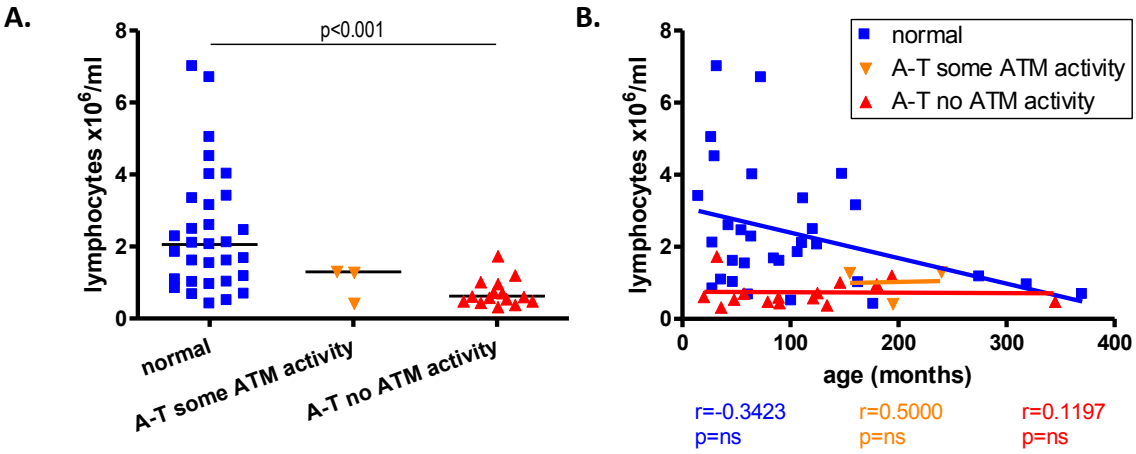


Fig 3:2:2: PBMC samples from A-T patients and normal controls were collected into heparinised syringes. Samples were separated using lymphoprep and matched lymphocyte and plasma samples collected and frozen.

A. A-T patients had a significantly lower number of lymphocytes per ml blood than normal controls. Lymphocytes were counted using a haemocytometer following separation of blood. Samples were then frozen.

B. There was no correlation between lymphocytes/ml blood and age at time of bleed in either A-T patients or normal controls.

3:3: Analysis of lymphocyte subsets by flow cytometry.

3:3:1: Lymphocyte subsets can be defined using an eleven colour antibody panel.

An eleven colour antibody panel was developed to allow analysis of lymphocyte subsets and CD95, FasL and CD127 levels in the PBMC samples. The chosen markers and fluorochromes are shown in Table 3:3:1.

The first step in the gating strategy was exclusion of doublets using a forward scatter height against forward scatter area plot. The lymphocyte population was then selected using forward scatter against side scatter followed by exclusion of dead (PI+ve) cells (Fig 3:3:1A). Gating on T cells (CD3+CD19-CD56-), B cells (CD19+CD3-CD56-), NK cells (CD56+CD19-CD3-), NKT cells (CD56+CD19-CD3+) and various subsets were carried out as shown in Fig 3:3:1B-D.

The cell surface markers CD27 and CD45RA were used to divide CD4+ and CD8+ T cells into naive (CD45RA+CD27+), central memory (CD45RA-CD27+), effector memory (CD45RA-CD27-) and TEMRA (CD45RA+CD27-) subsets (Fig 3:3:1B). CD27 is a member of the TNF family of receptors; it is expressed at high levels by central memory T cells but lost upon differentiation into effector memory T cells (Hintzen et al., 1993). Conventionally the chemokine receptor CCR7 and/or adhesion molecule CD62L are used along with CD45RA for definition of T cell subsets. Central memory T cells are CD45RA-ve and express CCR7 and high levels of CD62 which allows homing to lymph nodes, whereas effector memory T cells are CD45RA-CCR7-CD62low and are found in peripheral non-lymphoid tissues. TEMRA cells are CD45RA+CCR7-CD62low and naive cells are CD45RA+CCR7+.

However, as the panel was limited to a maximum of eleven colours CD27 was used as an alternative to CCR7 and CD62 as it can also distinguish between naive and memory B cell subsets (Fig 3:3:1C). This compromise means that my study's definition of central and

effector memory T cell subsets may not be as reliable as that of studies using CCR7 and CD62 but it was necessary in order to maximise the amount of data that could be generated from the panel.

NK cells were divided into two subsets. Although NK cells do not have distinct 'naive' and 'memory' subsets similar to T and B cells they can be subdivided according to the level of expression of the NK cell marker CD56 (Fig 3:3:1D). The majority of circulating NK cells belong to the CD56dim subset. These cells are cytotoxic and have been shown to arise from differentiation of the less mature CD56bright NK cells which produce cytokines and have an immunoregulatory function (Chan et al., 2007).

NKT cells (natural killer T cells) are a rare subset of T cells which express both CD3 and CD56 (Fig 3:3:1D). They have an unusual TCR which confers specificity to glycolipid antigens and they produce a broad range of cytokines in response to antigenic stimulation. NKT cells have roles in modulating the immune system in response to cancer, autoimmunity, infection, allergies, allograft rejection and graft versus host disease (Godfrey et al., 2010). They have not previously been investigated in Ataxia-telangiectasia patients.

Cell surface expression levels of CD95, FasL and CD127 on each cell subset were also analysed. 'All minus one plus isotype' (all antibodies in the panel apart from the antibody of interest which is replaced with appropriate isotype control) or 'all minus one' controls (when appropriate isotype was not available) were used to determine negative and positive populations (Fig 3:3:1E).

Table 3:3:1: The eleven colour antibody panel.

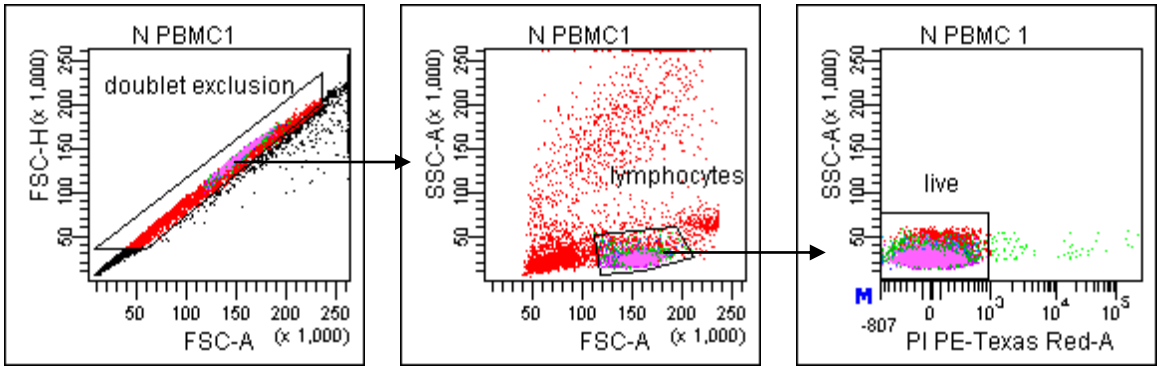
marker	fluorochrome
CD127	PerCP-Cy5.5
FasL	FITC
CD95	PE
CD3	Amcyan
CD4	APC
CD8	Q dot 655
CD56	PCy7
CD19	eflour 450
CD45RA	AF700
CD27	APC AF780
PI	-

Table 3:3:1: Antibodies used in the eleven colour antibody panel.

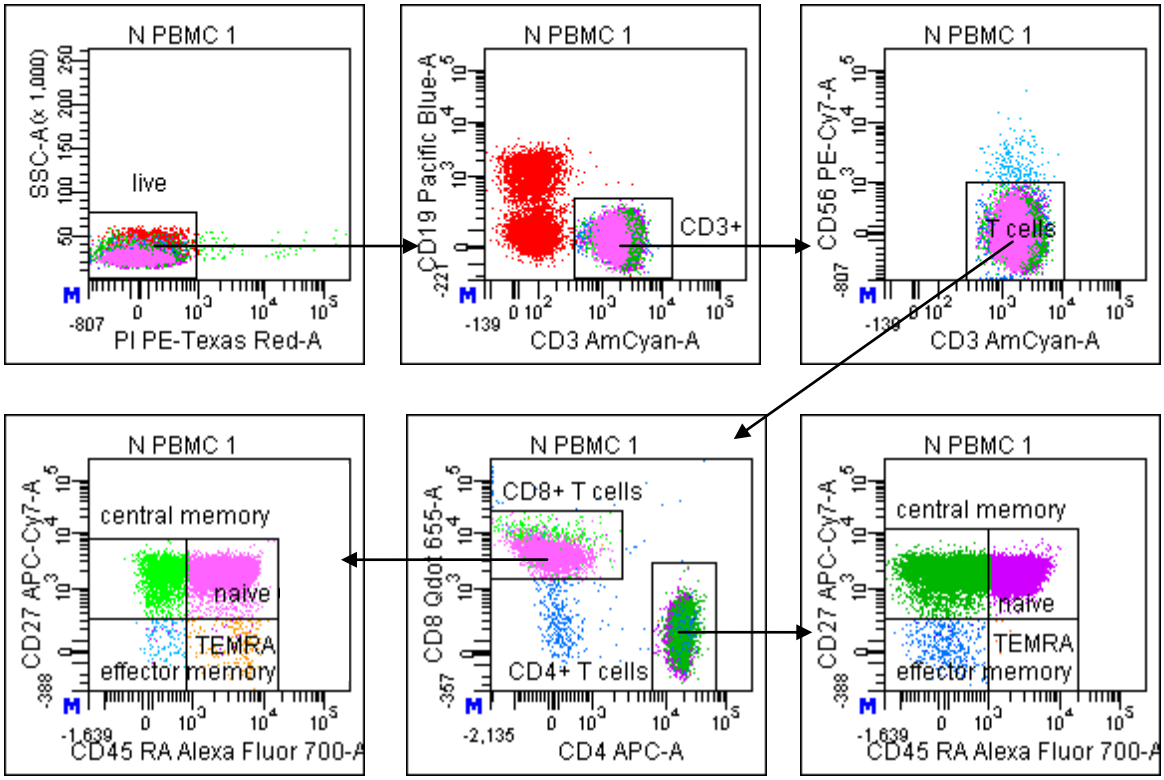
The panel consisted of 10 antibodies specific to the markers shown in the table and conjugated to the indicated fluorochromes as well as the dead cell exclusion dye propidium iodide (PI).

Fig 3:3:1: Gating to define lymphocyte subsets using the eleven colour antibody panel.

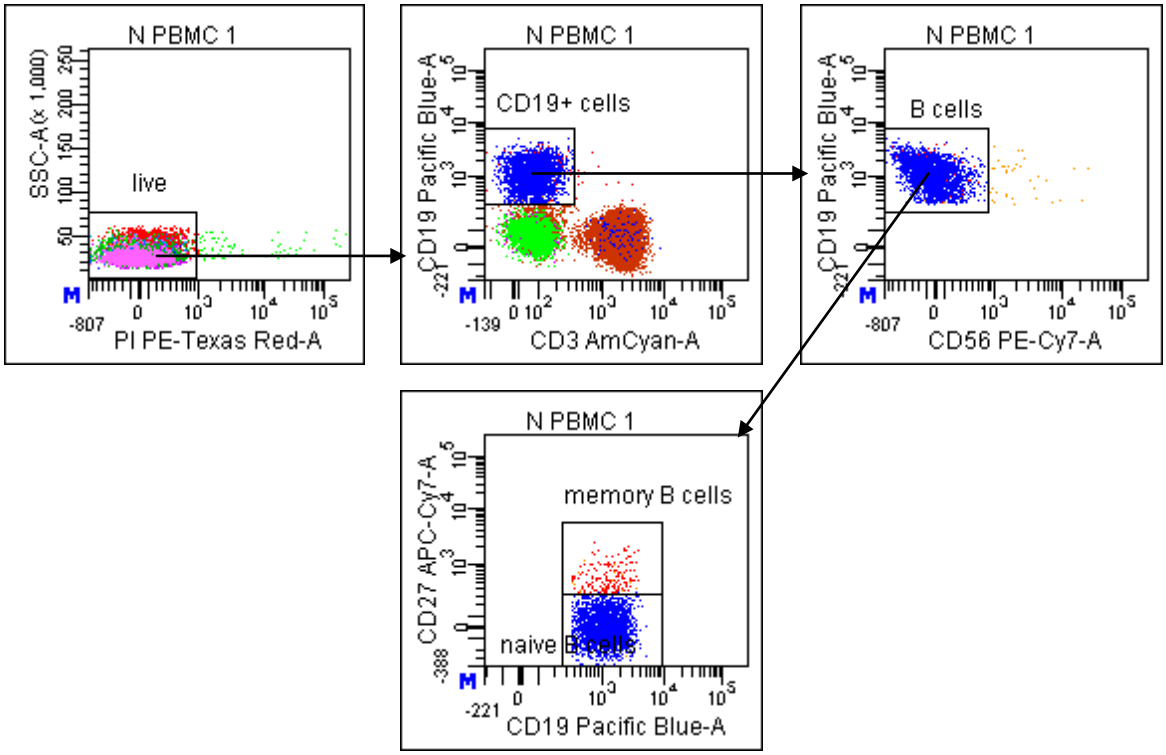
A. lymphocyte population.



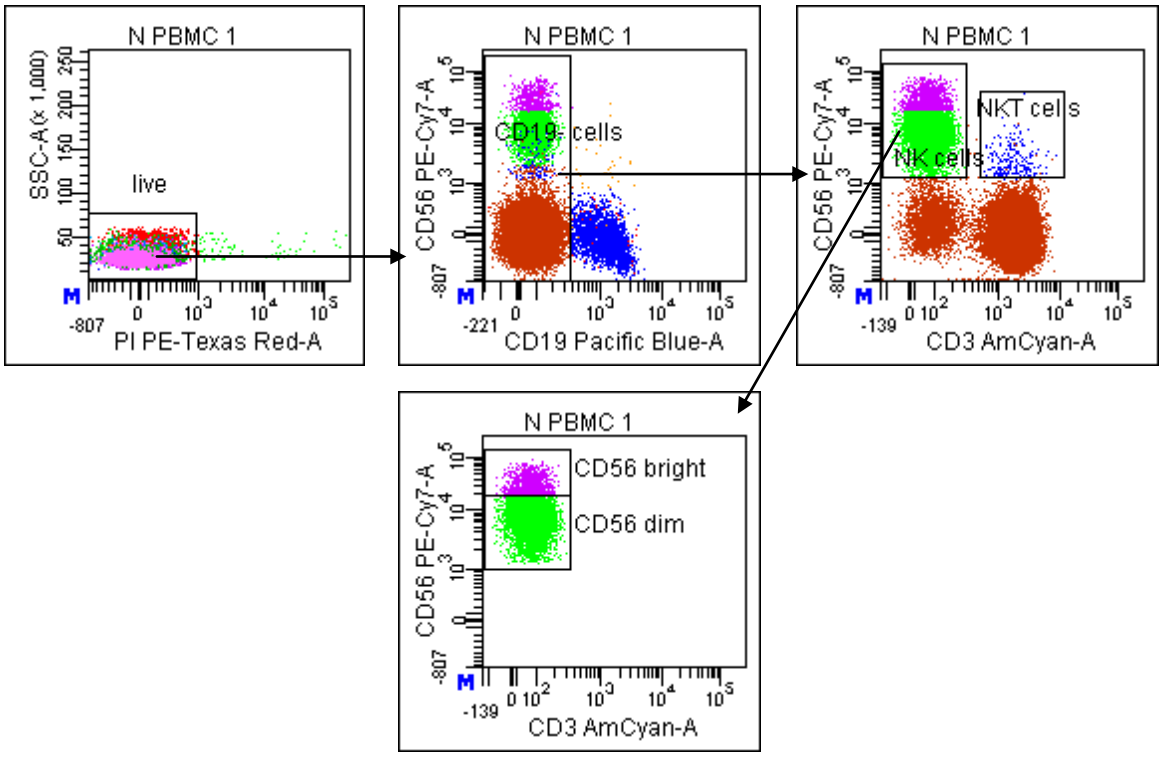
B. T cells and T cell subsets.



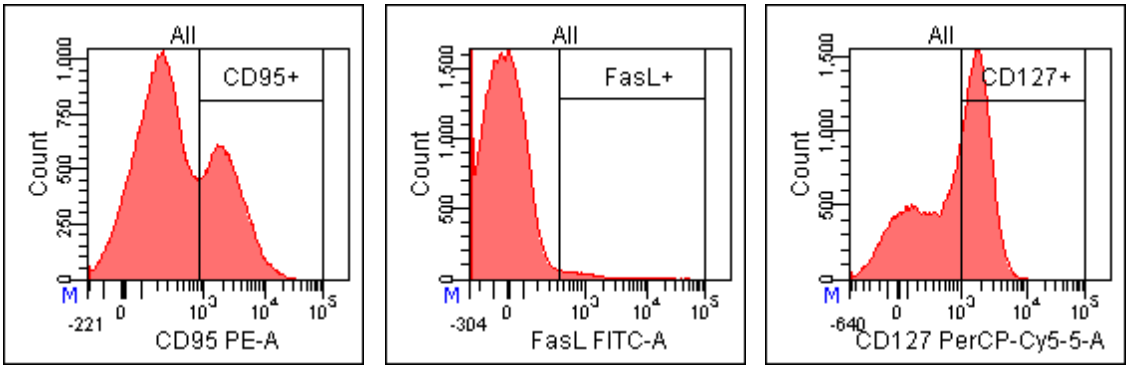
C. B cells and B cell subsets.



D. NK cells, NKT cells and NK cell subsets.



E. 11 colour panel: CD95, FasL and CD127 staining.



'all minus one plus isotype' and 'all minus one' controls

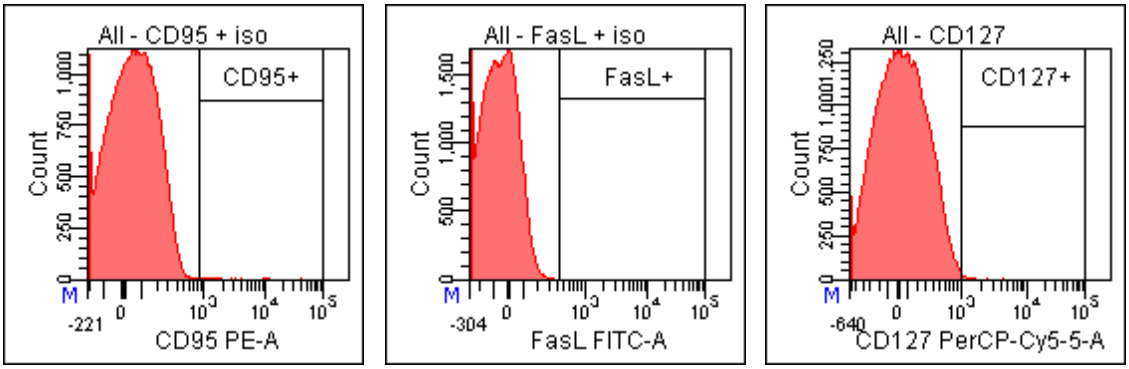


Fig 3:3:1: An 11 colour antibody panel was developed to analyse lymphocyte subsets and CD95, FasL and CD127 expression. Example plots from a normal control sample (N PBMC 1).

A. Gating strategy for definition of the lymphocyte population. Firstly exclusion of doublets was carried out by gating on a forward scatter height against forward scatter area plot. The lymphocyte population was then selected using a forward scatter against side scatter plot and any dead (PI+) cells excluded by gating on a side scatter against PI plot.

B. Selection of T cells and T cell subsets. Following selection of the live lymphocyte population a CD3 against CD19 plot was used for selection of CD3+ cells. Any NKT cells (CD56+) in the CD3+ gate were then excluded by gating on a CD3 against CD56 plot. The resulting pure T cell population was then further subdivided into CD4+ and CD8+ T cells by gating on a plot of CD4 against CD8. CD4+ and CD8+ T cells were divided into naive

(CD45RA+CD27+), central memory (CD45RA-CD27+), effector memory (CD45RA-CD27-) and TEMRA (CD45RA+CD27-) subsets by plotting CD45RA against CD27.

C. Selection of B cells and B cell subsets. CD19+ cells were selected from the live lymphocyte population by plotting CD19 against CD3. A plot of CD19 against CD56 was then used to further purify the B cell population. Finally a CD19 against CD27 plot was used to define naive (CD27-) and memory (CD27+) B cell subsets.

D. Selection of NKT cells, NK cells and subsets. CD19-ve cells were selected from the live lymphocyte population by plotting CD19 against CD56. NK (CD3-CD56+) and NKT cells (CD3+CD56-) were then defined using a CD3 against CD56 plot. Finally the NK cells were further subdivided into CD56bright and CD56dim populations using another CD3 against CD56 plot.

E. Example staining of CD95, FasL and CD127 on the live lymphocyte population. The top row of histograms show the results of staining with the complete eleven colour antibody panel and the bottom row shows the corresponding 'all minus one plus isotype' (CD95 and FasL) or 'all minus one' controls (CD127).

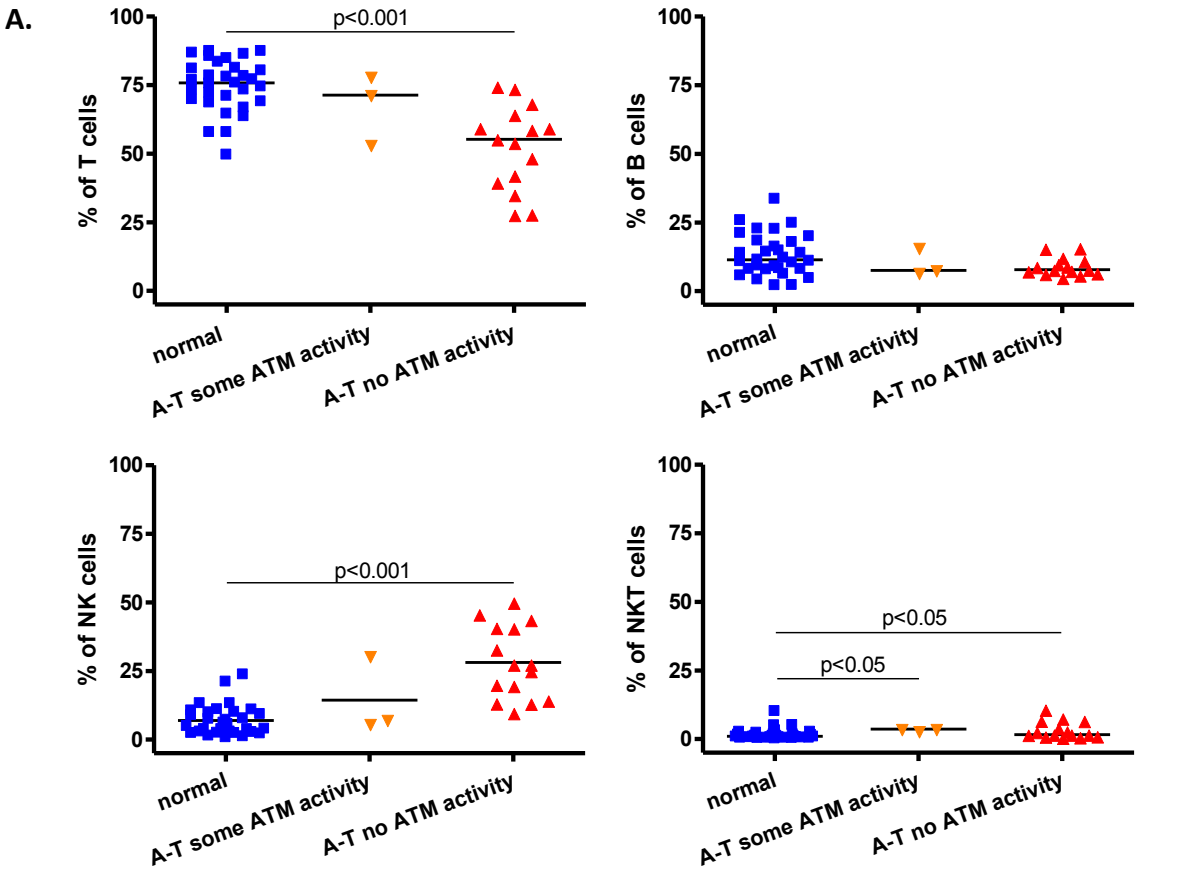
3:3:2: Lymphocyte subsets.

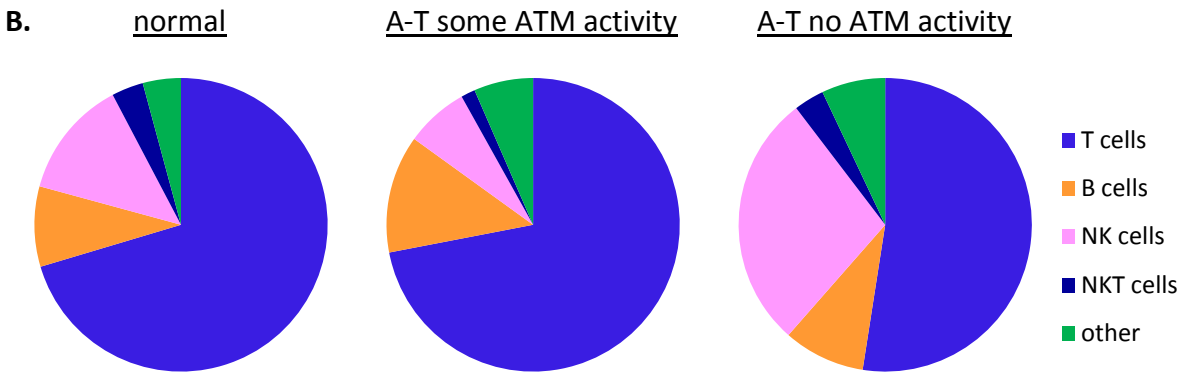
Analysis of lymphocyte subsets in A-T and normal control samples revealed significant differences between the proportions of different cell types. A-T patients with no ATM activity had a significantly lower percentage of T cells ($p<0.001$) and higher percentages of NK cells ($p<0.001$) and NKT cells ($P<0.05$) than normal controls (Fig 3:3:2A). In general A-T patients with some ATM activity were more similar to normal controls than A-T patients with no ATM activity in terms of their lymphocyte subsets, however they did have a significantly higher percentage of NKT cells than normal controls ($p<0.05$). The mean percentages of each cell type in the three groups are shown in Fig 3:3:2B.

As whole blood counts for each sample were not known it was not possible to determine absolute numbers of cells in each subset. Therefore it is unclear whether the increased proportions of NK and NKT cells in A-T patients is the result of an increase in production of these cells in order to compensate for T cell lymphopenia or a proportional increase due to the reduction in T cell number with no difference in NK cell number between A-T patients and normal controls.

The percentages of lymphocytes changed significantly with age in both normal controls and A-T patients. In the normal controls there were significant increases in the percentages of NK and NKT cells with age, whilst in the A-T patients with no ATM activity the percentage of T cells increased and NK cells decreased significantly with age (Fig 3:3:2C).

Fig 3:3:2: A-T patients with no ATM activity had a significantly lower percentage of T cells and higher percentage of NK cells than normal controls.





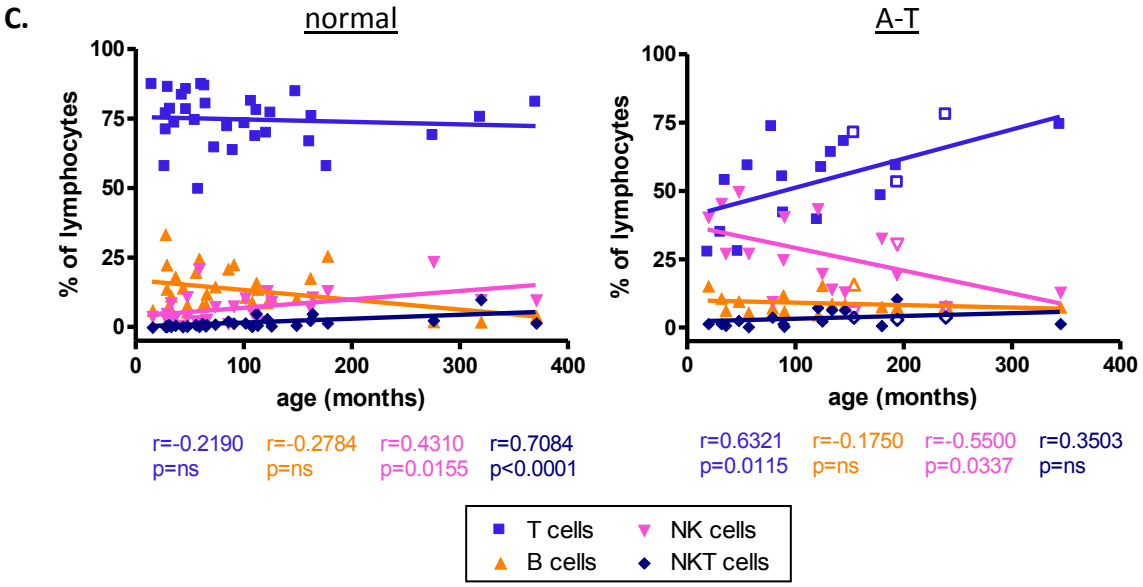


Fig 3:3:2: PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRII flow cytometer. Gating was carried out as described in Fig 3:3:1.

A. T cells, B cells, NK cells and NKT cells as a percentage of live lymphocytes in A-T patients and normal controls.

B. Mean percentages of different lymphocyte subsets in normal and A-T samples. (Mean of data shown in part A).

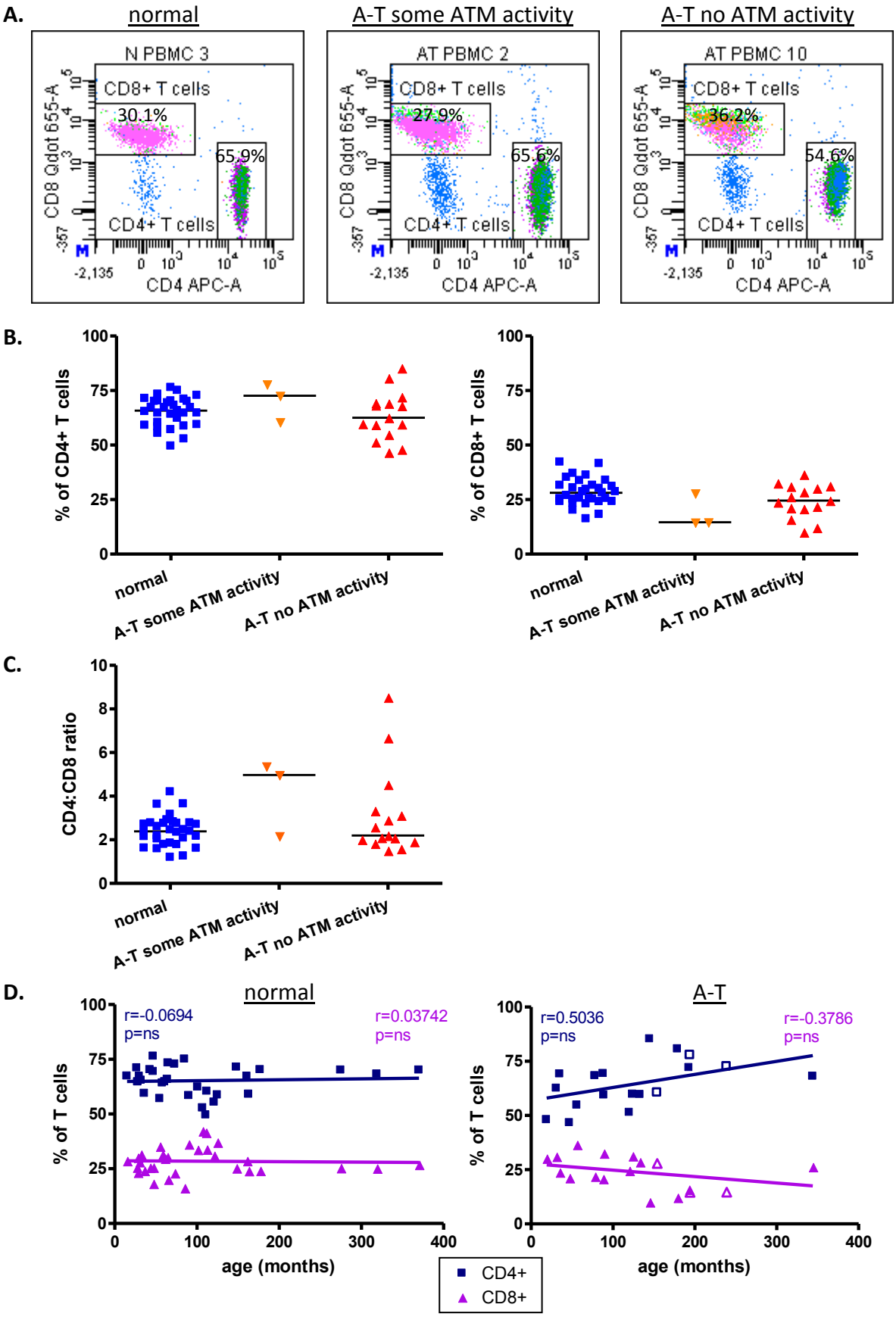
C. There were significant positive correlations between the percentages of NK cells and NKT cells and age in the normal controls. A-T patients with no ATM activity showed a significant positive correlation between age and percentage of T cells and a significant negative correlation between age and percentage of NK cells. A-T patients with no ATM activity and those with some ATM activity were analysed separately, however to improve clarity and as the sample size was very small linear regression lines and p and r values for the A-T patients with some ATM activity are not shown. In the A-T plot filled symbols = no ATM activity, open symbols = some ATM activity.

3:3:3: CD4+ and CD8+ T cells.

As the percentage of T cells in A-T patients with no ATM activity is clearly reduced compared to normal controls (Fig 3:3:2A) they were analysed further to determine if this reduction affected CD4+, CD8+ or both groups of T cells (Fig 3:3:3A). The analysis clearly showed that the proportional decrease in T cells affected both CD4+ and CD8+ cells as there was no significant difference between the percentage of T cells which expressed CD4 or CD8 in normal controls compared to A-T patients (Fig 3:3:3B) and no significant difference between the CD4:CD8 T cell ratio of A-T T cells compared to normal controls (Fig 3:3:3C).

There were no correlations between the percentages of CD4+ or CD8+ T cells and age (Fig 3:3:3D) or CD4:CD8 ratio and age (Fig 3:3:3E) in either A-T patients or normal controls.

Fig 3:3:3: There was no significant difference between the percentage of CD4+ or CD8+ T cells in A-T patients and normal controls.



E.

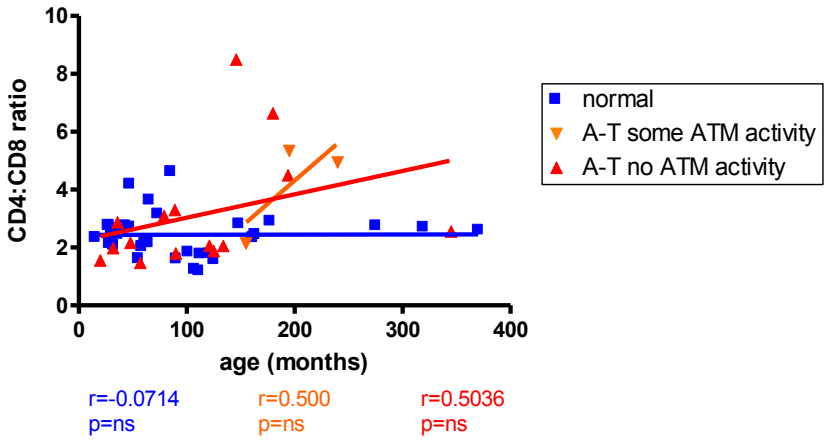


Fig 3:3:3: PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRII flow cytometer. Gating was carried out as described in Fig 3:3:1.

A. Representative plots of CD4+ and CD8+ T cells in the normal controls (N PBMC3 - 5 years 4 months), A-T patients with some ATM activity (AT PBMC2 - 12 years 10 months) and A-T patients with no ATM activity (AT PBMC10 - 4 years 8 months). Each plot represents 20,000 events. Gates are labelled with the percentage of total T cells expressing CD4 or CD8.

B. There was no significant difference between the percentages of total T cells that express either CD4 or CD8 in A-T patients and normal controls.

C. There was no significant difference between the CD4:CD8 ratios (% of CD4+ T cells/% of CD8+ T cells) of A-T patients and normal controls.

D. There was no correlation between the percentage of T cells which express either CD4 or CD8 and age in normal (left) or A-T samples (right). A-T patients with no ATM activity and those with some ATM activity were analysed separately, however to improve clarity and as the sample size was very small linear regression lines and p and r values for the A-T patients with some ATM activity are not shown. In the A-T plot filled symbols = no ATM activity, open symbols = some ATM activity.

E. There was no significant correlation between CD4:CD8 ratio (% of CD4+ T cells/% of CD8+ T cells) and age in A-T patients or normal controls.

3:3:4: Naive and memory T cells.

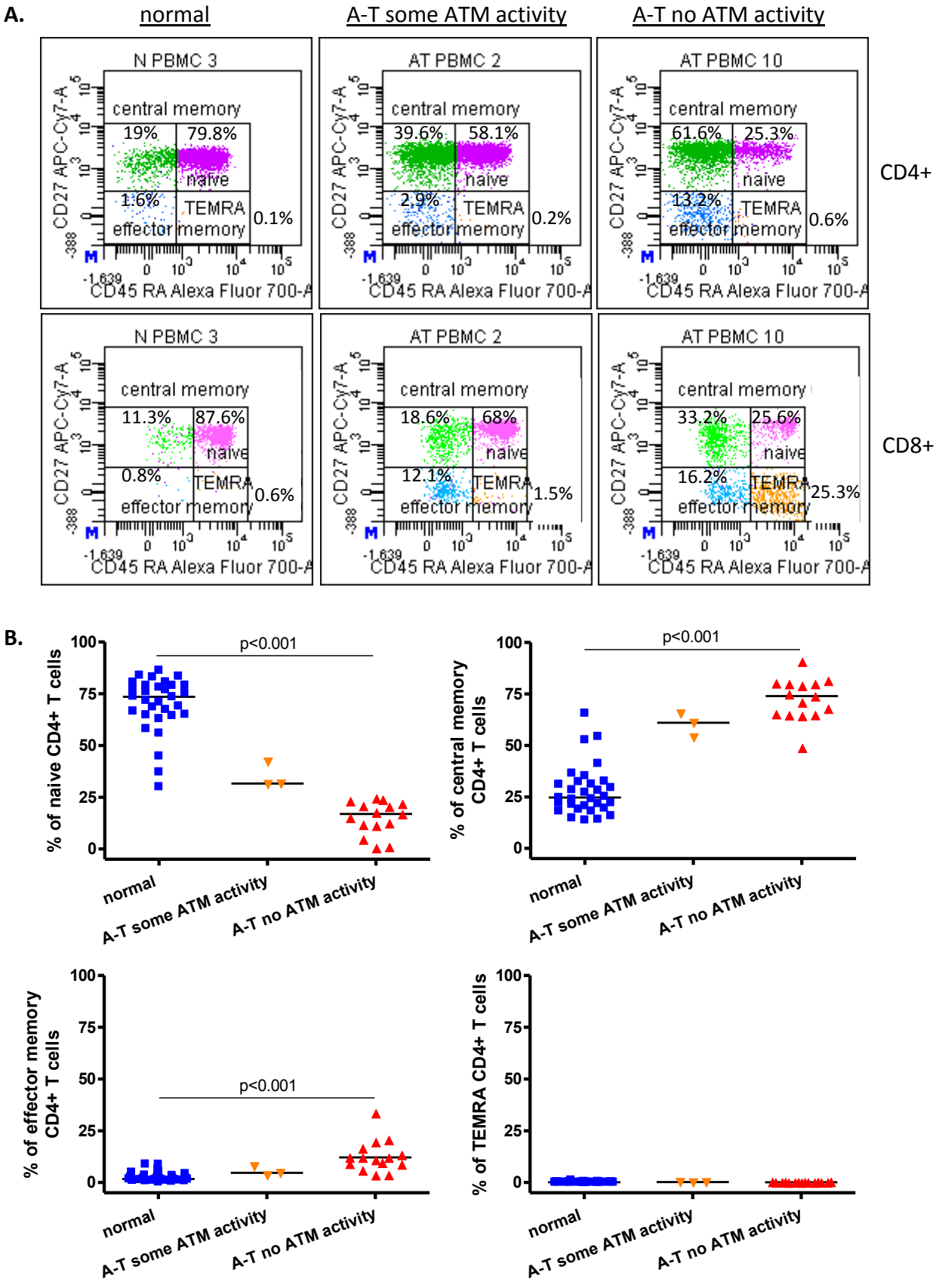
CD4+ve and CD8+ve T cells were further subdivided into naive, central memory, effector memory, and TEMRA subsets (Fig 3:3:4A). There were very obvious differences between the proportions of T cells in each subset in A-T patients and normal controls. A-T patients with no ATM activity had a significantly lower percentage of naive T cells (CD4+ - $p<0.001$, CD8+ - $p<0.001$) and significantly higher percentages of central memory (CD4+ - $p<0.001$, CD8+ - $p<0.001$) and effector memory (CD4+ - $p<0.001$, CD8+ - $p<0.001$) T cells than the normal controls. This effect was seen in both CD4+ (Fig 3:3:4A, B&D) and CD8+ (Fig 3:3:4A, C&D) T cells.

The immune system phenotype of the relatively young A-T patients with no ATM activity was similar to that of elderly individuals who also have a reduced proportion of naive T cells and increased memory T cells (Pawelec et al., 2010;Sansoni et al., 2008). Therefore the A-T patients could be described as immunologically aged. Interestingly the patients who had some ATM activity showed an intermediate phenotype between that of the normal controls and A-T patients with no ATM activity. This was especially apparent when the mean proportions of T cells in each subset were analysed (Fig 3:3:4D).

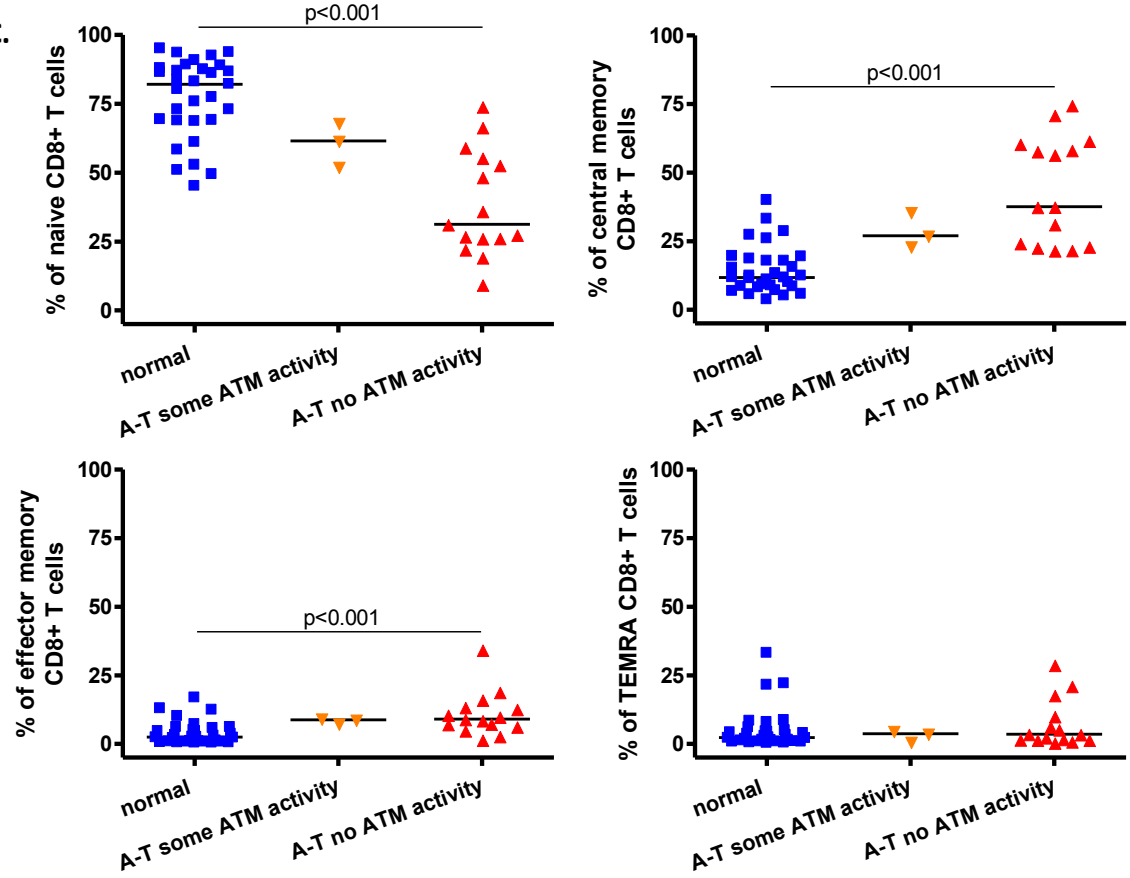
Further evidence for an 'immunologically aged' phenotype of A-T patients came from analysis of correlations between the percentage of T cells in naive and memory subsets and age of patients (Fig 3:3:4E). In the normal controls the percentages of CD4+ and CD8+ naive T cells decreased significantly with age whilst the percentages of central memory, effector memory and TEMRA cells significantly increased. This is unsurprising as thymic involution begins during early childhood (Steinmann, 1986) leading to a reduction in the production of naive T cells as an individual ages. However, the A-T patients showed no correlations between percentage of T cells in each subset and age. This is similar to an elderly group of

normal patients with a low stable output of naive cells from the thymus and a high proportion of memory cells resulting from oligoclonal expansions of T cells in response to previously encountered antigens (Akbar and Fletcher, 2005).

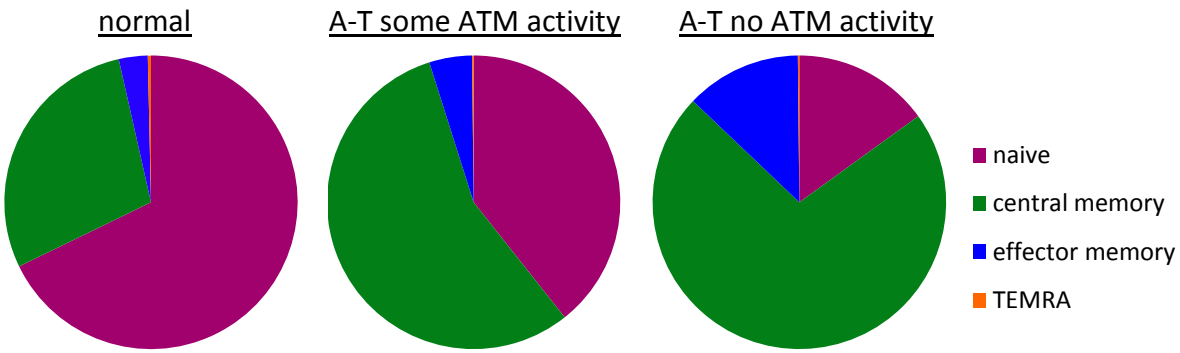
Fig 3:3:4: A-T patients with no ATM activity had a significantly smaller percentage of naive T cells and higher percentage of memory T cells than normal controls.



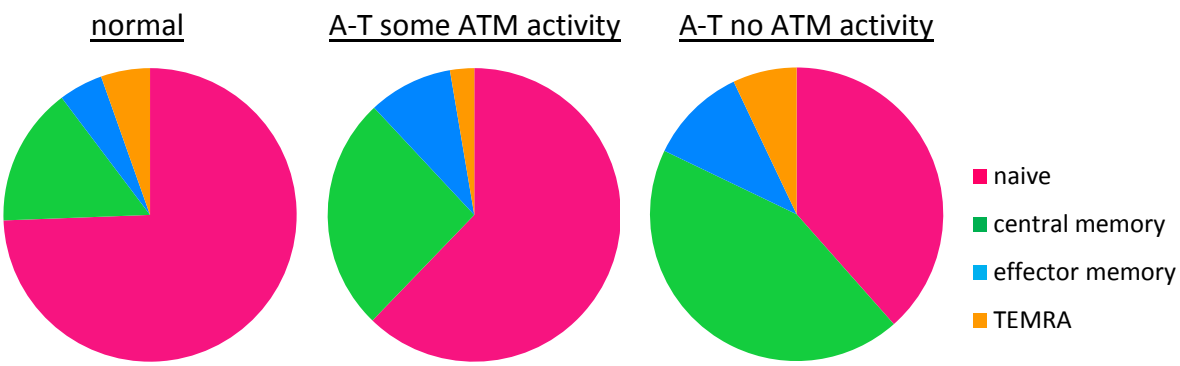
C.



D. CD4+ T cell subsets



CD8+ T cell subsets



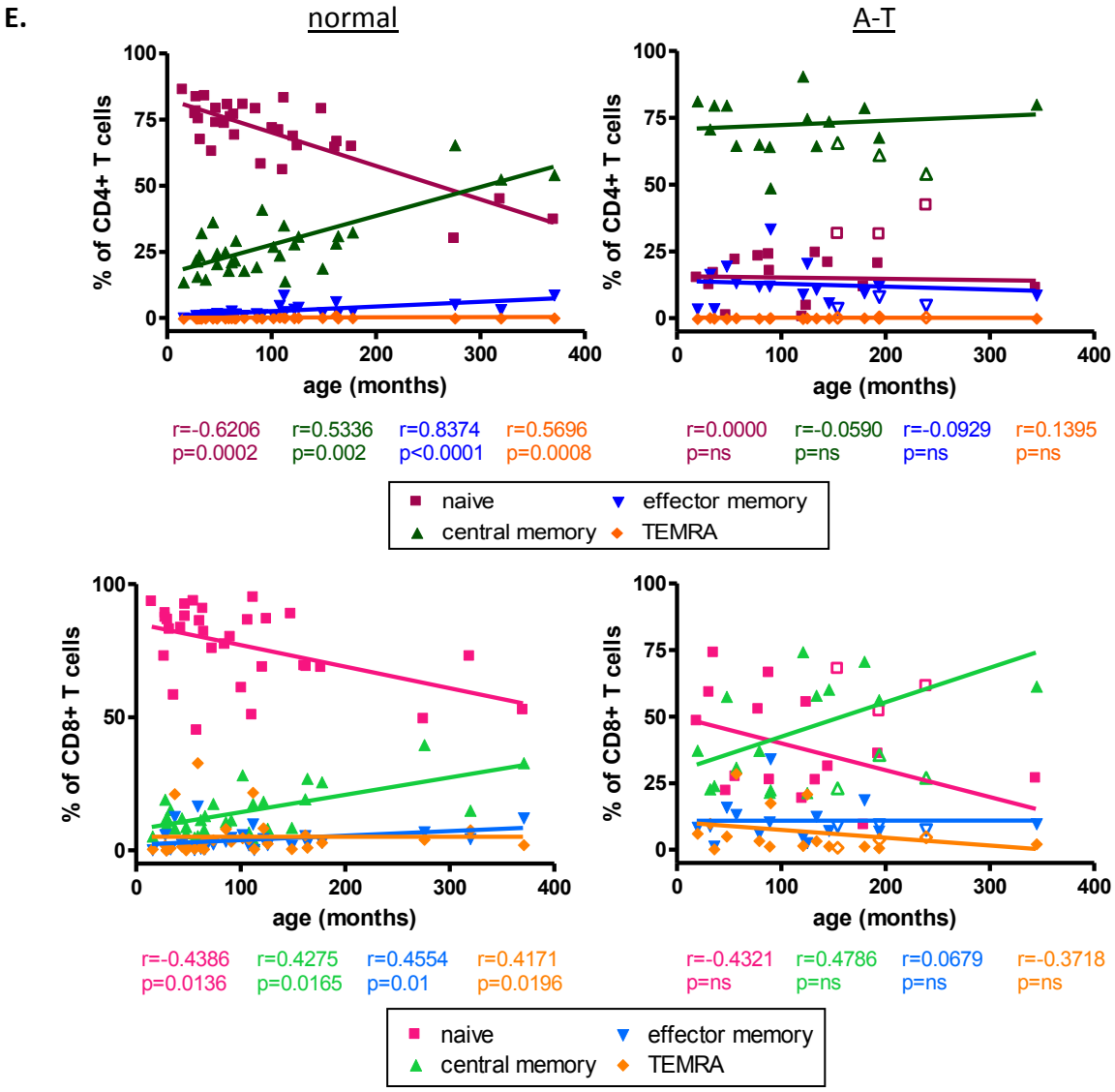


Fig 3:3:4: PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRII flow cytometer. Gating was carried out as described in Fig 3:3:1.

A. Representative plots of CD4+ve (top) and CD8+ve (bottom) T cell subsets in normal controls (N PBMC3 - 5 years 4 months), A-T patients with some ATM activity (AT PBMC2 - 12 years 10 months) and A-T patients with no ATM activity (AT PBMC10 - 4 years 8 months). Each plot represents 20,000 events, gates are labelled with the percentage of CD4+ve or CD8+ve T cells in each subset.

B. The percentage of CD4+ve T cells in each subset in normal controls and A-T samples.

C. The percentage of CD8+ve T cells in each subset in normal controls and A-T samples.

D. Summary of T cell subsets in normal controls and A-T samples (as percentage of either CD4+ve or CD8+ve T cells). Mean values of data shown in part B (CD4+) and C (CD8+).

E. There were significant correlations between increasing memory and decreasing naive T cells with age in the normal controls. A-T patients showed no change in the percentage of different T cell subsets with age. A-T patients with no ATM activity and those with some ATM activity were analysed separately, however to improve clarity and as the sample size was very small linear regression lines and p and r values for the A-T patients with some ATM activity are not shown. In the A-T plot filled symbols = no ATM activity, open symbols = some ATM activity.

3:3:5: Naive and memory B cells.

Although there was no significant difference in the percentage of total B cells between normal controls and A-T patients (Fig 3:3:2A) the percentages of naive (CD27-) and memory (CD27+) B cells in each group were analysed (Fig 3:3:5A) to determine whether the 'immunologically aged' phenotype of A-T patients applied to their B cells as well as to their T cells.

As the volume of haematopoietic tissue in the bone marrow decreases with age it is likely that B cell lymphopoesis also decreases (Ogawa et al., 2000). The B cell phenotype of A-T patients with no ATM activity was consistent with the hypothesis of immunological ageing as there was a significantly lower percentage of naive B cells ($p<0.001$) and a significantly higher percentage of memory B cells ($p<0.001$) than in the normal controls (Fig 3:3:5B). As was the case for T cells the A-T patients with some ATM activity showed an intermediate phenotype between that of the normal control and A-T patients with no ATM activity. Similarly to T cell subsets the percentage of naive B cells decreased and memory B cells increased significantly with age in the normal controls. There was no correlation between percentage of naive or memory B cells and age in the A-T patients (Fig 3:3:5C).

Fig 3:3:5: A-T patients with no ATM activity had a reduced percentage of naive B cells and increased memory B cells compared to normal controls.

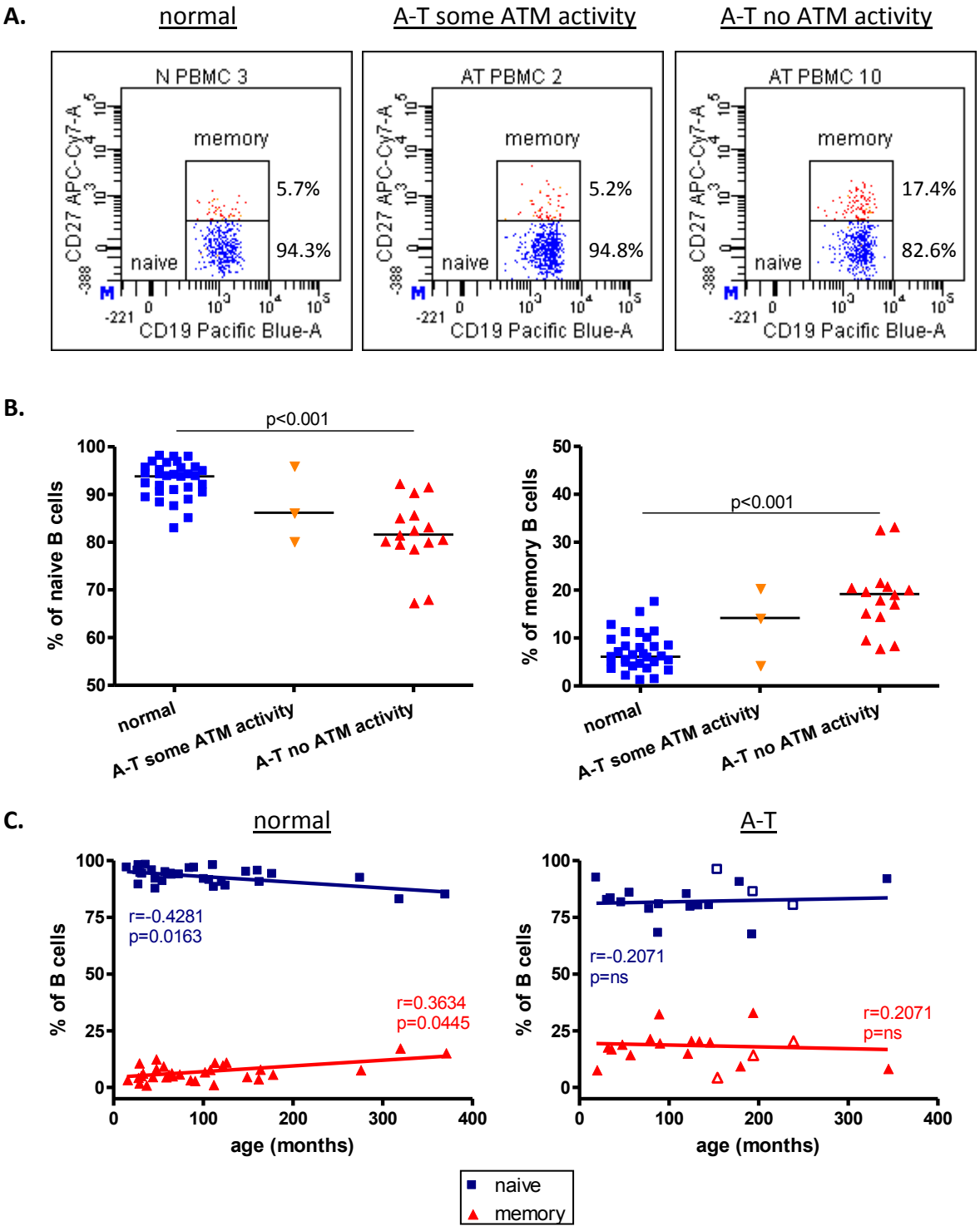


Fig 3:3:5: PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRII flow cytometer. Gating was carried out as described in Fig 3:3:1.

A. Representative plots of naive and memory B cell subsets in the normal controls (N PBMC3 - 5 years 4 months), A-T patients with some ATM activity (AT PBMC2 - 12 years 10 months) and A-T patients with no ATM activity (AT PBMC10 - 4 years 8 months). Each plot represents 20,000 events and gates are labelled with the percentage of total B cells in each subset.

B. Naive and memory B cells as a percentage of total B cells in normal control and A-T patient samples.

C. In the normal controls the percentage of naive B cells decreased and memory B cells increased significantly with age. There was no correlation between the percentage of memory or naive B cells and age in A-T patients. A-T patients with no ATM activity and A-T patients with some ATM activity were analysed separately, however to improve clarity and as the sample size was very small linear regression lines and p and r values for the A-T patients with some ATM activity are not shown. In the A-T plot filled symbols = no ATM activity, open symbols = some ATM activity.

3:3:6: CD56bright and CD56dim NK cells.

Finally NK cell subsets in normal controls and A-T patients were analysed (Fig 3:3:6A). Although A-T patients with no ATM activity had a significantly higher percentage of NK cells than normal controls (Fig 3:3:2A) there was no significant difference in the percentages of CD56bright and CD56dim NK cells (Fig 3:3:6B). There were also no correlations between the percentages of CD56bright or CD56dim NK cells and age in A-T patients or normal controls (Fig 3:3:6C).

Fig 3:3:6: There was no significant difference between the percentages of CD56dim or CD56bright NK cells in A-T patients and normal controls.

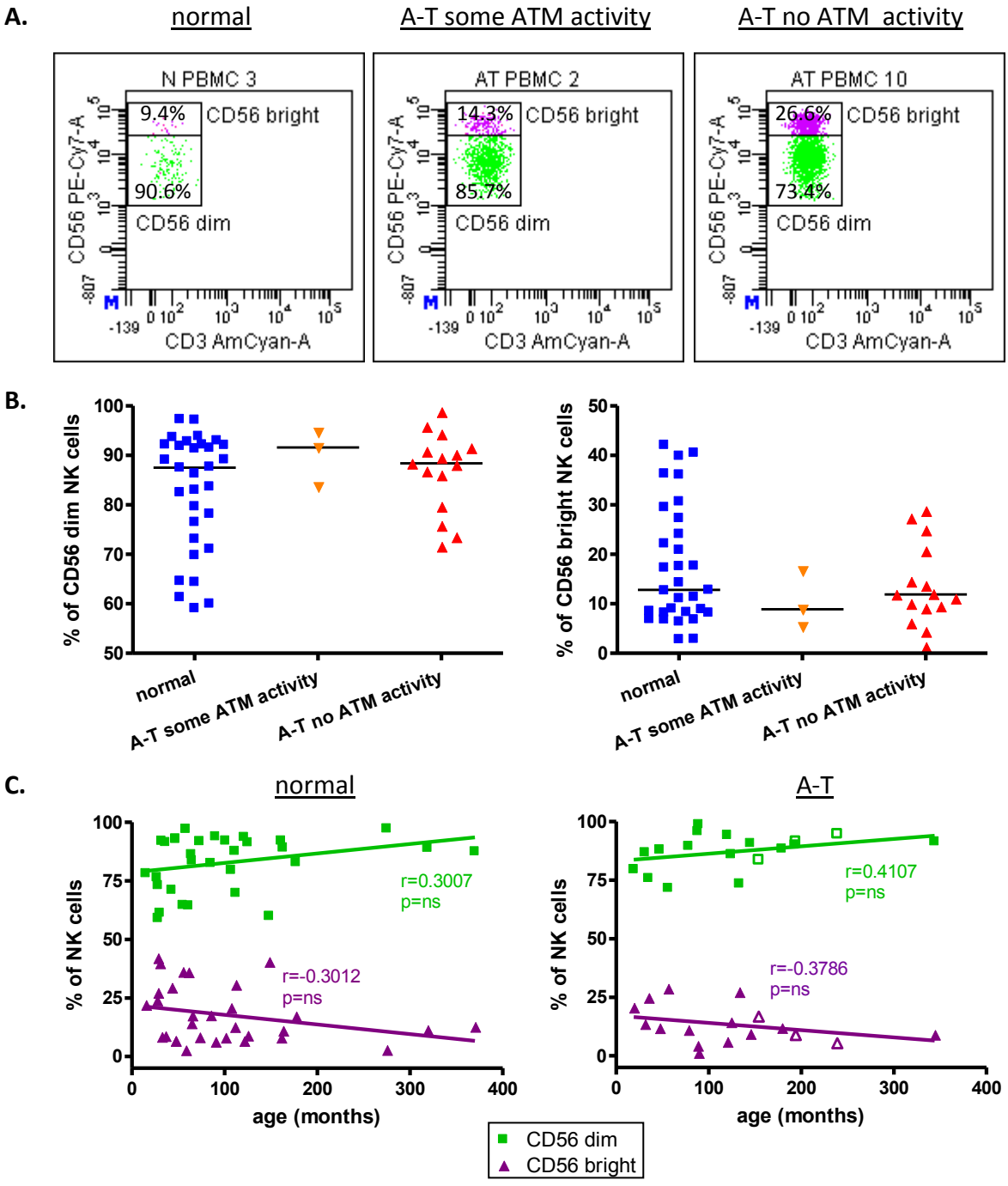


Fig 3:3:6: PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRII flow cytometer. Gating was carried out as described in Fig 3:3:1.

A. Representative plots of CD56bright and CD56dim NK cell subsets in the normal controls (N PBMC3 - 5 years 4 months), A-T patients with some ATM activity (AT PBMC2 - 12 years 10

months) and A-T patients with no ATM activity (AT PBMC10 - 4 years 8 months). Each plot represents 20,000 events. Gates are labelled with the percentage of total NK cells in each subset.

B. There was no significant difference between the percentage of total NK cells with CD56bright or CD56dim expression in normal controls and A-T patients.

C. There were no correlations between age and the percentage of CD56 dim or CD56 bright NK cells in normal controls or A-T patients. A-T patients with no ATM activity and A-T patients with some ATM activity were analysed separately, however to improve clarity and as the sample size was very small linear regression lines and p and r values for the A-T patients with some ATM activity are not shown. In the A-T plot filled symbols = no ATM activity, open symbols = some ATM activity.

3:3:7: A-T patients with no ATM activity had a significantly lower percentage of naive cells and higher percentage of memory cells than normal controls.

Significant differences between the percentages of lymphocyte subsets in normal controls and A-T patients as well as significant correlations between the percentages of lymphocyte subsets and age are summarised in Table 3:3:7.

A-T patients with no ATM activity had a significantly lower percentage of T cells than normal controls; however there was no significant difference between the percentages of CD4 and CD8 T cells in the two groups. There was also no significant difference in the percentage of B cells between A-T patients with no ATM activity and normal controls. The percentages of NK and NKT cells were significantly increased in the A-T patients with no ATM activity compared to normal controls.

Comparisons of the percentages of T and B cells belonging to naive and memory subsets in normal controls and A-T patients clearly showed that A-T patients with no ATM activity had significantly lower percentages of naive cells and significantly higher percentages of central and effector memory T cells and memory B cells than the normal controls. There was no significant difference in the percentage of CD56bright or CD56dim NK cells between A-T patients and normal controls.

With the exception of an increased percentage of NKT cells in A-T patients with some ATM activity compared to normal controls there were no significant differences in the percentage of lymphocyte subsets between A-T patients with some ATM activity and either A-T patients with no ATM activity or normal controls. However in general when there was a significant difference between normal controls and A-T patients with no ATM activity the median value of the A-T patients with some ATM activity tended to be between that of the other two

groups, suggesting an intermediate phenotype. The sample size of the A-T patients with some ATM activity was very small (3 patients) and it seems likely that if the sample had been larger these differences may have been significant.

In the normal controls the percentages of memory T and B cells correlated positively with age and the percentages of naive T and B cells correlated negatively with age. There were no correlations between age and the percentages of total T cells, CD4+ T cells, CD8+ T cells, B cells or CD56bright or CD56dim NK cells in the normal controls, however there were positive correlations between the percentages of NK and NKT cells and age. These changes are consistent with normal aging of the immune system.

Perhaps due to the consistently low thymic output of A-T patients, age did not have as much effect on the percentages of lymphocyte subsets in A-T patients as in the normal controls. There were no significant correlations between age and percentages of lymphocyte subsets in the A-T patients with some ATM activity. However in A-T patients with no ATM activity the percentage of T cells correlated positively and the percentage of NK cells correlated negatively with age.

These results showed an immune system phenotype of A-T patients which was consistent with the published literature (Nowak-Wegrzyn et al., 2004;Reichenbach et al., 2002) and similar to that of elderly individuals (Pawelec et al., 2010;Sansoni et al., 2008).

Table 3:3:7: Significant differences between the percentages of lymphocyte subsets and correlations with age in normal controls and A-T patients.

	significant differences in percentages of lymphocyte subsets			correlations between percentages of lymphocyte subsets and age		
	normal	A-T some ATM activity	A-T no ATM activity	normal	A-T some ATM activity	A-T no ATM activity
T cells	+	ns	-	ns	ns	+
all CD4+	ns	ns	ns	ns	ns	ns
CD4+ naive	+	ns	-	-	ns	ns
CD4+ central memory	-	ns	+	+	ns	ns
CD4+ effector memory	-	ns	+	+	ns	ns
CD4+ TEMRA	ns	ns	ns	+	ns	ns
all CD8+	ns	ns	ns	ns	ns	ns
CD8+ naive	+	ns	-	-	ns	ns
CD8+ central memory	-	ns	+	+	ns	ns
CD8+ effector memory	-	ns	+	+	ns	ns
CD8+ TEMRA	ns	ns	ns	+	ns	ns
B cells	ns	ns	ns	ns	ns	ns
naive	+	ns	-	-	ns	ns
memory	-	ns	+	+	ns	ns
NK cells	-	ns	+	+	ns	-
CD56dim	ns	ns	ns	ns	ns	ns
CD56bright	ns	ns	ns	ns	ns	ns
NKT cells	-	+	+	+	ns	ns

Table 3:3:7: Summary of significant differences between the percentages of lymphocyte subsets and correlations between percentages of lymphocyte subsets and age in A-T patients and normal controls.

In the first part of the table (significant differences in percentages of lymphocyte subsets) cells containing a positive symbol (+) and highlighted in yellow indicate a significantly higher percentage of the lymphocyte subset in the corresponding group compared to the group with a cell containing a negative symbol (-) and highlighted in blue, 'ns' refers to no significant difference compared to any group. The second part of the table (correlations between percentages of lymphocyte subsets and age) summarises significant correlations between the percentage of each lymphocyte subset and age. The yellow positive (+) cells indicate a positive correlation and blue negative (-) cells indicate a negative correlation, 'ns' indicates no significant correlation.

3:4: CD95 expression.

3:4:1: CD95 expression on lymphocyte subsets.

In normal individuals both CD95 expression on T cells and their sensitivity to CD95-mediated apoptosis increases with age, this may contribute to the age associated failing of adaptive immunity known as immune senescence (Aggarwal & Gupta, 1998;Gupta & Gollapudi, 2008;Potestio et al., 1999). The immune system phenotypes of A-T patients and the elderly are very similar and it is possible that increased CD95 expression and consequent sensitivity to CD95-mediated apoptosis may also be involved in shaping the immune system phenotype of A-T patients.

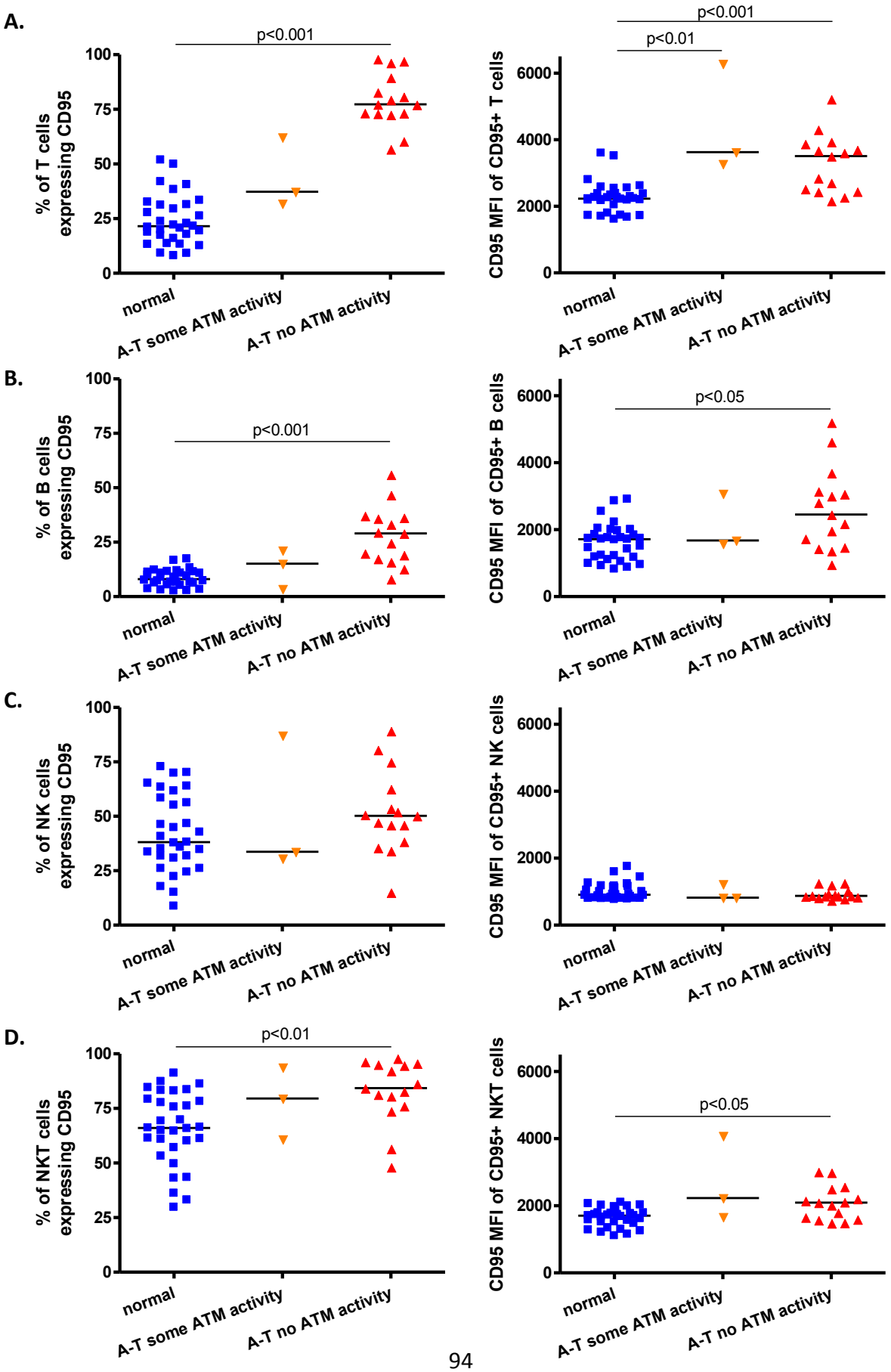
Expression of CD95 was compared on lymphocyte subsets from A-T patients and normal controls. CD95 expression measured both as the percentage and the CD95 MFI of CD95+ve cells was significantly higher on T cells (% - $p<0.001$, MFI - $p<0.001$), B cells (% - $p<0.001$, MFI - $p<0.05$) and NKT cells (% - $p<0.01$, MFI - $p<0.05$) in A-T patients with no ATM activity than normal controls (Fig 3:4:1A, B&D).

In general there were no significant differences between CD95 expression on lymphocytes from A-T patients with some ATM activity compared to normal controls or A-T patients with no ATM activity. The exception to this was the CD95 MFI of CD95+ T cells which was significantly higher in A-T patients with some ATM activity than in normal controls ($p<0.01$) (Fig 3:4:1A).

Perhaps because CD95 expression was already high there was no increase in expression with age in the A-T patients. However, the normal controls showed significant positive correlations between age and the percentages of CD95+ T cells, B cells and NKT cells and the CD95 MFI of CD95+ T cells and NKT cells (Fig 3:4:1E). This increase in CD95 expression on

lymphocytes with age over the 1 to 30 year age range was consistent with the published literature (Potestio et al., 1999).

Fig 3:4:1: CD95 expression was significantly increased on lymphocytes from A-T patients with no ATM activity compared to normal controls.



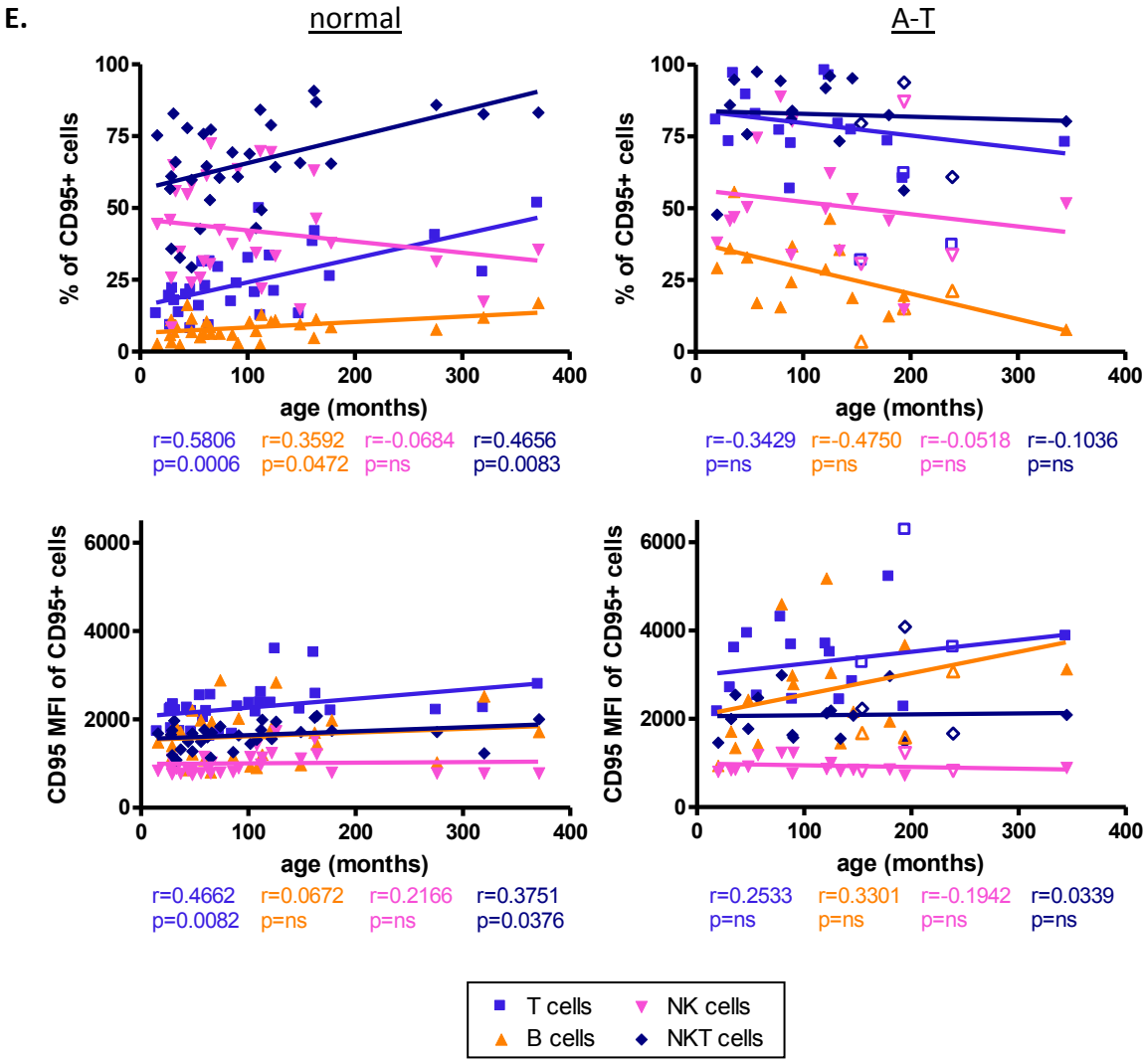


Fig 3:4:1: PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRII flow cytometer. Gating was carried out as described in Fig 3:3:1.

A. CD95 expression on T cells as the percentage of total T cells which express CD95 and the CD95 MFI of CD95+ve T cells.

B. CD95 expression on B cells as the percentage of total B cells which express CD95 and the CD95 MFI of CD95+ve B cells.

C. CD95 expression on NK cells as the percentage of total NK cells which express CD95 and the CD95 MFI of CD95+ve NK cells.

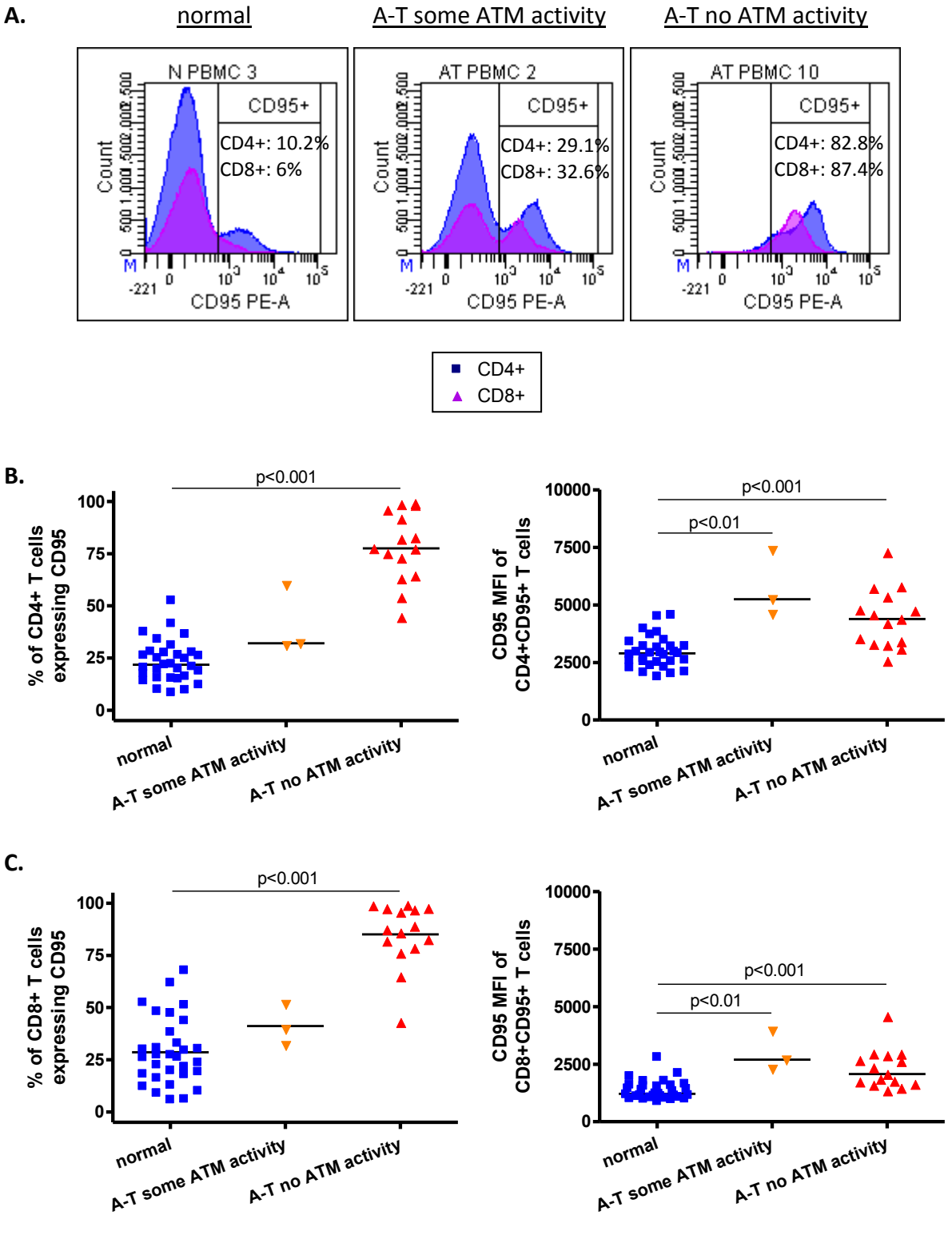
D. CD95 expression on NKT cells as the percentage of total NKT cells which express CD95 and the CD95 MFI of CD95+ve NKT cells.

E. There were no significant correlations between the percentage or CD95 MFI of CD95+ve lymphocytes of different types and age in A-T patients (top and bottom right). However in normal controls there were significant positive correlations between the percentages of CD95+ve T, B and NKT cells and age (top left). There were also positive correlations between age and the CD95 MFI of CD95+ve T and NKT cells. To improve clarity and as there were no significant correlations linear regression trend lines and r and p values for the A-T patients with some ATM activity are not shown, however results of the correlation analysis for this group are summarised in Table 3:4:7. (A-T plots: open symbols = some ATM activity, closed symbols = no ATM activity).

3:4:2: CD95 expression on CD4+ and CD8+ T cells.

Analysis of CD95 expression on CD4+ and CD8+ T cells gave similar results to analysis of expression on total T cells. CD95 expression measured as both the percentage and CD95 MFI of CD95+ve T cells was significantly higher on CD4+ve (% $p<0.001$, MFI $p<0.001$) (Fig 3:4:2A&B) and CD8+ve T cells (% $p<0.001$, MFI $p<0.001$) (Fig 3:4:2A&C) in A-T patients with no ATM activity than the normal controls. There was no significant difference between the percentages of CD4+CD95+ or CD8+CD95+ T cells from A-T patients with some ATM activity compared to either normal controls or A-T patients with no ATM activity. However the CD95 MFI of CD4+CD95+ and CD8+CD95+ T cells was significantly higher in the A-T patients with some ATM activity than the normal controls (CD4+ $p<0.01$, CD8+ $p<0.01$) (Fig 3:4:2B&C).

Fig 3:4:2: CD95 expression was significantly increased on CD4+ and CD8+ T cells from A-T patients with no ATM activity compared to normal controls.



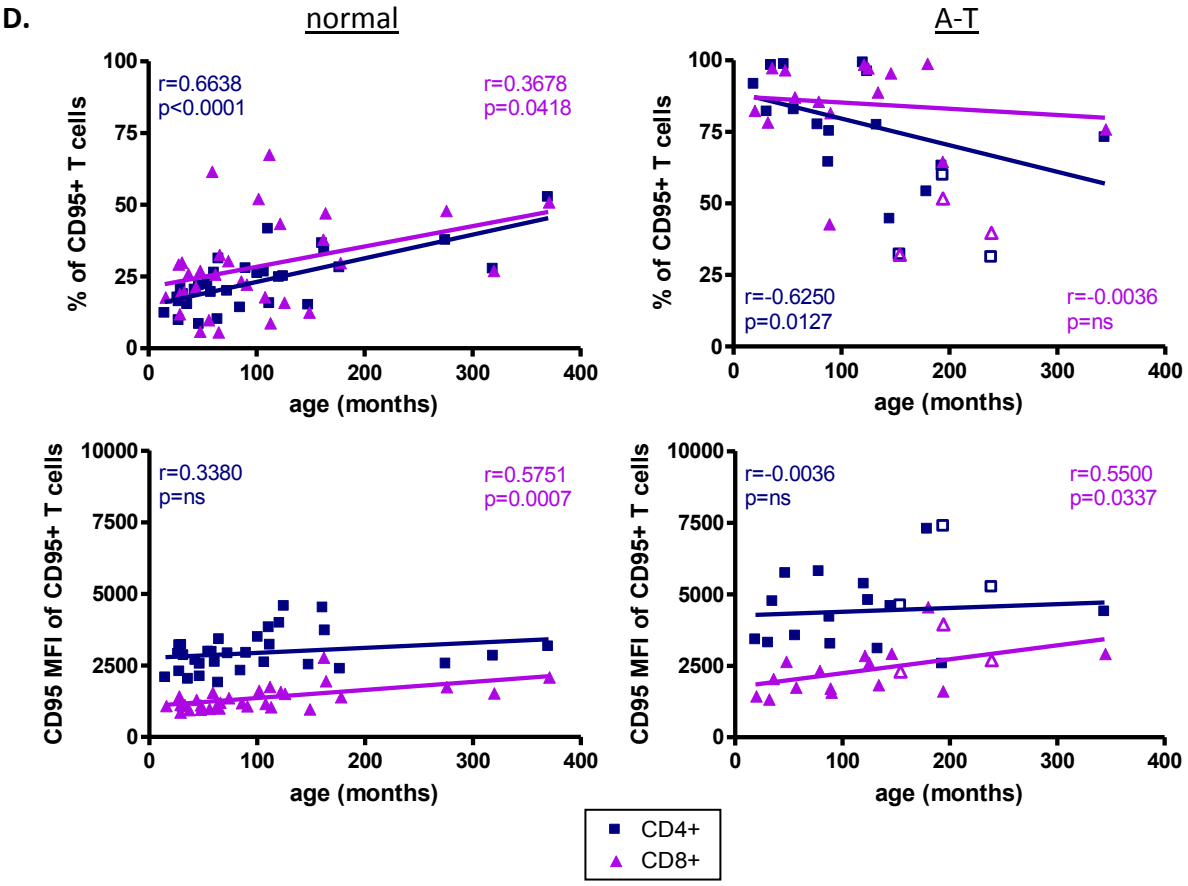


Fig 3:4:2: PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRll flow cytometer. Gating was carried out as described in Fig 3:3:1.

A. Representative histograms showing CD95 expression on CD4+ and CD8+ T cells in the normal controls (N PBMC3 - 5 years 4 months), A-T patients with some ATM activity (AT PBMC2 - 12 years 10 months) and A-T patients with no ATM activity (AT PBMC10 - 4 years 8 months). Percentages of CD4+CD95+ and CD8+CD95+ T cells are shown. Plots represent an equal number of events.

B. CD95 expression on CD4+ve T cells as the percentage of CD4+ve T cells that express CD95 and the CD95 MFI of CD4+CD95+ T cells.

C. CD95 expression on CD8+ve T cells as the percentage of CD8+ve T cells that express CD95 and the CD95 MFI of CD8+CD95+ T cells.

D. Normal CD4+ve and CD8+ve T cells showed a significant increase in the percentage of CD95+ve cells with age (top left). In A-T patients with no ATM activity there was a significant

negative correlation between the percentage of CD4+CD95+ T cells and age (top right). However in both normal controls and A-T patients with no ATM activity there were significant positive correlations between the CD95 MFI of CD8+CD95+ T cells and age (bottom left and right). To improve clarity and as there were no significant correlations linear regression trend lines and r and p values for the A-T patients with some ATM activity are not shown, however results of the correlation analysis for this group are summarised in Table 3:4:6. (A-T plots: open symbols = some ATM activity, closed symbols = no ATM activity).

3:4:3: CD95 expression on naive and memory T cells.

CD95 is expressed on memory T cells, however naive T cells do not upregulate the receptor until they are activated (Klas et al., 1993). Therefore CD95 expression on T cell subsets was analysed to investigate whether the high CD95 expression on T cells from A-T patients with no ATM activity compared to normal controls was solely due to the reduced proportion of naive T cells.

The normal controls had very low CD95 expression on naive CD4+ve and CD8+ve T cells and high expression on CD4+ and CD8+ memory T cell subsets as expected (Fig 3:4:3A). However the median percentages of naive CD4+CD95 and CD8+CD95+ T cells in the A-T patients with no ATM activity were very high (CD4+CD95+ - 35.9%, CD8+CD95+ - 74.6%) compared to the normal controls (CD4+CD95+ - 3.9%, CD8+CD95+ - 10.8%) and A-T patients with some ATM activity (CD4+CD95+ - 2.5%, CD8+CD95+ - 7.4%) (Fig 3:4:3B). There were significant differences in the percentages of naive CD95+ve T cells between the A-T patients with no ATM activity and normal controls (CD4+ - $p<0.001$, CD8+ - $p<0.001$) and the two groups of A-T patients (CD4+ - $p<0.05$, CD8+ - $p<0.001$).

Although there was no significant difference in the CD95 MFI of CD4+CD95+ naive T cells, the CD95 MFI of CD8+CD95 naive T cells was significantly higher in A-T patients with no ATM activity than normal controls ($p<0.001$). The CD95MFI of both CD4+CD95+ and CD8+CD95+ naive T cells were increased in A-T patients with some ATM activity compared to normal controls (CD4+CD95+ - $p<0.01$, CD8+CD95+ - $p<0.05$) (Fig 3:4:3B).

CD95 expression on memory T cells was also increased in A-T patients. The percentages of both CD95+CD4+ and CD95+CD8+ central memory, effector memory and TEMRA T cells were significantly higher in A-T patients with no ATM activity than normal controls (central

memory: CD4+ - p<0.01, CD8+ - p<0.001, effector memory: CD4+ - p<0.01, CD8+ - p<0.05, TEMRA: CD4+ - p<0.01, CD8+ - p<0.01). Similarly the CD95 MFI of CD8+CD95+ memory T cells was increased in A-T patients with no ATM activity compared to normal controls (central memory - p<0.001, effector memory - p<0.05, TEMRA - p<0.01) but this was not the case for all CD4+ memory subsets. The CD95 MFI of CD4+CD95+ central memory T cells was significantly higher in A-T patients with no ATM activity than normal controls (P<0.001) but there was no significant difference in CD95 MFI of either effector memory or TEMRA T cells between the two groups (Fig 3:4:3C-E).

Although the sample size was small there were also some significant differences in CD95 expression on memory T cells between A-T patients with some ATM activity and A-T patients with no ATM activity or normal controls; the percentage of CD4+CD95+ central memory cells was low in A-T patients with some ATM activity compared to A-T patients with no ATM activity (p<0.05). The CD95 MFI of all subsets of memory CD4+CD95+ T cells and CD8+CD95+ T cells were significantly higher in A-T patients with some ATM activity than normal controls (central memory: CD4 - p<0.01, CD8 – p<0.01, effector memory: CD4 - p<0.01, CD8 – p<0.05 TEMRA: CD4 - p<0.01, CD8+ - p<0.01) but there were no significant differences in CD95 MFI of CD4+CD95+ or CD8+CD95+ memory T cells between A-T patients with some ATM activity and A-T patients with no ATM activity (Fig 3:4:3C-E).

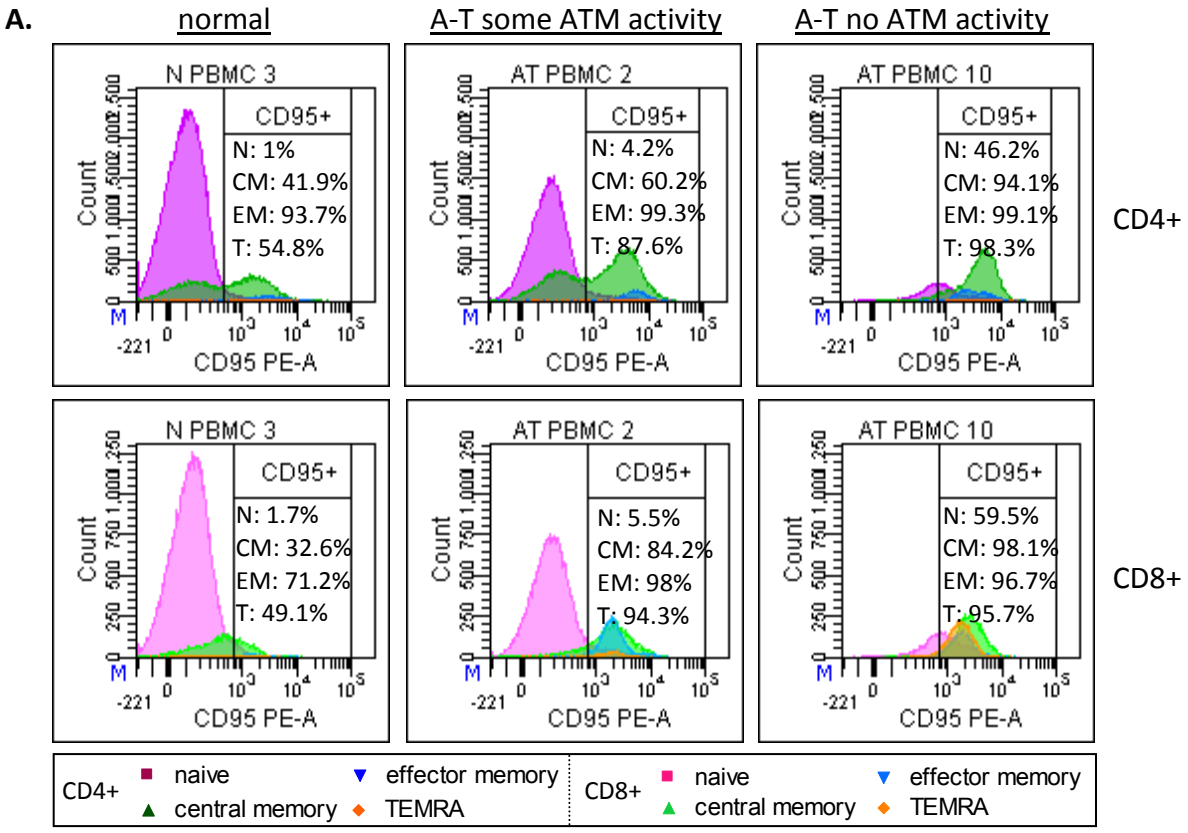
As CD95 expression was significantly increased on both naive and memory T cell subsets in A-T patients compared to normal controls the overall increased CD95 expression on T cells from A-T patients cannot be explained purely as a consequence of their naive T cell deficiency.

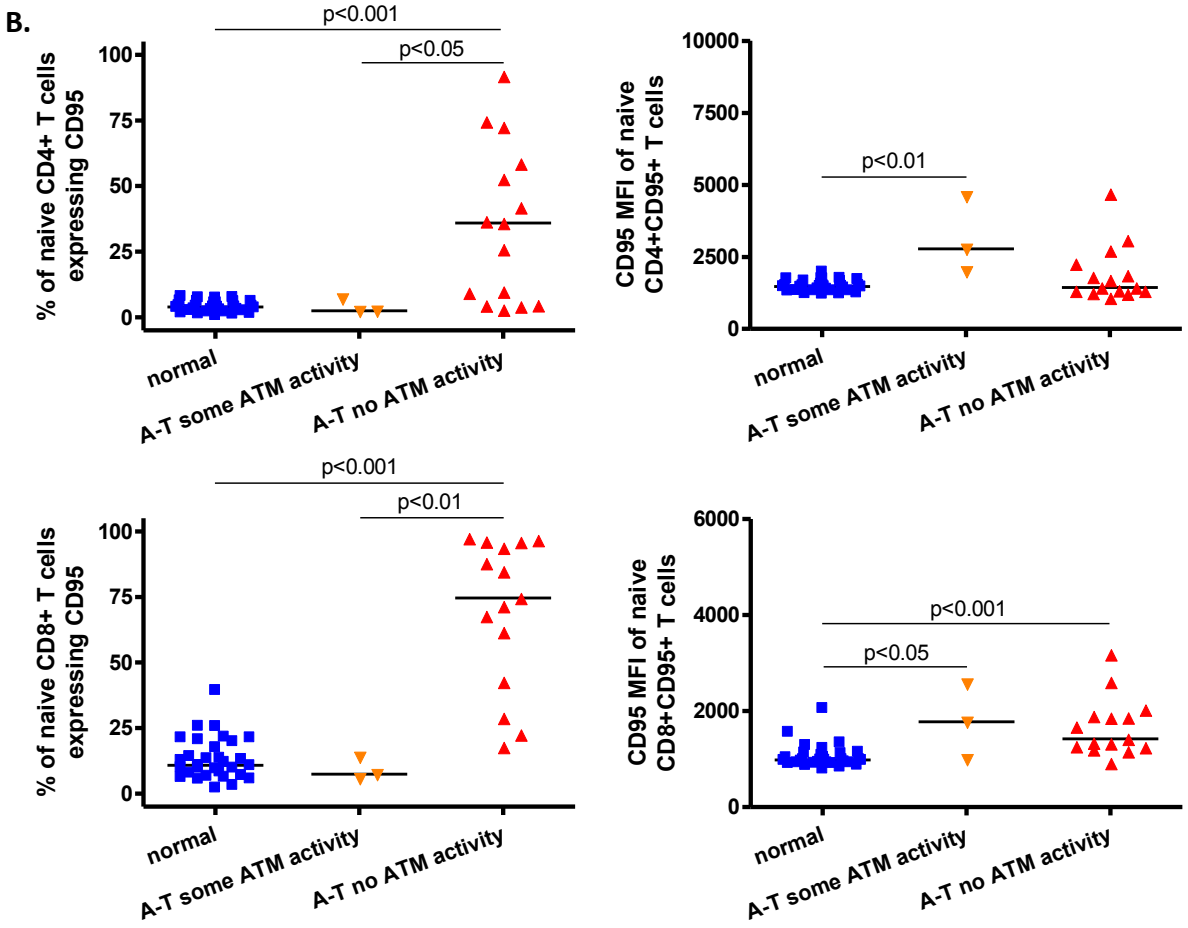
Finally correlations between age and CD95 expression on naive and memory T cell subsets were analysed to determine if the increase in CD95 expression on CD4+ and CD8+ T cells

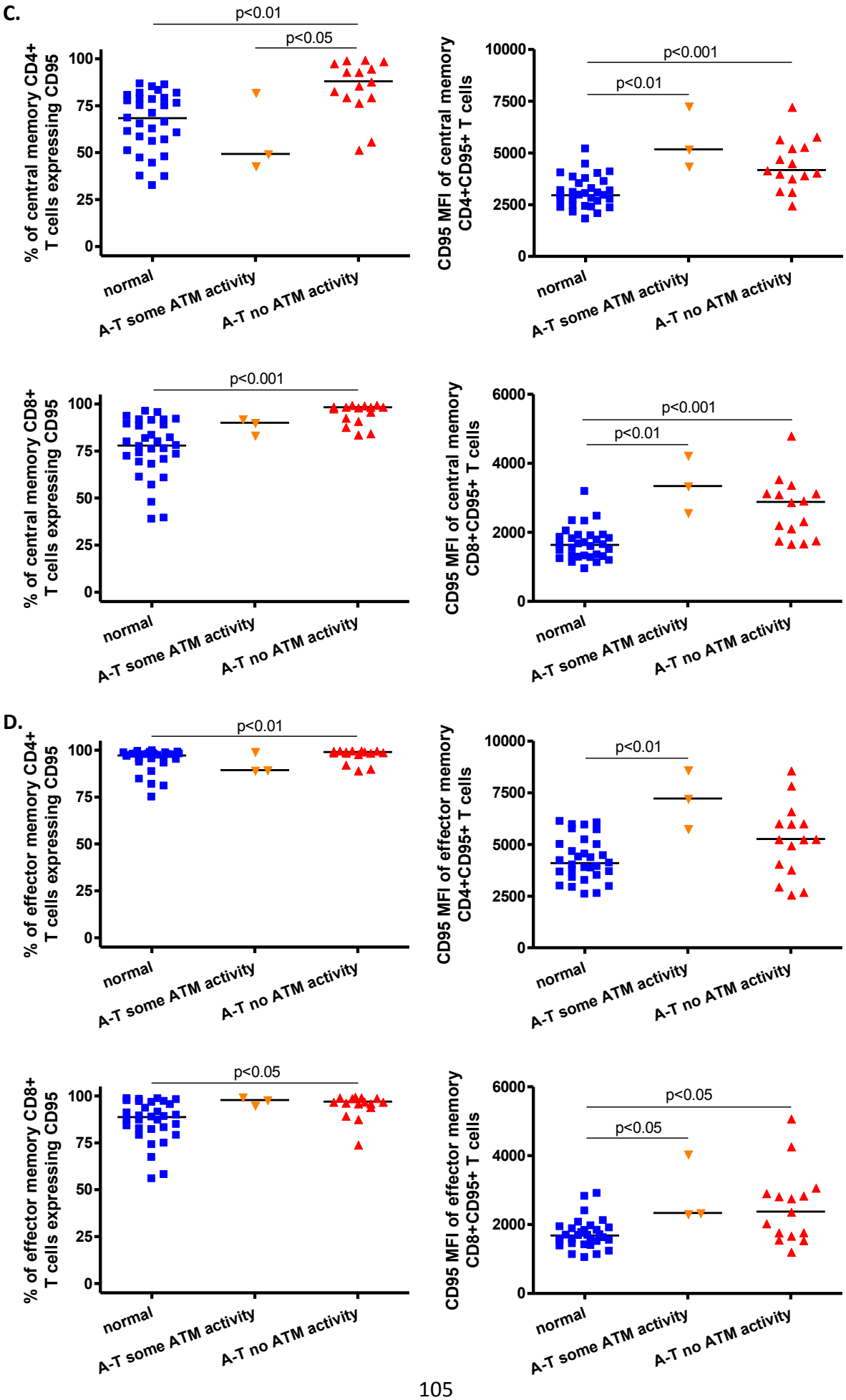
with age in the normal controls and decrease in CD95 expression on CD4+ T cells with age in A-T patients with no ATM activity (Fig 3:4:2C) were due to changes in expression on both naive and memory T cell subsets or on individual T cell subsets. In the normal controls the increase in the percentage of CD4+CD95+ T cells with age was the result of an increase in the percentage of TEMRA cells which express CD95, no other subset showed a positive correlation between percentage of CD4+CD95+ cells and age. Consistent with the lack of correlation on total CD4+ T cells there were no correlations between age and CD95 MFI of CD4+CD95+ T cells of any subset (Fig 3:4:3F). The significant positive correlations between age and both percentage and CD95 MFI of CD8+CD95+ T cells in the normal controls were due to significant increases in the percentages of both CD8+CD95+ effector memory and TEMRA T cells and the CD95 MFI of CD8+CD95+ T cells of all subsets (Fig 3:4:3G).

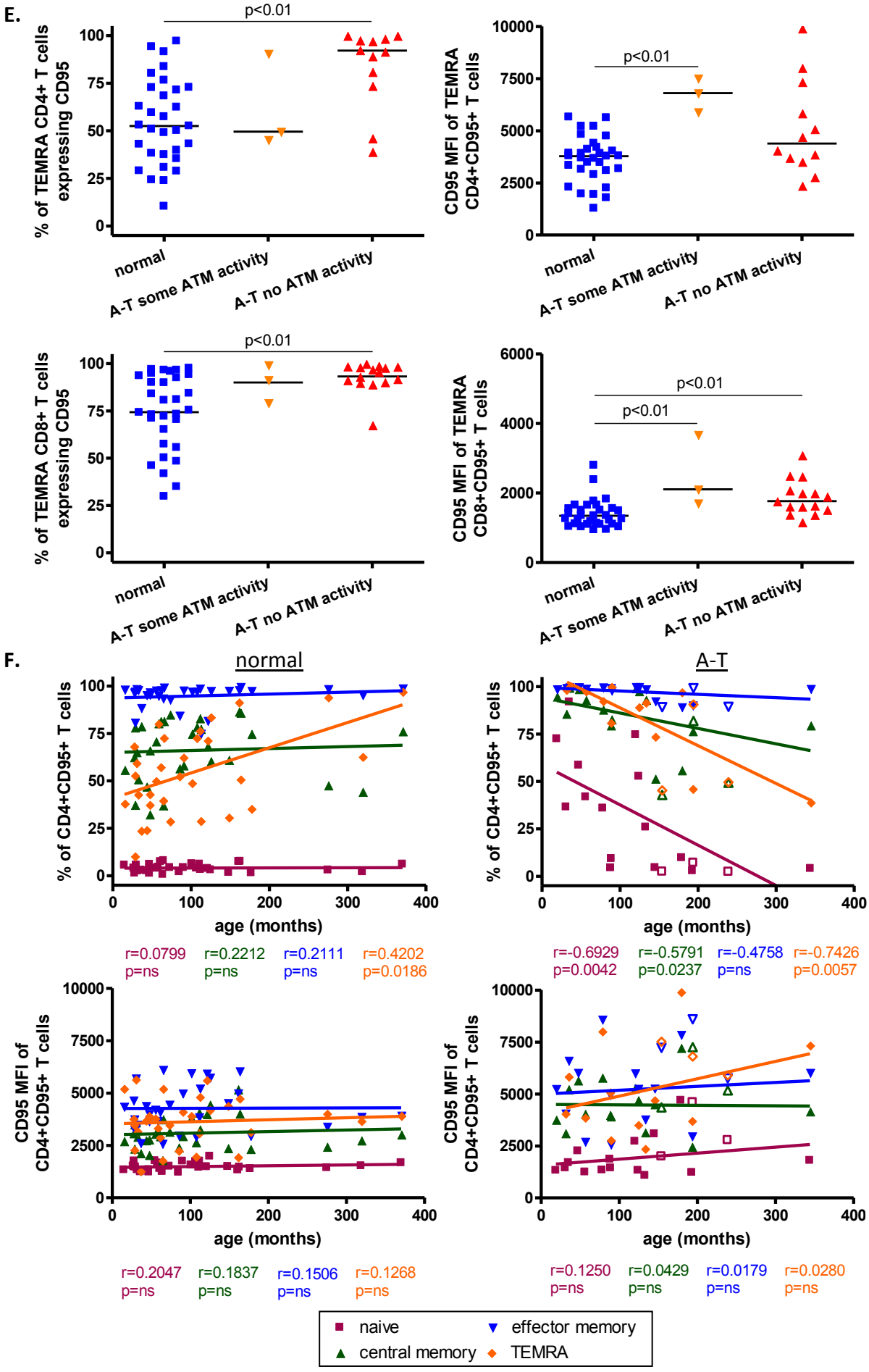
The negative correlation between the percentage of CD4+CD95+ T cells and age in the A-T patients with no ATM activity was due to significant decreases in the percentages of naive, central memory and TEMRA cells which expressed CD95. The percentage of CD4+CD95+ effector memory cells did not change significantly with age and there were no correlations between age and the CD95 MFI of CD4+CD95+ T cells of any subset (Fig 3:4:3F). Although A-T patients with no ATM activity showed a significant positive correlation between age and the CD95 MFI of all CD8+CD95+ T cells there were no significant correlations between age and CD95 expression on individual T cell subsets. However trend lines did suggest an increase in CD95 MFI of CD95+ T cells of all subsets with age (Fig 3:4:3G).

Fig 3:4:3: CD95 expression was significantly increased on naive and memory T cell subsets from A-T patients with no ATM activity compared to normal controls.






C.
p<0.01
p<0.05
% of central memory CD4+ T cells expressing CD95
100
75
50
25
0
normal
A-T some ATM activity
A-T no ATM activity
p<0.001
p<0.01
CD95 MFI of central memory CD4+CD95+ T cells
10000
7500
5000
2500
0
% of central memory CD8+ T cells expressing CD95
CD95 MFI of central memory CD8+CD95+ T cells
6000
4000
2000
D.
% of effector memory CD4+ T cells expressing CD95
CD95 MFI of effector memory CD4+CD95+ T cells
% of effector memory CD8+ T cells expressing CD95
CD95 MFI of effector memory CD8+CD95+ T cells


E.
% of TEMRA CD4+ T cells expressing CD95
p<0.01
normal
A-T some ATM activity
A-T no ATM activity
CD95 MFI of TEMRA CD4+CD95+ T cells
p<0.01
% of TEMRA CD8+ T cells expressing CD95
p<0.01
CD95 MFI of TEMRA CD8+CD95+ T cells
p<0.01
p<0.01
F.
normal
A-T
% of CD4+CD95+ T cells
age (months)
r=0.0799 p=ns
r=0.2212 p=ns
r=0.2111 p=ns
r=0.4202 p=0.0186
r=-0.6929 p=0.0042
r=-0.5791 p=0.0237
r=-0.4758 p=ns
r=-0.7426 p=0.0057
CD95 MFI of CD4+CD95+ T cells
r=0.2047 p=ns
r=0.1837 p=ns
r=0.1506 p=ns
r=0.1268 p=ns
r=0.1250 p=ns
r=0.0429 p=ns
r=0.0179 p=ns
r=0.0280 p=ns
naive
effector memory
central memory
TEMRA

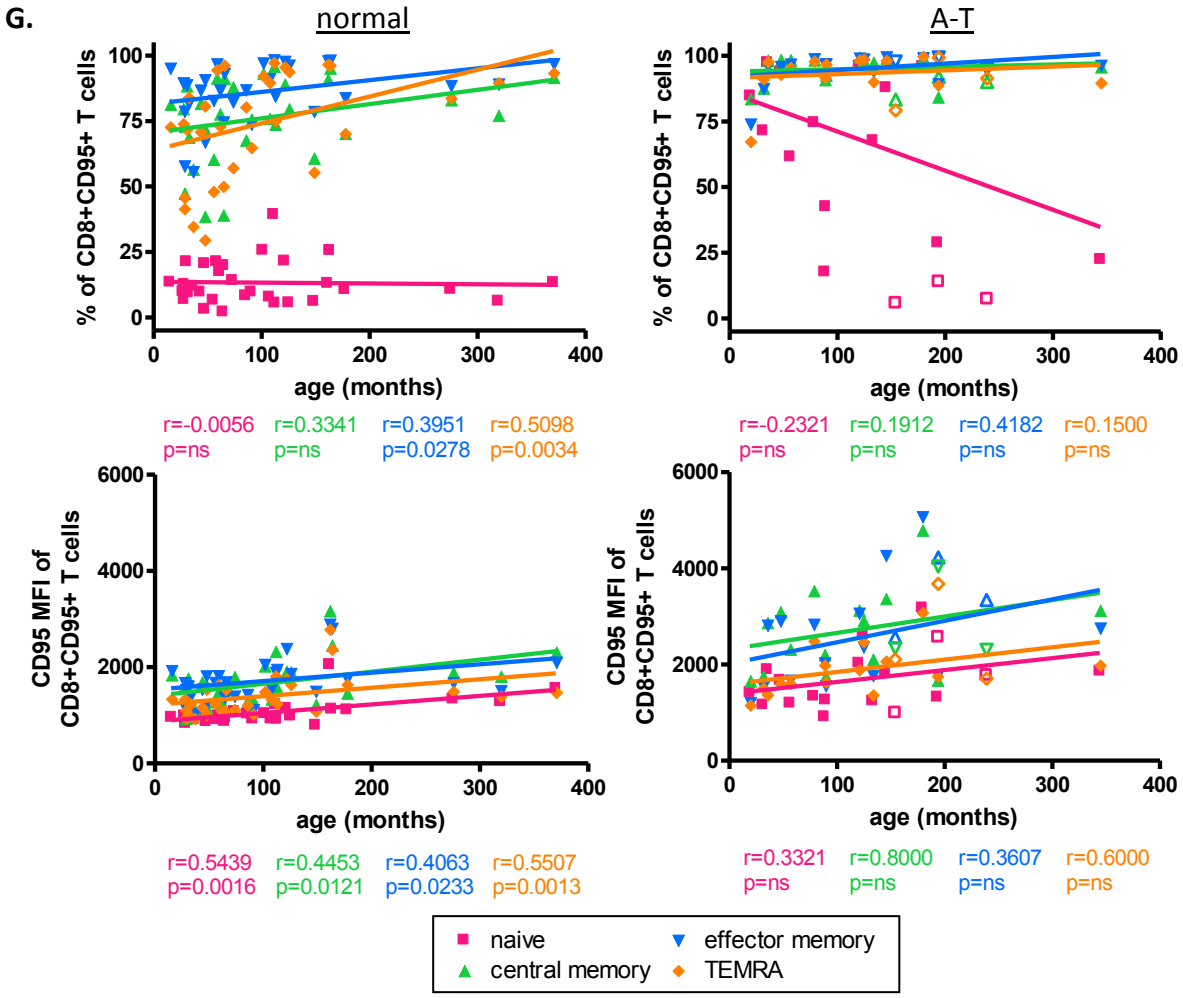


Fig 3:4:3: PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRII flow cytometer. Gating was carried out as described in Fig 3:3:1. In the correlation analysis to improve clarity and as there were no significant correlations linear regression trend lines and r and p values for the A-T patients with some ATM activity are not shown, however results of the correlation analysis for this group are summarised in Table 3:4:7. (A-T plots: open symbols = some ATM activity, closed symbols = no ATM activity).

A. Representative histograms showing CD95 expression on CD4+ve and CD8+ve T cell subsets in normal controls (N PBMC3 - 5 years 4 months), A-T patients with some ATM activity (AT PBMC2 - 12 years 10 months) and A-T patients with no ATM activity (AT PBMC10 - 4 years 8 months). Percentages of CD95+ve T cells in naive (N), central memory (CM),

effector memory (EM) and TEMRA (T) subsets are shown. Plots represent an equal number of events.

B. CD95 expression on naive CD4+ve and CD8+ve T cells as the percentage of naive CD4+ve and CD8+ve T cells that express CD95 and the CD95 MFI of naive CD4+CD95+ and CD8+CD95+ T cells.

C. CD95 expression on central memory CD4+ve and CD8+ve T cells as the percentage of central memory CD4+ve and CD8+ve T cells that express CD95 and the CD95 MFI of central memory CD4+CD95+ and CD8+CD95+ T cells.

D. CD95 expression on effector memory CD4+ and CD8+ T cells as the percentage of effector memory CD4+ve and CD8+ve T cells that express CD95 and the CD95 MFI of effector memory CD4+CD95+ and CD8+CD95+ T cells.

E. CD95 expression on TEMRA CD4+ T cells as the percentage of TEMRA CD4+ve and CD8+ve T cells that express CD95 and the CD95 MFI of TEMRA CD4+CD95+ and CD8+CD95+ T cells. As the number of CD4+ve TEMRA cells was very low samples with less than 20 TEMRA cell events were excluded from the analysis.

F. There was a significant positive correlation between the percentage of CD4+CD95+ TEMRA cells and age in normal controls (top left). However in A-T patients with no ATM activity there were significant negative correlations between percentages of CD4+CD95+ naive, central memory and TEMRA cells and age (top right). Neither normal nor A-T samples showed any correlation between CD95 MFI of CD95+ cells in CD4+ T cell subsets and age (bottom left and right).

G. There were significant correlations between the percentages of CD95+ve effector memory and TEMRA T cells and age in normal controls (top left) but no correlations in A-T patients (top right). In normal controls there were also significant correlations between

CD95 MFI and age on all CD8+ve T cell subsets (bottom left), but no correlations in A-T patients (bottom right).

3:4:4: CD95 expression on naive and memory B cells.

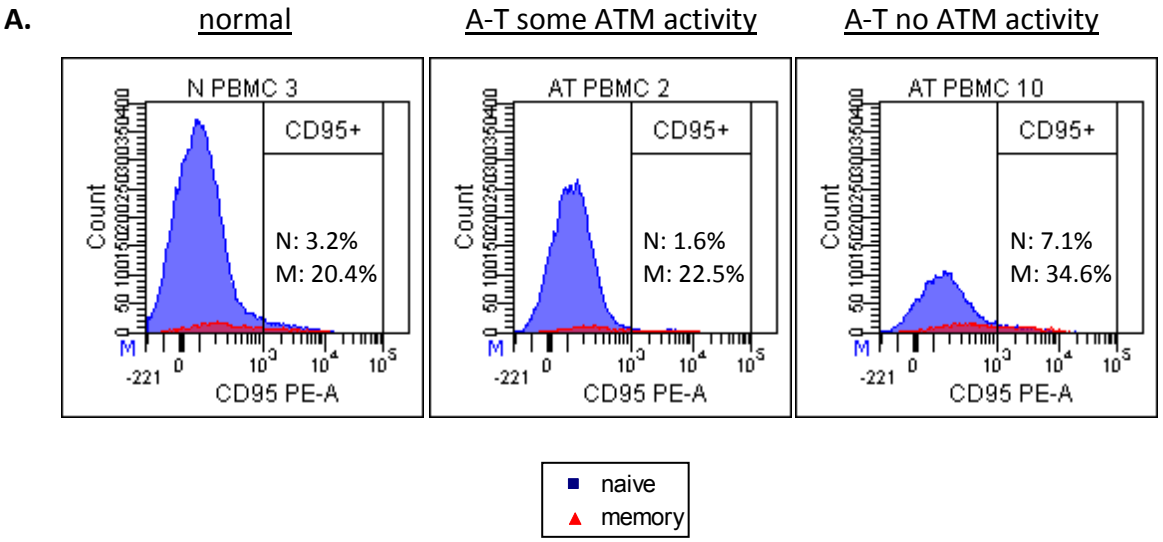
CD95 expression on naive and memory B cells was also analysed (Fig 3:4:4A). Unsurprisingly as CD95 upregulation is associated with activation (reviewed in (Mizuno et al., 2003)) expression on B cells in the normal controls was much higher on the memory (Fig 3:4:4C) than the naive subset (Fig 3:4:4B); a median of 45% of memory B cells were CD95+ve compared to a median of 5.2% of naive B cells.

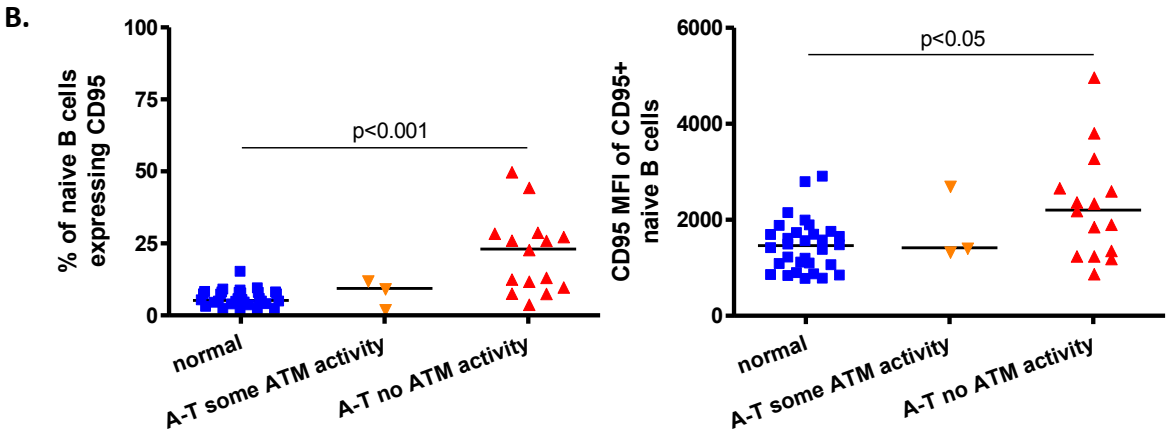
The B cell results were similar to that of T cells in that there was significantly higher expression of CD95 on both naive (Fig 3:4:4B) and memory B cells (Fig 3:4:4C) in the A-T patients with no ATM activity compared to normal controls. This applied to both the percentage (naive $p<0.001$, memory $p<0.05$) and CD95 MFI of CD95+ cells (naive $p<0.05$, memory $p<0.01$). There were no significant differences in CD95 expression on naive and memory B cells between A-T patients with some ATM activity and either normal controls or A-T patients with no ATM activity.

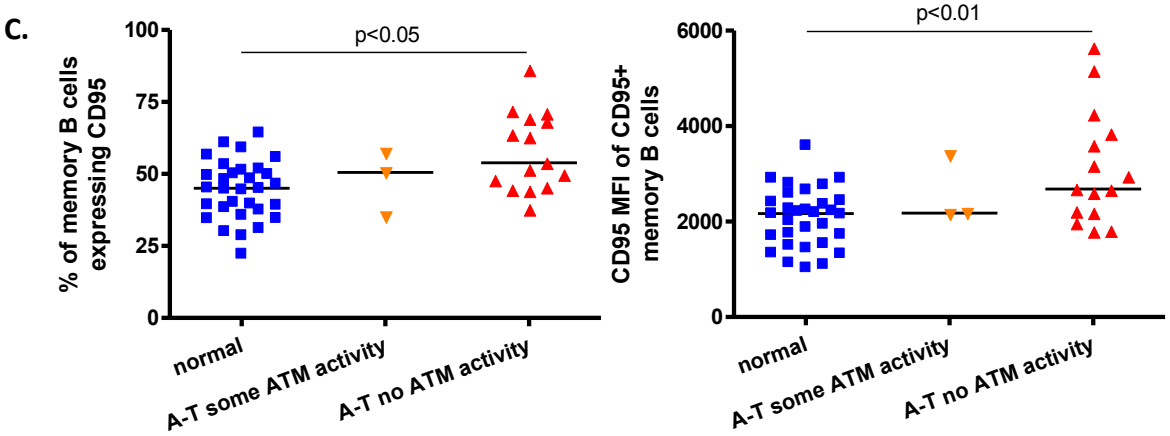
Interestingly, analysis of correlations between CD95 expression on B cells and age showed a positive correlation with the percentage of CD95+ memory B cells in the normal controls and a negative correlation in the A-T patients with no ATM activity. There was no correlation between the percentage of naive CD95+ve B cells and age or CD95 MFI of CD8+CD95+ naive or memory B cells in either group (Fig 3:4:4D).

As the increased CD95 expression on B cells from A-T patients with no ATM activity applies to both naive and memory B cells it cannot be simply a consequence of the increased proportion of memory B cells in A-T patients with no ATM activity compared to normal controls (Fig 3:3:5B).

Fig 3:4:4: CD95 expression was significantly increased on naive and memory B cell subsets from A-T patients with no ATM activity compared to normal controls.







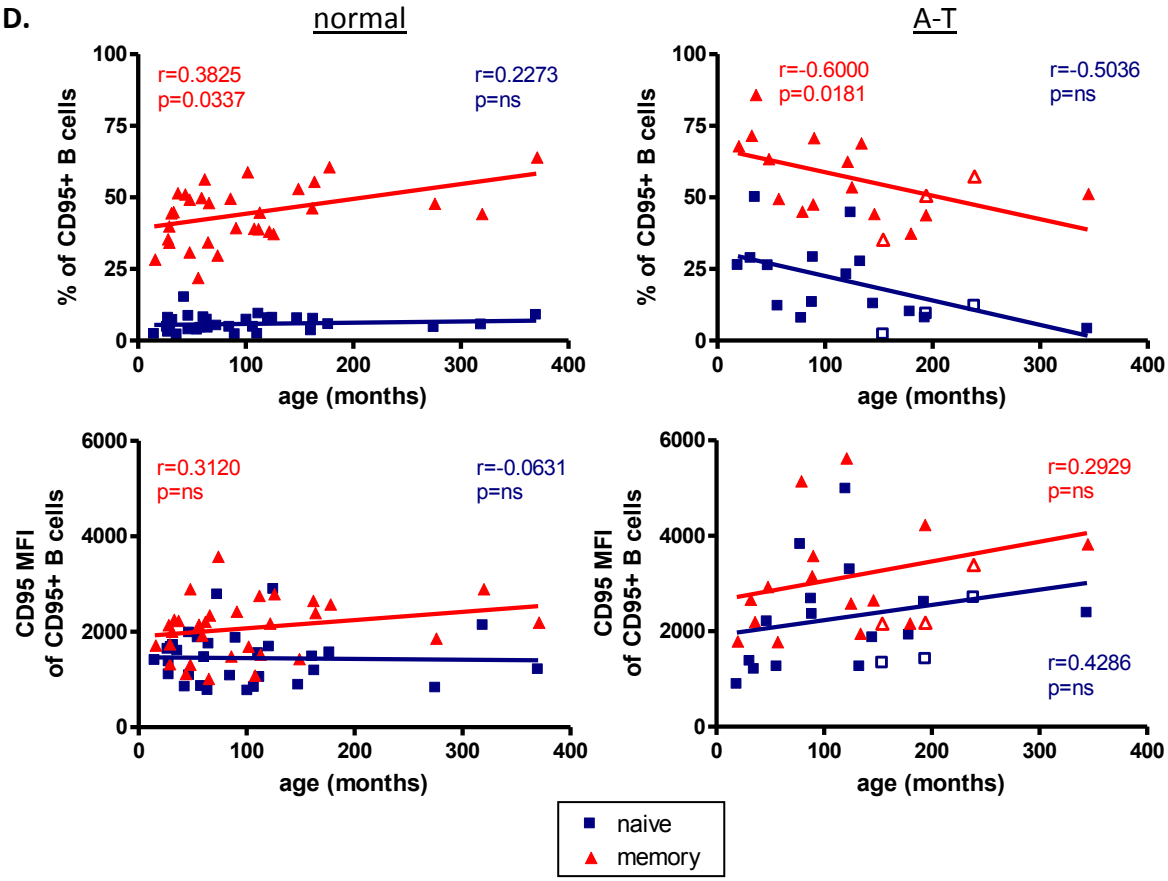


Fig 3:4:4: PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRll flow cytometer. Gating was carried out as described in Fig 3:3:1.

A. Representative histograms showing CD95 expression on B cell subsets in normal controls (N PBMC3 - 5 years 4 months), A-T patients with some ATM activity (AT PBMC2 - 12 years 10 months) and A-T patients with no ATM activity (AT PBMC10 - 4 years 8 months). Percentages of CD95+ B cells in naive (N) and memory (M), populations are shown.

B. CD95 expression on naive B cells as the percentage of naive B cells that express CD95 and the CD95 MFI of naive CD95+ve B cells

C. CD95 expression on memory B cells as the percentage of memory B cells that express CD95 and the CD95 MFI of memory CD95+ve B cells.

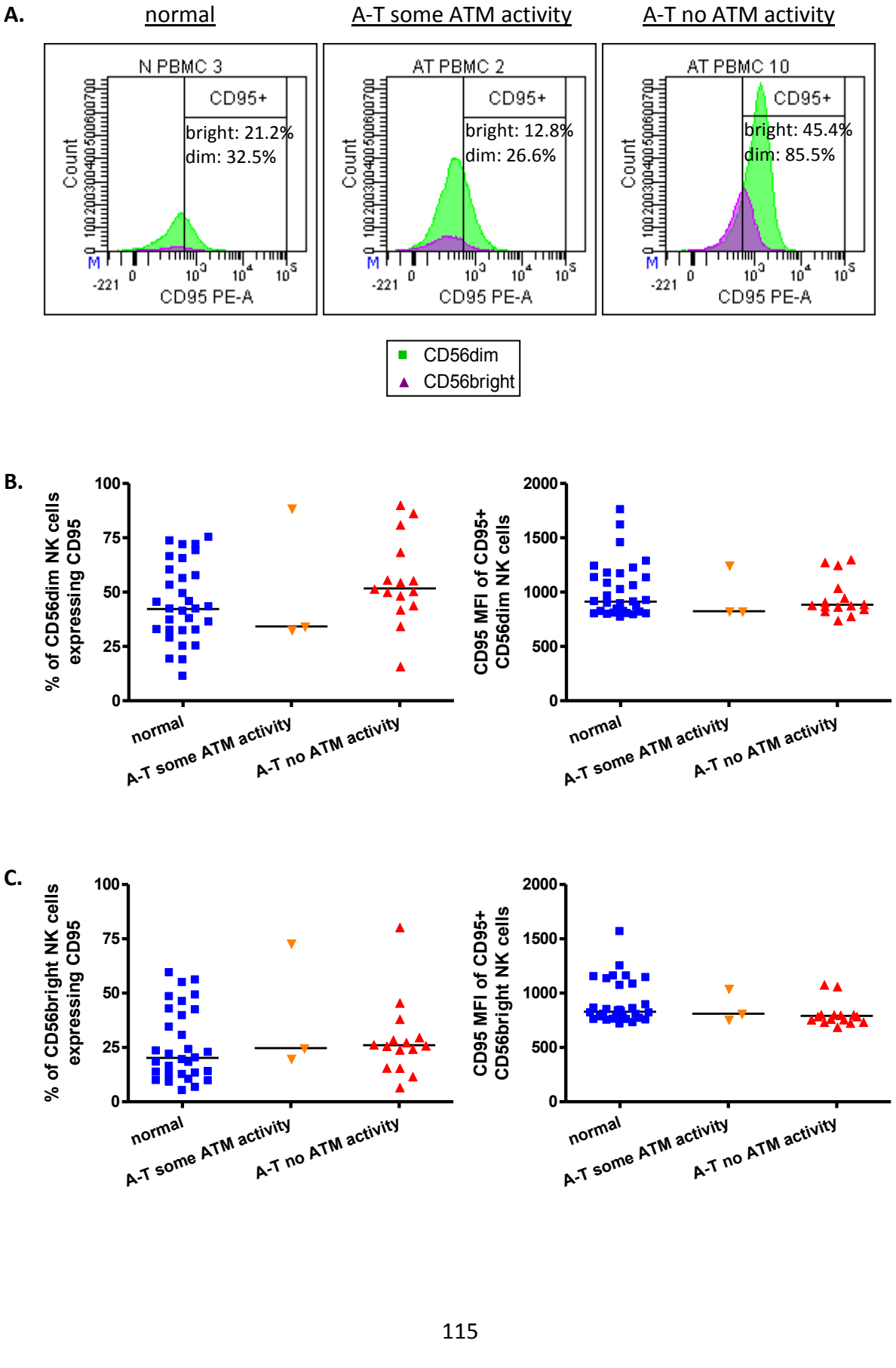
D. There were no significant correlations between the percentage of CD95+ve naive B cells and age in A-T patients or normal controls. However the percentage of CD95+ve memory B cells showed a significant positive correlation with age in normal controls (top left) and a

significant negative correlation with age in A-T patient with no ATM activity (top right). There were no significant correlations between CD95 MFI and age on CD95+ve naive or memory B cells in either group (bottom left and right). To improve clarity and as there were no significant correlations linear regression trend lines and r and p values for the A-T patients with some ATM activity are not shown, however results of the correlation analysis for this group are summarised in Table 3:4:6. (A-T plots: open symbols = some ATM activity, closed symbols = no ATM activity).

3:4:5: CD95 expression on CD56dim and CD56bright NK cells.

Finally CD95 expression on CD56bright and CD56dim NK cells was analysed (Fig 3:4:5A). Consistent with the findings on T and B cells, the more highly differentiated cytotoxic CD56dim NK cells expressed higher levels of CD95 than the less differentiated cytokine producing CD56bright NK cells (Fig 3:4:5B&C). In the normal controls a median percentage of 42.2% of CD56dim NK cells expressed CD95 compared to 20.2% of CD56bright NK cells. There was no significant difference in either the percentage or CD95 MFI of CD95+ve CD56bright or CD56dim NK cells between A-T patients and normal controls (Fig 3:4:5B&C). There was also no correlation between CD95 expression and age in any of the groups (Fig 3:4:5D).

Fig 3:4:5: CD95 expression was not increased on CD56dim and CD56bright NK cells from A-T patients compared to normal controls.



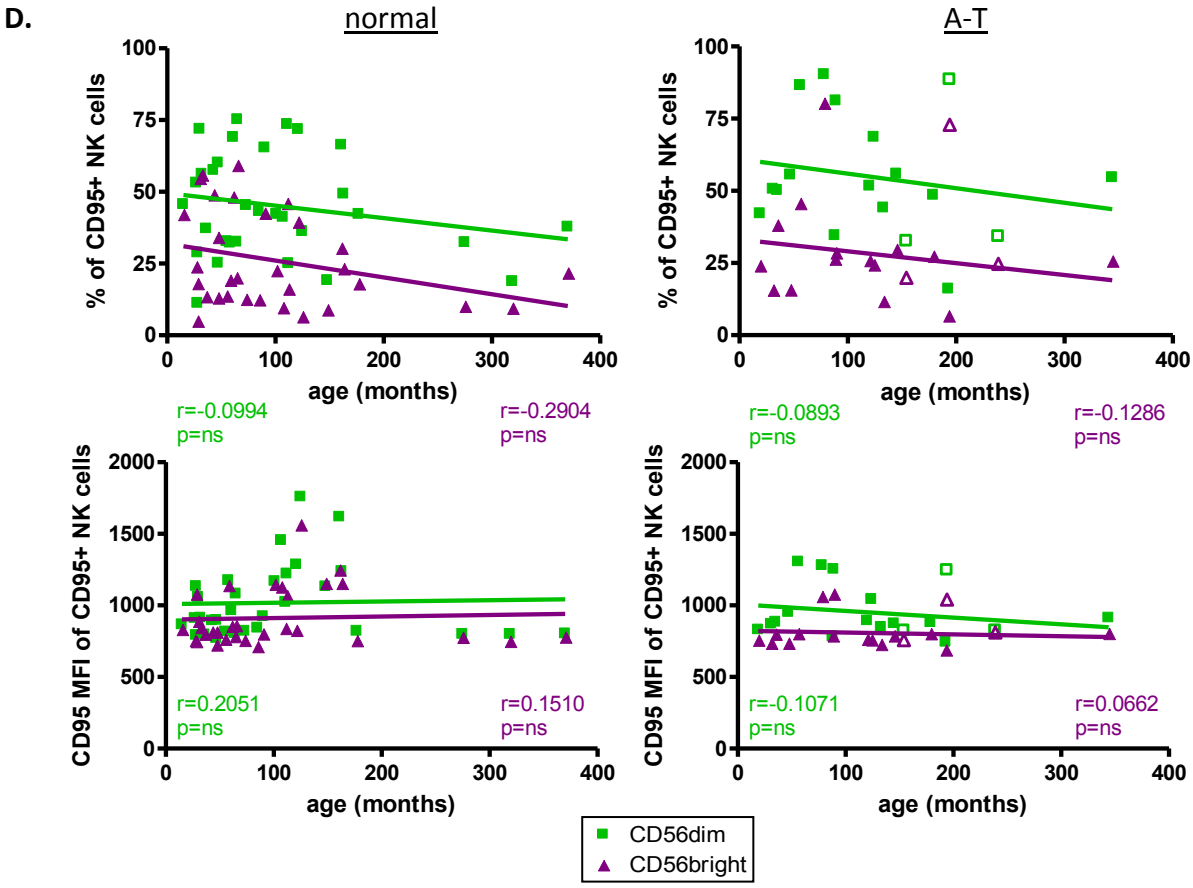


Fig 3:4:5: PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRll flow cytometer. Gating was carried out as described in Fig 3:3:1.

A. Representative histograms showing CD95 expression on NK cell subsets in normal controls (N PBMC3 - 5 years 4 months), A-T patients with some ATM activity (AT PBMC2 - 12 years 10 months) and A-T patients with no ATM activity (AT PBMC10 - 4 years 8 months). Percentages of CD95+ NK cells in CD56 bright (bright) and CD56dim (dim) populations are shown.

B. CD95 expression on CD56dim NK cells as the percentage of CD56dim NK cells that express CD95 and the CD95 MFI of CD56dimCD95+ NK cells

C. CD95 expression on CD56bright NK cells as the percentage of CD56bright NK cells that express CD95 and the CD95 MFI of CD56brightCD95+ NK cells.

D. There were no significant correlations between either percentage or CD95 MFI of CD95+ CD56dim or CD95+CD56bright NK cells and age in A-T patients or normal controls. To

improve clarity and as there were no significant correlations linear regression trend lines and r and p values for the A-T patients with some ATM are not shown, however results of the correlation analysis for this group are summarised in Table 3:4:6. (A-T plots: open symbols = some ATM activity, closed symbols = no ATM activity).

3:4:6: CD95 expression was significantly increased on A-T lymphocytes compared to normal controls.

The results described in section 3:4 including significant differences between the expression of CD95 (percentage and CD95 MFI of CD95+ve cells) and significant correlations between CD95 expression and age in A-T patients with and without ATM activity and normal controls are summarised in Table 3:4:6.

The percentage of CD95+ve cells of every subset with the exception of total NK cells and NK cell subsets was significantly increased in A-T patients with no ATM activity compared to normal controls. On most lymphocyte subsets there was no significant difference between the percentage of CD95+ve cells in A-T patients with some ATM activity compared to normal controls or A-T patients with no ATM activity. However the A-T patients with some ATM activity did have significantly lower percentages of CD95+ naive CD4+ and CD8+ T cells and CD4+ central memory T cells than A-T patients with no ATM activity.

The CD95 MFI of CD95+ve cells in every subset with the exception of total NK cells and NK cell subsets was significantly lower in the normal controls than in either one or both of the groups of A-T patients.

In general CD95 expression in the normal controls increased with age, there were positive correlations between the percentages of CD95+ T cells, CD4+ T cells, CD8+ T cells, CD8+ effector memory T cells, CD4+ and CD8+ TEMRA T cells, total and memory B cells and NKT cells and age. The CD95 MFI of CD95+ T cells, CD8+ T cells and subsets and NKT cells also correlated positively with age. In the A-T patients with no ATM activity the percentages of CD95+CD4+ total, naive, central memory and TEMRA T cells and memory B cells correlated negatively with age and the CD95 MFI of CD95+CD8+ T cells correlated positively with age.

The A-T patients with some ATM activity showed no correlations between percentage or CD95 MFI of CD95+ cells and age.

It was interesting that NK cells were the only lymphocyte subset on which CD95 expression was not increased in A-T patients with no ATM activity compared to normal controls. The percentage of NK cells was increased in A-T patients with no ATM activity (Fig 3:3:2A) but there were deficiencies in T and B cells, particularly naive cells, and CD95 expression was increased on all T cell and B cell subsets, with the greatest increase on the naive cells. These findings are consistent with the hypothesis that increased CD95 expression on A-T patient's lymphocytes increases their sensitivity to CD95-mediated and spontaneous apoptosis and contributes to their lymphopenia.

Table 3:4:6: Significant differences in CD95 expression on lymphocytes and correlations with age in A-T patients and normal controls.

	significant differences in CD95 expression on lymphocytes						correlations between CD95 expression on lymphocytes and age					
	% CD95+			CD95 MFI of CD95+ cells			% CD95+			CD95 MFI of CD95+ cells		
	normal	A-T some ATM activity	A-T no ATM activity	normal	A-T some ATM activity	A-T no ATM activity	normal	A-T some ATM activity	A-T no ATM activity	normal	A-T some ATM activity	A-T no ATM activity
T cells	-	ns	+	-	+	+	+	ns	ns	+	ns	ns
CD4+ T cells	-	ns	+	-	+	+	+	ns	-	ns	ns	ns
CD4+ naive	-	-	+	-	+	ns	ns	ns	-	ns	ns	ns
CD4+ central memory	-	-	+	-	+	+	ns	ns	-	ns	ns	ns
CD4+ effector memory	-	ns	+	-	+	ns	ns	ns	ns	ns	ns	ns
CD4+ TEMRA	-	ns	+	-	+	ns	+	ns	-	ns	ns	ns
CD8+ T cells	-	ns	+	-	+	+	+	ns	ns	+	ns	+
CD8+ naive	-	-	+	-	+	+	ns	ns	ns	+	ns	ns
CD8+ central memory	-	ns	+	-	+	+	ns	ns	ns	+	ns	ns
CD8+ effector memory	-	ns	+	-	+	+	+	ns	ns	+	ns	ns
CD8+ TEMRA	-	ns	+	-	+	+	+	ns	ns	+	ns	ns
B cells	-	ns	+	-	ns	+	+	ns	ns	ns	ns	ns
naive B cells	-	ns	+	-	ns	+	ns	ns	ns	ns	ns	ns
memory B cells	-	ns	+	-	ns	+	+	ns	-	ns	ns	ns
NK cells	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CD56dim NK cells	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CD56bright NK cells	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
NKT cells	-	ns	+	-	ns	+	+	ns	ns	+	ns	ns

Table 3:4:6: Summary of significant differences between the expression of CD95 on lymphocytes (percentage and CD95 MFI of CD95+ve cells) and significant correlations between CD95 expression and age in A-T patients and normal controls.

In the first part of the table (significant differences in CD95 expression on lymphocytes) cells containing a positive symbol (+) and highlighted in yellow indicate a significantly higher percentage or CD95 MFI of CD95+ve cells of the particular cell type in the corresponding group than in the group with a cell containing a negative symbol (-) and highlighted in blue, 'ns' refers to no significant difference with any group. The second part of the table summarises significant correlations between CD95 expression and age. Yellow (+) cells indicate a positive correlation and blue (-) cells indicate a negative correlation, 'ns' indicates no significant correlation.

3:5: FasL expression.

3:5:1: FasL expression on lymphocyte subsets.

CD95-mediated apoptosis can occur spontaneously due to overexpression of the receptor (in the absence of ligand) (Le Clorennec et al., 2008), or can be activated by binding of Fas ligand to CD95 on the surface of the target cell. Therefore the expression of Fas ligand on lymphocytes from A-T patients and normal controls was also analysed.

FasL is upregulated on T cells in response to activation (Latinis et al., 1997). According to the majority of the published literature FasL expression is restricted to activated T cells, activated NK cells (Wallin et al., 2003), certain tumour cells (Hahne et al., 1996b;O'Connell et al., 1996;Wallin et al., 2003;Xerri et al., 1997) and non-immune cells in immune privileged sites such as the eye and testis (Griffith et al., 1995). However, I also found expression of FasL on B cells and NKT cells from A-T patients and normal controls. As there was no background staining when FasL was replaced in the panel with the appropriate isotype control (all minus FasL plus isotype), this staining appeared to be genuine (Fig 3:5:1A).

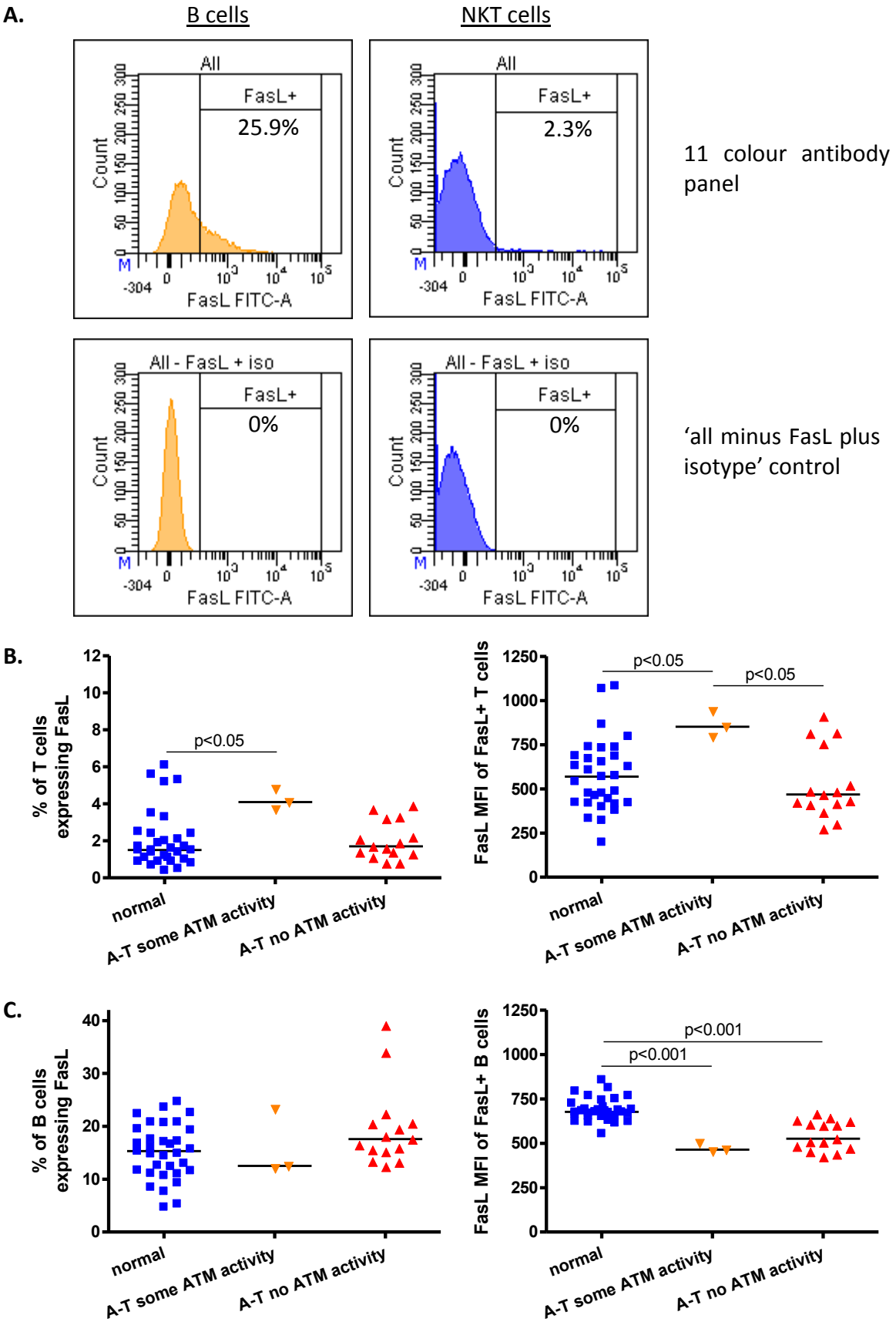
There was no significant difference between the percentages of FasL+ve T cells, B cells, NK cells or NKT cells in the normal controls and A-T patients with no ATM activity (Fig 3:5:1B-E). However the percentage of FasL+ T cells was significantly higher in the A-T patients with some ATM activity than the normal controls ($p<0.05$) (Fig 3:5:1B) and the percentage of FasL+ NK cells was significantly higher in A-T patients with some ATM activity than the other two groups (normal controls - $p<0.05$, A-T patients with some ATM activity - $p<0.05$) (Fig 3:5:1D).

The FasL MFI of FasL+ve T cells was significantly higher in A-T patients with some ATM activity than A-T patients with no ATM activity ($p<0.05$) or normal controls ($p<0.05$) (Fig

3:5:1B). However, the FasL MFI of FasL+ve B cells (Fig 3:5:1C) and NKT cells (Fig 3:5:1E) was significantly higher in the normal controls than both groups of A-T patients (B cells: A-T some ATM activity – $p<0.001$, A-T no ATM activity – $p<0.001$, NKT cells: A-T some ATM activity – $p<0.05$, A-T no ATM activity – $p<0.001$).

Analysis of correlations between FasL expression on different cell types and age showed a significant decrease in the percentage of FasL+ NKT cells and a significant increase in the FasL MFI of FasL+ T and B cells in the normal controls. The A-T patients with no ATM activity showed significant positive correlations between the percentages of FasL+ T and NK cells and age and a decrease in the FasL MFI of FasL+ B cells with age (Fig 3:4:1F).

Fig 3:5:1: FasL expression was reduced on B cells and NKT cells from A-T patients with no ATM activity compared to normal controls.



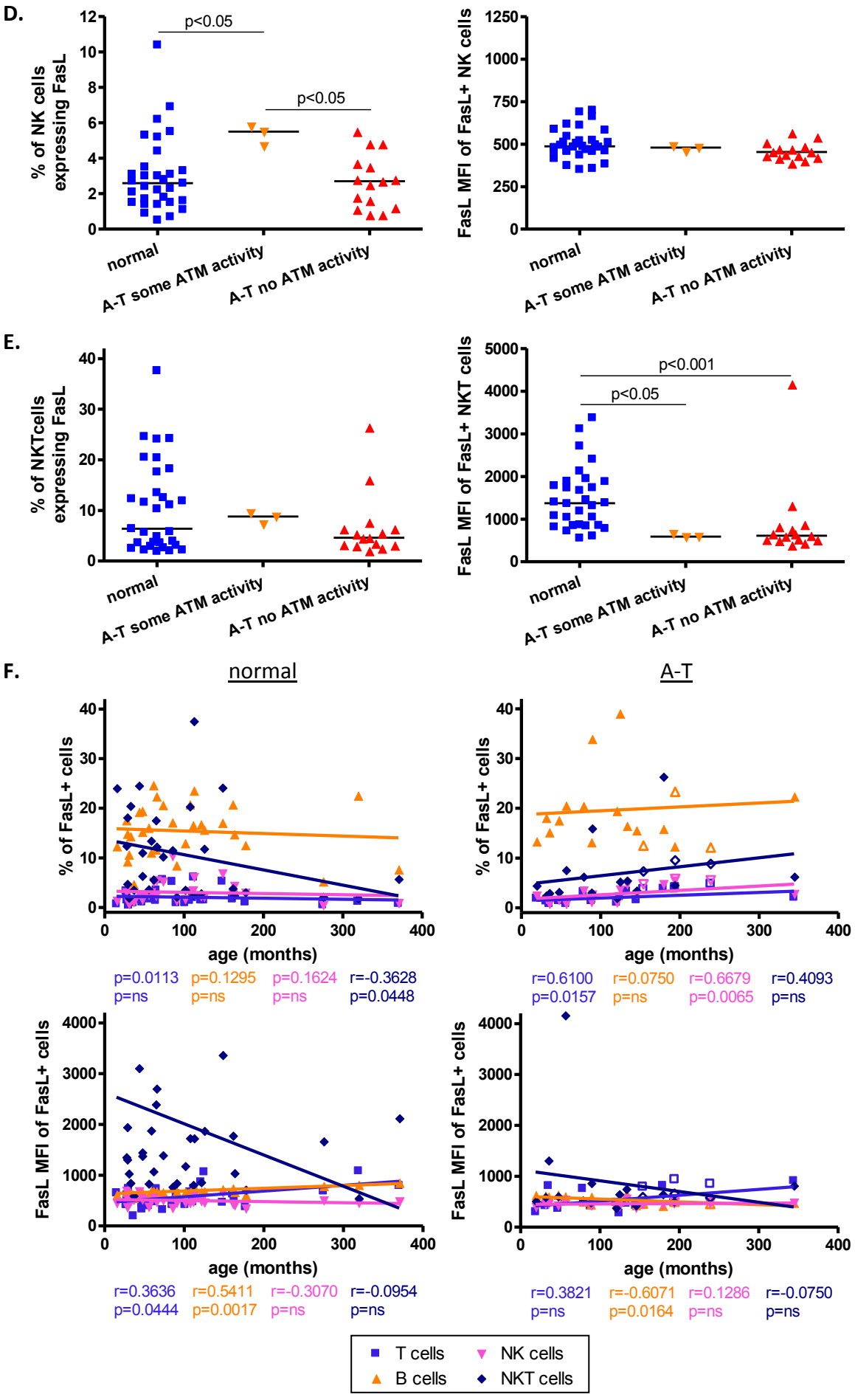

D.
% of NK cells expressing FasL
p<0.05
p<0.05
normal
A-T some ATM activity
A-T no ATM activity
FasL MFI of FasL+ NK cells
E.
% of NKTcells expressing FasL
FasL MFI of FasL+ NKT cells
p<0.001
p<0.05
F.
normal
A-T
% of FasL+ cells
FasL MFI of FasL+ cells
age (months)
p=0.0113 p=0.1295 p=0.1624 r=-0.3628
p=ns p=ns p=ns p=0.0448
r=0.6100 r=0.0750 r=0.6679 r=0.4093
p=0.0157 p=ns p=0.0065 p=ns
r=0.3636 r=0.5411 r=-0.3070 r=-0.0954
p=0.0444 p=0.0017 p=ns p=ns
r=0.3821 r=-0.6071 r=0.1286 r=-0.0750
p=ns p=0.0164 p=ns p=ns
T cells
B cells
NK cells
NKT cells

Fig 3:5:1: PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRll flow cytometer. Gating was carried out as described in Fig 3:3:1.

A. Example plots showing FasL+ve B cells and NKT cells in a normal control sample (N PBMC31). The top row shows the result obtained from staining with the complete 11 colour antibody panel and the bottom row shows the 'all minus FasL plus isotype' control.

B. FasL expression on T cells as the percentage of T cells that express FasL and the FasL MFI of FasL+ve T cells.

C. FasL expression on B cells as the percentage of B cells that express FasL and the FasL MFI of FasL+ve B cells.

D. FasL expression on NK cells as the percentage of NK cells that express FasL and the FasL MFI of FasL+ve NK cells.

E. FasL expression on NKT cells as the percentage of NKT cells that express FasL and the FasL MFI of FasL+ve NKT cells.

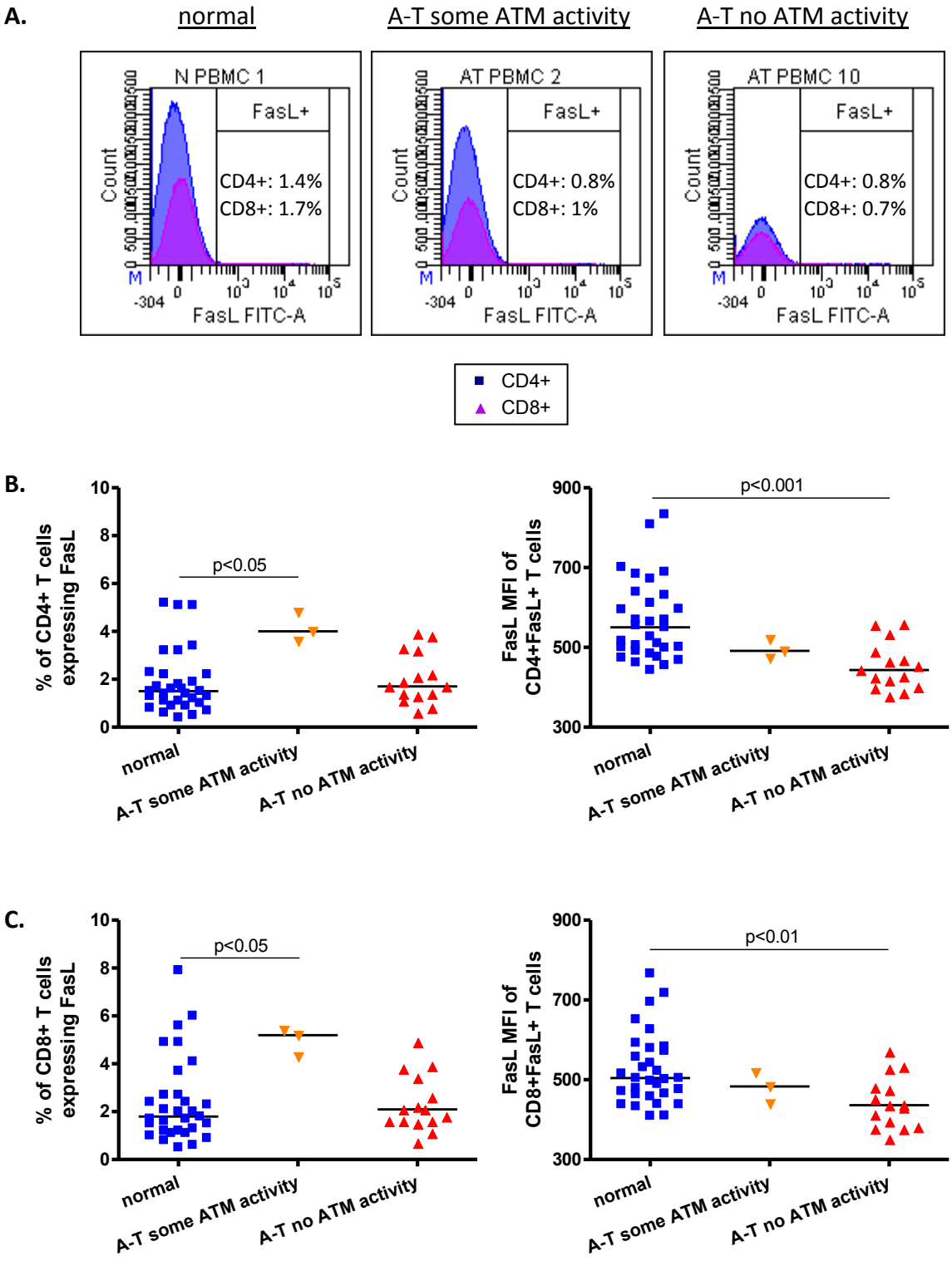
F. There was a significant negative correlation between age and the percentage of FasL+ve NKT cells in normal controls (top left) and positive correlations with the percentages of FasL+ve T cells and NK cells in the A-T patients with no ATM activity (top right). There were also positive correlations between age and the FasL MFI of FasL+ve B and T cells in normal controls (bottom left) and a negative correlation with FasL MFI of FasL+ve B cells in A-T patients with no ATM activity (bottom right). To improve clarity and as there were no significant correlations linear regression trend lines and r and p values for the A-T patients with some ATM activity are not shown, however results for this group are summarised in Table 3:5:6. (A-T plots: open symbols = some ATM activity, closed symbols = no ATM activity).

3:5:2: Fas Ligand expression on CD4+ and CD8+ T cells.

Unsurprisingly, as FasL is expressed only on activated T cells the percentages of FasL+CD4+ and FasL+CD8+ T cells in the normal controls were very low (median of 1.5% of CD4+ve and 1.8% of CD8+ve T cells) (Fig 3:5:2A-C). There was no significant difference between the percentages of CD4+FasL+ (Fig 3:5:2B) or CD8+FasL+ T cells (Fig 3:5:2C) in the normal controls and A-T patients with no ATM activity. However the FasL MFI of both CD4+FasL+ and CD8+FasL+ T cells in the normal controls was significantly higher than in A-T patients with no ATM activity (CD4+ $p<0.001$, CD8+ $p<0.01$). There was a significantly higher percentage of both CD4+FasL+ ($p<0.05$) and CD8+FasL+ T cells ($p<0.05$) in A-T patients with some ATM activity compared to normal controls but no difference in FasL expression between the two groups of A-T patients.

Interestingly, although there was no correlation between FasL expression on CD4+ or CD8+ T cells and age in the normal controls, the percentages of both CD4+FasL+ and CD8+FasL+ T cells increased significantly with age in the A-T patients with no ATM activity (Fig 3:5:2D).

Fig 3:5:2: Fas Ligand expression was reduced on CD4+ and CD8+ T cells from A-T patients with no ATM activity compared to normal controls.



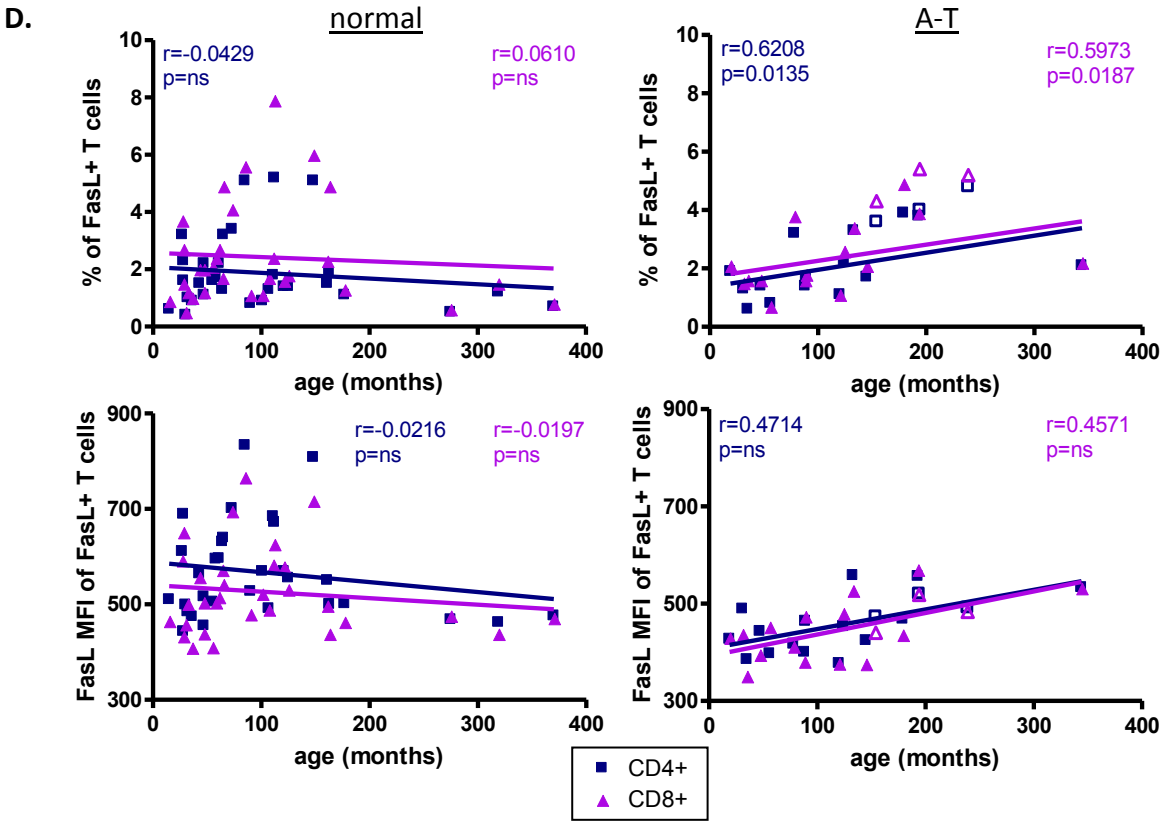


Fig 3:5:2: PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRll flow cytometer. Gating was carried out as described in Fig 3:3:1.

A. Representative histograms showing FasL expression on CD4+ve and CD8+ve T cells in normal controls (N PBMC3 - 5 years 4 months), A-T patients with some ATM activity (AT PBMC2 - 12 years 10 months) and A-T patients with no ATM activity (AT PBMC10 - 4 years 8 months). Percentages of CD4+ve and CD8+ve cells which express FasL are shown.

B. FasL expression on CD4ve T cells as the percentage of CD4+ve T cells that express CD95 and the CD95 MFI of CD4+FasL+ T cells.

C. FasL expression on CD8+ve T cells as the percentage of CD8+ve T cells that express CD95 and the CD95 MFI of CD8+FasL+ T cells.

D. There was no significant correlation between the percentages of CD4+FasL+ or CD8+FasL+ T cells and age in normal controls (top left). However in A-T patients with no ATM activity there were significant increases in the percentages of CD4+FasL+ and CD8+ FasL+ T cells with age (top right). There were no correlations between FasL MFI of

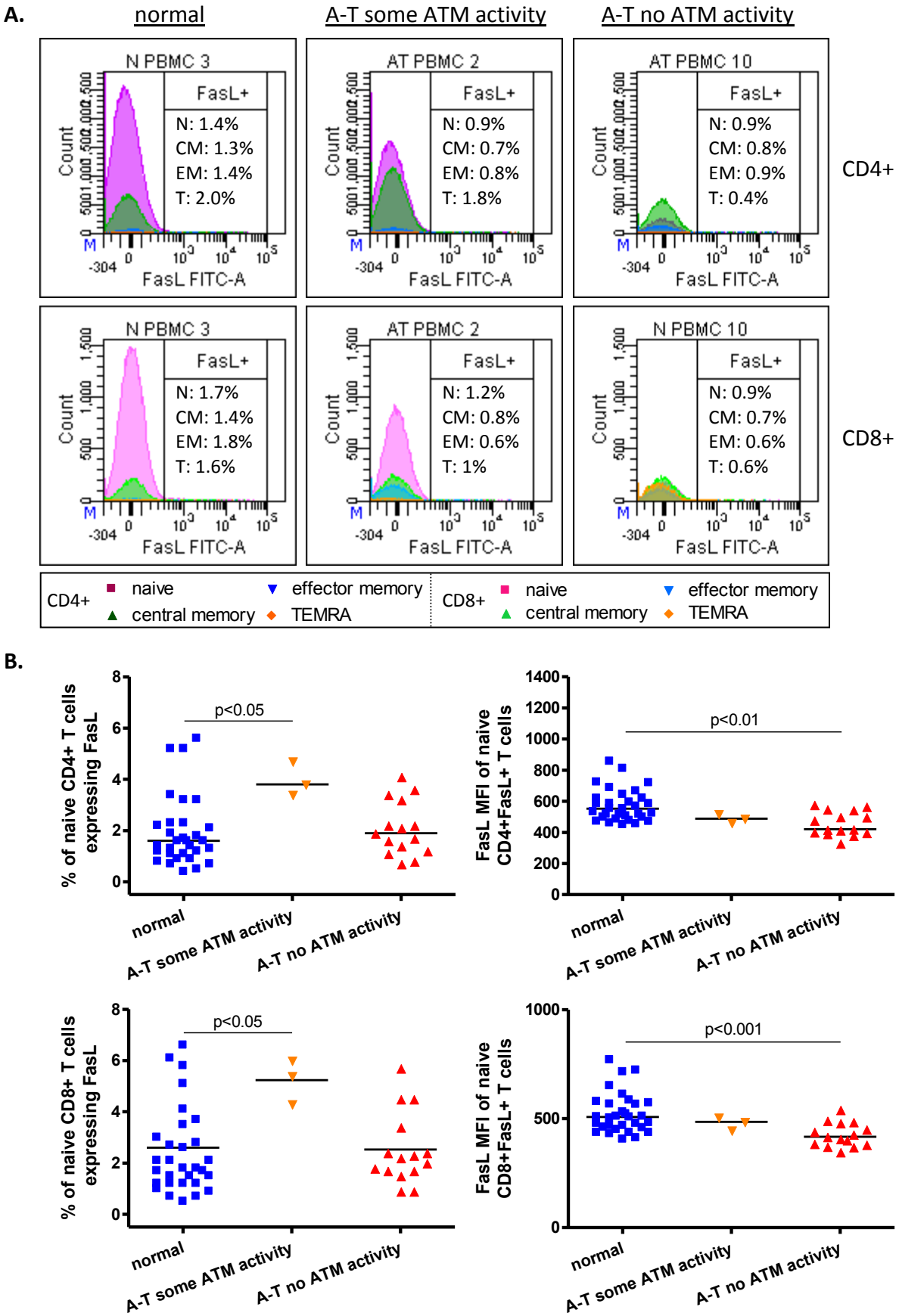
CD4+FasL+ or CD8+FasL+ T cells and age in either A-T patients or normal controls (bottom). To improve clarity and as there were no significant correlations linear regression trend lines and r and p values for the A-T patients with some ATM activity are not shown, however results of the correlation analysis for this group are summarised in Table 3:5:6. (A-T plots: open symbols = some ATM activity, closed symbols = no ATM activity).

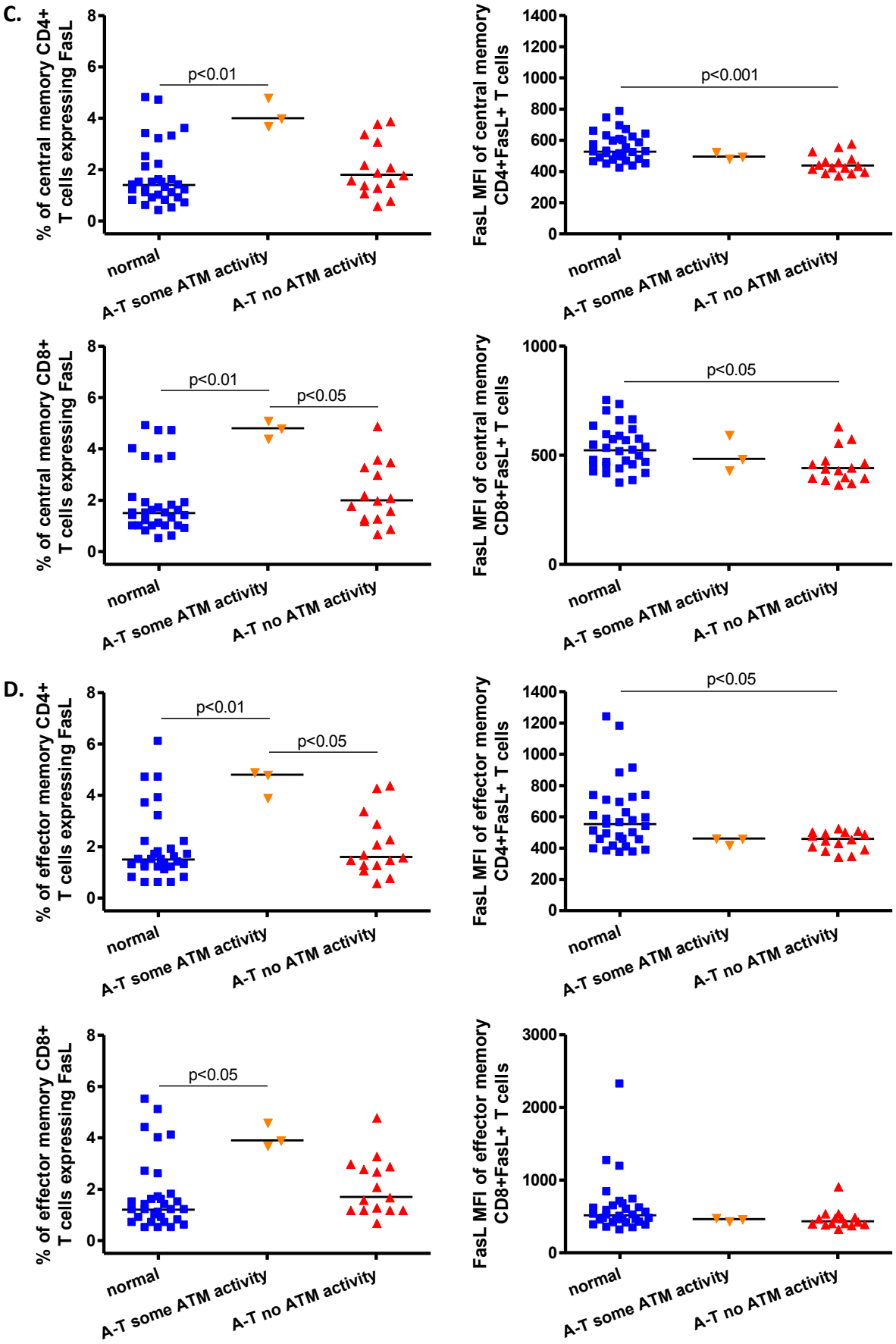
3:5:3: Fas Ligand expression on naive and memory T cells.

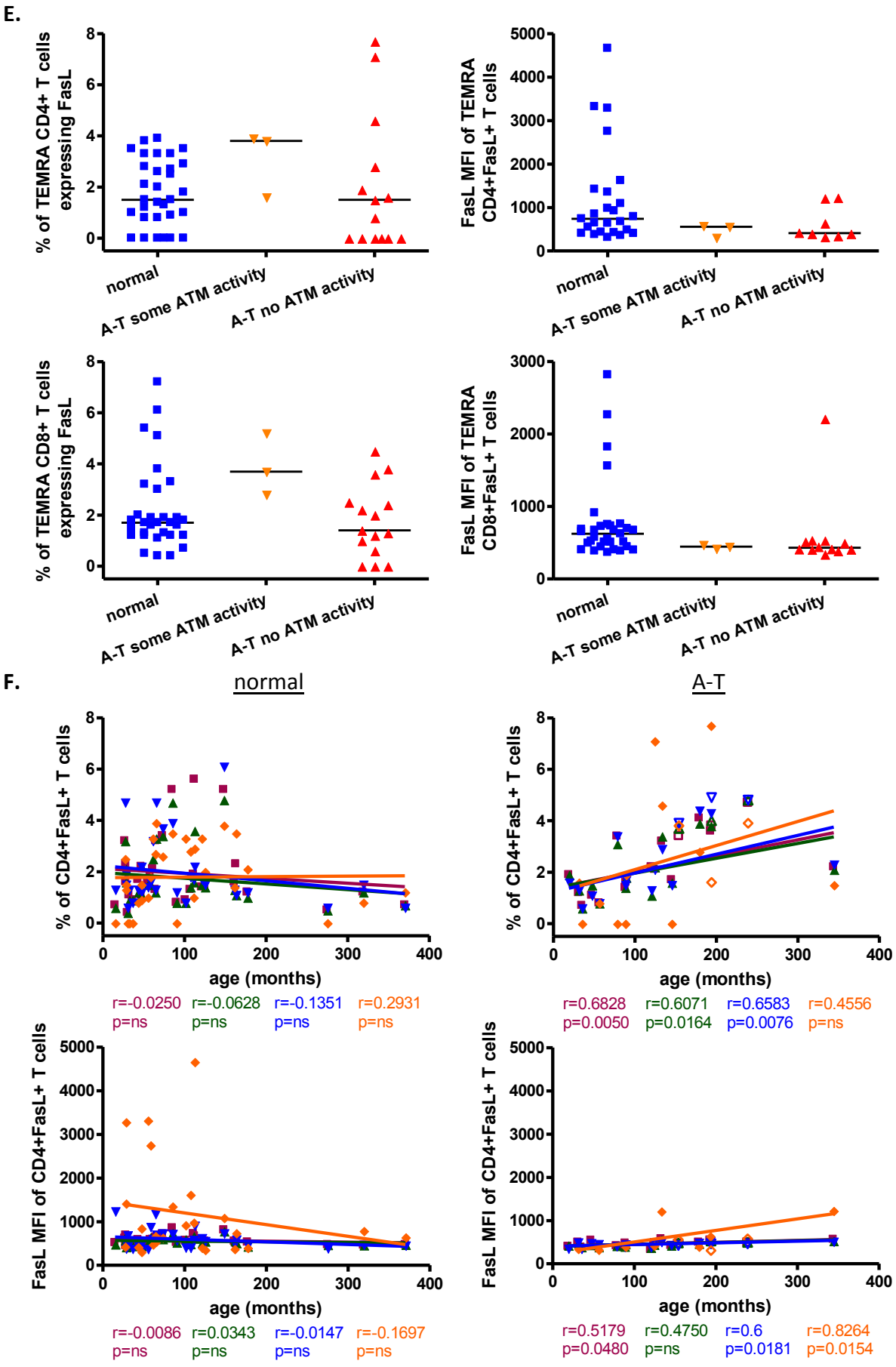
FasL expression on CD4+ and CD8+ naive and memory T cell subsets was similar (Fig 3:5:3A). There were no significant differences between the percentages of CD4+FasL+ or CD8+FasL+ T cells of any subset in the normal controls and A-T patients with no ATM activity. However, the percentages of both CD4+ve and CD8+ve FasL+ve naive (CD4+ - $p<0.05$, CD8+ - $p<0.05$), central memory (CD4+ - $p<0.01$, CD8+ - $p<0.01$) and effector memory (CD4+ - $p<0.01$, CD8+ - $p<0.05$) T cells were significantly higher in A-T patients with some ATM activity than normal controls (Fig 3:5:3B-E). The FasL MFI of CD4+FasL+ and CD8+FasL+ naive (Fig 3:5:3B) and central memory T cells (Fig 3:5:3C) and CD4+FasL+ effector memory (Fig 3:5:3D) T cells was significantly lower in the A-T patients with no ATM activity than the normal controls (naive: CD4 - $p<0.01$, CD8 - $p<0.001$, central memory: CD4 - $p<0.001$, CD8 – $p<0.05$, effector memory: CD4 - $p<0.05$).

There were no correlations between FasL expression on CD4+ or CD8+ T cell subsets and age in the normal controls. However in the A-T patients with no ATM activity the percentages of CD4+FasL+ and CD8+FasL+ naive, central memory and effector memory T cells increased significantly with age (Fig 3:5:3F&G). There were also positive correlations between age and the FasL MFI of CD4+FasL+ naive, effector memory and TEMRA T cells and CD8+FasL+ naive T cells in the A-T patients with no ATM activity (Fig 3:5:3F&G).

Fig 3:5:3: Fas Ligand MFI was reduced on naive, and memory T cell subsets from A-T patients with no ATM activity compared to normal controls.




C.
% of central memory CD4+ T cells expressing FasL
p<0.01
normal
A-T some ATM activity
A-T no ATM activity
FasL MFI of central memory CD4+FasL+ T cells
p<0.001
% of central memory CD8+ T cells expressing FasL
p<0.01
p<0.05
FasL MFI of central memory CD8+FasL+ T cells
p<0.05
D.
% of effector memory CD4+ T cells expressing FasL
p<0.01
p<0.05
FasL MFI of effector memory CD4+FasL+ T cells
p<0.05
% of effector memory CD8+ T cells expressing FasL
p<0.05
FasL MFI of effector memory CD8+FasL+ T cells


E.
% of TEMRA CD4+ T cells expressing FasL
FasL MFI of TEMRA CD4+FasL+ T cells
% of TEMRA CD8+ T cells expressing FasL
FasL MFI of TEMRA CD8+FasL+ T cells
normal
A-T some ATM activity
A-T no ATM activity
F.
normal
A-T
% of CD4+FasL+ T cells
FasL MFI of CD4+FasL+ T cells
age (months)
r=-0.0250 r=-0.0628 r=-0.1351 r=0.2931
p=ns p=ns p=ns p=ns
r=0.6828 r=0.6071 r=0.6583 r=0.4556
p=0.0050 p=0.0164 p=0.0076 p=ns
r=-0.0086 r=0.0343 r=-0.0147 r=-0.1697
p=ns p=ns p=ns p=ns
r=0.5179 r=0.4750 r=0.6 r=0.8264
p=0.0480 p=ns p=0.0181 p=0.0154
naive
effector memory
central memory
TEMRA

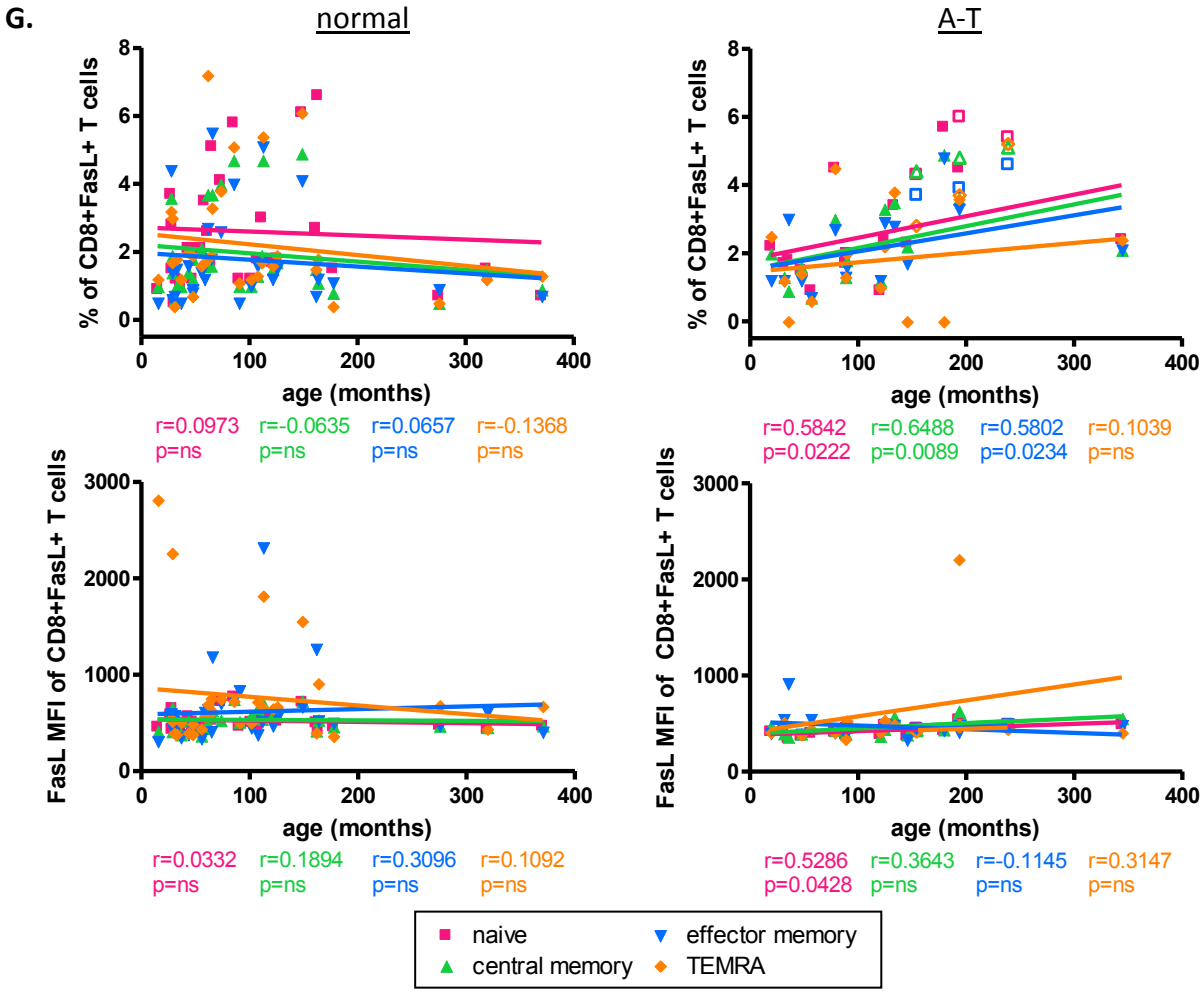


Fig 3:5:3: PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRII flow cytometer. Gating was carried out as described in Fig 3:3:1. In the correlation analysis to improve clarity and as there were no significant correlations linear regression trend lines and r and p values for the A-T patients with some ATM activity are not shown, however results of the correlation analysis for this group are summarised in Table 3:5:6. (A-T plots: open symbols = some ATM activity, closed symbols = no ATM activity).

A. Representative histograms showing FasL expression on CD4+ve and CD8+ve T cell subsets in normal controls (N PBMC3 - 5 years 4 months), A-T patients with some ATM activity (AT PBMC2 - 12 years 10 months) and A-T patients with no ATM activity (AT PBMC10 - 4 years 8 months). Percentages of CD4+FasL+ and CD8+FasL+ T cells in naive (N), central memory (CM), effector memory (EM) and TEMRA (T) subsets and the ages of the donors are shown.

B. FasL expression on naive CD4+ve and CD8+ve T cells as the percentage of naive CD4+ve and CD8+ve T cells that express CD95 and the CD95 MFI of naive CD4+FasL+ and CD8+FasL+ T cells.

C. FasL expression on central memory CD4+ve and CD8+ve T cells as the percentage of central memory CD4+ve and CD8+ve T cells that express CD95 and the CD95 MFI of central memory CD4+FasL+ and CD8+FasL+ T cells.

D. FasL expression on effector memory CD4+ve and CD8+ve T cells as the percentage of effector memory CD4+ve and CD8+ve T cells that express CD95 and the CD95 MFI of effector memory CD4+FasL+ and CD8+ FasL+ T cells.

E. FasL expression on TEMRA CD4+ve and CD8+ve T cells as the percentage of TEMRA CD4+ve and CD8+ve T cells that express CD95 and the CD95 MFI of TEMRA CD4+FasL+ and CD8+FasL+ T cells. Samples with less than 20 CD4+ve or CD8+ve TEMRA events were excluded.

F. Normal controls showed no correlation between age and the percentage of CD4+FasL+ T cells in any subset (top left), however in A-T patient with no ATM activity the percentage increased with age in naive, central memory and effector memory subsets (top right). There were no significant correlations between age and FasL MFI on CD4+FasL+ T cells of any subset in the normal controls (bottom left), however in A-T patients with no ATM activity there were significant increases in FasL MFI with age in naive, effector memory and TEMRA subsets.

G. In the normal controls there were no correlations between age and the percentage of CD8+FasL+ T cells in any subset (top left), however in A-T patients with no ATM activity the percentage increased with age in naive, central memory and effector memory subsets (top right). There were no significant correlations between age and FasL MFI on normal

CD8+FasL+ T cells of any subset, however in A-T patients with no ATM activity there was a significant increase in FasL MFI of naive CD8+FasL+ T cells with age.

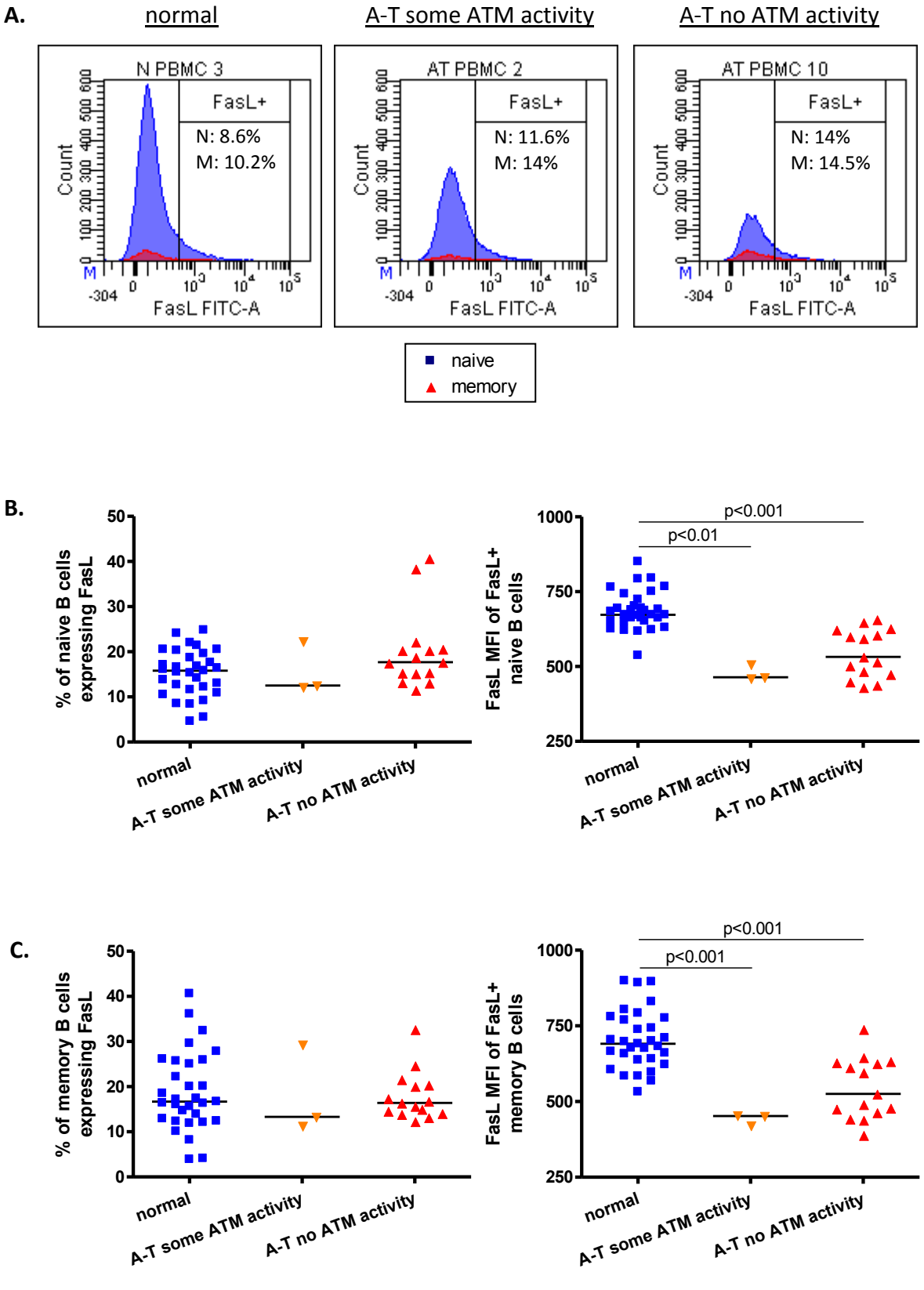
3:5:4: Fas ligand expression on naive and memory B cells.

Both naive and memory B cells from A-T patients and normal controls expressed FasL (Fig 3:5:4A). There was little difference in FasL expression between naive and memory B cells; in the normal controls a median of 15.8% of naive and 16.7% of memory B cells expressed FasL and the median FasL MFI of FasL+ cells was 673 on the naive and 691 on the memory cells (Fig 3:4:5B&C).

There was no significant difference in the percentage of FasL+ naive and memory B cells in normal controls and A-T patients. However, the FasL MFI of FasL+ naive and memory B cells was significantly higher in the normal controls than the A-T patients with no ATM activity (naive - $p<0.001$, memory - $p<0.001$) and A-T patients with some ATM activity (naive $p<0.01$, memory – $p<0.001$) (Fig 3:5:4B&C).

There were no correlations between the percentages of FasL+ naive or memory B cells and age in A-T patients or normal controls. However the FasL MFI of FasL+ naive B cells showed a positive correlation with age in the normal controls. The A-T patients with no ATM activity showed the opposite trend, the FasL MFI of both FasL+ naive and memory B cells decreased significantly with age (Fig 3:5:4D).

Fig 3:5:4: Fas ligand expression was reduced on naive and memory B cells from A-T patients compared to normal controls.



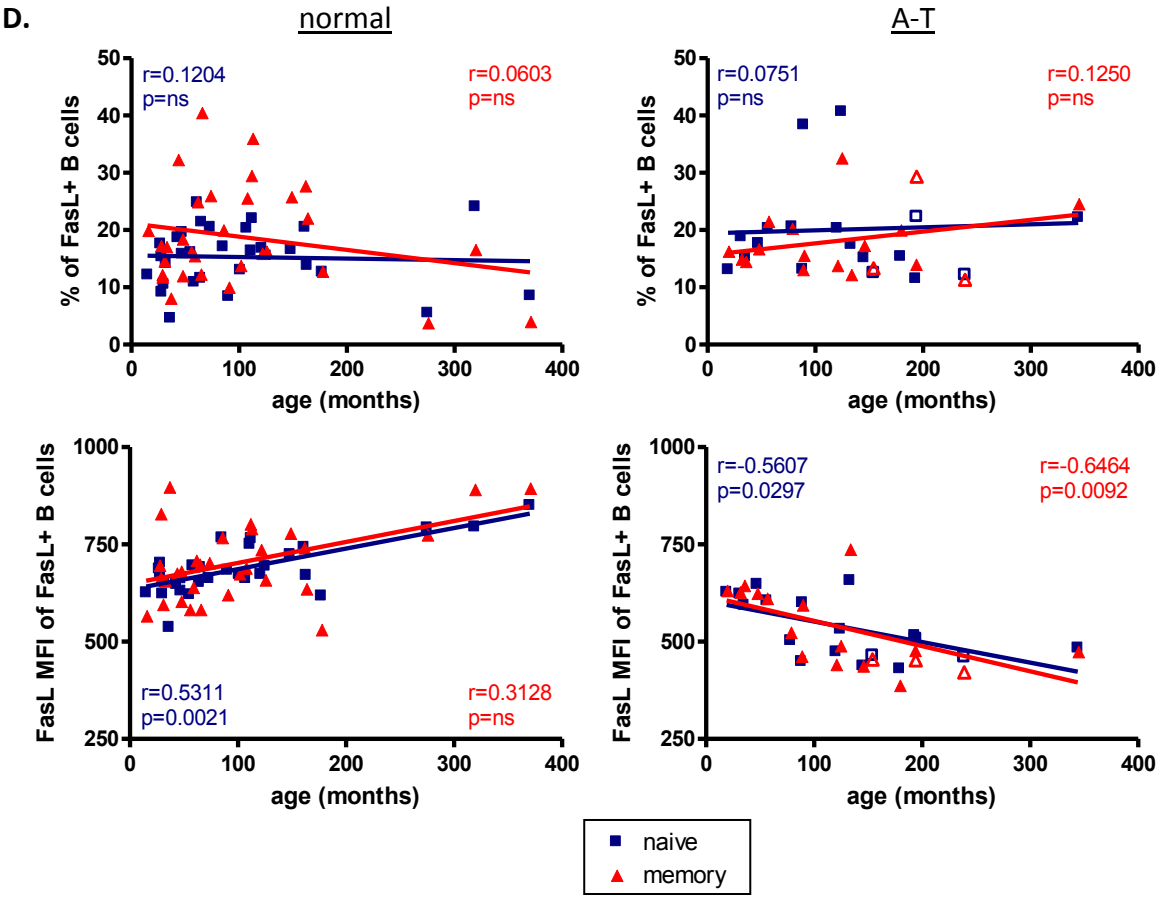


Fig 3:5:4: PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRll flow cytometer. Gating was carried out as described in Fig 3:3:1.

A. Representative histograms showing FasL expression on B cell subsets in normal controls (N PBMC3 - 5 years 4 months), A-T patients with some ATM activity (AT PBMC2 - 12 years 10 months) and A-T patients with no ATM activity (AT PBMC10 - 4 years 8 months). Percentages of FasL+ B cells in naive (N) and memory (M) populations are shown.

B. FasL expression on naive B cells as the percentage of naive B cells that express FasL and the FasL MFI of naive FasL+ve B cells.

C. FasL expression on memory B cells as the percentage of memory B cells that express FasL and the FasL MFI of memory FasL+ve B cells.

D. There were no correlations between the percentages of FasL+ve naive or memory B cells and age in A-T patients with no ATM activity (top right) or normal controls (top left), however the FasL MFI of FasL+ve naive B cells increased with age in normal controls (bottom

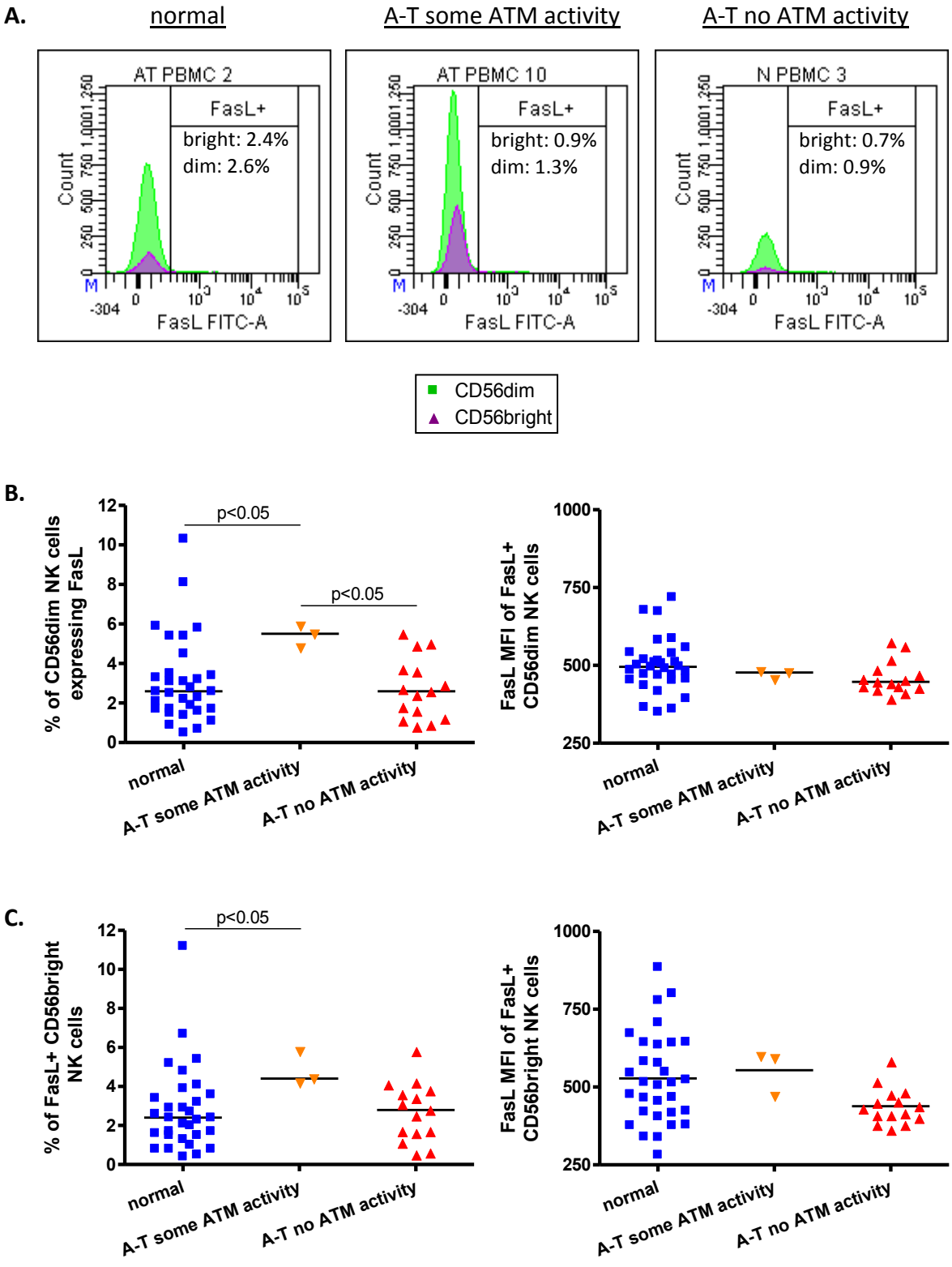
left). In A-T patients with no ATM activity there were significant negative correlations between age and the FasL MFI of both FasL+ve naive and memory B cells (bottom right). To improve clarity and as there were no significant correlations linear regression trend lines and r and p values for the A-T patients with some ATM activity are not shown, however results of the correlation analysis for this group are summarised in Table 3:5:6. (A-T plots: open symbols = some ATM activity, closed symbols = no ATM activity).

3:5:5: FasL expression on CD56bright and CD56dim NK cells.

FasL expression on CD56bright and CD56dim NK cells was very low; in the normal controls a median of 2.4% of CD56bright and 2.6% of CD56dim NK cells expressed FasL (Fig 3:5:5A). There was no significant difference between the FasL MFI of FasL+ cells in A-T patients and normal controls (Fig 3:5:5B&C). However, a significantly higher percentage of CD56dim NK cells in the A-T patients with some ATM activity expressed FasL than in the normal controls ($p<0.05$) or A-T patients with no ATM activity ($p<0.05$). The percentage of CD56brightFasL+ NK cells was also significantly higher in the A-T patients with some ATM activity than the normal controls ($p<0.05$).

The results were similar to those for T cells in that whilst there were no correlations between age and percentage of FasL+ cells in the normal controls, the percentages of both CD56dimFasL+ and CD56brightFasL+ NK cells increased with age in the A-T patients with no ATM activity. (Fig 3:5:5D). There was also a significant negative correlation between the FasL MFI of CD56brightFasL+ NK cells and age in the normal controls.

Fig 3:5:5: There was no significant difference in FasL expression on NK cells from A-T patients with no ATM activity and normal controls.



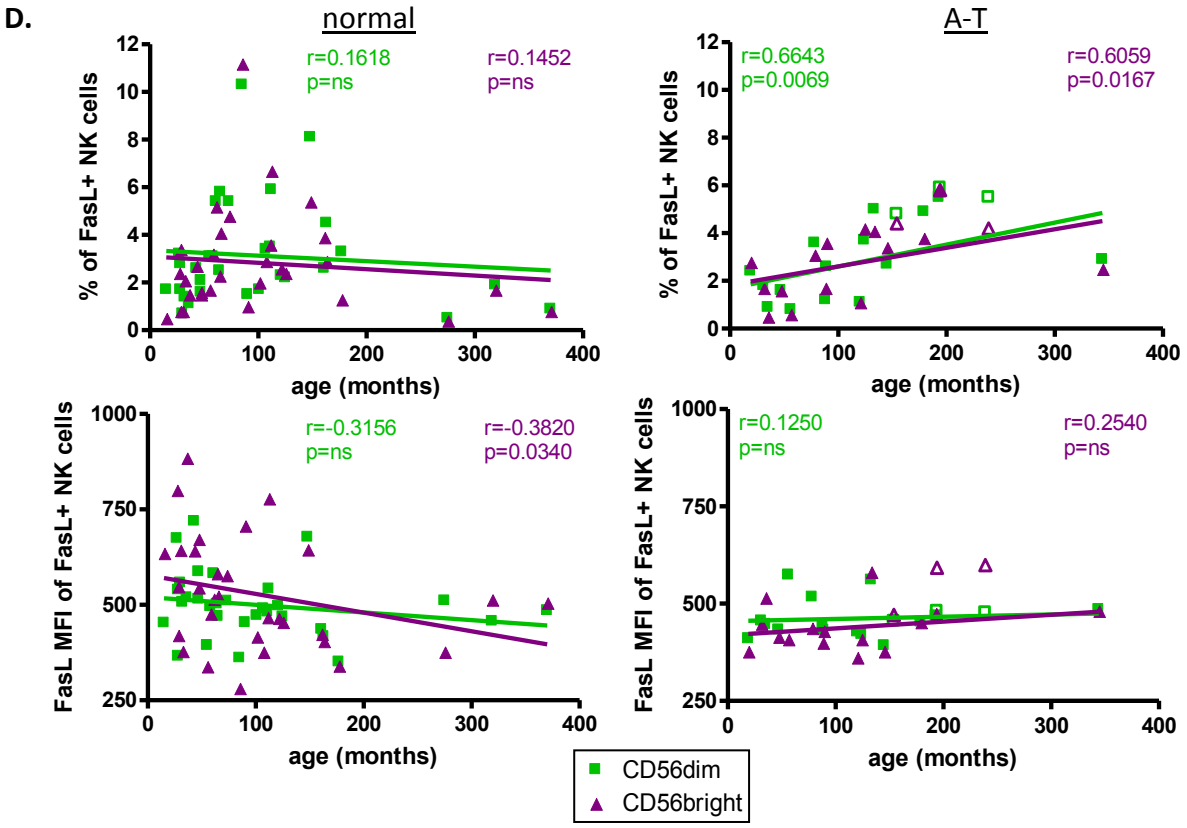


Fig 3:5:5: PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRll flow cytometer. Gating was carried out as described in Fig 3:3:1.

A. Representative histograms showing FasL expression on NK cell subsets in normal controls and A-T patients. Percentages of FasL+ve NK cells in CD56 bright (bright) and CD56dim (dim) populations are shown.

B. FasL expression on CD56dim NK cells as the percentage of CD56dim NK cells that express FasL and the FasL MFI of CD56dimFasL+ NK cells.

C. FasL expression on CD56bright NK cells as the percentage of CD56bright NK cells that express FasL and the FasL MFI of CD56brightFasL+ NK cells.

D. There were no significant correlation between the percentages of FasL+ve CD56dim or CD56bright NK cells and age in the normal controls (top left), however in the A-T patients with no ATM activity the percentages of both CD56dimFasL+ and CD56brightFasL+ NK cells increased with age (top right). There were no correlations between FasL MFI of CD56dim or CD56bright FasL+ve NK cells and age in A-T patients with no ATM activity (bottom right), but

the FasL MFI of CD56brightFasL+ cells decreased with age in the normal controls. To improve clarity and as there were no significant correlations linear regression trend lines and r and p values for the A-T patients with some ATM activity are not shown, however results of the correlation analysis for this group are summarised in Table 3:5:6. (A-T plots: open symbols = some ATM activity, closed symbols = no ATM activity).

3:5:6: FasL MFI was reduced on lymphocytes from A-T patients with no ATM activity compared to normal controls.

The results described in section 3:5 including significant differences between the expression of FasL on lymphocytes (percentage and FasL MFI of FasL+ cells) and significant correlations between FasL expression and age in A-T patients and normal controls are summarised in Table 3:5:6.

There was no significant difference in the percentage of FasL+ve cells of any type between normal controls and A-T patients with no ATM activity. However, the percentages of CD4+FasL+ effector memory T cells, CD8+FasL+ central memory T cells, FasL+NK cells and CD56dimFasL+ NK cells were significantly higher in A-T patients with some ATM activity than normal controls or A-T patients with no ATM activity. In addition the percentages of FasL+ve T cells, CD4+FasL+ T cells, CD4+FasL+ naive and central memory T cells, CD8+FasL+ naive and effector memory T cells and CD56brightFasL+ NK cells were significantly higher in A-T patients with some ATM activity than in the normal controls.

With the exception of NK cells and NK cell subsets, total T cells, CD4+ and CD8+ TEMRA T cells, and CD8+ effector memory T cells, the FasL MFI of FasL+ cells was increased in the normal controls compared to A-T patients with no ATM activity. The FasL MFI of FasL+ve NKT cells, B cells and B cell subsets were also significantly higher in normal controls than A-T patients. However, the FasL MFI of FasL+ total T cells was increased in A-T patients with some ATM activity compared to A-T patients with no ATM activity and normal controls.

A-T patients with no ATM activity showed positive correlations between age and the percentage of FasL+ cells in all NK and T cell subsets with the exception of CD4+ and CD8+ TEMRA T cells. A-T patients with some ATM activity showed no significant effect of age on

the percentage of FasL+ cells, although this may be due to the small sample size. The normal controls showed a negative correlation between age and the percentage of FasL+ NKT cells.

The effect of age on FasL MFI of FasL+ cells was less clear. In the normal controls there were positive correlations between age and FasL MFI of FasL+ total T cells and total and naive B cells and a negative correlation between age and FasL MFI of FasL+CD56bright NK cells. A-T patients with some ATM activity showed no significant correlations between FasL MFI of FasL+ cells and age. However in the A-T patients with no ATM activity the FasL MFI of FasL+ total B cells and B cell subsets decreased and the FasL MFI of FasL+ CD4+ and CD8+ naive T cells and CD4+ effector memory and TEMRA T cells increased with age.

Table 3:5:6: Significant differences in FasL expression and correlations with age in A-T patients and normal controls.

	significant differences in FasL expression on lymphocytes						correlations between FasL expression on lymphocytes and age					
	% FasL+			FasL MFI of FasL+ cells			% FasL+			FasL MFI of FasL+ cells		
	normal	A-T some ATM activity	A-T no ATM activity	normal	A-T some ATM activity	A-T no ATM activity	normal	A-T some ATM activity	A-T no ATM activity	normal	A-T some ATM activity	A-T no ATM activity
T cells	-	+	ns	-	+	-	ns	ns	+	+	ns	ns
CD4+ T cells	-	+	ns	+	ns	-	ns	ns	+	ns	ns	ns
CD4+ naive	-	+	ns	+	ns	-	ns	ns	+	ns	ns	+
CD4+ central memory	-	+	ns	+	ns	-	ns	ns	+	ns	ns	ns
CD4+ effector memory	-	+	-	+	ns	-	ns	ns	+	ns	ns	+
CD4+ TEMRA	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	+
CD8+ T cells	ns	ns	ns	+	ns	-	ns	ns	+	ns	ns	ns
CD8+ naive	-	+	ns	+	ns	-	ns	ns	+	ns	ns	+
CD8+ central memory	-	+	-	+	ns	-	ns	ns	+	ns	ns	ns
CD8+ effector memory	-	+	ns	ns	ns	ns	ns	ns	+	ns	ns	ns
CD8+ TEMRA	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
B cells	ns	ns	ns	+	-	-	ns	ns	ns	+	ns	-
naive B cells	ns	ns	ns	+	-	-	ns	ns	ns	+	ns	-
memory B cells	ns	ns	ns	+	-	-	ns	ns	ns	ns	ns	-
NK cells	-	+	-	ns	ns	ns	ns	ns	+	ns	ns	ns
CD56dim NK cells	-	+	-	ns	ns	ns	ns	ns	+	ns	ns	ns
CD56bright NK cells	-	+	ns	ns	ns	ns	ns	ns	+	-	ns	ns
NKT cells	ns	ns	ns	+	-	-	-	ns	ns	ns	ns	ns

Table 3:5:6: Summary of significant differences between the expression of FasL (percentage and FasL MFI of FasL+ cells) and significant correlations between FasL expression and age in A-T patients and normal controls.

In the first part of the table (significant differences in FasL expression on lymphocytes) cells containing a positive symbol (+) and highlighted in yellow indicate a significantly higher percentage or FasL MFI of FasL+ cells of the particular cell type in the corresponding group than in the group with a cell containing a negative symbol (-) and highlighted in blue, 'ns' refers to no significant difference with any group. The second part of the table summarises significant correlations between FasL expression and age. Yellow (+) cells indicate a positive correlation and blue (-) cells indicate a negative correlation, 'ns' indicates no significant correlation.

3:6: CD127 expression.

3:6:1: CD127 expression on lymphocyte subsets.

The final antibody included in the 11 colour panel was CD127 which binds to an important component of the IL-7 receptor (IL-7Rα). Cells which lack CD127 expression cannot bind IL-7; therefore expression of CD127 regulates the proliferative response of cells to the cytokine. As A-T patients are lymphopenic they are likely to show increased availability of IL-7, however a deficiency in CD127 expression on A-T lymphocytes could prevent the cells from responding to the cytokine and potentially contribute to lymphopenia.

Abnormal CD127 expression on T cells from A-T patients could potentially influence sensitivity to CD95-mediated apoptosis as IL-7 may be involved in upregulation of CD95 on T cells (Brugnoni et al., 1999;Rethi et al., 2008). A high level of CD127 expression could therefore lead to increased binding of IL-7 causing upregulation of CD95, increased sensitivity to CD95-mediated apoptosis and potentially lymphopenia. Conversely CD127 deficiency could potentially prevent CD95 upregulation in response to IL-7 and so increase the resistance of T cells to CD95-mediated apoptosis.

CD127 expression was analysed on lymphocyte subsets from normal controls and A-T patients. As expression was low and it was difficult to clearly distinguish positive and negative populations, the CD127 MFI of all cells was compared rather than the CD127 MFI of CD127+ve cells.

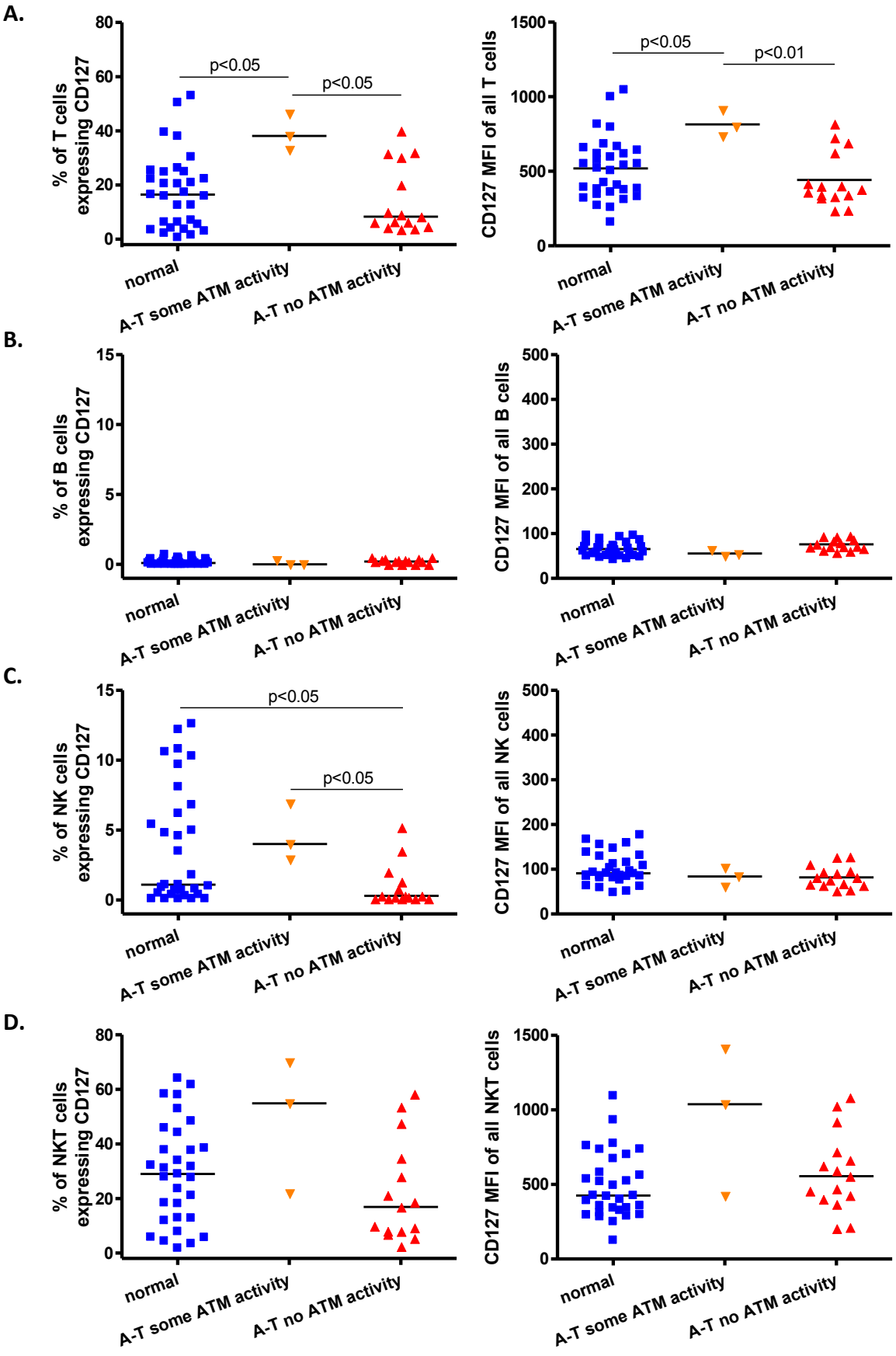
A median of 15.5% of T cells in the normal controls expressed CD127. There was no significant difference in CD127 expression on T cells between the normal controls and A-T patients with no ATM activity. However, a significantly higher percentage of T cells in A-T patients with some ATM activity expressed CD127 than normal controls ($p<0.05$) and A-T

patients with no ATM activity ($p<0.05$). The CD127 MFI of all T cells was also significantly higher in the A-T patients with some ATM activity than in the A-T patients with no ATM activity ($p<0.01$) and the normal controls ($p<0.05$) (Fig 3:6:1A).

Although CD127 is expressed on pro- and pre-B cells it is not expressed on mature B cells (reviewed in (Mazzucchelli and Durum, 2007)), therefore it was unsurprising that CD127 expression was not detected on B cells in A-T patients or normal controls (Fig 3:6:1B). Expression was low on NK cells (median of 1.1% CD127+ve in the normal controls) but the percentage of CD127+ve cells was significantly higher in the normal controls ($p<0.05$) and A-T patients with some ATM activity ($p<0.05$) than in the A-T patients with no ATM activity (Fig 3:6:1C). CD127 expression was highest on NKT cells (median of 29% CD127+ in the normal controls) but there was no significant difference between A-T patients and normal controls (Fig 3:6:1D).

There was no significant correlation between CD127 expression and age on the majority of cell types. However in the normal controls both the percentage of CD127+ T cells and CD127 MFI of all T cells increased significantly with age (Fig 3:6:1E). This suggests that the ability of normal T cells from healthy individuals to proliferate in response to IL-7 may increase with age over the 0 to 30 year age range.

Fig 3:6:1: CD127 expression was increased on T cells from A-T patients with some ATM activity.



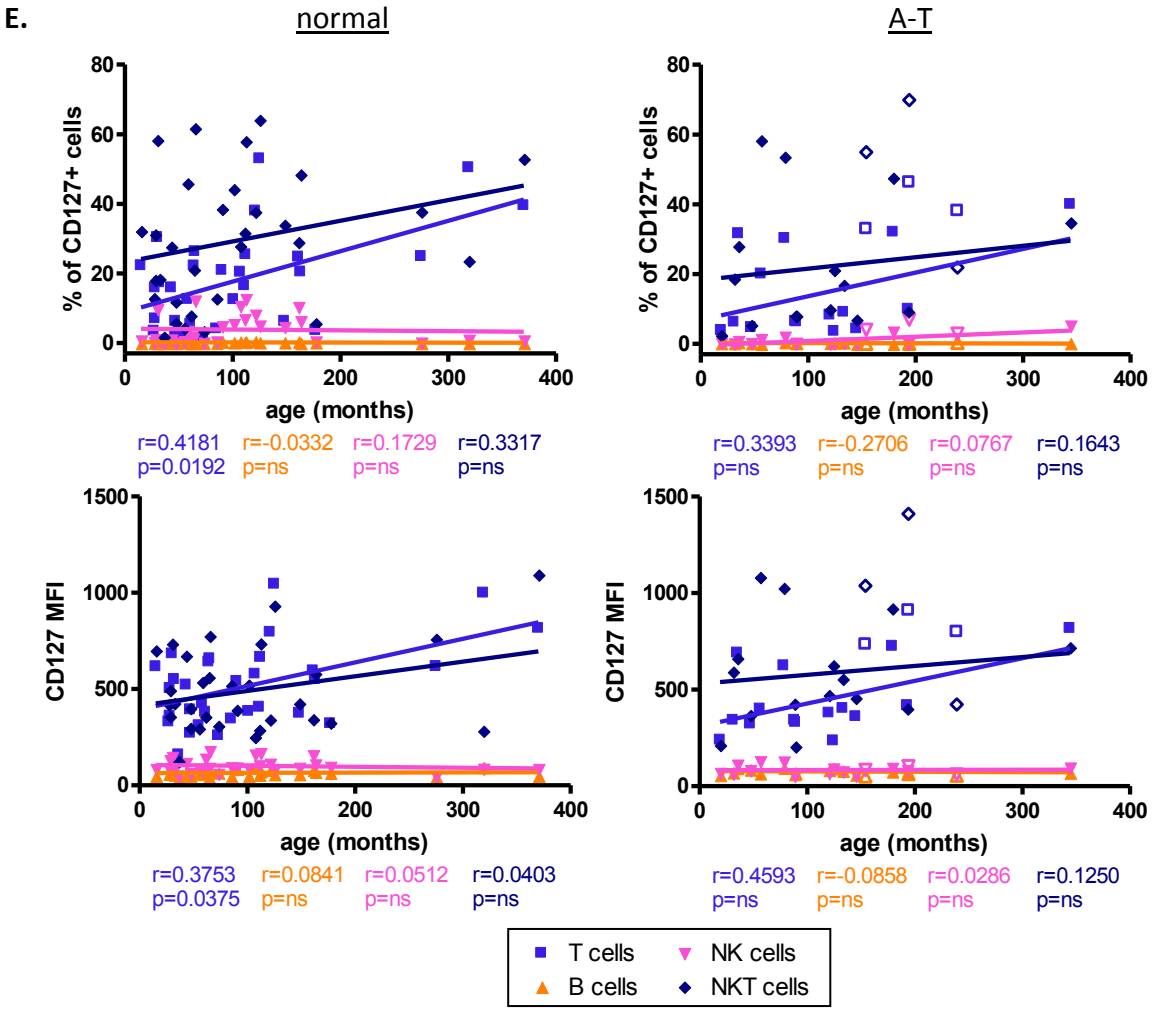


Fig 3:6:1: PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRII flow cytometer. Gating was carried out as described in Fig 3:3:1.

A. CD127 expression on T cells as the percentage of T cells that express CD127 and the CD127 MFI of all T cells.

B. CD127 expression on B cells as the percentage of B cells that express CD127 and the CD127 MFI of all B cells.

C. CD127 expression on NK cells as the percentage of NK cells that express CD127 and the CD127 MFI of all NK cells.

D. CD127 expression on NKT cells as the percentage of NKT cells that express CD127 and the CD127 MFI of all NKT cells.

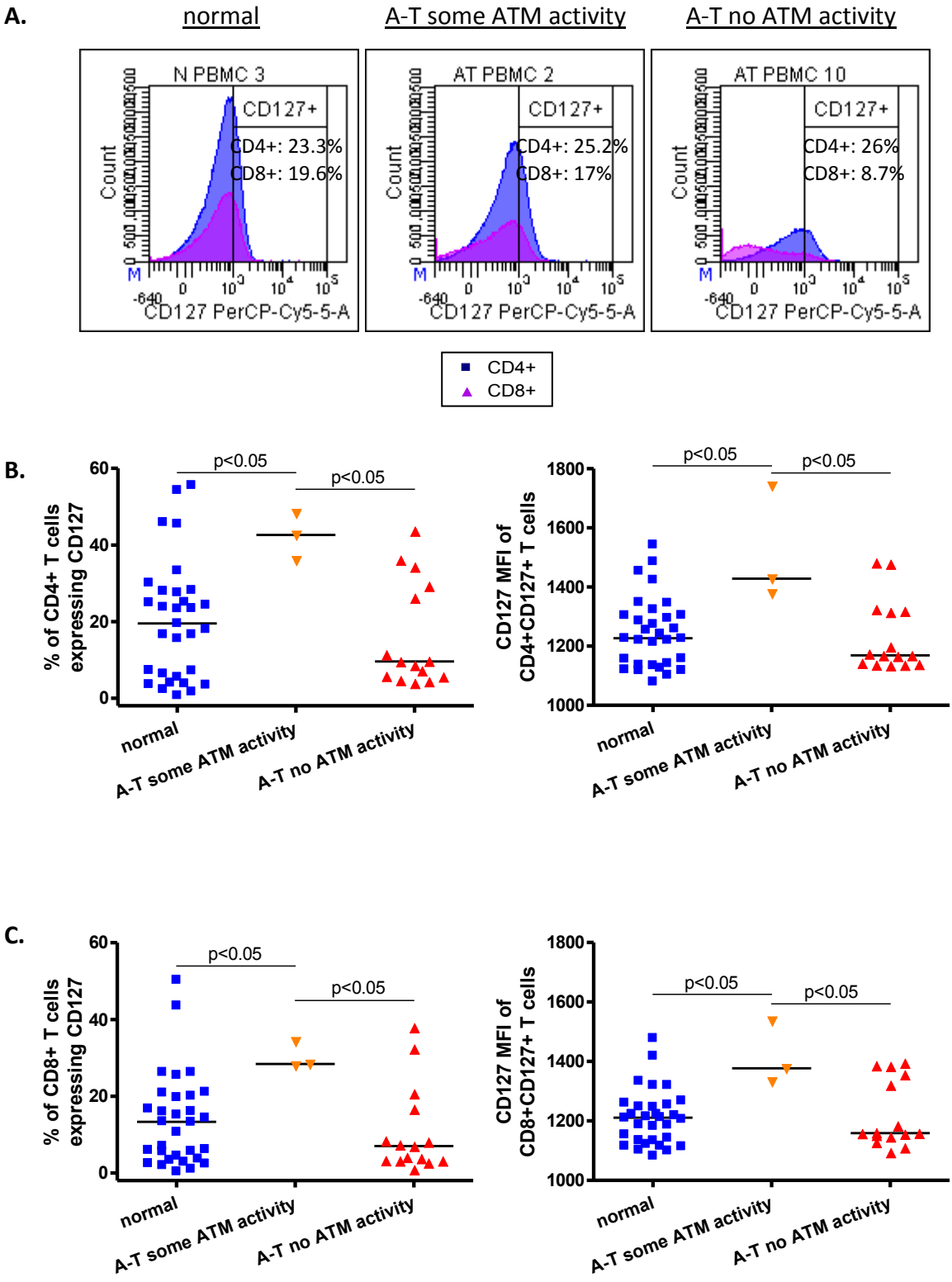
E. In the normal controls both the percentage of CD127+ve T cells (top left) and the CD127 MFI of all T cells (bottom left) increased significantly with age. There were no other correlations between percentage of CD127+ve cells or CD127 MFI of cells of any cell type and age in A-T patients or normal controls. To improve clarity and as there were no significant correlations linear regression trend lines and r and p values for the A-T patients with some ATM activity are not shown, however results of the correlation analysis for this group are summarised in Table 3:6:5. (A-T plots: open symbols = some ATM activity, closed symbols = no ATM activity).

3:6:2: CD127 expression on CD4+ and CD8+ T cells.

CD127 expression on CD4+ and CD8+ T cells was very similar (Fig 3:6:2A-C). There was no significant difference in CD127 expression on CD4+ve or CD8+ve T cells from A-T patients with no ATM activity compared to normal controls (Fig 3:6:2B&C). However, both the percentages and CD127MFI of CD4+CD127+ and CD8+CD127+ T cells were significantly higher in A-T patients with some ATM activity than A-T patients with no ATM activity or normal controls (all $p<0.05$).

The A-T patients with no ATM activity showed no correlations between CD127 expression on CD4+ or CD8+ T cells and age, however in the normal controls both the percentage and CD127 MFI of CD4+CD127+ T cells and the CD127 MFI of CD8+CD127+ T cells increased with age (Fig 3:6:2D).

Fig 3:6:2: CD127 expression on CD4+ve and CD8+ve T cells was increased in A-T patients with some ATM activity.



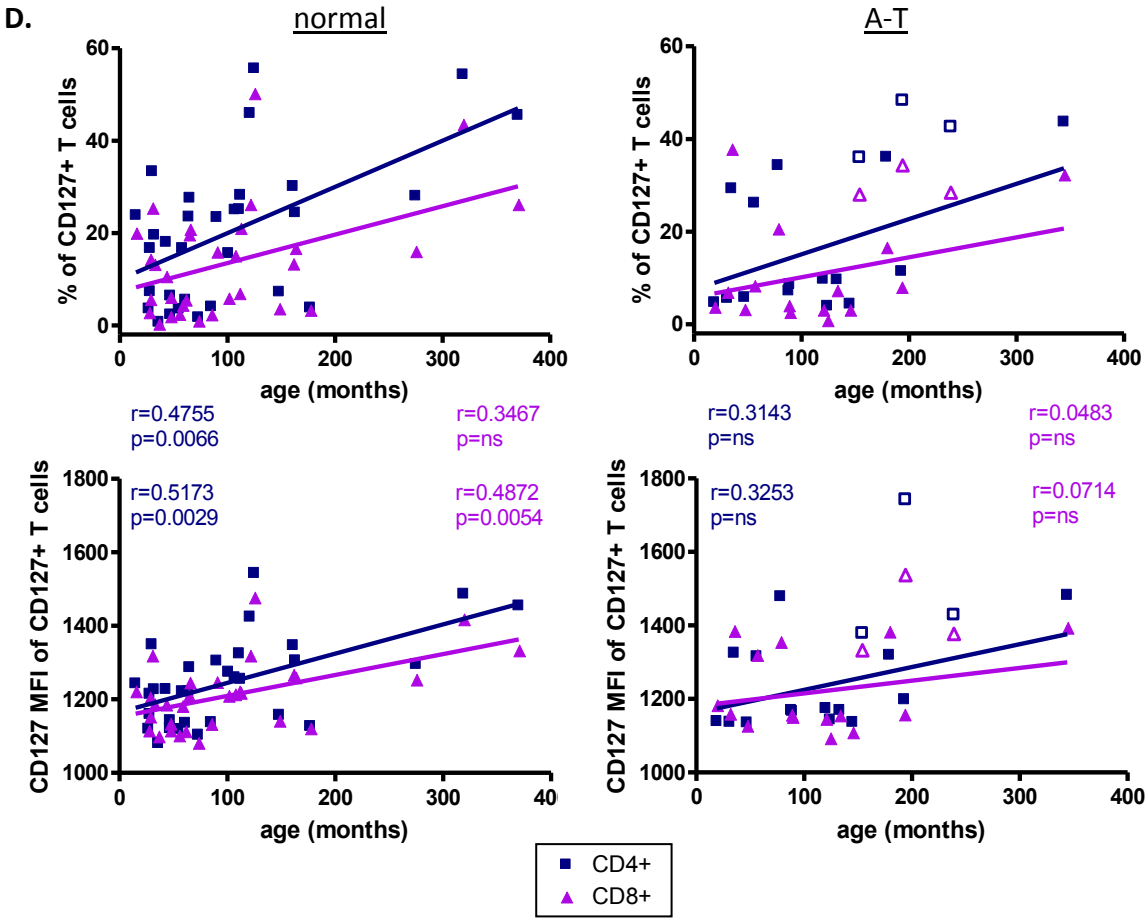


Fig 3:6:2: PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRll flow cytometer. Gating was carried out as described in Fig 3:3:1.

A. Representative histograms showing CD127 expression on CD4+ve and CD8+ve T cells in normal controls (N PBMC3 - 5 years 4 months), A-T patients with some ATM activity (AT PBMC2 - 12 years 10 months) and A-T patients with no ATM activity (AT PBMC10 - 4 years 8 months). Percentages of CD4+ve and CD8+ve cells which express CD127 are shown.

B. CD127 expression on CD4+ve T cells as the percentage of CD4+ve cells that express CD127 and the CD127 MFI of CD4+CD127+ T cells.

C. CD127 expression on CD8+ve T cells as the percentage of CD8+ve cells that express CD127 and the CD127 MFI of CD8+CD127+ T cells.

D. There were significant positive correlations between age and the percentage (top left) and CD127 MFI (bottom left) of CD4+CD127+ T cells and also the CD127 MFI of CD8+CD127+ T cells in the normal controls. There were no correlations between the percentage or CD127

MFI of CD4+CD127+ or CD8+CD127+ T cells and age in A-T patient (top and bottom right). To improve clarity, and as there were no significant correlations, linear regression trend lines and r and p values for the A-T patients with some ATM activity are not shown, however results of the correlation analysis for this group are summarised in Table 3:6:5. (A-T plots: open symbols = some ATM activity, closed symbols = no ATM activity).

3:6:3: CD127 expression on naive and memory T cell subsets.

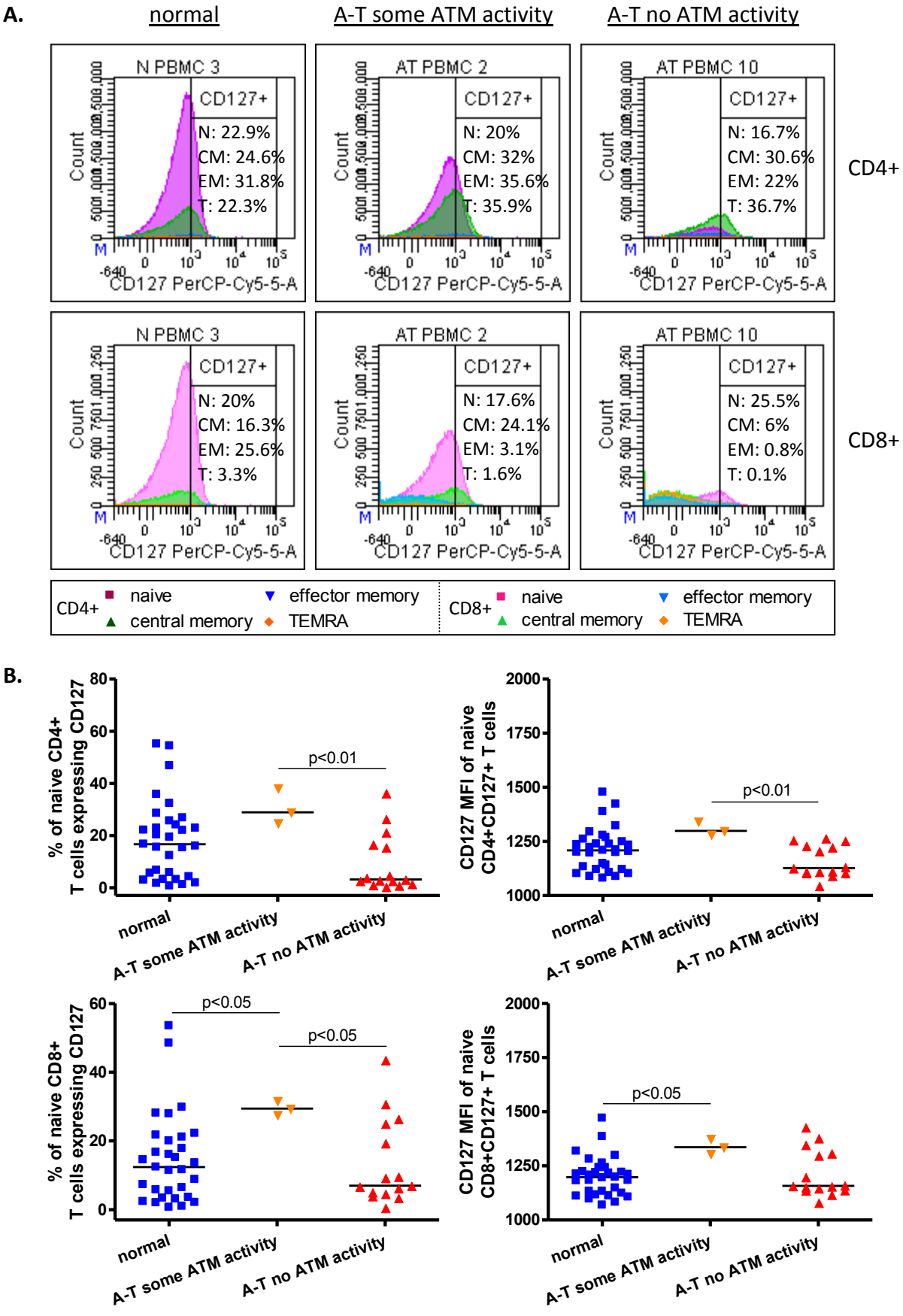
Analysis of CD127 expression on naive and memory T cells subsets revealed differences between CD4+ve and CD8+ve T cells. Overall CD127 expression was higher on the CD4+ve T cell subsets than CD8+ve T cell subsets. CD127 expression on CD4+ve T cells (measured both as percentage of positive cells and CD127 MFI) was high on central and effector memory T cells and low on naive cells whereas on CD8+ve T cells CD127 expression was highest on the naive cells and lower on the memory cells. This was true of both the normal controls and A-T patients with no ATM activity (Fig 3:6:3A&F).

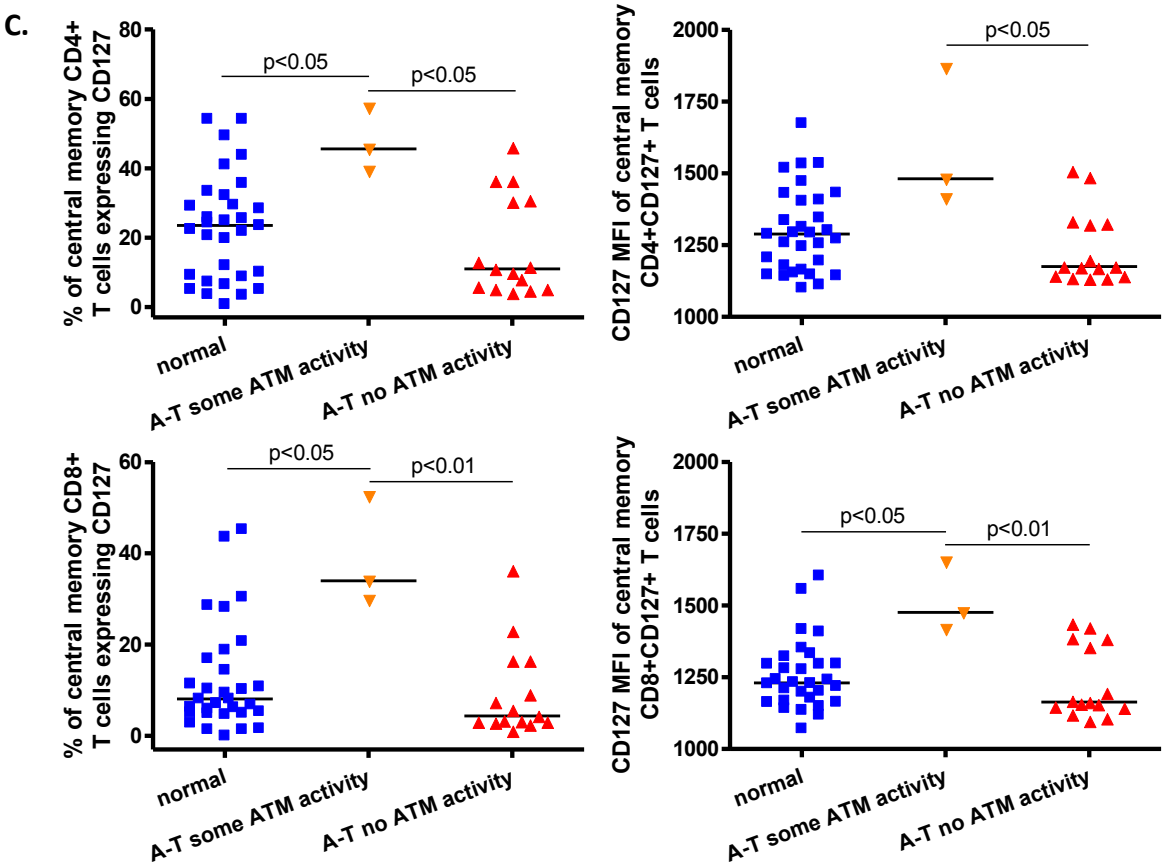
There was no significant difference in CD127 expression on CD4+ve or CD8+ve T cells of any subset between the normal controls and A-T patients with no ATM activity. However, the percentages of CD8+CD127+ naive (normal - $p<0.05$, A-T no ATM activity - $p<0.05$) and central memory (normal - $p<0.05$, A-T no ATM activity - $p<0.01$) and CD4+CD127+ central memory (normal - $p<0.05$, A-T no ATM activity - $p<0.05$) T cells were significantly higher in the A-T patients with some ATM activity than A-T patients with no ATM activity or normal controls. The same was true of the CD127 MFI of CD8+CD127+ central (normal - $p<0.05$, A-T no ATM activity - $p<0.01$) and effector memory (normal - $p<0.05$, A-T no ATM activity - $p<0.01$) T cells. In addition the A-T patients with some ATM activity had significantly higher CD127 expression (percentage, CD127MFI or both) on all CD4+ve T cell subsets than A-T patients with no ATM activity (Fig 3:6:3B-E) and the CD127 MFI of CD8+CD127+ naive T cells was higher in A-T patients with some ATM activity than in the normal controls ($p<0.05$) (Fig 3:6:3C).

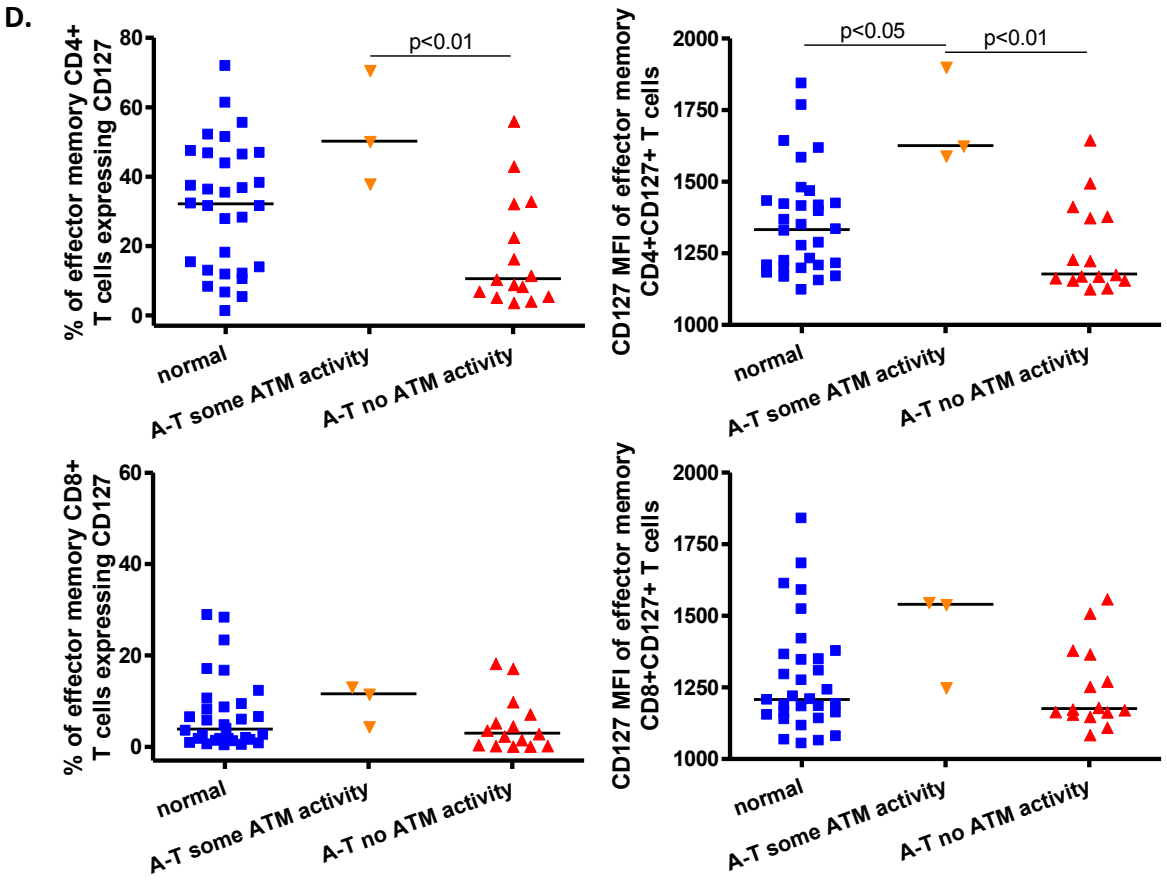
Consistent with the increase in CD127 expression on CD4+ and CD8+ T cells with age (Fig 3:6:2D) the percentages of CD4+CD127+ naive, central memory and TEMRA cells and CD8+CD127+ central memory, effector memory and TEMRA cells in the normal controls

correlated positively with age. There were also positive correlations between age and the CD127 MFI of all CD4+ve T cells subsets and the CD127 MFI of CD8+CD127+ naive and central memory cells. There were no correlations between CD127 expression and age on any CD4+ve T cell subset in the A-T patients, however there was a positive correlation between age and the percentage of CD8+CD127+ effector memory T cells in the A-T patients with no ATM activity (Fig 3:6:3G&H).

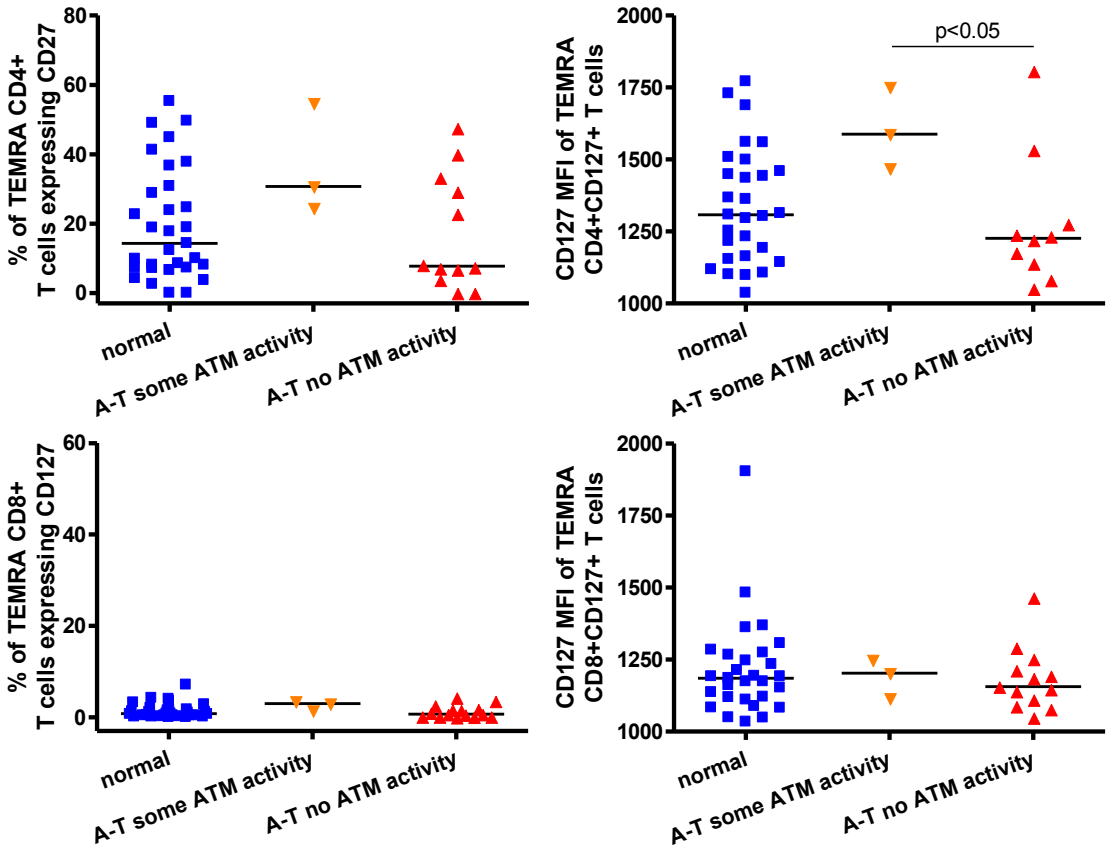
Fig 3:6:3: CD127 expression on T cell subsets in normal controls increased with age.




C.
% of central memory CD4+ T cells expressing CD127
p<0.05
p<0.05
normal
A-T some ATM activity
A-T no ATM activity
CD127 MFI of central memory CD4+CD127+ T cells
p<0.05
% of central memory CD8+ T cells expressing CD127
p<0.05
p<0.01
CD127 MFI of central memory CD8+CD127+ T cells
p<0.05
p<0.01

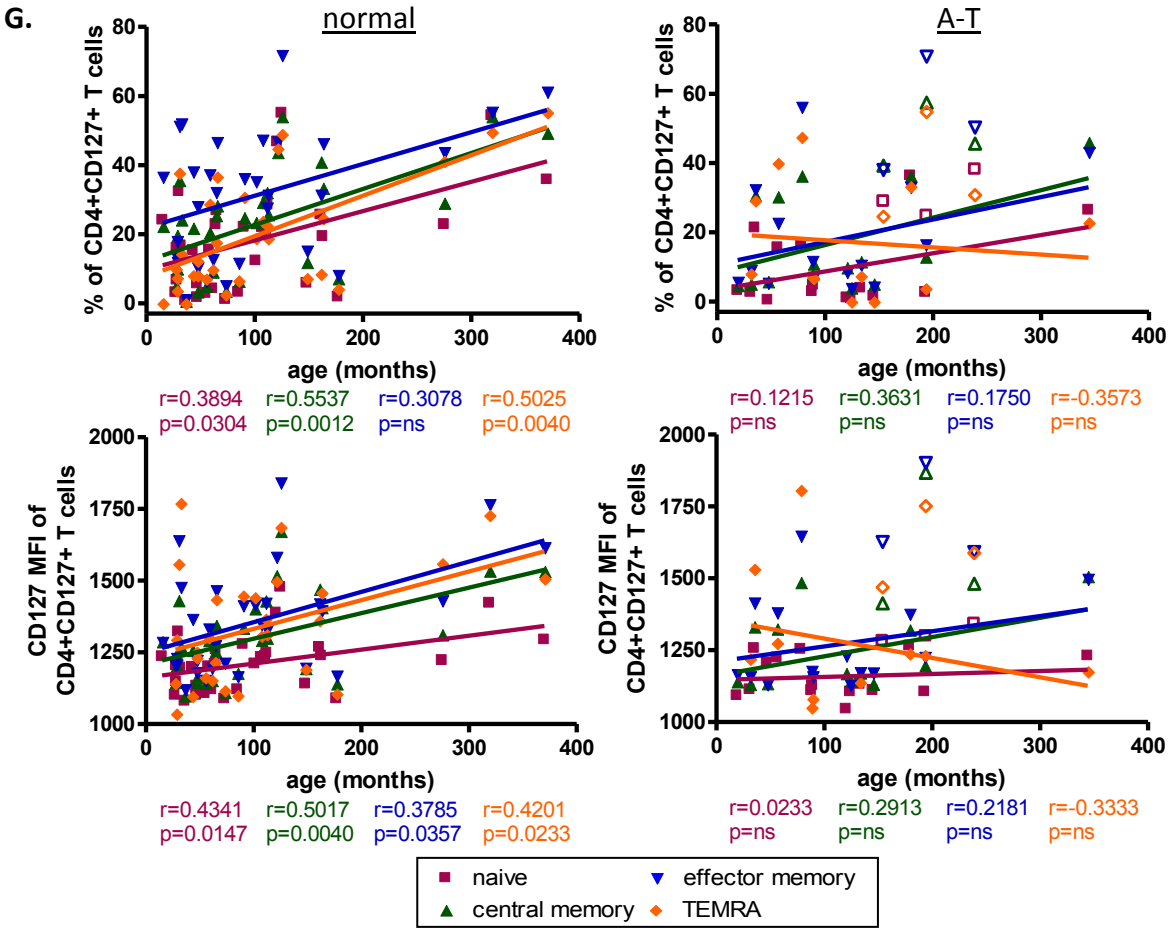

D.
% of effector memory CD4+ T cells expressing CD127
p<0.01
normal
A-T some ATM activity
A-T no ATM activity
CD127 MFI of effector memory CD4+CD127+ T cells
p<0.05
p<0.01
% of effector memory CD8+ T cells expressing CD127
CD127 MFI of effector memory CD8+CD127+ T cells

E.



F.

	normal				A-T some ATM activity				A-T no ATM activity			
	naive	central memory	effector memory	TEMRA	naive	central memory	effector memory	TEMRA	naive	central memory	effector memory	TEMRA
median % CD4+CD127+	16.7	23.5	32.2	14.3	28.9	45.6	50.2	30.7	3.2	11.0	10.6	7.75
median CD127 MFI of CD4+ cells	1209	1289	1333	1308	1299	1481	1626	1588	1127	1175	1178	1227
median % CD8+CD127+	12.4	8.1	3.9	0.8	29.4	34.0	11.6	3.0	7.0	4.4	3.0	0.7
median CD127 MFI of CD8+ cells	1198	1231	1208	1185	1336	1476	1540	1203	1158	1164	1176	1156


G.
normal
A-T
% of CD4+CD127+ T cells
CD127 MFI of CD4+CD127+ T cells
age (months)
r=0.3894 r=0.5537 r=0.3078 r=0.5025
p=0.0304 p=0.0012 p=ns p=0.0040
r=0.1215 r=0.3631 r=0.1750 r=-0.3573
p=ns p=ns p=ns p=ns
r=0.4341 r=0.5017 r=0.3785 r=0.4201
p=0.0147 p=0.0040 p=0.0357 p=0.0233
r=0.0233 r=0.2913 r=0.2181 r=-0.3333
p=ns p=ns p=ns p=ns
naive
effector memory
central memory
TEMRA

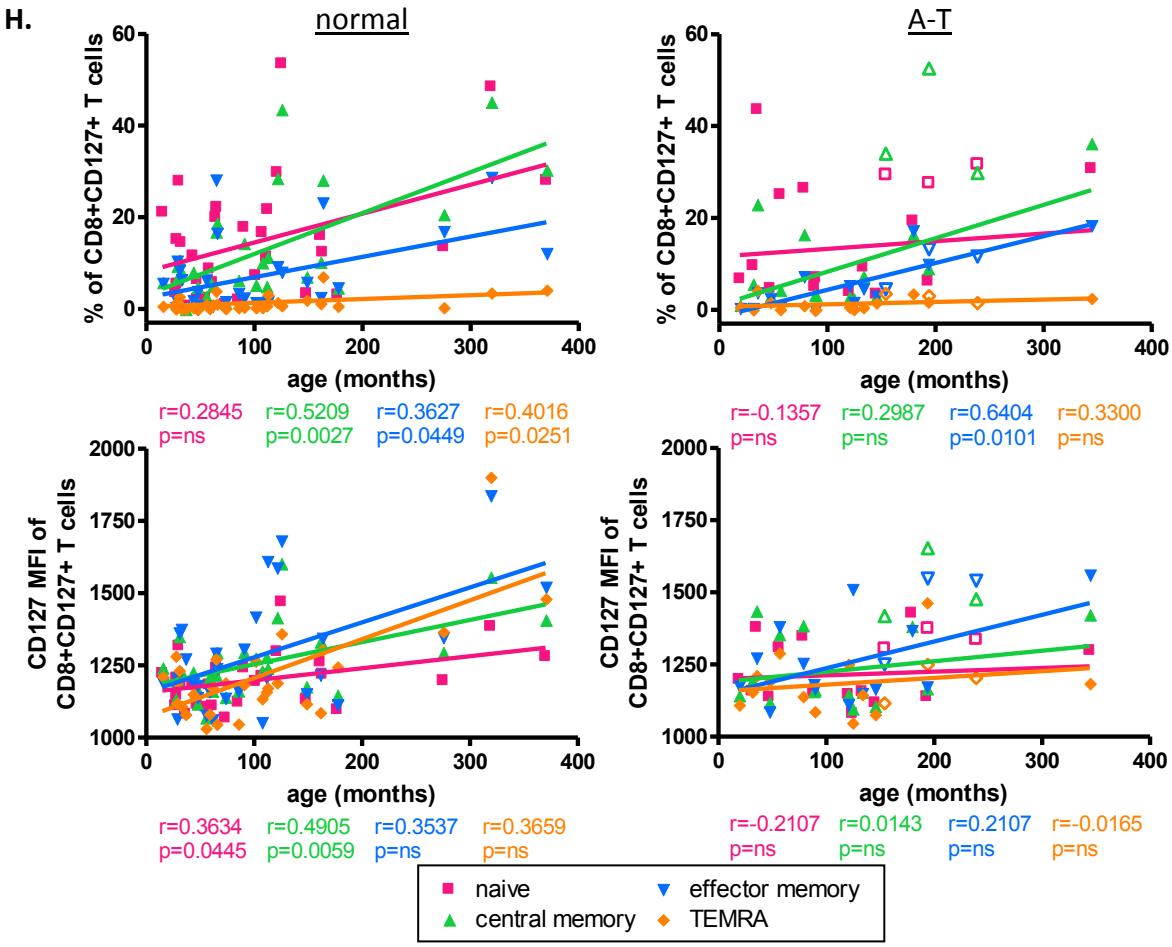

H.
normal
A-T
% of CD8+CD127+ T cells
CD127 MFI of CD8+CD127+ T cells
age (months)
r=0.2845 r=0.5209 r=0.3627 r=0.4016
p=ns p=0.0027 p=0.0449 p=0.0251
r=-0.1357 r=0.2987 r=0.6404 r=0.3300
p=ns p=ns p=0.0101 p=ns
r=0.3634 r=0.4905 r=0.3537 r=0.3659
p=0.0445 p=0.0059 p=ns p=ns
r=-0.2107 r=0.0143 r=0.2107 r=-0.0165
p=ns p=ns p=ns p=ns
naive
effector memory
central memory
TEMRA

Fig 3:6:3: PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRII flow cytometer. Gating was carried out as described in Fig 3:3:1. For correlation analysis, to improve clarity and as there were no significant correlations, linear regression trend lines and r and p values for the A-T patients with some ATM activity are not shown, however results of the correlation analysis for this group are summarised in Table 3:6:5. (A-T plots: open symbols = some ATM activity, closed symbols = no ATM activity).

A. Representative histograms showing CD127 expression on CD4+ve and CD8+ve T cell subsets in normal controls (N PBMC3 - 5 years 4 months), A-T patients with some ATM activity (AT PBMC2 - 12 years 10 months) and A-T patients with no ATM activity (AT PBMC10 - 4 years 8 months). Percentages of CD127+ve T cells in naive (N), central memory (CM), effector memory (EM) and TEMRA (T) subsets are shown.

B. CD127 expression on naive CD4+ve and CD8+ve T cells as the percentage of naive CD4+ve and CD8+ve T cells that express CD127 and the CD127 MFI of naive CD4+CD127+and CD8+CD127+ T cells.

C. CD127 expression on central memory CD4+ve and CD8+ve T cells as the percentage of central memory CD4+ve and CD8+ve cells that express CD127 and the CD127 MFI of central memory CD4+CD127+ and CD8+CD127+ T cells.

D. CD127 expression on effector memory CD4+ve and CD8+ve T cells as the percentage of effector memory CD4+ve and CD8+ve T cells that express CD127 and the CD127 MFI of effector memory CD4+CD127+ and CD8+CD127+ T cells.

E. CD127 expression on TEMRA CD4+ve and CD8+ve T cells as the percentage of TEMRA CD4+ve and CD8+ve T cells that express CD127 and the CD127 MFI of TEMRA CD4+CD127+ and CD8+CD127+ T cells.

F. Median percentages of naive and memory CD4+ve and CD8+ve T cells expressing CD127 and median CD127 MFI of naive and memory CD4+ve and CD8+ve T cells in normal controls and A-T patients.

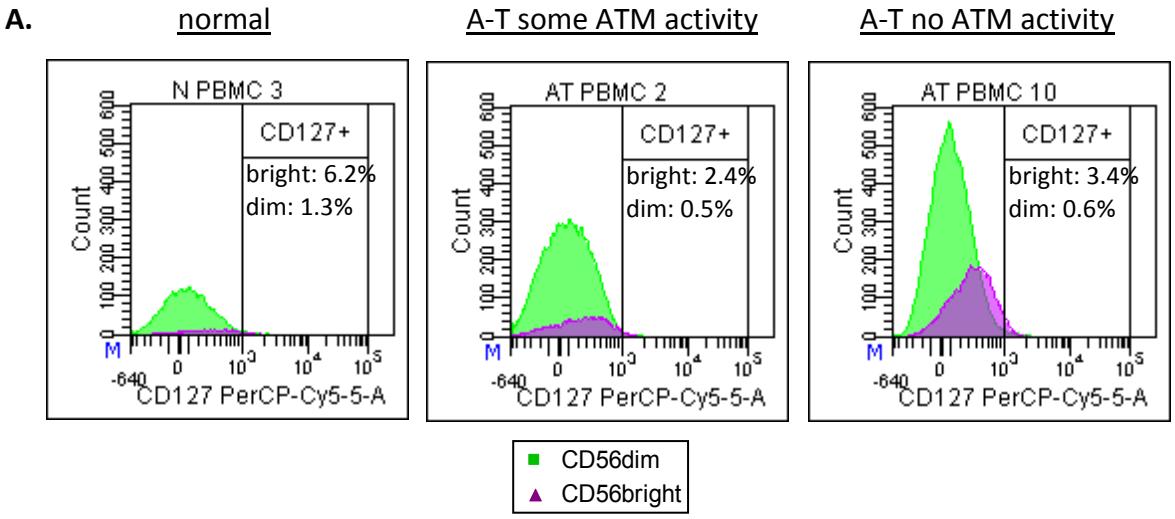
G. There were significant positive correlations between age and the percentages of CD4+CD127+ naive, central memory and TEMRA cells (top left) and the CD127 MFI of CD4+CD127+ cells of all subsets (bottom left) in the normal controls. The A-T patients showed no correlations between CD127 expression and age (top and bottom right).

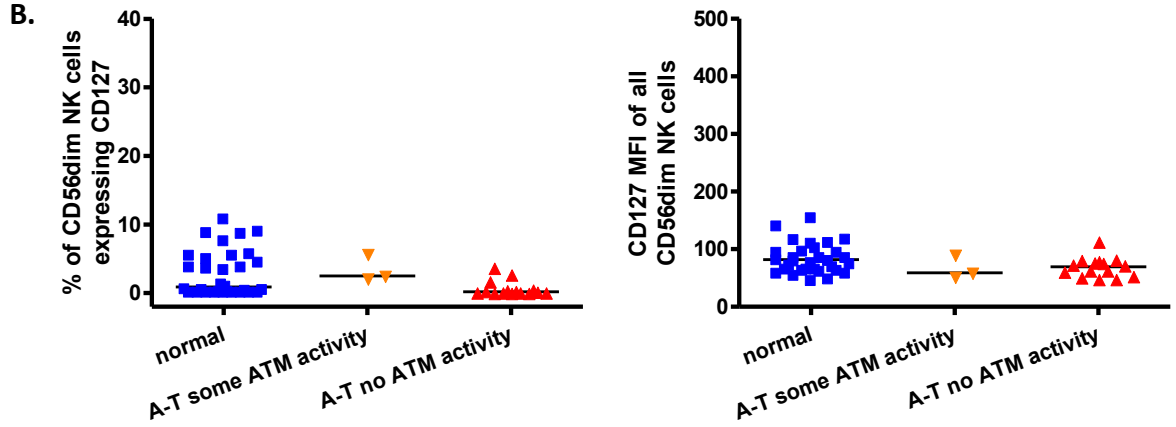
H. There were significant positive correlations between age and the percentages of CD8+CD127+ central memory, effector memory and TEMRA T cells and also the CD127 MFI of CD8+CD127+ naive and central memory T cells in the normal controls. In the A-T patients with no ATM activity there was a significant correlation between age and the percentage of CD8+CD127+ effector memory T cells but no correlations with CD127 MFI of CD8+CD127+ T cells of any subset.

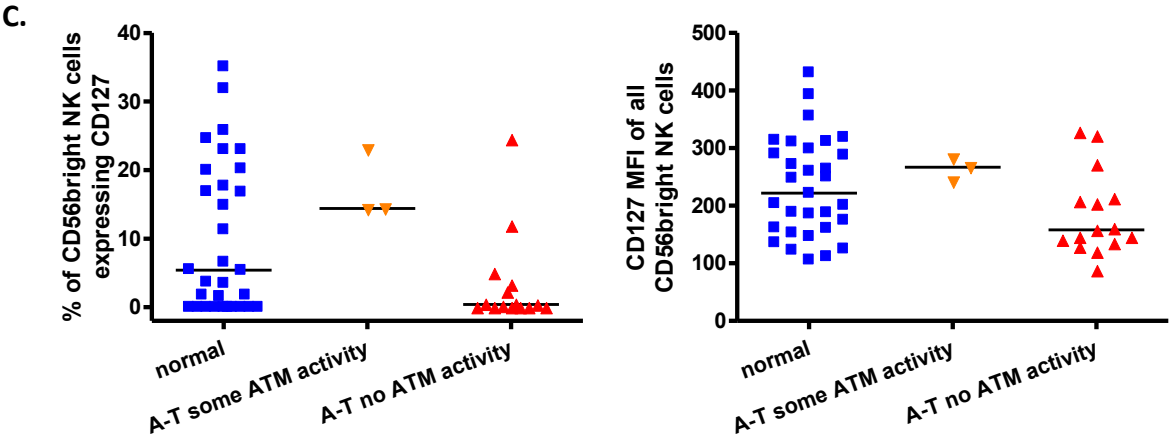
3:6:4: CD127 expression on CD56dim and CD56bright NK cells.

Finally CD127 expression on CD56dim and CD56bright NK cells was analysed. CD127 expression was higher on the CD56bright cells (Fig 3:6:4A&C), however there were no significant differences between CD127 expression on either CD56dim (Fig 3:6:4B) or CD56bright (Fig 3:6:4C) NK cells between the A-T patients and normal controls. There were positive correlation between age and both the percentage of CD56brightCD127+ NK cells and CD127 MFI of all CD56bright NK cells in the normal controls but no significant correlations in the A-T patients (Fig 3:6:5D).

Fig 3:6:4: There was no significant difference in CD127 expression on NK cells from A-T patients and normal controls.







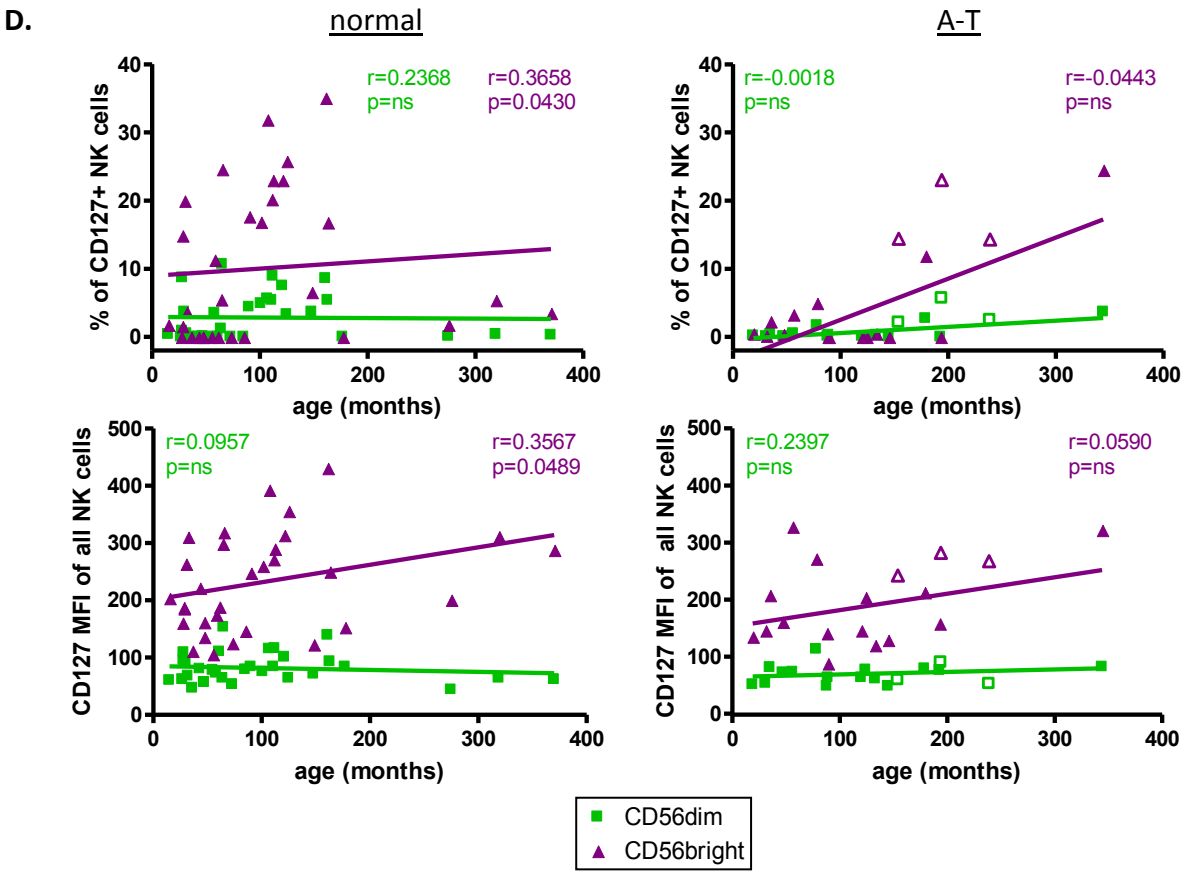


Fig 3:6:4: PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRll flow cytometer. Gating was carried out as described in Fig 3:3:1.

A. Representative histograms showing CD127 expression on NK cell subsets in normal controls (N PBMC3 - 5 years 4 months), A-T patients with some ATM activity (AT PBMC2 - 12 years 10 months) and A-T patients with no ATM activity (AT PBMC10 - 4 years 8 months). Percentages of FasL+ve NK cells in CD56 bright (bright) and CD56dim (dim) populations are shown.

B. CD127 expression on CD56dim NK cells as the percentage of CD56dim NK cells that express CD127 and the CD127 MFI of all CD56dim NK cells.

C. CD127 expression on CD56bright NK cells as the percentage CD56bright NK cells that express CD127 and the CD127 MFI of all CD56bright NK cells.

D. There were significant correlations between age and both the percentage of CD56brightCD127+ NK cells and CD127MFI of all CD56bright NK cells in the normal controls

(top and bottom left) but no correlations in A-T patients (top and bottom right). To improve clarity, and as there were no significant correlations, linear regression trend lines and r and p values for the A-T patients with some ATM activity are not shown, however results of the correlation analysis for this group are summarised in Table 3:6:5. (A-T plots: open symbols = some ATM activity, closed symbols = no ATM activity).

3:6:5: Lymphocytes from A-T patients with no ATM activity express normal levels of CD127.

The results of section 3:6 including significant differences between the expression of CD127 on lymphocyte subsets (percentage of CD127+ve cells and CD127 MFI) and significant correlations between CD127 expression and age in A-T patients and normal controls are summarised in Table 3:6:5.

The percentage of CD127+ve NK cells was increased in normal controls compared to A-T patients with no ATM activity, however in all other lymphocyte subsets there was no significant difference between these two groups in terms of CD127 MFI and percentage of CD127+ cells.

CD127 expression was increased on lymphocytes of several subsets in A-T patients with some ATM activity. The percentages of CD127+ve T cells, CD4+CD127+ T cells, CD4+CD127+ central memory T cells, CD8+CD127+ T cells and CD8+CD127+ naive and central memory T cells were all significantly increased in A-T patients with some ATM activity compared to both A-T patients with no ATM activity and normal controls. The percentages of CD4+CD127+ naive and effector memory T cells were also significantly higher in A-T patients with some ATM activity than A-T patients with no ATM activity and the percentage of CD127+ NK cells was significantly higher in both normal controls and A-T patients with some ATM activity than A-T patients with no ATM activity.

Analysis of the CD127 MFI of CD127+ lymphocytes gave similar results. The CD127 MFI of total T cells, CD4+ T cells, CD4+ effector memory T cells, CD8+ T cells and CD8+ central memory T cells were significantly higher in A-T patients with some ATM activity than both A-T patients with no ATM activity and normal controls. The CD127 MFI of CD4+CD127+ naive,

central memory and TEMRA T cells were also significantly higher in A-T patients with some ATM activity than A-T patients with no ATM activity and the CD127 MFI of CD8+CD127+ naive T cells was significantly higher in A-T patients with some ATM activity than normal controls. However as there were only three A-T patients with some ATM activity and their median age was older than that of the other two groups the increased CD127 expression may not be representative of a larger group of A-T patients with some ATM activity.

In the normal controls CD127 expression on T cells increased with age. There were positive correlations between age and the percentages of CD127+ T cells, CD4+CD127+ T cells, CD4+CD127+ naive, central memory and TEMRA T cells, CD8+ central memory, effector memory and TEMRA T cells. There were also positive correlations between age and the CD127 MFI of T cells, CD4+ T cells and subsets and CD8+ naive and central memory T cells in the normal controls. CD127 expression on CD56bright NK cells (both percentage of CD127+ cells and CD127MFI) also correlated positively with age in the normal controls.

The A-T patients with some ATM activity showed no correlations between age and CD127 expression on any lymphocyte subset, however the age range of the patients in this group (12 years 10 months to 19 years 11 months) was considerably smaller than in the normal controls (1 year 3 months to 30 years 10 months). In A-T patients with no ATM activity the percentage of CD127+ CD8+ effector memory T cells correlated positively with age. These findings suggest that in healthy individuals the ability of T cells to bind IL-7 and proliferate in response to the cytokine may increase with age, perhaps in order to compensate for the age related decline in output of T cells from the thymus.

As CD127 expression tended to increase with age it is possible that the increased expression on lymphocytes from A-T patients with some ATM activity compared to normal controls and A-T patients with no ATM activity is related to their older median age. If the sample size of

the A-T patients with some ATM activity was increased significant correlations between CD127 expression and age may be seen.

There was no evidence that either the high CD95 expression or lymphopenia of A-T patients was related to abnormal expression of CD127 resulting in increased or decreased ability to respond to IL-7.

Table 3:6:5: Significant differences in CD127 expression on lymphocytes and correlations with age in A-T patients and normal controls.

	significant differences in CD127 expression on lymphocytes						correlations between CD127 expression on lymphocytes and age					
	% CD127+			CD127 MFI			% CD127+			CD127 MFI		
	normal	A-T some ATM activity	A-T no ATM activity	normal	A-T some ATM activity	A-T no ATM activity	normal	A-T some ATM activity	A-T no ATM activity	normal	A-T some ATM activity	A-T no ATM activity
T cells	-	+	-	-	+	-	+	ns	ns	+	ns	ns
CD4+ T cells	-	+	-	-	+	-	+	ns	ns	+	ns	ns
CD4+ naive	ns	+	-	ns	+	-	+	ns	ns	+	ns	ns
CD4+ central memory	-	+	-	ns	+	-	+	ns	ns	+	ns	ns
CD4+ effector memory	ns	+	-	-	+	-	ns	ns	ns	+	ns	ns
CD4+ TEMRA	ns	ns	ns	ns	+	-	+	ns	ns	+	ns	ns
CD8+ T cells	-	+	-	-	+	-	ns	ns	ns	ns	ns	ns
CD8+ naive	-	+	-	-	+	ns	ns	ns	ns	+	ns	ns
CD8+ central memory	-	+	-	-	+	-	+	ns	ns	+	ns	ns
CD8+ effector memory	ns	ns	ns	ns	ns	ns	+	ns	+	ns	ns	ns
CD8+ TEMRA	ns	ns	ns	ns	ns	ns	+	ns	ns	ns	ns	ns
B cells												
naive B cells												
memory B cells												
NK cells	+	+	-	ns	ns	ns	ns	ns	ns	ns	ns	ns
CD56dim NK cells	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CD56bright NK cells	ns	ns	ns	ns	ns	ns	+	ns	ns	+	ns	ns
NKT cells	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Table 3:6:5: Summary of significant differences between the expression of CD127 (percentage of CD127+ve cells and CD127 MFI of all cells) and significant correlations between CD127 expression and age in A-T patients and normal controls.

In the first part of the table (significant differences in CD127 expression on lymphocytes) cells containing a positive symbol (+) and highlighted in yellow indicate a significantly higher percentage of CD127+ve cells or CD127 MFI of all cells in the corresponding group than in the group with a cell containing a negative symbol (-) and highlighted in blue, 'ns' refers to no significant difference with any group. The second part of the table summarises significant correlations between CD127 expression and age. Yellow (+) cells indicate a positive correlation and blue (-) cells indicate a negative correlation, 'ns' indicates no significant correlation.

3:7: Measurement of plasma cytokine concentrations by ELISA.

3:7:1: Plasma cytokine concentrations of IL-7, IL-15 and IL-21.

The cytokines IL-7, IL-15 and IL-21 have important roles in proliferation and lymphocyte homeostasis, therefore a deficiency in one of these cytokines could contribute to the lymphopenia of A-T patients. The concentrations of the cytokines IL-7, IL-15 and IL-21 in the plasma samples collected during blood preparation were measured by ELISA, however the concentration of IL-21 was too low to allow detection.

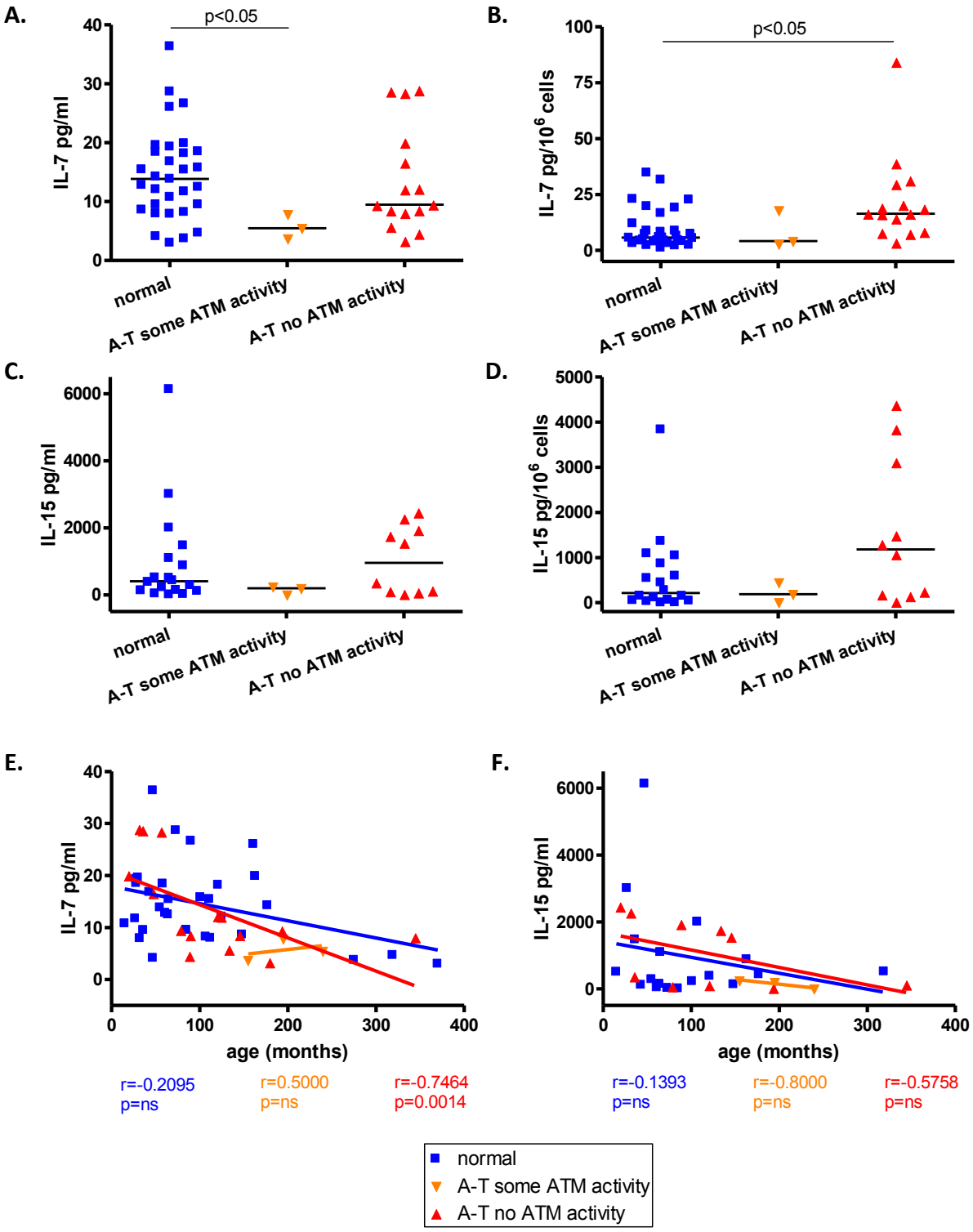
Despite the lymphopenia of the A-T patients there was no increase in plasma IL-7 or IL-15 concentration compared to normal controls (Fig 3:7:1A&C). The IL-7 plasma concentration was significantly lower in A-T patients with some ATM activity than normal controls ($p<0.05$) although it was not abnormally low compared to the control samples with the lowest IL-7 concentrations. However, as the total cell number was significantly reduced in A-T patients with no ATM activity ($p<0.001$) (Fig 3:2:2A) the amount of IL-7 available per cell was significantly higher than in normal controls ($p<0.05$) (Fig 3:7:1B). There was no significant difference in the amount of IL-15 available per cell in A-T patients and normal controls (Fig 3:7:1D).

Interestingly there was a significant negative correlation between IL-7 concentration and age in A-T patients with no ATM activity ($r=-0.7464$, $p=0.0014$) but no correlation in the normal controls or A-T patients with some ATM activity (Fig 3:7:1E). It is unlikely that the decrease in IL-7 concentration with age in A-T patients with no ATM activity is the result of increased usage of the cytokine by lymphocytes as with the exception of CD8+ effector memory T cells the expression of CD127 on lymphocytes from these patients did not increase with age

(Table 3:6:5). Therefore it seems more likely that production of IL-7 by the stromal cells of the thymus and bone marrow may decrease with age in A-T patients.

There were no correlations between plasma IL-15 concentration and age (Fig 3:7:1F) or IL-7 or IL-15 concentration and cell count (Fig 3:7:1G&H) in either A-T patients or normal controls.

Fig 3:7:1: A-T patients did not have IL-7 or IL-15 deficiencies, however IL-7 concentration decreased significantly with age in A-T patients with no ATM activity.



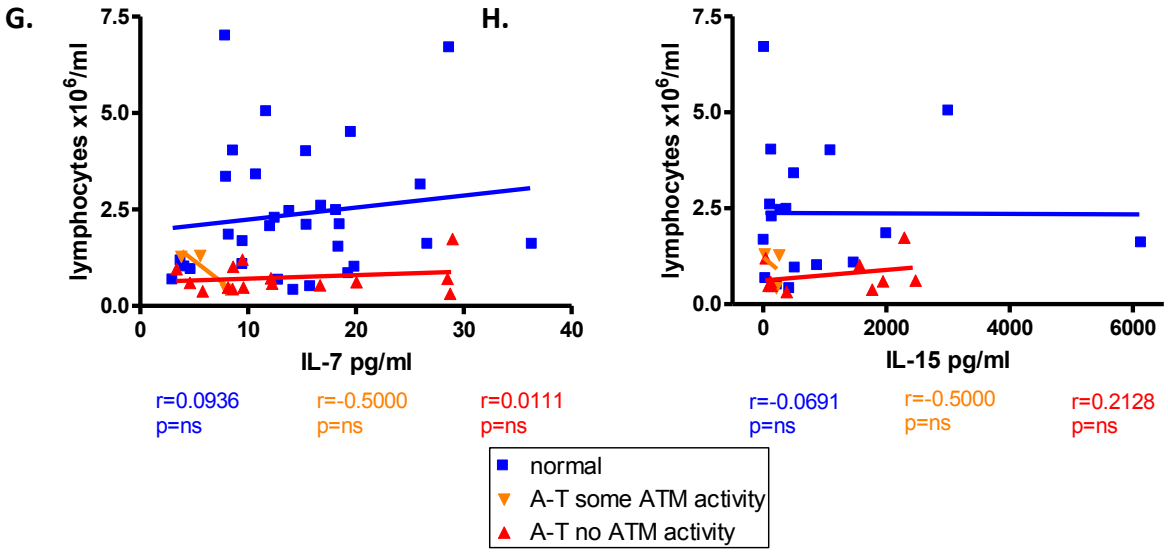


Fig 3:7:1: Plasma IL-7, IL-15 and IL-21 concentrations were measured in triplicate by ELISA. Data for IL-21 is not shown as the concentration in the plasma samples was below the detection limit of the assay.

A. There was no significant difference in plasma IL-7 concentration between A-T patients and normal controls.

B. The amount of IL-7 available per lymphocyte was significantly higher in A-T patients with no ATM activity than normal controls.

C. There was no significant difference in plasma IL-15 concentration between A-T patients and normal controls. Samples with IL-15 concentrations lower than the detection limit of the assay are not shown.

D. There was no significant difference in the amount of IL-15 available per lymphocyte in A-T patients and normal controls.

E. There was a significant negative correlation between increasing age and decreasing plasma IL-7 concentration in A-T patients with no ATM activity. Normal controls and A-T patients with some ATM activity did not show a similar correlation.

F. There were no significant correlations between plasma IL-15 concentration and age in normal controls or A-T patients.

G. There was no correlation between plasma IL-7 concentration and cell count in any group.

H. There was no correlation between plasma IL-15 concentration and cell count in any group.

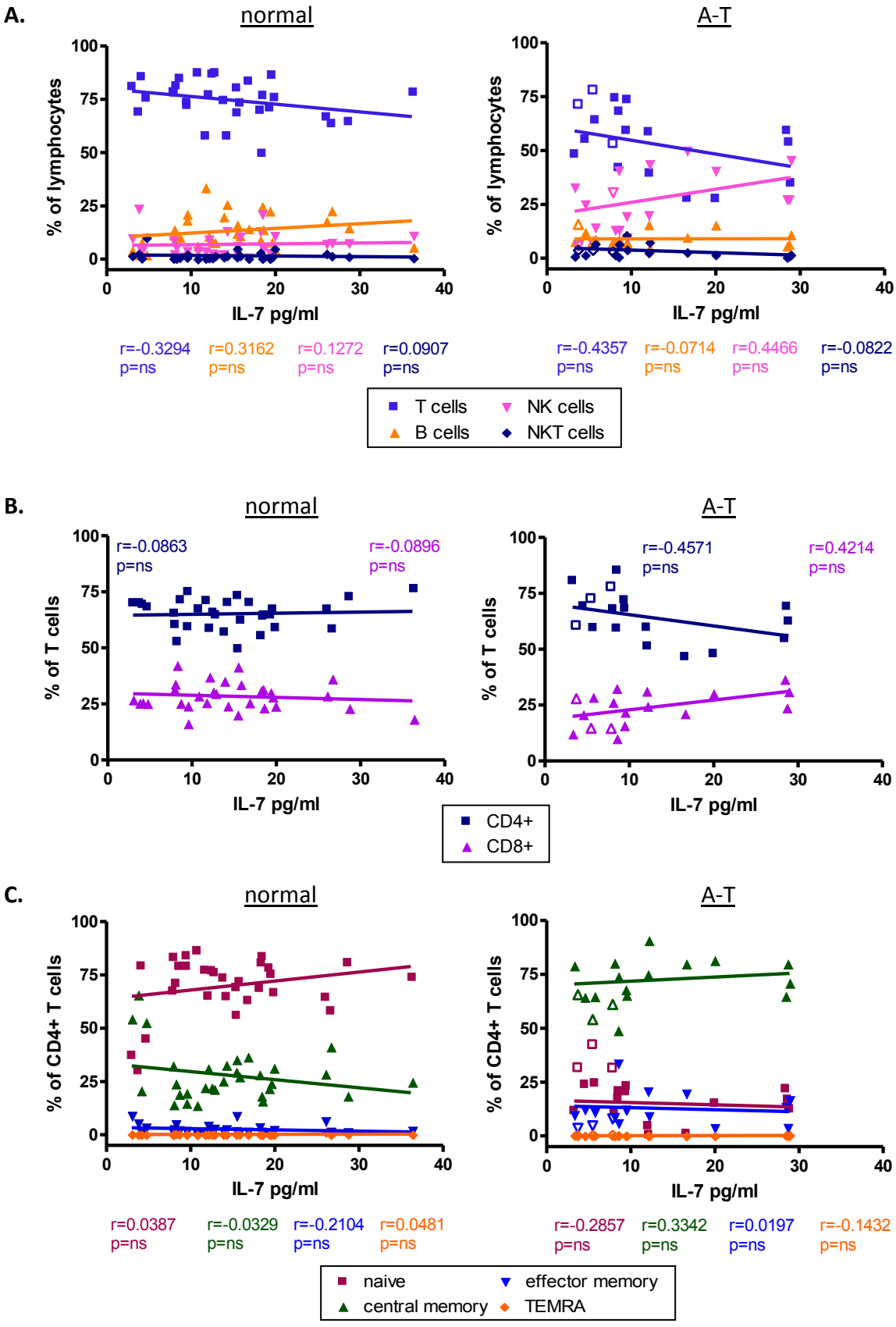
3:8: Correlations between IL-7 concentrations, percentages of lymphocyte subsets and expression of CD95, FasL and CD127.

3:8:1: IL-7 concentration and lymphocyte subsets.

As IL-7 is able to induce proliferation of lymphocytes, particularly naive T cells, correlations between IL-7 concentration in the plasma samples and the percentages of T cells, B cells, NK cells, NKT cells and subsets in matched PBMC samples from A-T patients and normal controls were analysed. However, there were no significant correlations between IL-7 concentration and percentage of any lymphocyte subset in either A-T patients or normal controls (Fig 3:8:1A-F).

Under normal conditions IL-7 acts to maintain a homeostatic balance in T cell number, therefore the lack of correlation was unsurprising in the non-lymphopenic normal controls. However, as A-T patients are lymphopenic and consequently the amount of IL-7 available per lymphocyte is relatively high (Fig 3:7:1B) it would be expected that increasing IL-7 concentration would induce increasing proliferation of naive T cells. This would result in a positive correlation between IL-7 concentration and the percentages of both naive and total T cells. The lack of correlation (Fig 3:8:1C&D) despite normal CD127 expression on naive T cells (Fig 3:6:3B) suggests that either the IL-7 concentration was not sufficient to induce proliferation, which seems unlikely, or that the IL-7 did not have a proliferative effect on naive T cells from A-T patients.

Fig 3:8:1: There were no correlations between percentages of lymphocyte subsets and IL-7 concentration in A-T patients or normal controls.



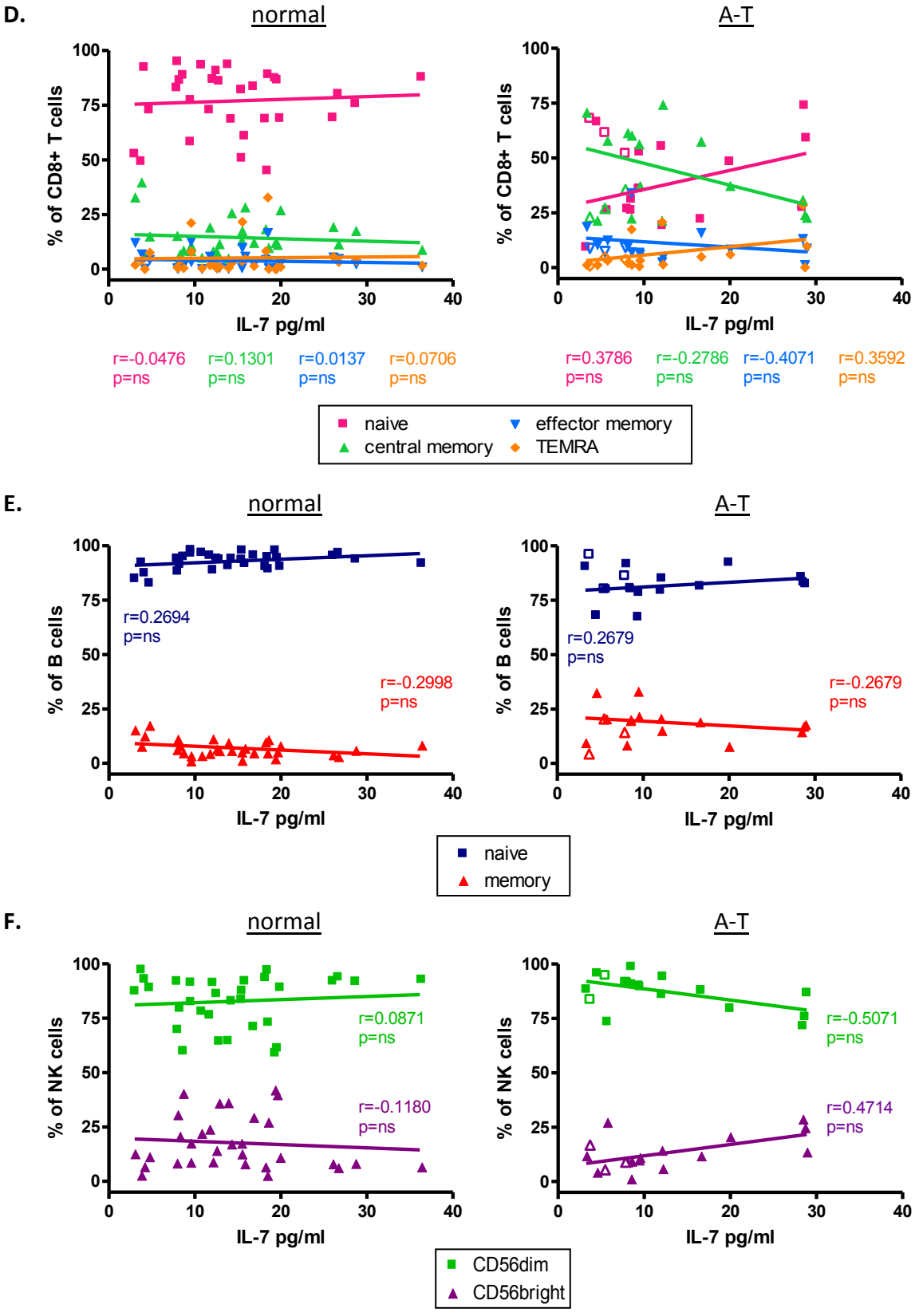


Fig 3:8:1: PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRII flow cytometer. Gating was carried out as described in Fig 3:3:1. IL-7 concentration in matched plasma samples was measured by ELISA. To improve clarity and as

there were no significant correlations linear regression trend lines and r and p values for the A-T patients with some ATM activity are not shown, however results of the correlation analysis for this group are summarised in Table 3:8:5. (A-T plots: open symbols = some ATM activity, closed symbols = no ATM activity).

A. There were no correlations between IL-7 concentration and the percentage of total T cells, B cells, NK cells or NKT cells in the normal controls (left) or A-T patients (right).

B. There were no correlations between percentage of CD4+ or CD8+ T cells and IL-7 concentration in the normal controls (left) or A-T patients (right).

C. There were no correlations between the percentages of naive, central memory, effector memory or TEMRA CD4+ T cells and IL-7 concentration in the normal controls (left) or A-T patients (right).

D. There were no correlations between the percentages of naive, central memory, effector memory or TEMRA CD8+ T cells and IL-7 concentration in the normal controls (left) or A-T patients (right).

E. There were no correlations between the percentage of naive or memory B cells and IL-7 concentration in the normal controls (left) or A-T patients (right).

F. There were no correlations between the percentage of CD56dim or CD56bright NK cells and IL-7 concentration in the normal controls (left) or A-T patients (right).

3:8:2: IL-7 concentration and CD95 expression on lymphocyte subsets.

Although IL-7 is able to induce proliferation of naive T cells, increased availability of the cytokine as a result of lymphopenia may induce upregulation of CD95 leading to sensitivity to CD95-mediated apoptosis and contributing to the lymphopenia (Brugnoni et al., 1999;Rethi et al., 2008). This could explain the lack of correlation between IL-7 concentration and the percentage of naive T cells in A-T patients. To test this hypothesis correlations between plasma concentrations of IL-7 and CD95 expression on lymphocyte subsets in A-T patients and normal controls were analysed.

Firstly correlations between IL-7 concentration and CD95 expression on T cells, B cells, NK cells and NKT cells were analysed. In the normal controls there were positive correlations between IL-7 concentration and the percentages of CD95+ve NK cells and CD56dimCD95+ NK cells (Fig 3:8:2A&F) whereas the A-T patients with no ATM activity showed a positive correlation between the percentage of CD95+ve T cells and IL-7 concentration (Fig 3:8:2A).

When CD4+ve and CD8+ve T cells were analysed separately there were no correlations between CD95 expression and IL-7 concentration in the normal controls. However, there was a significant positive correlation between the percentage of CD4+CD95+ T cells and IL-7 concentration in the A-T patients with no ATM activity (Fig 3:8:2B).

Further analysis of CD4+ve T cells according to subset revealed further correlations between IL-7 plasma concentration and CD95 expression. In the normal controls there were positive correlations between IL-7 concentration and the percentages of CD4+CD95+ naive and central memory T cells and the CD95 MFI of CD4+CD95+ central and effector memory T cells. The A-T patients with no ATM activity showed positive correlations between IL-7 concentration and the percentages of CD4+CD95+ naive, central memory and effector

memory T cells. These correlations were more significant (higher r values and lower p values) than those seen in the normal controls. There were no correlations between the CD95 MFI of CD4+CD95+ T cells and IL-7 concentration on any T cell subset in the A-T patients with no ATM activity (Fig 3:8:2C).

The effect of increasing IL-7 concentration on CD95 expression on CD8+ve T cell subsets was not as clear as for CD4+ve T cell subsets. There was a significant positive correlation between the percentage of CD8+CD95+ naive T cells and IL-7 concentration in the normal controls. In the A-T patients with no ATM activity there was a negative correlation between CD95 MFI of CD8+CD95+ TEMRA cells and IL-7 concentration (Fig3:8:2D).

In the normal controls increasing IL-7 concentration had no effect on CD95 expression on naive or memory B cells. However A-T patients with no ATM activity showed a significant positive correlation between IL-7 concentration and the percentage of CD95+ve memory B cells (Fig 3:8:2E). As the B cells did not express CD127 (Fig 3:6:1B) so could not bind IL-7 it is unlikely that this correlation is genuine.

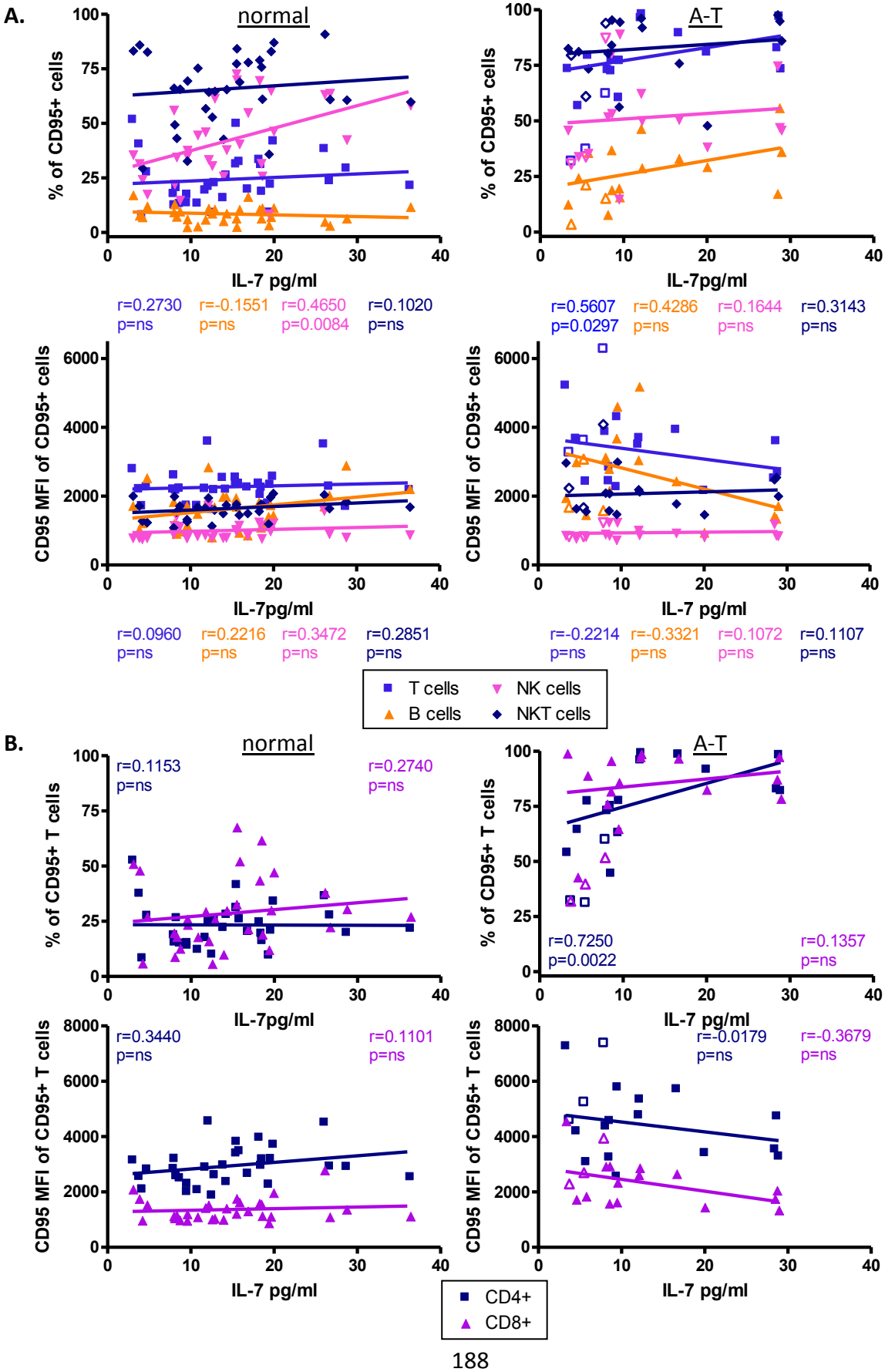
The most significant effect of increasing IL-7 concentration on CD95 expression was the increase in CD95 expression on CD4+ve T cell subsets; this was more dramatic in the A-T patients with no ATM activity than the normal controls (Fig 3:8:2C) and suggests that the increased availability of IL-7 in A-T patients with no ATM activity may induce upregulation of CD95 on CD4+ T cells. This could contribute to the CD4+ T cell deficiency of A-T patients by increasing their sensitivity to CD95-mediated apoptosis and spontaneous apoptosis.

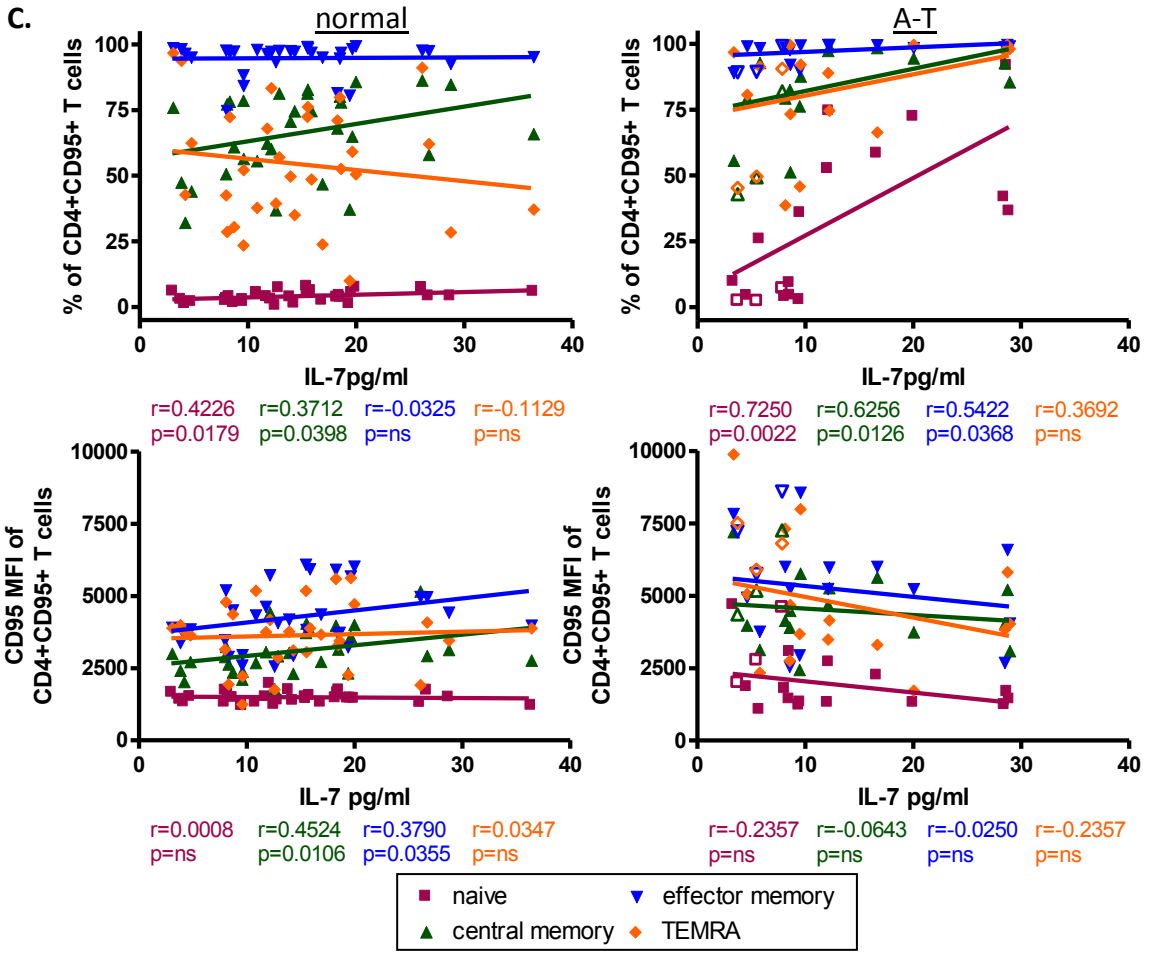
It is interesting that there were no correlations between IL-7 concentration and the percentage of CD8+CD95+ T cells of any subset. As CD95 expression is increased on both CD4+ and CD8+ T cells from A-T patients with no ATM activity (Fig 3:4:2) this suggests that

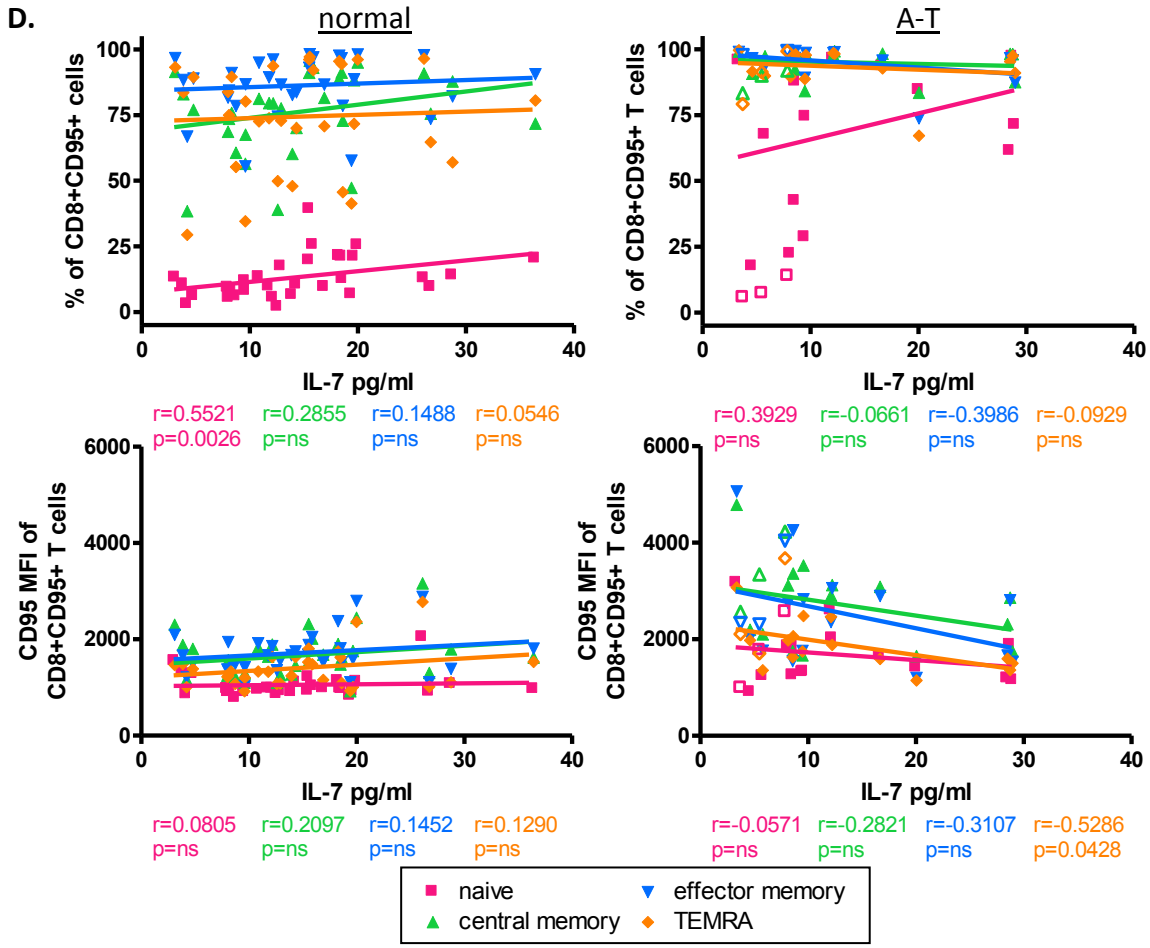
increased IL-7 availability is not the only factor responsible for increased CD95 expression on A-T T cells.

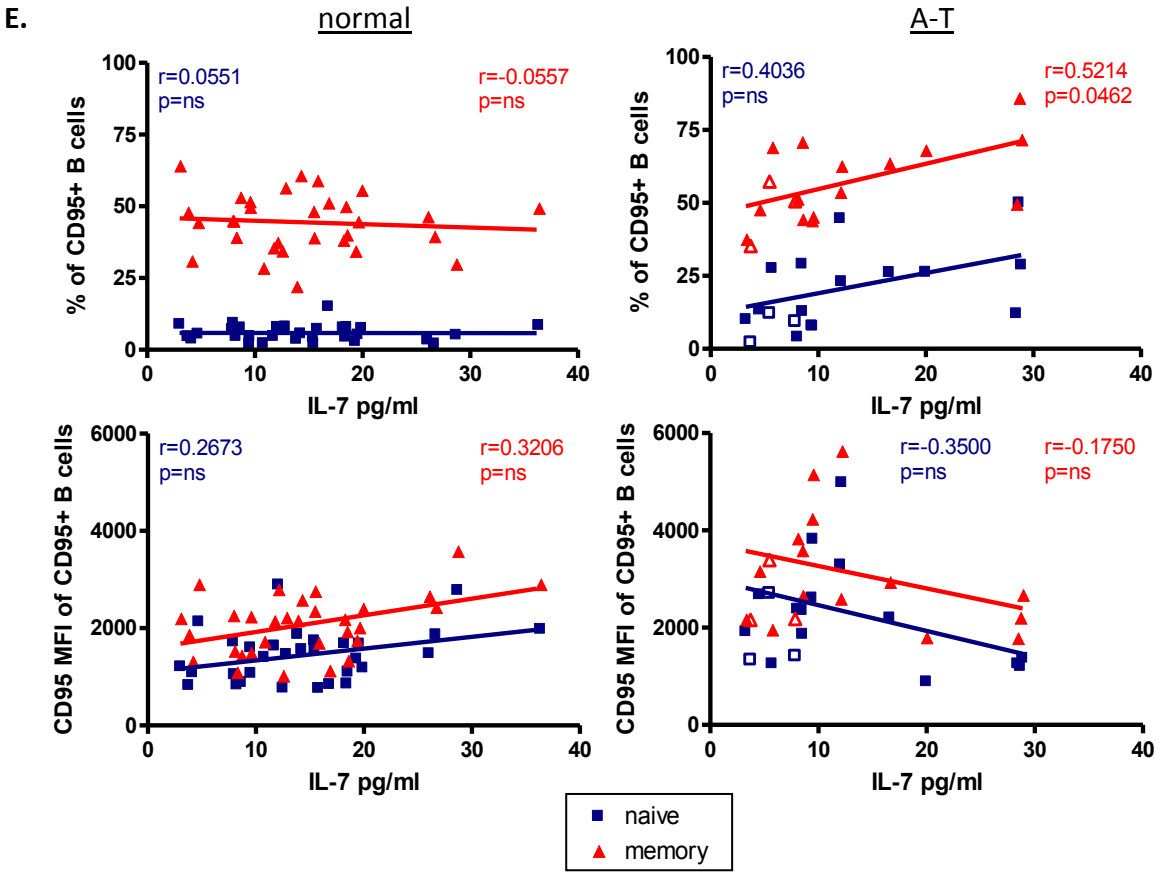
CD95 upregulation in response to IL-7 also does not explain the increased CD95 expression on B cells (which do not express CD127) or NKT cells from A-T patients with no ATM activity compared to normal controls (Fig 3:4:1).

Fig 3:8:2: IL-7 concentration correlated positively with CD95 expression on CD4+ T cells but not CD8+ T cells or NKT cells.




C.
normal
A-T
% of CD4+CD95+ T cells
IL-7pg/ml
r=0.4226 r=0.3712 r=-0.0325 r=-0.1129
p=0.0179 p=0.0398 p=ns p=ns
r=0.7250 r=0.6256 r=0.5422 r=0.3692
p=0.0022 p=0.0126 p=0.0368 p=ns
CD95 MFI of CD4+CD95+ T cells
IL-7 pg/ml
r=0.0008 r=0.4524 r=0.3790 r=0.0347
p=ns p=0.0106 p=0.0355 p=ns
r=-0.2357 r=-0.0643 r=-0.0250 r=-0.2357
p=ns p=ns p=ns p=ns
naive
effector memory
central memory
TEMRA


D.
normal
A-T
% of CD8+CD95+ cells
IL-7 pg/ml
r=0.5521 r=0.2855 r=0.1488 r=0.0546
p=0.0026 p=ns p=ns p=ns
r=0.3929 r=-0.0661 r=-0.3986 r=-0.0929
p=ns p=ns p=ns p=ns
CD95 MFI of CD8+CD95+ T cells
r=0.0805 r=0.2097 r=0.1452 r=0.1290
p=ns p=ns p=ns p=ns
r=-0.0571 r=-0.2821 r=-0.3107 r=-0.5286
p=ns p=ns p=ns p=0.0428
naive
effector memory
central memory
TEMRA


E.
normal
A-T
% of CD95+ B cells
CD95 MFI of CD95+ B cells
IL-7 pg/ml
r=0.0551
p=ns
r=-0.0557
p=ns
r=0.4036
p=ns
r=0.5214
p=0.0462
r=0.2673
p=ns
r=0.3206
p=ns
r=-0.3500
p=ns
r=-0.1750
p=ns
naive
memory

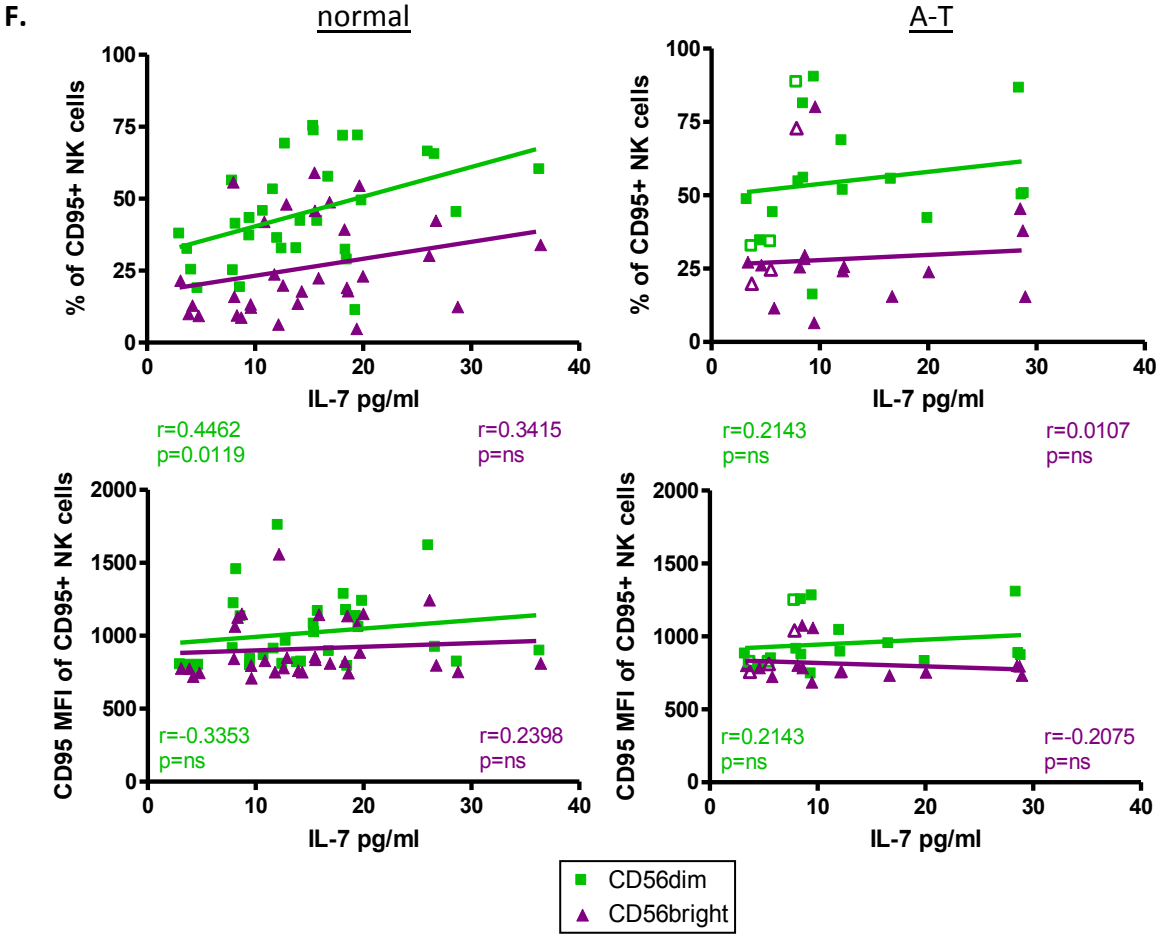

F.
normal
A-T
% of CD95+ NK cells
CD95 MFI of CD95+ NK cells
IL-7 pg/ml
r=0.4462
p=0.0119
r=0.3415
p=ns
r=0.2143
p=ns
r=0.0107
p=ns
r=-0.3353
p=ns
r=0.2398
p=ns
r=0.2143
p=ns
r=-0.2075
p=ns
CD56dim
CD56bright

Fig 3:8:2: PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRII flow cytometer. Gating was carried out as described in Fig 3:3:1. To improve clarity, and as there were no significant correlations, linear regression trend lines and r and p values for the A-T patients with some ATM activity are not shown, however the results of the correlation analysis for this group are summarised in Table 3:8:5. (A-T plots: open symbols = some ATM activity, closed symbols = no ATM activity).

A. Analysis of correlations between IL-7 concentration and CD95 expression on T cells, B cells, NK cells and NKT cells. The normal controls showed a positive correlation between the percentage of CD95+ve NK cells and IL-7 concentration (top left). The A-T patients with no ATM activity showed a positive correlation between the percentage of CD95+ve T cells and IL-7 concentration (top right). There were no correlations between CD95 MFI of CD95+ve cells of any type in either A-T patients or normal controls (bottom left and right).

B. There were no correlations between either percentage of CD4+CD95+ or CD8+CD95+ T cells (top left) or CD95 MFI of CD4+CD95+ or CD8+CD95+ T cells (bottom left) and IL-7 concentration in the normal controls. The A-T patients with no ATM activity showed a significant positive correlation between the percentage of CD4+CD95+ T cells and IL-7 concentration (top right).

C. CD4+ve T cells in the normal controls showed positive correlations between IL-7 concentration and the percentages of CD4+CD95+ naive and central memory T cells (top left) and CD95 MFI of CD4+CD95+ central and effector memory T cells (bottom left). The A-T patients with no ATM activity showed positive correlations between the percentages of CD4+CD95+ naive, central memory and effector memory T cells and IL-7 concentration. There were no correlations between CD95 MFI of CD4+CD95+ T cells and IL-7 concentration on any T cell subset in the A-T patients with no ATM activity (bottom right).

D. There was a significant positive correlation between IL-7 concentration and the percentage of CD8+CD95+ naive T cells in the normal controls (top left) and a significant negative correlation between the CD95 MFI of CD8+CD95+ TEMRA cells and IL-7 concentration in the A-T patients with no ATM activity (bottom right).

E. Increasing IL-7 concentration had no effect on CD95 expression on naive or memory B cells in the normal controls (top and bottom left). There was a significant positive correlation between IL-7 concentration and the percentage of CD95+ve memory B cells in the A-T patients with no ATM activity (top right) but no correlations with CD95 MFI of CD95+ve naive or memory B cells (bottom right).

F. There was a significant positive correlation between the percentage of CD56dimCD95+ NK cells and IL-7 concentration in the normal controls (top left) but no correlations in the A-T patients with no ATM activity (top right). There were also no correlations between CD95 MFI of CD56brightCD95+ or CD56dimCD95+ NK cells and IL-7 concentration in either normal controls or A-T patients (bottom left and right).

3:8:3: IL-7 concentration and FasL expression on lymphocyte subsets.

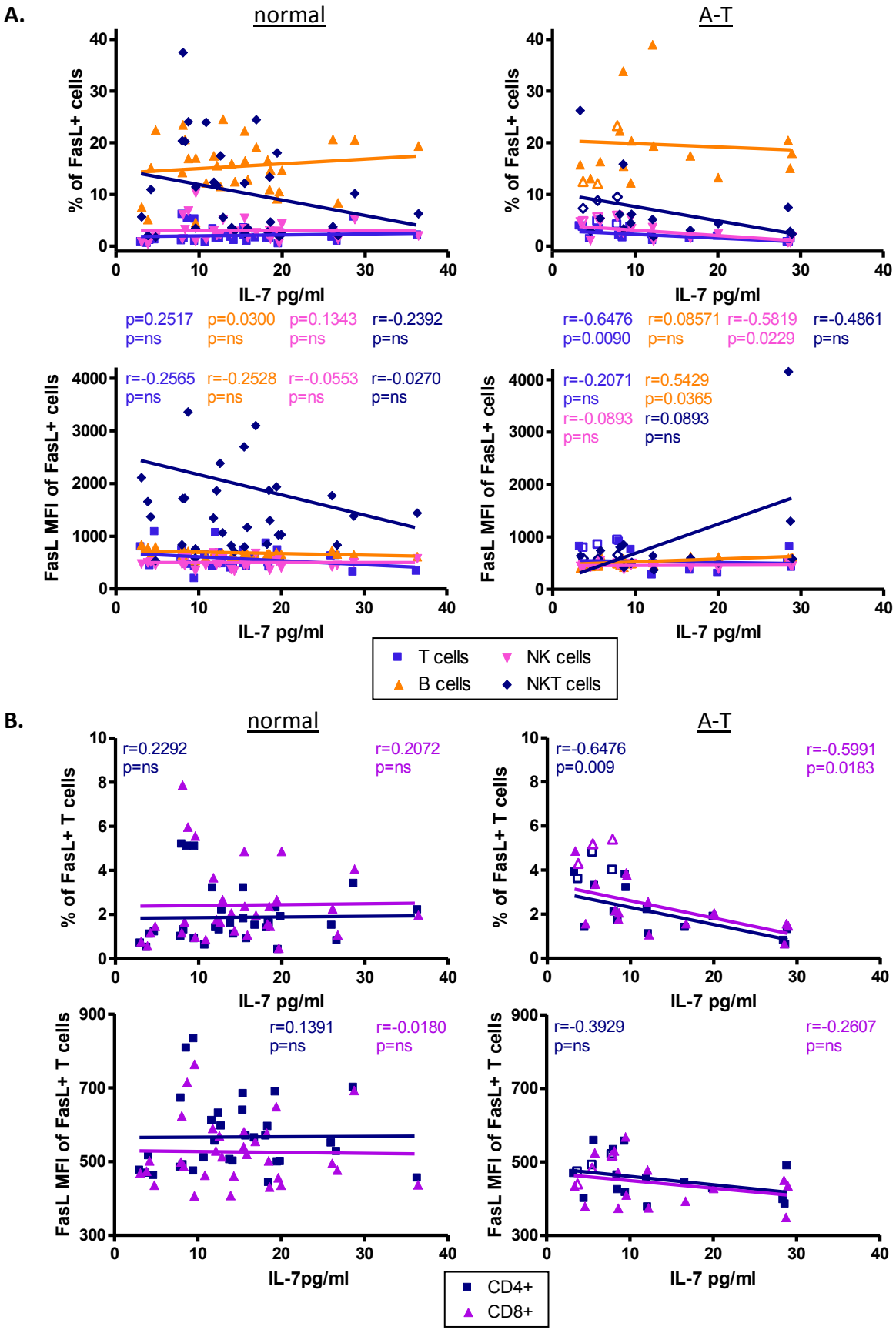
As FasL expression may also affect the sensitivity of lymphocytes to CD95-mediated apoptosis the effect of increasing IL-7 plasma concentration on FasL expression was analysed. There were no correlations between IL-7 concentration and FasL expression on T cells, B cells, NK cells or NKT cells in the normal controls. However A-T patients with no ATM activity showed negative correlations between IL-7 concentration and the percentages of FasL+ve T and NK cells and a positive correlation with the FasL MFI of FasL+ B cells (Fig 3:8:3A).

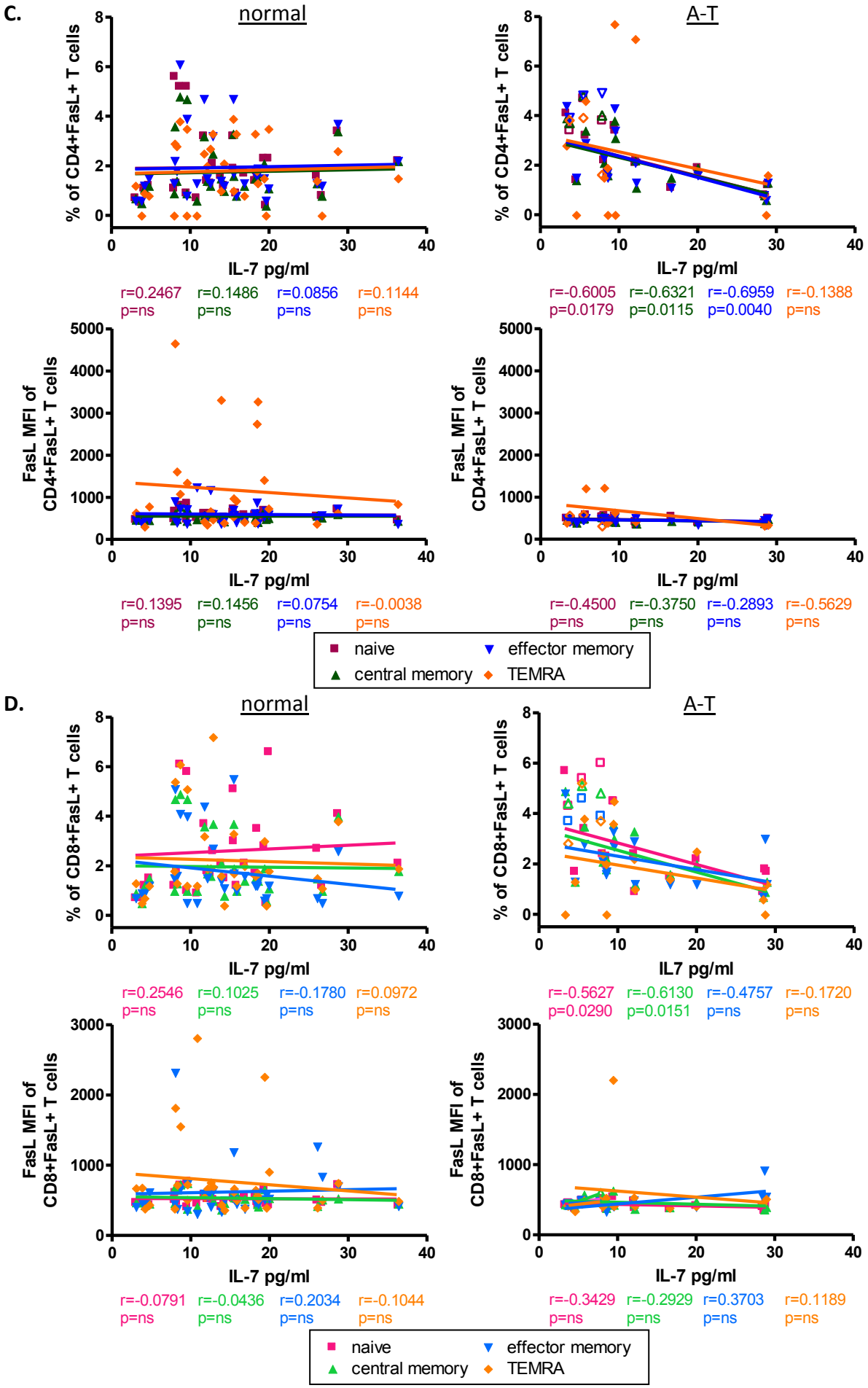
There were no correlations between IL-7 concentration and FasL expression on CD4+ve and CD8+ve T cells and subsets in the normal controls (Fig 3:8:3B-D). However in the A-T patients with no ATM activity the percentages of both CD4+FasL+ and CD8+FasL+ T cells decreased significantly with increasing IL-7 concentration (Fig 3:8:3B). This was the result of significant negative correlations between IL-7 concentration and the percentages of CD4+FasL+ naive, central memory and effector memory T cells and CD8+FasL+ naive and central memory T cells (Fig 3:8:3C&D).

Naive and memory B cells showed no correlations between the percentages of FasL+ve cells and IL-7 concentration in either normal controls or A-T patients. However, the normal controls did show a negative correlation between FasL MFI of FasL+ve memory B cells and IL-7 concentration (Fig 3:8:2E). There were also no correlations between IL-7 concentration and FasL expression on CD56dim or CD56bright NK cells in the normal controls, whereas the A-T patients with no ATM activity showed significant negative correlations between the percentage of FasL+ve CD56dim and CD56bright NK cells and plasma IL-7 concentration (Fig 3:8:2F).

With the exception of a negative correlation between IL-7 concentration and FasL expression on memory B cells the normal controls showed no correlations between IL-7 concentration and FasL expression on any of the cell types or subsets analysed. However, in general the A-T patients with no ATM activity showed decreasing FasL expression on the majority of cell types as IL-7 concentration increased. As IL-7 concentration decreased with age in the A-T patients with no ATM activity this could possibly contribute to the increase in FasL expression on lymphocytes with age in these patients (Table 3:5:6).

Fig 3:8:3: There were negative correlations between IL-7 concentration and FasL expression on several lymphocyte subsets in A-T patients with no ATM activity.




C.
normal
A-T
% of CD4+FasL+ T cells
IL-7 pg/ml
r=0.2467 r=0.1486 r=0.0856 r=0.1144
p=ns p=ns p=ns p=ns
r=-0.6005 r=-0.6321 r=-0.6959 r=-0.1388
p=0.0179 p=0.0115 p=0.0040 p=ns
FasL MFI of CD4+FasL+ T cells
r=0.1395 r=0.1456 r=0.0754 r=-0.0038
p=ns p=ns p=ns p=ns
r=-0.4500 r=-0.3750 r=-0.2893 r=-0.5629
p=ns p=ns p=ns p=ns
naive
effector memory
central memory
TEMRA
D.
normal
A-T
% of CD8+FasL+ T cells
IL-7 pg/ml
IL7 pg/ml
r=0.2546 r=0.1025 r=-0.1780 r=0.0972
p=ns p=ns p=ns p=ns
r=-0.5627 r=-0.6130 r=-0.4757 r=-0.1720
p=0.0290 p=0.0151 p=ns p=ns
FasL MFI of CD8+FasL+ T cells
r=-0.0791 r=-0.0436 r=0.2034 r=-0.1044
p=ns p=ns p=ns p=ns
r=-0.3429 r=-0.2929 r=0.3703 r=0.1189
p=ns p=ns p=ns p=ns
naive
effector memory
central memory
TEMRA

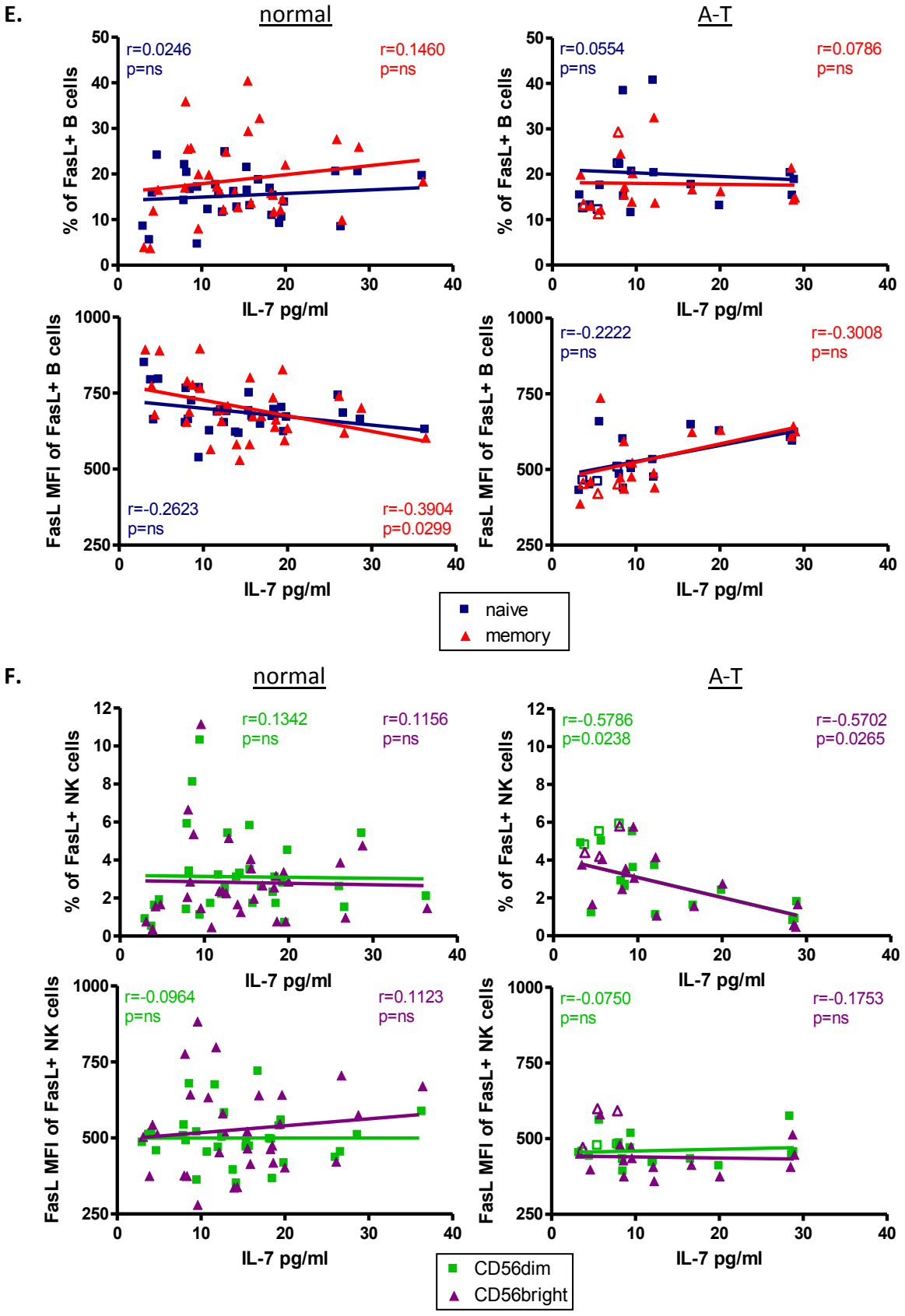


Fig 3:8:3: PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRII flow cytometer. Gating was carried out as described in Fig 3:3:1. To improve clarity, and as there were no significant correlations, linear regression trend lines

and r and p values for the A-T patients with some ATM activity are not shown, however results of the correlation analysis for this group are summarised in Table 3:8:5 (A-T plots: open symbols = some ATM activity, closed symbols = no ATM activity).

A. There were no significant correlations between IL-7 concentration and the percentage or FasL MFI of FasL+ve T cells, B cells, NK cells or NKT cells in normal controls (top & bottom left), however in A-T patients with no ATM activity the percentages of both FasL+ve T cells and NK cells decreased with increasing IL-7 concentration (top right) and the FasL MFI of FasL+ve B cells increased with increasing IL-7 (bottom right).

B. There were no correlations between IL-7 concentration and FasL expression on CD4+ve and CD8+ve T cells in the normal controls (top and bottom left). In the A-T patients with no ATM activity the percentages of CD4+FasL+ and CD8+FasL+ T cells showed a negative correlation with IL-7 concentration (top right).

C. There were no correlations between FasL expression on CD4+ve T cell subsets and IL-7 concentration in the normal controls (top & bottom left). In the A-T patients with no ATM activity there were significant negative correlations between the percentages of CD4+FasL+ T cells and IL-7 concentration in naive, central memory and effector memory subsets (top right).

D. There were no correlations between FasL expression on CD8+ve T cell subsets and IL-7 concentration in the normal controls (top & bottom left). In the A-T patients with no ATM activity there were significant negative correlations between the percentages of CD8+FasL+ naive and central memory T cells and IL-7 concentration (top right).

E. There were no correlations between the percentages of FasL+ve naive or memory B cells and IL-7 concentration in the normal controls (top left), however the FasL MFI of FasL+ve memory B cells decreased with increasing IL-7 concentration (bottom left). In the A-T

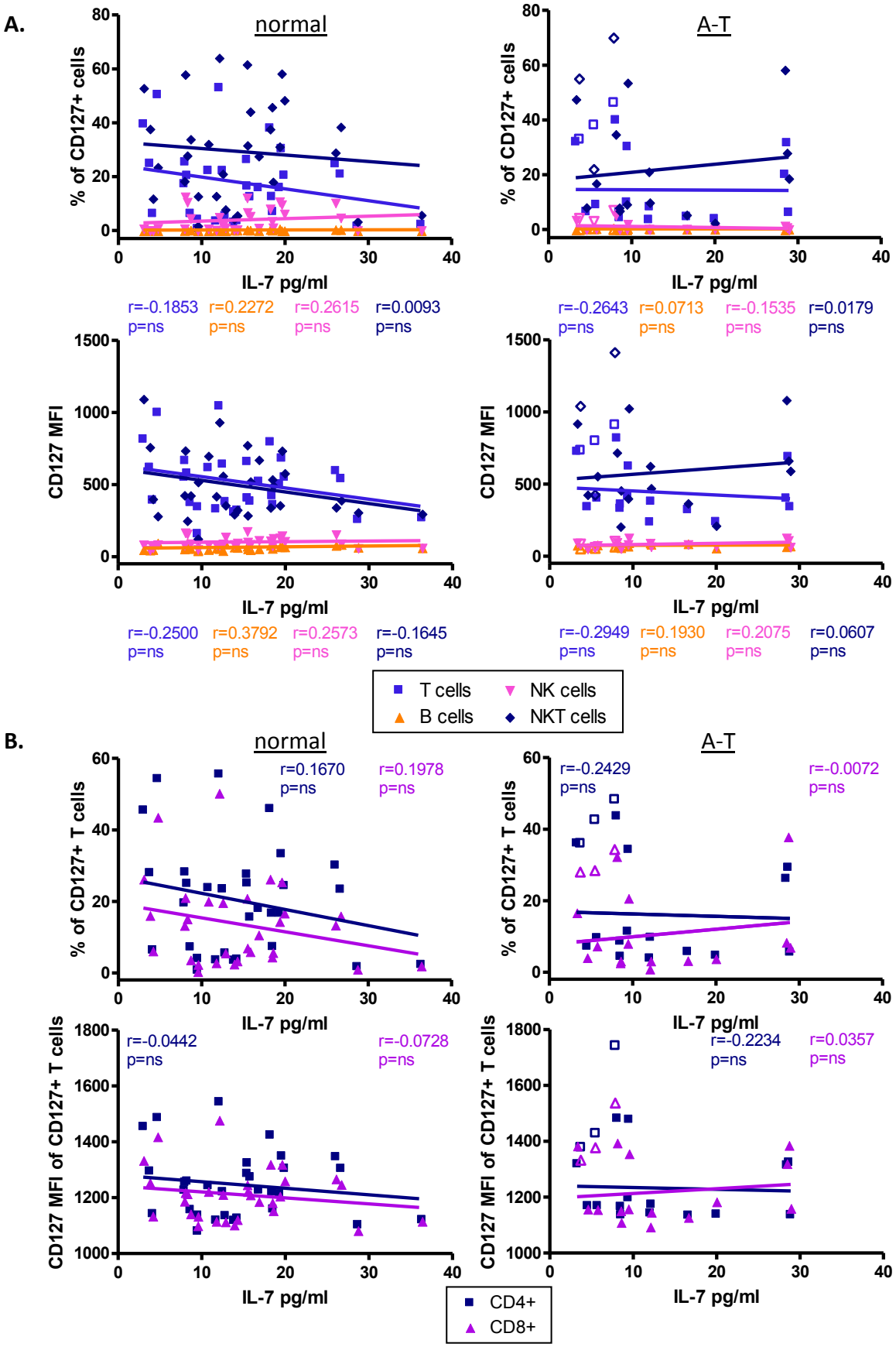
patients with no ATM activity there were no correlations between FasL expression on naive or memory B cells and IL-7 concentration (top and bottom right).

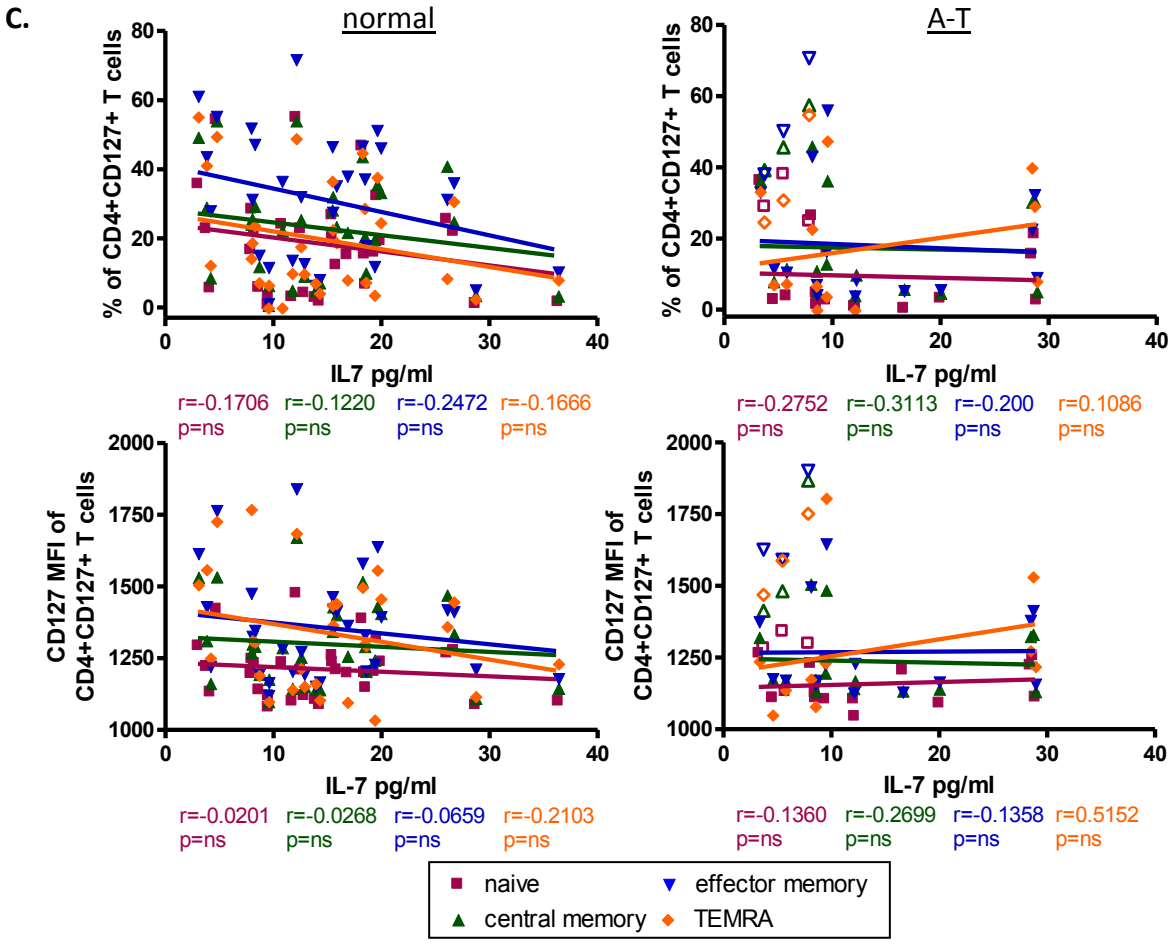
F. There were no correlations between FasL expression on CD56dim or CD56bright NK cells and IL-7 plasma concentration in the normal controls (top and bottom left). However in the A-T patients with no ATM activity there were significant negative correlations between the percentages of CD56dimFasL+ and CD56brightFasL+ NK cells and plasma IL-7 concentration (top right).

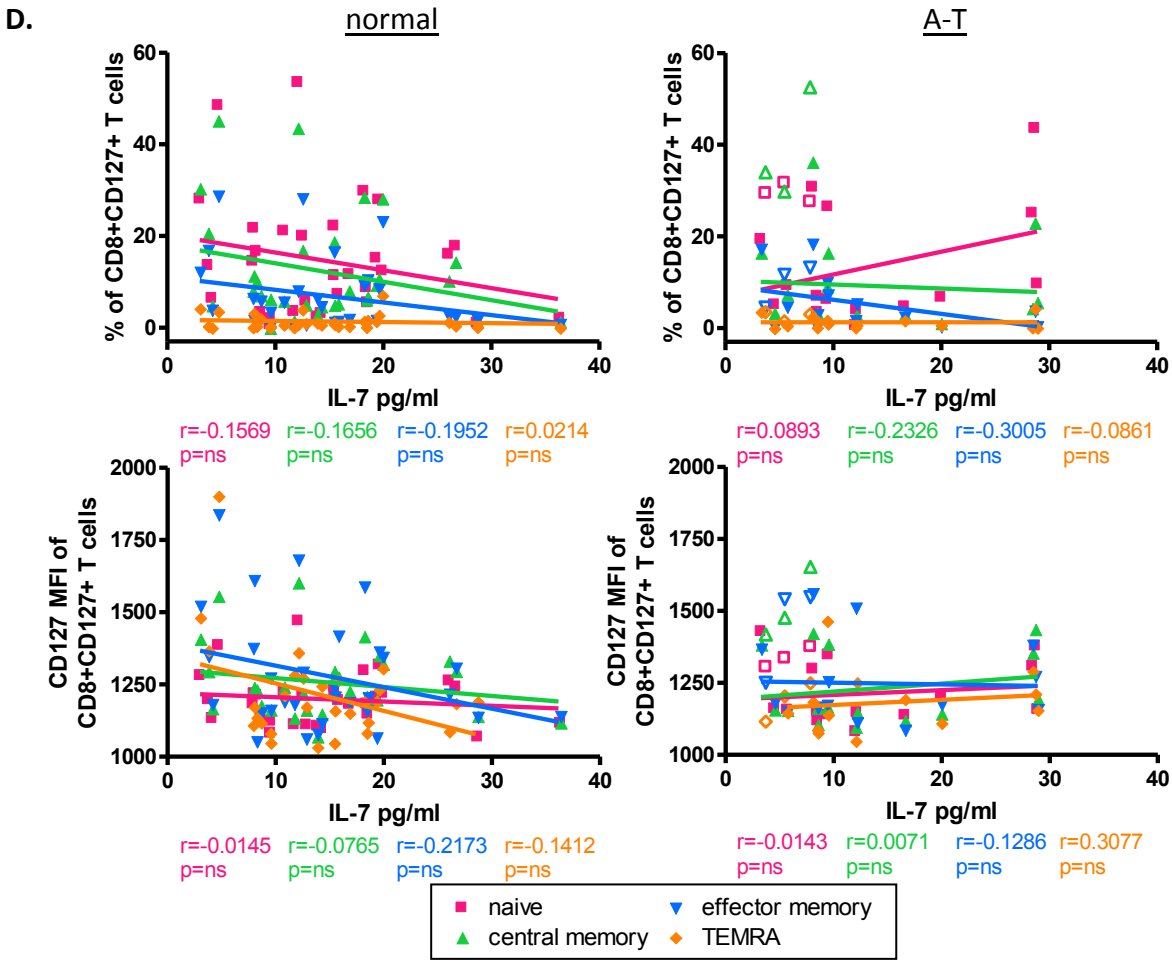
3:8:4: IL-7 concentration and CD127 expression on lymphocyte subsets.

Finally, as CD127 expression regulates consumption of IL-7 (reviewed in (Mazzucchelli & Durum, 2007)) correlations between CD127 expression on lymphocyte subsets and IL-7 plasma concentration were analysed. There were no correlations between IL-7 concentration and CD127 expression on total T cells, B cells, NK cells or NKT cells (Fig 3:8:4A), CD4+ or CD8+ T cells (Fig 3:8:4B), naive and memory CD4+ or CD8+ T cell subsets (Fig 3:8:4C&D), or CD56dim and CD56bright NK cells (Fig 3:8:4E) in either A-T patients or normal controls. This finding together with the absence of an increase in CD127 expression on lymphocytes (excluding CD8+ve effector memory T cells) with age in A-T patients with no ATM activity (Table 3:6:5) suggests that the decrease in IL-7 concentration with age in these patients (Fig 3:7:1E) is not due to increased consumption of the cytokine by CD127+ve lymphocytes.

Fig 3:8:4: There were no correlations between IL-7 concentration and cell surface CD127 expression on lymphocytes from A-T patients or normal controls.




C.
normal
A-T
% of CD4+CD127+ T cells
IL7 pg/ml
IL-7 pg/ml
r=-0.1706 r=-0.1220 r=-0.2472 r=-0.1666
p=ns p=ns p=ns p=ns
r=-0.2752 r=-0.3113 r=-0.200 r=0.1086
p=ns p=ns p=ns p=ns
CD127 MFI of CD4+CD127+ T cells
r=-0.0201 r=-0.0268 r=-0.0659 r=-0.2103
p=ns p=ns p=ns p=ns
r=-0.1360 r=-0.2699 r=-0.1358 r=0.5152
p=ns p=ns p=ns p=ns
naive
effector memory
central memory
TEMRA


D.
normal
A-T
% of CD8+CD127+ T cells
IL-7 pg/ml
r=-0.1569 r=-0.1656 r=-0.1952 r=0.0214
p=ns p=ns p=ns p=ns
r=0.0893 r=-0.2326 r=-0.3005 r=-0.0861
p=ns p=ns p=ns p=ns
CD127 MFI of CD8+CD127+ T cells
r=-0.0145 r=-0.0765 r=-0.2173 r=-0.1412
p=ns p=ns p=ns p=ns
r=-0.0143 r=0.0071 r=-0.1286 r=0.3077
p=ns p=ns p=ns p=ns
naive
effector memory
central memory
TEMRA

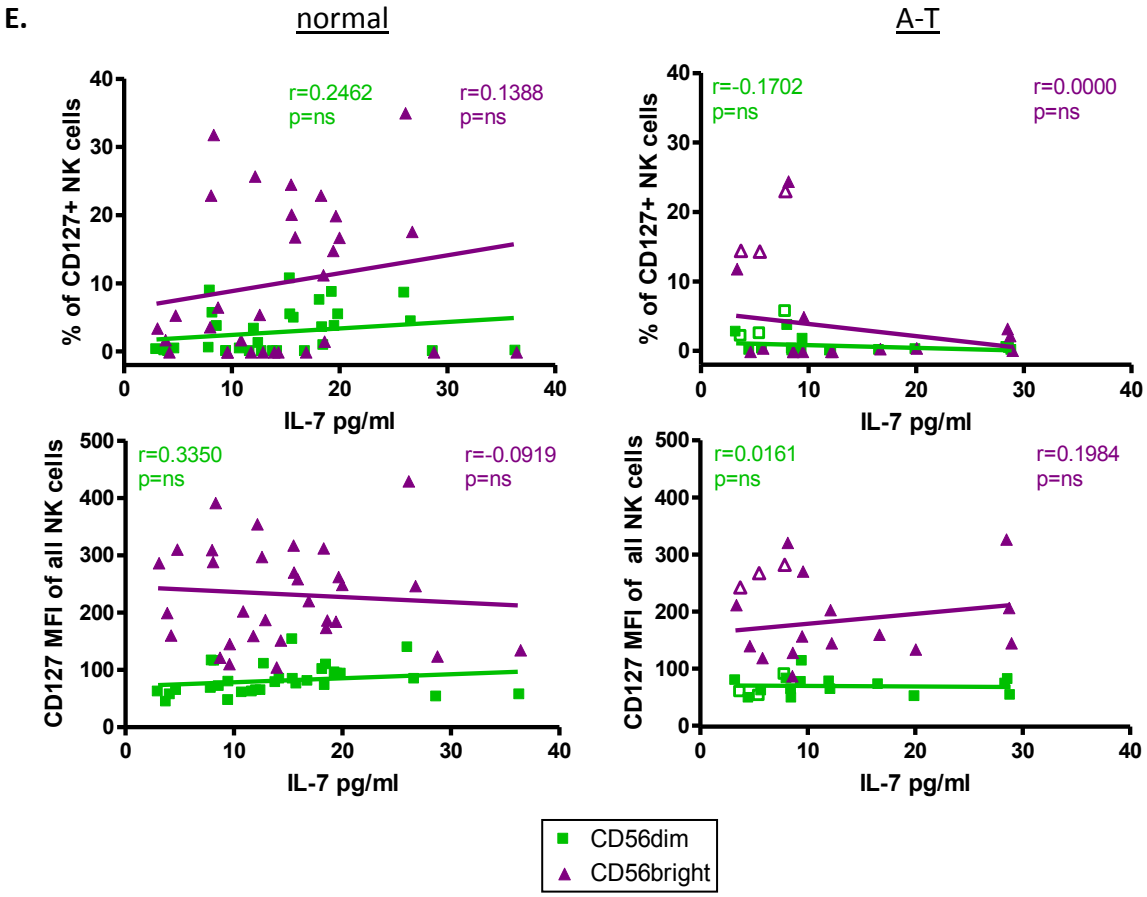


Fig 3:8:4: Analysis of correlations between plasma IL-7 concentrations and cell surface CD127 expression (percentage of CD127+ve cells and CD127 MFI of all cells) on lymphocyte subsets. To improve clarity, and as there were no significant correlations, linear regression trend lines and r and p values for A-T patients with some ATM activity are not shown however, results of the correlation analysis for this group are summarised in Table 3:8:5. (A-T plots: open symbols = some ATM activity, closed symbols = no ATM activity).

A. There were no correlations between plasma IL-7 concentration and cell surface CD127 expression on T cell, B cells, NK cells or NKT cells in either A-T patients or normal controls.

B. There were no correlations between plasma IL-7 concentration and cell surface CD127 expression on CD4+ve or CD8+ve T cells in either normal controls or A-T patients.

C. There were no correlations between plasma IL-7 concentration and cell surface CD127 expression on CD4+ve T cell subsets in either normal controls or A-T patients.

D. There were no correlations between plasma IL-7 concentration and cell surface CD127 expression on naive, effector memory or TEMRA CD8+ve T cell subsets in either normal controls or A-T patients.

E. There were no correlations between plasma IL-7 concentration and cell surface CD127 expression on CD56dim or CD56bright NK cells in either normal controls or A-T patients.

3:8:5: IL-7 concentration correlated positively with CD95 expression on CD4+ve T cells and negatively with FasL expression on CD4+ve and CD8+ve T cells in A-T patients with no ATM activity.

The results of the analysis of correlations between plasma IL-7 concentration and percentages of lymphocyte subsets, CD95, FasL and CD127 expression are summarised in Table 3:8:5.

Although IL-7 has an important role in inducing proliferation of naive T cells there were no correlations between IL-7 concentration and the percentages of different cell types or subsets in either A-T patients or normal controls.

It has been suggested that IL-7 may be involved in regulation of CD95 expression (Brugnoni et al., 1999;Rethi et al., 2008) and there were a number of positive correlations between IL-7 concentration and the percentages of CD95+ve lymphocytes of various subsets. In the normal controls the percentages of CD4+CD95+ naive and central memory and CD8+CD95+ naive T cells as well as CD95+ve NK cells and CD56dimCD95+ NK cells correlated positively with IL-7 concentration. The A-T patients with no ATM activity showed positive correlations between IL-7 concentration and the percentages of CD95+ve T cells, CD4+CD95+ T cells and CD4+CD95+ naive, central memory and effector memory T cells. This suggests that the age related decrease in the percentage of CD4+CD95+ T cells and subsets in A-T patients with no ATM activity (Table 3:4:7) may be due to the decrease in IL-7 plasma concentration with age in these patients.

There were also some correlations between the CD95 MFI of CD95+ve lymphocytes and IL-7 concentration. In the normal controls the CD95 MFI of CD4+CD95+ central and effector memory T cells correlated positively with IL-7 concentration and in the A-T patients with no

ATM activity the CD95 MFI of CD8+CD95+ TEMRA T cells correlated negatively and the CD95 MFI of CD95+ve naive B cells correlated positively with IL-7 concentration.

IL-7 concentration had no effect on the percentages of FasL+ve lymphocytes of any subset in the normal controls or A-T patients with some ATM activity. However in the A-T patients with no ATM activity the percentage of FasL+ve cells tended to decrease as IL-7 concentration increased. There were significant negative correlations between IL-7 concentration and FasL expression on NK cells and NK cell subsets, total T cells and all T cell subsets with the exception of CD4+ve and CD8+ve TEMRA T cells and CD8+ve effector memory T cells. IL-7 concentration had little effect on the FasL MFI of FasL+ve cells although there was a significant negative correlation between the FasL MFI of FasL+ve memory B cells and IL-7 concentration in the normal controls and a significant positive correlation between the FasL MFI of FasL+ B cells and IL-7 concentration in the A-T patients with no ATM activity.

There was no evidence of any effect of IL-7 concentration on expression of its receptor CD127.

Table 3:8:5: Correlations between IL-7 concentration and percentage of lymphocytes, CD95, FasL, and CD127 expression and age.

	correlations with IL-7 concentration																						
	% lymphocytes			% CD95+			CD95 MFI			% FasL+			FasL MFI			% CD127+			CD127 MFI			age		
	normal	A-T some ATM activity	A-T no ATM activity	normal	A-T some ATM activity	A-T no ATM activity	normal	A-T some ATM activity	A-T no ATM activity	normal	A-T some ATM activity	A-T no ATM activity	normal	A-T some ATM activity	A-T no ATM activity	normal	A-T some ATM activity	A-T no ATM activity	normal	A-T some ATM activity	A-T no ATM activity	normal	A-T some ATM activity	A-T no ATM activity
T cells	ns	ns	ns	ns	ns	+	ns	ns	ns	ns	ns	-	ns	ns	ns	ns	ns	ns	ns	ns	ns			
CD4+ T cells	ns	ns	ns	ns	ns	+	ns	ns	ns	ns	ns	-	ns	ns	ns	ns	ns	ns	ns	ns	ns			
CD4+ naive	ns	ns	ns	+	ns	+	ns	ns	ns	ns	ns	-	ns	ns	ns	ns	ns	ns	ns	ns	ns			
CD4+ central memory	ns	ns	ns	+	ns	+	+	ns	ns	ns	ns	-	ns	ns	ns	ns	ns	ns	ns	ns	ns			
CD4+ effector memory	ns	ns	ns	ns	ns	+	+	ns	ns	ns	ns	-	ns	ns	ns	ns	ns	ns	ns	ns	ns			
CD4+ TEMRA	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns			
CD8+ T cells	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	-	ns	ns	ns	ns	ns	ns	ns	ns	ns			
CD8+ naive	ns	ns	ns	+	ns	ns	ns	ns	ns	ns	ns	-	ns	ns	ns	ns	ns	ns	ns	ns	ns			
CD8+ central memory	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	-
CD8+ effector memory	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns			
CD8+ TEMRA	ns	ns	ns	ns	ns	ns	ns	ns	-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns			
B cells	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	+									
naive B cells	ns	ns	ns	ns	ns	ns	ns	ns	+	ns	ns	ns	ns	ns	ns									
memory B cells	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	-	ns	ns									
NK cells	ns	ns	ns	+	ns	ns	ns	ns	ns	ns	ns	-	ns	ns	ns	ns	ns	ns	ns	ns	ns			
CD56dim NK cells	ns	ns	ns	+	ns	ns	ns	ns	ns	ns	ns	-	ns	ns	ns	ns	ns	ns	ns	ns	ns			
CD56bright NK cells	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	-	ns	ns	ns	ns	ns	ns	ns	ns	ns			
NKT cells	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns			

Table 3:8:5: Summary of significant correlations between plasma IL-7 concentration and age, percentages of lymphocyte subsets and expression of CD95, FasL and CD127 in blood samples from A-T patients and normal controls.

The table summarises significant correlations between IL-7 plasma concentration and percentages of lymphocyte subsets, IL-7 plasma concentration and expression of CD95, FasL and CD127 (both percentage of positive cells and MFI) and plasma IL-7 concentration and age in normal controls and A-T patients. Yellow (+) cells indicate a positive correlation and blue (-) cells indicate a negative correlation in the corresponding group, 'ns' indicates no significant correlation.

3:9: Discussion.

My phenotype analysis of A-T lymphocytes revealed what I believe is a congenitally aged immune system in A-T patients. The age associated failing of adaptive immunity is known as immune senescence and leads to increased incidence of infectious disease and reduced response to vaccination in the elderly. Low numbers of naive T cells and increased memory T cells (with reduced TCR diversity) are considered markers of immune senescence (reviewed in (Pawelec et al., 2010)) but reduced overall T and B cell numbers and increased NK cells are also characteristic of the aging immune system (Sansoni et al., 2008).

3:9:1: A-T patients were deficient in naive lymphocytes and had increased NK and NKT cells.

My analysis of 18 A-T patients showed that they were lymphopenic with deficiencies in CD4+ve naive T cells, CD8+ve naive T cells and naive B cells and increased percentages of memory T cells, memory B cells, NK cells and NKT cells compared to normal controls. A decrease in the absolute number of naive T cells but normal numbers of memory T cells and increased NK cell number in A-T patients has previously been reported (Schubert et al., 2002). Therefore the lymphopenia in A-T patients appears to be due to a deficiency in naive cells.

Although the percentage of NK cells was increased in A-T patients I did not find any difference in the proportions of CD56dim or CD56bright NK cell subsets compared to normal controls. The majority of circulating NK cells in both A-T patients and normal controls belonged to the cytotoxic CD56dim subset rather than the cytokine producing CD56bright subset. NK cells are particularly important in the defence against viral infection and an increase in their number in A-T patients (Schubert et al., 2002) may compensate for naive T

cell deficiency and reduced antigen receptor repertoire. This could explain why A-T patients do not more commonly suffer from severe viral or opportunistic infections despite their T cell deficiency (Nowak-Wegrzyn et al., 2004).

NKT cells were also increased in A-T patients compared to normal controls. These are a rare subset of highly cytotoxic T cells which have an unusual γδ TCR which confers specificity to glycolipid antigens (Godfrey et al., 2010). They are involved in the response to bacterial, viral and parasitic infections and also have an immunoregulatory function (reviewed in (Yu and Porcelli, 2005)). Like conventional T cells NKT cells develop in the thymus and undergo V(D)J recombination. Although NKT cells have not been investigated previously in A-T patients an increased proportion of γδ TCR bearing T cells has been reported (Carbonari et al., 1990). Increased NKT cells may explain or at least contribute to the increased proportion of γδ TCR bearing T cells in A-T patients. However, this does not invalidate the hypothesis that the defect in V(D)J recombination caused by the absence of functional ATM is more detrimental to the rearrangement of conventional αβ TCRs than γδ TCRs (Carbonari et al., 1990). Glycolipid vaccine adjuvants which activate NKT cells are soon to enter clinical trials (Li et al., 2010). My finding of increased NKT cells in A-T patients suggests that such adjuvants could be beneficial in improving their response to vaccination.

In general I found that A-T patients with some ATM activity had an intermediate phenotype between that of the normal controls and A-T patients with no ATM activity, this suggests that residual ATM kinase activity may reduce the severity of the immunological deficiency in A-T. A similar finding was reported by Staples et al. (2008) who compared lymphopenia, immunoglobulin deficiency and frequency of recurrent sinopulmonary infections in A-T patients with and without residual ATM activity and found that patients with residual ATM activity had a less severe immune deficiency (Staples et al., 2008).

My results showed clear differences in the way the lymphocyte phenotypes of normal controls and A-T patients changed with age over the 1 to 30 year age range. In the normal controls the proportions of T cells and B cells did not change but the proportions of memory T cells, memory B cells, NK cells and NKT cells increased and naive T cells and naive B cells decreased with age. In contrast there was an increase in the proportion of T cells and a decrease in NK cells in A-T patients of different ages but no change in the proportions of B or T cell subsets. Therefore the lymphocyte phenotypes of even the youngest A-T patients were more similar to that of the oldest normal controls than to normal controls of comparable age. This suggested differential aging of the immune system in A-T patients and normal controls.

The age related decrease in the proportion of naive T cells and increase in the proportions of memory T cells subsets in the normal controls was unsurprising as thymic involution begins in early childhood, leading to a decrease in production of naive T cells, whilst increasing numbers of antigen encounters over time leads to oligoclonal expansion of memory T cells (Giovannetti et al., 2002). These changes will result in a proportional decrease in naive T cells and increase in memory T cells during normal aging. The increase in the percentage of NK cells with age may compensate for a reported decrease in the absolute number of total T cells with age (Sansoni et al., 1993).

In contrast to normal individuals, thymic output in A-T patients is low even in infants (Micheli et al., 2003). Therefore it is possible that the effect of thymic involution on T cell repertoire in A-T patients is small as it starts from a low baseline. Although I did not find a significant increase in the percentage of any individual T cell subset with age in A-T patients it is likely that the increase in total T cells was due to oligoclonal expansions of memory cells. It would be interesting to look at how absolute cell numbers change with age as it is possible

that the decrease in the percentage of NK cells with age is a proportional change due to the increase in percentage of T cells rather than an actual change in absolute number of NK cells.

My finding of no significant change in the proportions of B and T cell subsets in A-T patients of different ages suggested a congenitally aged rather than prematurely aged immune system.

3:9:2: A-T patients had increased CD95 expression.

I showed that CD95 expression on all lymphocyte subsets except NK cells was increased in A-T patients compared to normal controls. This was true of the proportion and in many cases also the median fluorescence intensity of positive cells. The difference in CD95 expression was especially dramatic on naive CD4+ve and CD8+ve T cells and naive B cells; CD95 expression was low on these cells in the normal controls (medians of 3.9%, 10.8% and 5.2% CD95+ve cells respectively) but high in the A-T patients with no ATM activity (medians of 35.9%, 74.6% and 23% CD95+ve cells).

CD95 is upregulated on T and B cells in response to activation (Klas, 1993; Mizuno, 2003). Therefore CD95-ve T cells have never encountered antigen and decrease as a direct result of ageing (Sansoni et al., 2008). Naive CD95-ve T cells are important for defense against new pathogens so their age-related decline weakens the immune system. They have been described as a 'hallmark of senescence' (Sansoni et al., 2008) as their number can be used to predict mortality in the very old (Luciani et al., 2001). Naive T cells are CD95-ve on exit from the thymus and upregulate CD95 on activation, therefore the low percentage of CD95-ve naive T cells and high percentage of CD95+ve naive T cells that I have shown in A-T patients is consistent with low thymic output. Similarly to the elderly, A-T patients with a CD95-ve naive T cell deficiency will have an impaired ability to respond to new antigens compared to

young healthy individuals, this may contribute to their increased susceptibility to sinopulmonary infections.

My non-longitudinal analysis showed that CD95 expression on T cells, B cells and NKT cells increased with age in the normal controls whilst there was a decrease in expression on CD4+ve T cells and memory B cells in A-T patients with no ATM activity. The increase in CD95 expression on lymphocytes during normal ageing was unsurprising as increased activation of the immune system (due to unknown stimuli) (Fagnoni et al., 1996;Sansoni et al., 1993;Sansoni et al., 2008) and increased CD95 expression on CD4+ve and CD8+ve T cells with age have previously been reported (Aggarwal & Gupta, 1998;Potestio et al., 1999).

Interestingly Potestio et al (1999) found a differential effect of aging on CD95 expression on CD4+ve and CD8+ve T cells. Whilst CD95 expression on CD8+ve T cells in healthy individuals increased with age from 0 (cord blood) to 102 years, CD95 expression on CD4+ve T cells increased from 0 to 74 years but then decreased with age in individuals over the age of 75. This is consistent with my finding of a significant positive correlation between both proportion and CD95 MFI of CD8+CD95+ T cells and age and the proportion of CD4+CD95+ T cells and age over the 0 to 30 year age range in the normal controls. However the A-T patients with no ATM activity showed a significant positive correlation between age and the CD95 MFI of CD8+CD95+ T cells and a significant negative correlation between age and the percentage of CD4+CD95+ cells over the same age range.

It has been reported that CD4+ve T cell subsets preferentially undergo CD95-mediated apoptosis in response to anti-CD95 (CH11) treatment compared to CD8+ve T cell subsets in both young and elderly individuals (Aggarwal & Gupta, 1998). Potestio et al (1999) suggested that the increase in CD4+CD95+ T cells during normal aging is a marker of immune senescence as a slightly decreased percentage of these cells in very old people (85-102)

compared to younger elderly people (75-84) in their non-longitudinal study indicated successful aging. In contrast, the increase in CD8+CD95+ T cells is a marker of aging as it continued to increase across all the age groups analysed including the very old. The apparent reduction in CD95 expression on CD4+ve T cells in the oldest elderly patients (85 to 102 years) could be due to selection of long lived healthy individuals with a slow rate of immune senescence as those with a faster rate and therefore a higher percentage of CD4+CD95+ T cells may have increased morbidity and mortality (Potestio et al., 1999). It is possible that a similar selection pressure could explain the negative correlation between CD95 expression on CD4+ve T cells and age that I have reported in A-T patients with no ATM activity, as those with the highest CD95 expression on their CD4+ve T cells may also have increased morbidity and mortality. Alternatively the decreasing CD95 expression on CD4+ve T cells with age in different A-T patients may be related to increasing FasL expression or decreasing plasma IL-7 concentration as discussed in sections 3:9:3 and 3:9:5 respectively.

3:9:3: A-T patients had reduced FasL expression.

My results showed expression of FasL on T cells, B cells, NK cells and NKT cells in all A-T patients and all normal controls. FasL expression on B cells was unexpected as studies investigating FasL expression on normal lymphocytes have generally concentrated on T cells and NK cells, perhaps due to the size limitations of antibody panels. However FasL can be expressed on immature B lymphocytes, CD19+ve cells in normal human bone marrow and on some human B cell lines (Nilsson et al., 2000) and functional FasL expression on activated B cells has been shown in the mouse (Hahne et al., 1996a). In addition upregulation of FasL on human B cells has been reported in response to bacterial and viral infections including HIV and EBV and constitutive expression has been described on B cells from systemic lupus erythematosus patients (reviewed in (Lundy, 2009)).

My finding of FasL expression on B cells in both A-T patients and normal controls was convincing due to the significant percentage and FasL MFI of the positive cells. It is unlikely that the result was a false positive as the antibody panel was carefully selected and optimized to avoid compensation problems and there was no background when the FasL antibody was replaced with its isotype control in the eleven colour panel. It is possible that the FasL expression may be due to infection of the B cells with EBV or other viruses, however further work would be required to confirm if this is the case.

I did not find a significant difference in the percentage of FasL+ve cells of any type between normal controls and A-T patients with no ATM activity, however on the majority of cell types the FasL MFI of FasL+ve cells was significantly higher in the normal controls. The difference was especially dramatic on naive and memory B cells.

I also found differences in the effect of aging on FasL expression on lymphocytes from A-T patients and normal controls. The percentages of FasL+ve NK cells and T cells of all subsets excluding TEMRA and the FasL MFI of CD4+FasL+ T cell subsets and CD8+FasL+ naive T cells increased, and the FasL MFI of FasL+ B cells decreased significantly with age in A-T patients with no ATM activity. In the normal controls there was no change in FasL expression with age on the majority of subsets. However there was a decrease in the percentage of FasL+ve NKT cells, an increase in the FasL MFI of FasL+ve total T cells and FasL+ve total and naive B cells and a decrease in the FasL MFI of FasL+ve CD56bright NK cells with age.

It is unclear if reduced FasL MFI has a significant effect on the immune system of A-T patients. It could be argued that the reduction in FasL expression will result in reduced CD95-mediated apoptosis, however a low FasL MFI does not prevent a FasL expressing cell from binding to CD95 and initiating apoptosis. The lymphopenic phenotype of A-T patients suggests that CD95-mediated apoptosis is not impaired as this would lead to an

accumulation of lymphocytes similar to that seen in autoimmune lymphoproliferative syndrome (ALPS) patients whose cells are resistant to CD95-mediated apoptosis due to *Fas* mutation. It may be interesting to investigate soluble FasL concentration in plasma or serum samples from A-T patients and normal controls as a decrease in membrane bound FasL may correlate with an increase in its soluble form. As soluble FasL is able to bind to CD95 and initiate apoptosis this could negate any potential effect of reduced apoptosis due to reduced membrane bound FasL.

The increase in the percentage of FasL+ve T cells and NK cells with age that I have shown in A-T patients with no ATM activity may contribute to the age-related decrease in the percentage of CD4+CD95+ naive, central memory and TEMRA T cells and CD95+ve memory B cells by increasing CD95-mediated apoptosis of these cells.

3:9:4: A-T patients had normal CD127 expression.

My results showed that A-T patients with no ATM activity were not deficient in CD127 (IL-7Rα) expression compared to normal controls. However whilst CD127 expression on all T cell subsets increased with age in normal controls the only increase in expression with age on A-T patients was on CD8+ve effector memory T cells. This suggests that in healthy individuals the ability of T cells to bind IL-7 and proliferate in response to the cytokine may increase with age, perhaps in order to compensate for the age related decline in output of T cells from the thymus. If CD127 expression is related to thymic output this could explain why a similar age related increase in CD127 expression on T cells is not seen in A-T patients as their thymic output is very low even in infancy.

Interestingly CD127 expression on the majority of T cell subsets was significantly higher in A-T patients with some ATM activity than A-T patients with no ATM activity and normal

controls. However, as only three A-T patients with some ATM activity were analysed this finding may not be representative of a larger group of patients with residual ATM activity. The increased CD127 expression may be related to the older mean age of the A-T patients with some ATM activity (16 years 3.7 months) compared to normal controls (8 years 6.4 months) and A-T patients with no ATM activity (9 years 4 months) as I found that CD127 expression increased with age in the normal controls.

As A-T T cells are not deficient in CD127 expression it is unlikely that their ability to bind and respond to IL-7 is impaired.

3:9:5: A-T patients had normal plasma concentrations of IL-7.

Despite their lymphopenia I did not find elevated concentrations of IL-7 or IL-15 in A-T patients' plasma compared to normal controls. However, there was a negative correlation between plasma IL-7 concentration and age in A-T patients with no ATM activity. This finding was surprising, even in the context of premature aging, as a study of IL-7 expression in healthy individuals including centenarians showed no decline with age (Nasi et al., 2006). The decrease in IL-7 concentration with age that I have shown in A-T patients is more likely to be the result of a decrease in production of the cytokine than an increase in its consumption as the number of lymphocytes did not increase with age. The reduction in available IL-7 appeared to have no effect on A-T T cell repertoire as the proportions of T cell subsets did not change and the total percentage of T cells increased with age.

Increased IL-7 concentration has been implicated in CD95 upregulation on T cells (Brugnoni et al., 1999;Rethi et al., 2008) and I have shown increased CD95 expression on A-T lymphocytes. Although A-T patients did not have elevated plasma IL-7 concentrations they were lymphopenic so the amount of IL-7 available per T cell was considerably greater than in

the normal controls. Therefore a role for the cytokine in CD95 upregulation on A-T lymphocytes cannot be ruled out.

Interestingly I found that in A-T patients with no ATM activity both plasma IL-7 concentration and CD95 expression on CD4+ve T cells and memory B cells decreased with age and IL-7 concentration correlated positively with expression of CD95 on total T cells, CD4+ve T cells (excluding TEMRA cells) and naive B cells. This suggests that the decrease in plasma IL-7 concentration could be a contributing factor in the decrease in CD95 expression with age. In the normal controls positive correlations between IL-7 plasma concentration and CD95 expression on CD4+ve naive, central and effector memory T cells, CD8+ve naive T cells and both total and CD56dim NK cells also suggested a role for IL-7 in regulation of CD95 expression.

The normal level of CD127 expression in A-T patients along with the normal plasma concentration of IL-7 suggests that the lymphopenic phenotype of A-T patients is not due to a deficiency in expression of the cytokine or its receptor.

3:9:6: The immune system of A-T patients is congenitally aged.

The immune system phenotype of A-T patients has striking similarities to that of healthy elderly individuals. Both groups have a naive T and B cell deficiency and increased NK cells compared to healthy young individuals (Giovannetti et al., 2002;Gupta & Gollapudi, 2008;Sansoni et al., 2008). CD95 expression on lymphocytes *in vivo* is increased in A-T patients and increases with normal aging (Aggarwal & Gupta, 1998;Potestio et al., 1999) and both the elderly (Chen et al., 2009;Frasca et al., 2008) and some A-T patients show decreased production of specific antibodies and decreased antibody responses to vaccination (Nowak-Wegrzyn et al., 2004;Sadighi Akha et al., 1999). In addition both A-T

patients (Reichenbach et al., 2002) and the elderly normal population (reviewed in (Oliveira et al., 2010)) have low thymic output and elevated levels of oxidative stress. These similarities suggest a congenitally aged immune system in A-T.

Despite the similarities there may also be differences between the immune system phenotypes of A-T patients and the elderly. Gupta (2005) analysed the effect of normal aging on T cell subsets and found no change in the percentage but a decrease in the absolute number of central memory T cells in the elderly (≤65 years) (Gupta, 2005). Another study by the same group showed similar CD95 expression on naive and memory T cells from young and elderly individuals (Gupta & Gollapudi, 2008) and an increase in the percentage of FasL+ve CD4+ve and CD8+ve T cells in the elderly has also been reported (Aggarwal & Gupta, 1998).

In contrast, I found that in A-T patients (1 to 28 years) the percentage of central memory T cells was significantly increased compared to normal controls (1 to 30 years), CD95 expression was lower on naive T cells than on memory T cell subsets and there was no significant difference in the percentage of FasL+ve CD4+ve and CD8+ve T cells but the FasL MFI of FasL+ve CD4+ve and CD8+ve T cells was decreased compared to normal controls. Interestingly CD95 expression on T cells in A-T patients with no ATM activity was higher than on both the young adult and elderly cohorts in the Gupta & Gollapudi (2008) study.

However these differences may be the result of variations in experimental design rather than indicitative of real differences in the immune system phenotypes of A-T patients and the elderly. In my study central memory T cells were defined as CD45RA-CD27+ and in the Gupta study (2005) they were defined as CD45RA-CCR7+. Although CD27 and CCR7 are coexpressed on naive T cells (Ferrando-Martinez et al., 2010), the populations of memory T cells defined by these sets of markers are not identical (Fritsch et al., 2005;Tomiyama et al.,

2002). Therefore the apparent difference in the proportion of central memory T cells in A-T patients and the elderly may be due to the difference in markers. If the antibody panels had been the same it is likely that the analysis would have shown similar proportions of central memory T cells in A-T patients and the elderly.

Gupta & Gollapudi (2008) found comparable levels of CD95 expression on all T cell subsets whereas I found that in A-T patients and normal controls CD95 expression was lower on naive than memory T cells. However, Gupta & Gollapudi (2008) analysed CD95 expression following activation with anti-CD3 and I analysed CD95 expression on unstimulated lymphocytes. As CD95 is upregulated on naive T cells in response to activation it is unsurprising that there was no difference in CD95 expression on naive and memory T cell subsets in the Gupta & Gollapudi (2008) study. Therefore I do not believe that the difference in results represents a real difference in CD95 expression on T cell subsets in A-T patients and the elderly. The artificial activation in Gupta & Gollapudi (2008) explains why in contrast to previous studies (Aggarwal & Gupta, 1998;Potestio et al., 1999) they did not report an increase in CD95 expression on T cells with age. However, despite the activation-induced CD95 upregulation, expression on T cell subsets in the young adult and elderly cohorts of Gupta & Gollapudi (2008) was not as high as the expression I found on T cell subsets in A-T patients with no ATM activity.

My finding of reduced FasL expression on T cells in A-T patients with no ATM activity compared to normal controls also appears to be inconsistent with an 'immunologically aged' immune system phenotype as an increased percentage of FasL+ve CD4+ve and CD8+ve T cells in the elderly has been reported (Aggarwal & Gupta, 1998). However, in the latter study PBMCs were activated with PHA and IL-2 followed by PMA and ionomycin to upregulate FasL prior to analysis whereas I analysed FasL expression on unstimulated

lymphocytes from normal controls and A-T patients. It would be informative to analyse FasL expression on unstimulated lymphocytes from the elderly in order to directly compare with A-T patients.

As all of the differences I have discussed may be explained by differences in experimental protocol I do not believe that they represent real differences in phenotype between the immune systems of A-T patients and the elderly.

It is clear that lymphocytes in A-T patients and the elderly are similar in terms of CD95 expression. Interestingly Gupta & Gollapudi (2008) showed that naive and central memory T cells were significantly more sensitive to CD95-mediated apoptosis in elderly individuals than in the young. They concluded that deficiencies in naive and central memory T cell subsets in aged humans were partly due to this increased sensitivity. Effector memory and TEMRA T cells from both young and elderly groups were resistant to CD95-mediated apoptosis.

It would be interesting to compare the sensitivity of A-T T cells of different subsets to CD95-mediated apoptosis. It is probable that similarly to healthy elderly individuals, effector memory and TEMRA T cells from A-T patients would be resistant, but naive and central memory T cells would show increased sensitivity to CD95-mediated apoptosis. As well as its role in apoptosis CD95 can provide co-stimulatory signals, which in combination with TCR signalling can induce proliferation of T cells recognising low-affinity antigens. This raises the possibility that increased CD95 expression could contribute to both the naive T cell deficiency of A-T patients (by increasing apoptosis) and the predominance of activated memory T cells (by increasing proliferation).

The immune system phenotype of A-T patients with no ATM activity illustrates the effect of complete loss of ATM protein kinase activity on the immune system. The primary cause of

immune deficiency in A-T and immune senescence during normal aging is probably the naive cell deficiency. In A-T this is undoubtedly the result of a failure in the DNA damage response due to ATM deficiency. Interestingly mouse studies have suggested that a decline in ATM expression and function occurs during normal aging (Fang 2007, Panda 2007) and a reduction in DNA double strand break repair capacity has been reported in PBMCs from elderly people (Frasca et al., 1999). A defect in DNA repair would help to explain the many similarities between the aged immune system and the immune system of A-T patients, it may also help to explain why the ATM mutant tumour T-PLL is associated with aging.

The result of my phenotyping experiment in combination with the published literature suggests that defective DNA damage response due to loss of ATM function results in some or all of the following features which contribute to the congenitally aged immune system phenotype of A-T patients, and possibly also to normal immune senescence in the context of declining DNA damage repair function with age:

1. A deficiency of both T and B cell progenitors. ATM has a role in telomere maintenance and its deficiency has been implicated in telomere dysfunction in humans (Metcalfe et al., 1996) and mice (Wong et al., 2003). Wong et al. (2003) showed increased apoptosis of haematopoietic progenitor cells in the bone marrow of ATM-/- mice. They suggested that defective telomere maintenance due to ATM deficiency caused increased apoptosis and limited proliferation of the cells leading to a deficiency of both T and B cell progenitors. This may explain the finding of Xu et al. (1996) who reported a pre-B cell deficiency in ATM-/- mice (Xu et al., 1996). It could also contribute to the naive B and T cell deficiency that I and others (Giovannetti et al., 2002;Schubert et al., 2002) have shown in A-T patients. Interestingly the proliferative and regenerative capacity of haematopoietic stem cells

decreases with age (reviewed in (Rossi et al., 2008)), this may result in a similar deficiency in T and B cell progenitors in the elderly.

2. Defective V(D)J recombination. T cells in the thymus undergo a process of V(D)J recombination which creates functional antigen receptor genes. In normal individuals this process relies on ATM-dependent NHEJ to repair the double strand breaks in DNA created during the process (Bredemeyer et al., 2006;Bredemeyer et al., 2008). In the absence of ATM activity relatively few cells achieve functional TCR rearrangements (Giovannetti et al., 2002); therefore the recombination defect may explain the low thymic output of A-T patients (Giovannetti et al., 2002;Micheli et al., 2003). Failure of conventional V(D)J recombination may also explain the relative increase in T cells with γδ rather than αβ T cell receptors in A-T patients (Carbonari et al., 1990;Micheli et al., 2003). However, I have shown an increased percentage of NKT cells in A-T patients. As these cells also express γδ TCRs they may contribute to or explain the increase in the proportion of T cells with γδ TCRs in A-T patients.

3. Low thymic output. This may be related to defective V(D)J recombination. Low thymic output contributes to both the overall naive T cell deficiency and the deficiency in CD95-ve naive T cells that I have shown in A-T patients. The cause of thymic involution during normal aging and consequent low thymic output in the elderly is not yet clear (Lynch et al., 2009).

4. CD95 upregulation on lymphocytes. It is not possible to determine from my lymphocyte phenotyping experiment whether or not increased CD95 expression on A-T lymphocytes is a direct consequence of lack of ATM activity. However it is likely to be the result of a combination of increased activation, oxidative stress (McKallip et al., 2010) resulting from the inability to repair DNA damage caused by ROS and increased IL-7 availability as a result of naive T cell deficiency and lymphopenia. Increased CD95 expression on lymphocytes in

the elderly may be related to prolonged *in vivo* activation (Aggarwal & Gupta, 1998). However oxidative stress is also increased in the elderly (reviewed in (Oliveira et al., 2010)) and this could contribute to CD95 upregulation.

5. Naive T and B cell deficiency. In A-T patients this may be the result of a combination of T and B cell progenitor deficiency (Wong et al., 2003), low thymic output of naive T cells (Giovannetti et al., 2002;Micheli et al., 2003), pre-B cell deficiency (Xu et al., 1996) and increased spontaneous and FasL induced CD95-mediated apoptosis of CD95+ve naive T and CD95+ve naive B cells, as suggested by the high level of CD95 expression that I have shown on these cells. Similar factors may account for naive B cell (Veneri et al., 2009) and naive T cell deficiency (Giovannetti et al., 2002;Gupta & Gollapudi, 2008;Sansoni et al., 2008) in the elderly.

6. Oligoclonal expansions of memory T cells. Dramatic expansions of memory cell populations may be detrimental as they reduce the ability of the immune system to fight heterologous infection (Khan et al., 2002). In A-T patients peripheral T cell oligoclones occur due to the limited antigen receptor repertoire which results from a reduction in the number of thymocytes which achieve functional TCR rearrangements in the absence of ATM (Giovannetti et al., 2002). Although I have shown that memory T cells in A-T patients express high levels of CD95 it is likely that similar to effector memory and TEMRA T cells in the elderly (Gupta & Gollapudi, 2008) they are resistant to CD95-mediated apoptosis and may proliferate in response to TCR signalling in combination with co-stimulation through CD95. Oligoclonal expansions of memory T cells in the elderly result from chronic viral infection, CMV infection in particular results in very large expansions (Khan et al., 2002).

7. Immunoglobulin deficiencies. Class switch recombination is required to produce antibodies of different isotypes and effector function but the same specificities as the

original IgD and IgM antibodies. The process involves the generation and repair of DNA double strand breaks and requires ATM (Lumsden et al., 2004). Therefore failure of the ATM-dependent joining process may account for the low serum antibody levels of A-T patients (Reina-San-Martin et al., 2004). Interestingly a defect in class switch recombination resulting in immunoglobulin deficiency has also been reported in the elderly (Frasca et al., 2008).

In conclusion the striking similarities between the immune system phenotypes of A-T patients and the elderly suggest a congenitally aged immune system in A-T. This is supported by my observation that there is little difference in the proportions of lymphocyte subsets in A-T patients of different age; they show the characteristics of an aged immune system from birth rather than a premature or accelerated rate of immune senescence starting from a normal baseline.

I have confirmed that A-T patients have a deficiency in naive T cells and naive B cells, increased proportions of memory T and B cells and increased NK cells. In addition I have shown for the first time that the proportions of CD56bright and CD56dim NK cells are normal and the proportion of NKT cells are increased A-T patients. It is likely that the increase in both NK and NKT cells in A-T patients compensates for the naive T cell deficiency and so reduces the frequency of severe viral and opportunistic infections in A-T patients.

I have also shown that the lymphopenia and naive T cell deficiency of A-T patients is not due to an IL-7 or CD127 deficiency because their levels of expression were normal in A-T patients. Interestingly I found a correlation between IL-7 plasma concentration and CD95 expression on CD4+ve T cells in A-T patients. This suggests that IL-7 may be involved in upregulation of CD95 on these cells. Therefore attempting to increase naive T cell proliferation in A-T patients using IL-7 therapy could be detrimental as it may result in

upregulation of CD95 on T cells leading to increased spontaneous and CD95-mediated apoptosis and worsening of the lymphopenia.

Low thymic output undoubtedly has an important role in shaping the immune system of A-T patients, however my finding of increased CD95 expression on all lymphocyte subsets excluding NK cells and the striking similarities to elderly patients who show both increased CD95 expression on lymphocytes and increased sensitivity to apoptosis of naive T cells suggests that increased CD95-mediated apoptosis may also contribute to the immune deficiency in A-T.

Chapter 4: Investigating the sensitivity of A-T cells to CD95-mediated apoptosis.

4:1: Introduction

A-T lymphocytes express very high levels of the death receptor CD95. This suggests a possible contribution of increased CD95-mediated apoptosis to the lymphopenia seen in A-T patients. Little is known about the function of ATM in extrinsic apoptotic pathways, such as CD95-mediated apoptosis. It is possible that abnormalities in these processes could contribute to the development of the lymphopenia and/or the lymphoid tumours that are common in A-T patients.

CD95-mediated apoptosis is initiated by the binding of Fas ligand to CD95 (Fas receptor). CD95 belongs to a subgroup of the tumour necrosis factor family of receptors (TNF-R) which also includes TRAIL. It contains an intracellular 'death domain' and binding of the ligand initiates a cell death pathway that activates caspases resulting in apoptosis of the CD95-expressing cell. The process can also be initiated by overexpression of CD95 on the cell surface leading to autoactivation and apoptosis (Le Clorennec et al., 2008). In certain situations, signalling through CD95 can also initiate non-apoptotic pathways leading to cellular activation, proliferation and differentiation (reviewed in (Strasser et al., 2009)).

CD95-mediated apoptosis has important roles in both the killing of pathogen-infected cells and in the regulation of the immune system. It is central to the maintenance of immune system homeostasis, especially in the termination of an immune response. The removal of surplus B cells by activated T cells towards the end of an immune response is CD95 dependent (Hao et al., 2008), and CD95 also has an important role in activation-induced cell death (AICD) of mature peripheral T cells. This reduces the number of reactive T cells during the down phase of an immune response and could help to prevent autoimmunity by deleting autoreactive T cells. CD95-mediated apoptosis may also be involved in the negative

selection of B cells in the germinal centres and of T cells in the thymus (Siegel et al., 2000;Takahashi et al., 2001).

Recent work has suggested a role for ATM in determining sensitivity to CD95-mediated apoptosis, although results in different cell types are conflicting. A recent study by Stagni et al. reported that although ATM inhibition did not alter the expression of CD95 in lymphoblastoid cell lines (LCLs), ATM deficiency caused resistance to CD95-mediated apoptosis (Stagni et al., 2008). This was reported to occur through upregulation of the anti-apoptotic protein cFLIP by the LCLs in the absence of ATM. Reconstitution of cells with ATM kinase decreased the cFLIP level and restored sensitivity to CD95-mediated apoptosis. In contrast, Ivanov et al. showed that inhibition of ATM kinase activity in melanoma cells prior to irradiation downregulated cFLIP. Inhibition had no effect on expression of CD95 but upregulated the TRAIL receptor DR5. This, together with cFLIP downregulation, enhanced TRAIL-mediated apoptosis (Ivanov et al., 2009). CD95-mediated apoptosis was not investigated in the study but cFLIP downregulation in melanoma cells has also been associated with increased sensitivity to CD95-mediated apoptosis (Geserick et al., 2008).

In order to clarify the effect of loss of ATM activity on sensitivity to apoptosis, CD95 and cFLIP protein expression, as well as sensitivity to CD95-mediated apoptosis, was investigated in LCLs derived from normal healthy donors and A-T patients. The A-T LCLs were significantly more sensitive to CD95-mediated apoptosis than normal LCLs but there was no evidence for a role of ATM in regulating either CD95 or cFLIP protein expression. The sensitivity to CD95-mediated apoptosis of several B-CLL and two T-PLL tumours was also analysed. The increased sensitivity to CD95-mediated apoptosis of A-T LCLs and the sensitivity of T-PLL tumours with *ATM* mutations, suggests that *ATM* mutations increase the sensitivity to CD95-

mediated apoptosis of lymphoid cells; this could contribute to the lymphopenic phenotype of A-T patients.

4:2: Development of assays for testing the sensitivity of LCLs to CD95-mediated apoptosis.

4:2:1: Lymphoblastoid cell lines

The sensitivity to apoptosis of lymphocytes from A-T patients and normal controls was analysed using a bank of lymphoblastoid cell lines (LCLs). A total of 24 A-T patient (AT1 - AT24) and 19 normal LCLs (N1 – N19) were used in the study. The A-T LCLs were generated by colleagues from blood samples collected from patients attending A-T clinics. Normal control LCLs were generated from lab donor PBMCs. Four of the A-T LCLs (AT4, AT7, AT10 and AT21) were from patients with residual ATM kinase activity; the remainder had no ATM activity. There were two sets of siblings in the A-T patient group (AT19 & AT20 and AT23 & AT24) in each set both siblings had the same ATM mutations.

Table 4:2:1: A-T LCLs.

	sex	residual ATM protein		*ATM* mutations	
		amount expressed	activity	mutation 1	mutation 2
AT1	F	0%	none	c.7638_7646del9; p.(Arg2547_Ser2549del)	c.6997_6998insA p.(Thr2333X)
AT2	F	0%	none	IVS40-1 G>C	c.216_217delAG; p.(Glu73fs)
AT3	M	0%	none	c.748C>T; p.(Arg250X)	c.5679delAG; p????
AT4	F	5%	yes	not identified	not identified
AT5	M	0%	none	c.3802delG; p.(Val1268fs)	not identified
AT6	F	5%	none	c.8520G>C; p (Leu2840Phe)	homozygous
AT7	F	20%	yes	not identified	not identified
AT8	F	10%	none	c.2932 (T>C); p.(Ser978Pro)	c.8395-840del 10
AT9	F	trace	none	c.2T>C; p.(Met1Thr)	c.9139 C>T; p.(Arg3047X)
AT10	M	5%	yes	c. 5623C>T; p.(1875Arg>X)	not identified
AT11	M	0%	none	c.216_217delAG; p.(Glu73fs)	c.8300T>C; p.(Leu2767Pro)
AT12	F	0%	none	IVS19-19delAAT	homozygous
AT13	M	0%	none	c.1262C>T; p.(Ser431X)	homozygous
AT14	M	0%	none	c.5290delC; p.(Leu1764fs)	c.2250G>A (loss of exon 16)
AT15	M	trace	none	c.9138C>T; p.(Arg3047X)	homozygous
AT16	F	0%	none	c.7886_7890del5(TATTA)	not identified
AT17	M	trace	none	c.8494 C>T; p.(Arg2832Cys)	c.1844T>C; p.(Leu615Pro)
AT18	F	0%	none	c.1563_1564delAG; p.(Glu522fs)	c7865C>T; p.(Ala2622Val)
AT19	F	5%	none	c.7638_7646del9; p.(Arg2547_ser2549del)	c.5285C>T; p.(Ala1942Val)
AT20	M	5%	none	c.7638_7646del9; p.(Arg2547_ser2549del)	c.5285C>T; p.(Ala1942Val)
AT21	F	trace	yes	c.1109insA	8517C>G; p.(Phe2839Leu)
AT22	M	0%	none	IVS36+1 G>C (exon 36 skip)	IVS19-19delAAT (exon 20 skip)
AT23	F	100%	none	c.1441/2delT	c.9022C>T; p(Arg3008Cys)
AT24	M	100%	none	c.1441/2delT	c.9022C>T; p(Arg3008Cys)

Table 4:2:1: A-T LCLs.

Amount of residual ATM protein (as percentage of normal protein expression), presence of ATM activity (measured by western blot) and ATM mutations in A-T LCLs. A-T patients with no ATM activity are highlighted in red and those with some ATM activity are highlighted in orange. AT19 & AT20 and AT23 & AT24 were sets of siblings.

4:2:2: Induction of cell death by CH11.

CH11 is a CD95-activating antibody which has been reported to mimic Fas ligand and activate the characteristic CD95-mediated caspase cascade leading to cleavage of caspase 3 and apoptosis (Tran, 2008). The use of CH11 rather than T cell clones in assays testing sensitivity to CD95-mediated apoptosis has the major advantage that any LCL can be used as a target in the assay regardless of HLA type.

In order to test whether CH11 would be effective in inducing cell death of normal and A-T LCLs and to determine the optimum concentration for further experiments, a CH11 titration was carried out on four normal and four A-T LCLs. Three of the A-T LCLs (AT1, AT2, AT6) had no ATM activity and one (AT7) retained some ATM activity. Cell death was measured using a chromium release assay in order to minimise the number of cells of each LCL and the volume of CH11 required for the experiment. LCLs were loaded with chromium and incubated for 15 hours in complete media containing varying concentrations of CH11. This is comparable to the incubation period used in CD95-mediated apoptosis assays with T cell clones.

As the chromium release assay measures the release of radiation by cells as they lyse it does not discriminate between necrotic cell death and apoptosis. In order to account for any non CH11-induced cell death a no-CH11 control for each LCL was analysed to measure spontaneous lysis. The radiation count of the control was then subtracted from the radiation count of the CH11-treated cells and of the maximum lysis control (LCL + 1% SDS) and the percentage of CH11-induced lysis calculated using the equation: 100 x (CH11 release - spontaneous release)/(maximum release - spontaneous release).

The assay results were reproducible (Fig 4:2:2A) and showed a broadly linear relationship between increasing CH11 concentration and increasing lysis over the concentration range

(Fig 4:2:2B). Interestingly three of the four 4 A-T LCLs underwent higher levels of lysis than the normal LCLs at the higher CH11 concentrations (Fig 4:2:2B). A CH11 concentration of 500ng/ml was chosen for further assays as it was estimated to induce lysis of approximately 50% of the cells of the most sensitive LCLs used in the assay (AT1 and AT7). Apoptosis of 100% of cells was undesirable for further assays as it would prevent comparisons being made between the sensitivity of different LCLs to CH11-induced apoptosis.

Fig 4:2:2: CH11 treatment induced lysis of normal and A-T LCLs in a dose dependent manner.

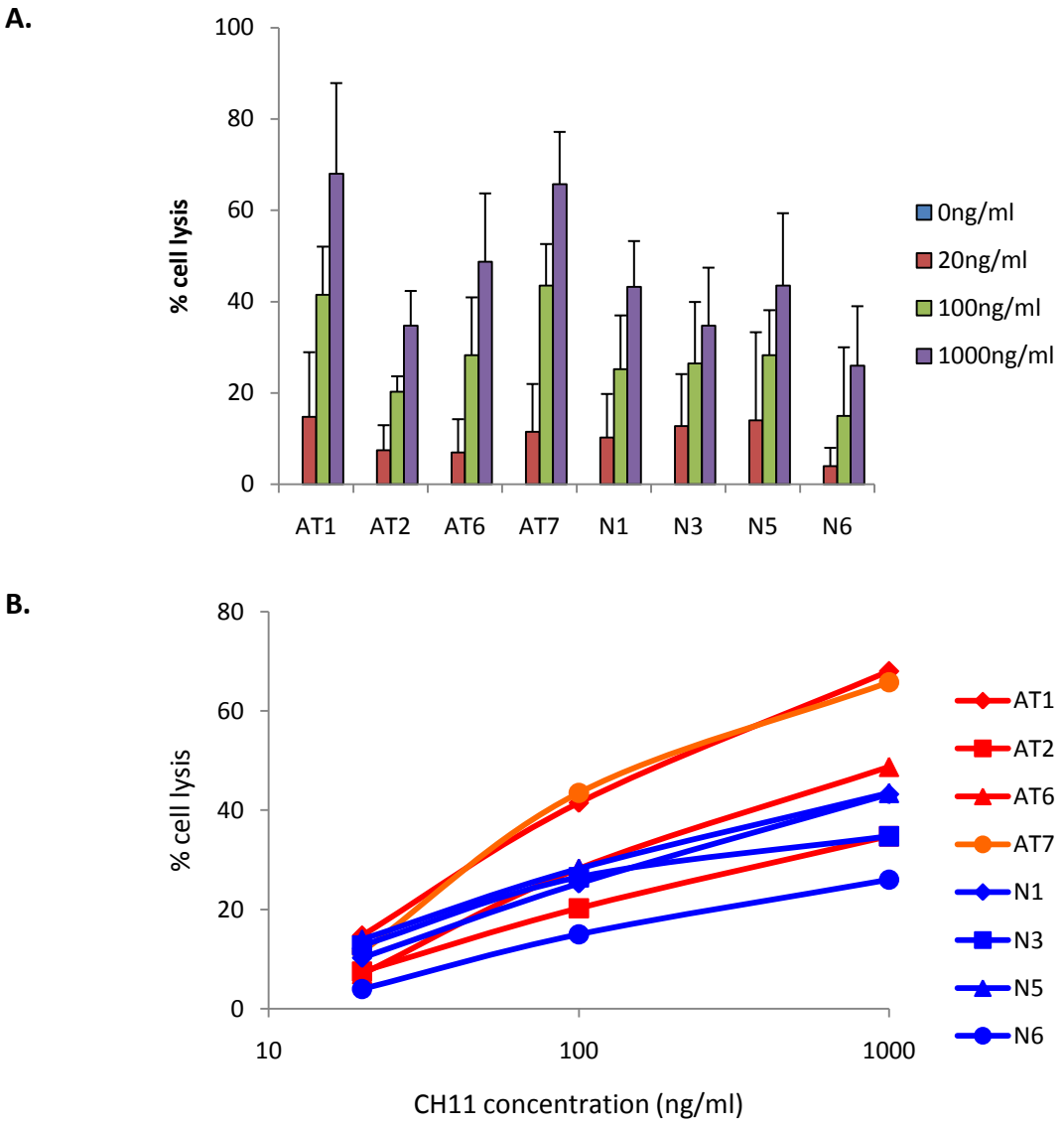


Fig 4:2:2: Cells were loaded with chromium and incubated with increasing concentrations of CH11. The percentage of CH11-induced cell lysis was calculated by chromium release.

A + B Percentage of CH11-induced cell lysis of LCLs at increasing concentrations of CH11 (mean of 4 experiments).

4:2:3: Confirmation of the mechanism of cytotoxicity of CH11.

To test the ability of CH11 to initiate a characteristic death-receptor dependent caspase cascade and initiate apoptosis, a western blot and Annexin V/PI apoptosis assay were carried out together on a normal LCL over a 24h timecourse. Cells were incubated with 500ng/ml CH11 and a sample of cells from each timepoint was stained with Annexin V and PI for analysis of apoptosis with the remaining cells being used to make protein lysates for western blotting.

The results of the Annexin V/PI apoptosis assay were analysed using the gating strategy shown in Fig 4:2:3C. FS/SS gates were drawn to include the lower right 'viable' population and the 'apoptotic' population on the far left. Cells in the extreme lower left corner were excluded as this population largely consists of cell debris. Dead cells which have fragmented during the final stages of apoptosis will form part of this population so the proportion of dead cells may be underestimated. However, as Annexin V and PI staining allows comparisons to be made between changes in viable (Annexin V-PI-) early apoptotic (Annexin V+PI-) and late apoptotic (Annexin V+PI+) cells this is not a major problem. In fact comparisons of the proportion of dead cells (Annexin V-PI-) may not be as robust as comparisons of the proportion of cells still undergoing apoptosis as once cells are dead the assay cannot distinguish between those that have undergone apoptosis and those that have died through necrosis.

The western blot membranes were probed for caspases 7 and 3 to detect activation of the caspase cascade. In order to analyse whether activation of CD95-mediated apoptosis resulted in activation of ATM the levels of total and phosphorylated ATM and its downstream targets SMC1 and Nbs1 were also analysed. Cells not treated with CH11 but

irradiated were used as a positive control for phosphorylation of ATM on Ser1981, SMC1 on Ser966 and Nbs1 on Ser343 and negative control for caspase cascade activation (Fig 4:2:3A).

The presence of a small amount of the cleavage products of caspase 7 and caspase 3 was observed after 2 hours of CH11 treatment. After 4 hours of treatment the majority of these caspases appeared to be in the cleaved state. Following 24 hours of treatment no further apoptosis was evident (Fig 4:2:3A). This result correlates well with the result of the Annexin V/PI assay (Fig 4:2:3B). Very little apoptosis was observed after 2 hours of treatment but by 4 hours apoptosis was well under way with a 20% increase in the percentage of cells in the early stages of apoptosis, this increased to 30% after 8 hours. Cells began to enter the late stages of apoptosis between 4 and 6 hours of treatment and an increase in dead cell number was seen between 8 and 15 hours. No further decrease in cell viability was observed after 8 hours of treatment indicating that a maximum level of apoptosis had been reached. This was in agreement with the western blot (Fig 4:2:3A) which showed a reduction in the amount of cleaved caspase 7 and 3 present after 15h of CH11 treatment. Together these results confirm that CH11 treatment initiates apoptosis by a caspase cascade involving caspase 7 and 3. This is characteristic of death receptor induced apoptosis. As maximum apoptosis appeared to be occurring between 8 and 15 hours of CH11 treatment 15h was chosen as the incubation period for further experiments.

Analysis of ATM expression showed the presence of two bands of ATM, the expected band of full length ATM (370 kDa) and a second smaller band of around 250kDa (Fig 4:2:3A). Cleavage of ATM in response to apoptotic stimuli including CD95 (Stagni et al., 2008), and inducers of DNA damage (Smith et al., 1999;Wang et al., 2006) has been described previously and is likely to be the result of caspase 3 activity (Smith et al., 1999;Stagni et al., 2008). This produces 2 fragments of ATM of approximately 240kDa and 100kDa (Smith et

al., 1999). Only the larger of these fragments could be detected on the western blot as the membrane was cut into sections and probed with individual antibodies (the section containing 100kDa proteins was incubated with the NBS1 antibody so the 100kDa ATM fragment was not detected).

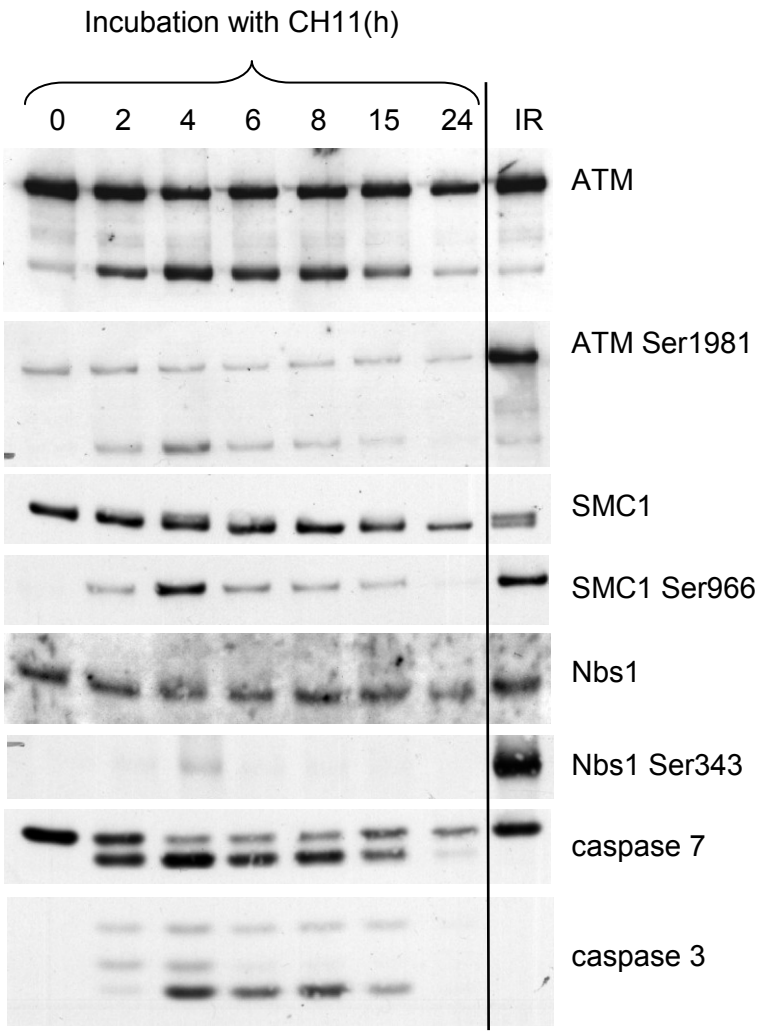
The strong correlation between the presence of cleaved activated caspase 3 and cleaved ATM over the timecourse supports the hypothesis that activity of this caspase is responsible for ATM cleavage. The cleavage of ATM in response to apoptotic stimuli has been shown to reduce its ability to phosphorylate p53 on ser15 but does not affect its DNA binding ability (Smith et al., 1999;Wang et al., 2006). This may prevent the initiation of DNA damage repair processes in response to the DNA double strand breaks generated during apoptosis (Smith et al., 1999). Interestingly Wang et al. (2006) showed that ATM deficient cells were more sensitive to apoptosis induced by the DNA damaging agent cisplatin than normal cells. They suggested that in their experimental model ATM was cytoprotective and that its cleavage by caspases may diminish its cytoprotective effects by reducing its ability to phosphorylate p53 on ser15 and thus facilitating the progression of apoptosis. ATM is also able to phosphorylate p53 at Ser46 in response to severe DNA damage leading to preferential transactivation of proapoptotic genes (Kodama et al., 2010). However to my knowledge the effect of ATM cleavage on phosphorylation of p53 at Ser46 has not yet been investigated.

There was evidence of phosphorylation of ATM, and its downstream substrate SMC1, after 2 hours of CH11 treatment. The maximum level of ATM and SMC1 phosphorylation as well as phosphorylation of Nbs1 was observed after 4 hours of CH11 treatment. However as apoptosis was well underway by 4 hours this suggests that ATM activation may not be a direct result of the CH11 treatment and may be a consequence of the cleavage of DNA during apoptosis.

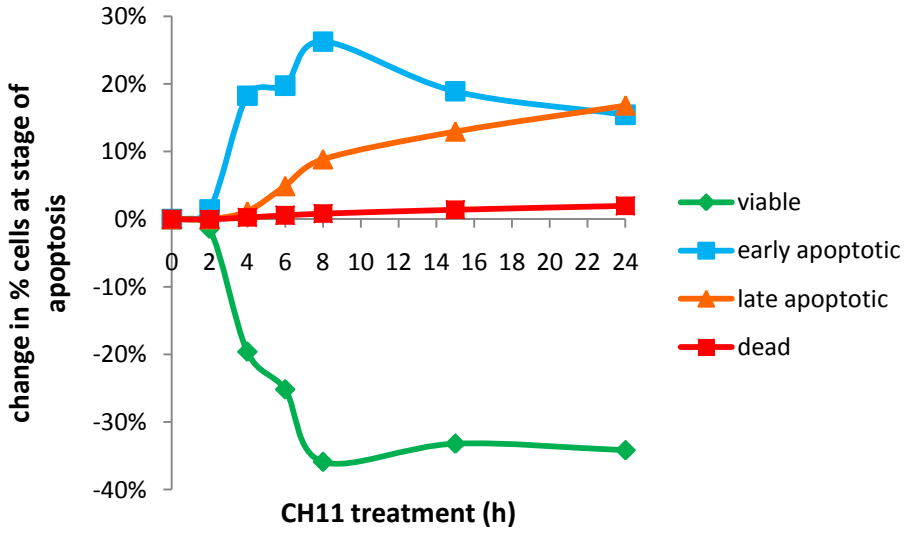
Taken together these results clearly show that CH11 activates a CD95-mediated caspase-dependent apoptotic pathway of cell death in LCLs rather than necrosis. Therefore it can be used in assays to assess the sensitivity of LCLs to CD95-mediated apoptosis. It is unlikely that ATM has a direct role in the CD95-mediated apoptotic pathway.

Fig 4:2:3: CH11 induces CD95-mediated apoptosis leading to a caspase cascade and phosphorylation of ATM.

A.



B.



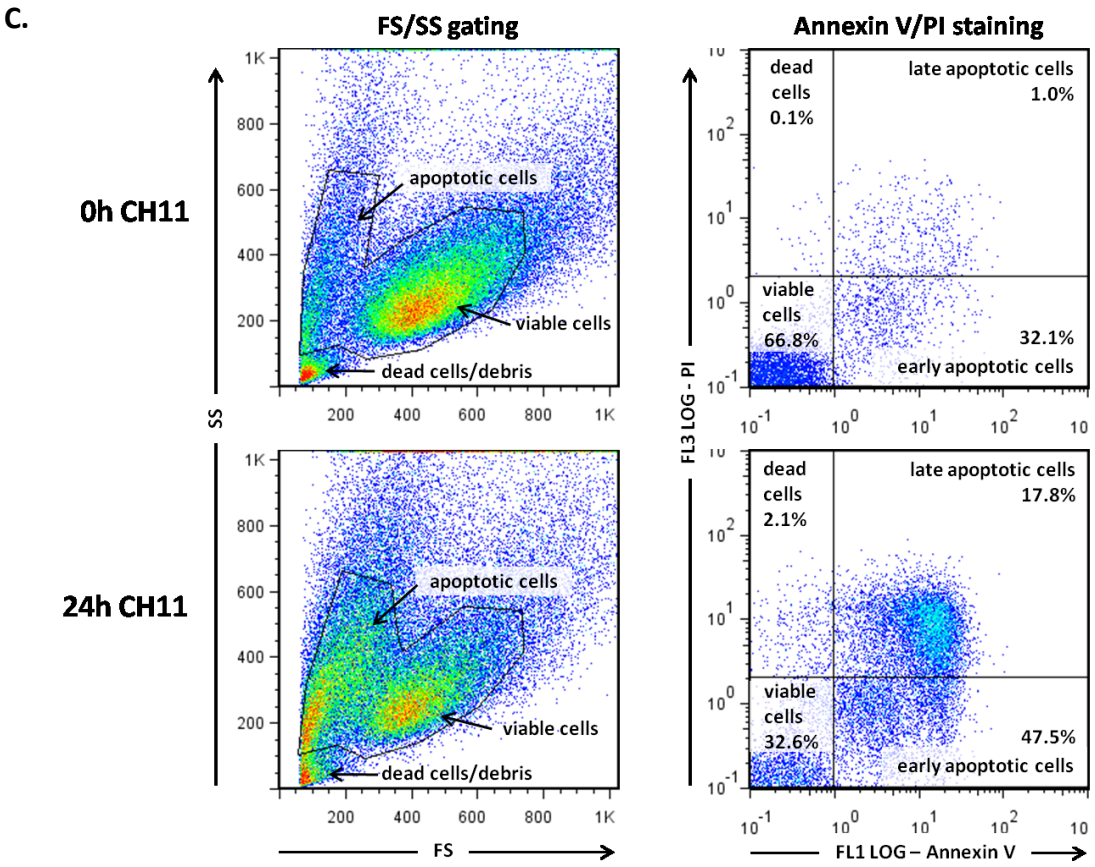


Fig 4:2:3: Timecourse of apoptosis and ATM activation following CH11 treatment. A normal LCL (N8) was seeded in tissue culture plates at a concentration of $1x10^6$cells/ml (8ml/well) in complete RPMI containing 500ng/ml CH11. Cells were harvested at indicated time points, approximately $0.5x10^6$ cells was analysed by flow cytometry and the remainder used to make lysates for western blotting. Untreated cells were irradiated (5Gy) and incubated at 37°C for 30min prior to lysis as a positive control for activation of ATM kinase.

A. Western blot of CH11 timecourse showing cleavage of caspases 7 and 3 and activation of ATM protein kinase (phosphorylation of downstream substrates SMC1 and Nbs1) in response to CH11 treatment.

B. Cells were stained with Annexin V-FITC and PI for analysis of apoptosis. The change in the percentage of cells at each stage of apoptosis was calculated as (% cells at stage of apoptosis following treatment - % cells at stage of apoptosis prior to treatment).

C. Example FACs plots and gating strategy for Annexin V/PI apoptosis assay. FS/SS gates (left) were drawn to include viable and apoptotic cell populations but exclude debris. Annexin V-FITC and PI staining (right) was used to differentiate between viable (Annexin V-PI-), early apoptotic (Annexin V+PI-), late apoptotic (Annexin V+PI-) and dead cells (Annexin V-PI+). Example plots of untreated (0h of CH11, top) and CH11-treated cells (24h of CH11, bottom) are shown.

4:2:4: Analysing the sensitivity of LCLs to CH11-induced apoptosis using flow cytometry.

Analysis of apoptosis using an Annexin V/PI assay is more informative than a chromium release assay as it allows the proportion of cells in different stages of apoptosis to be determined. The CH11 sensitivity of 5 normal LCLs, 4 A-T LCLs with no ATM activity and 3 A-T LCLs with some ATM activity were compared using the Annexin V/PI assay. The assay was repeated 3 times and was reproducible (Fig 4:2:4A).

This preliminary result suggested that the A-T LCLs which have no ATM activity were more sensitive to CH11-induced apoptosis than normal LCLs (Fig 4:2:4B) as there was a larger increase in cells in early and late apoptosis in these A-T LCLs than in the normal LCLs. The difference is not statistically significant but the small sample size is very small. An increased sensitivity of A-T compared to normal LCLs is consistent with the result of the CH11 titration chromium release assay (Fig 4:2:2).

Fig 4:2:4: The sensitivity of LCLs to CH11-induced apoptosis can be analysed by flow cytometry.

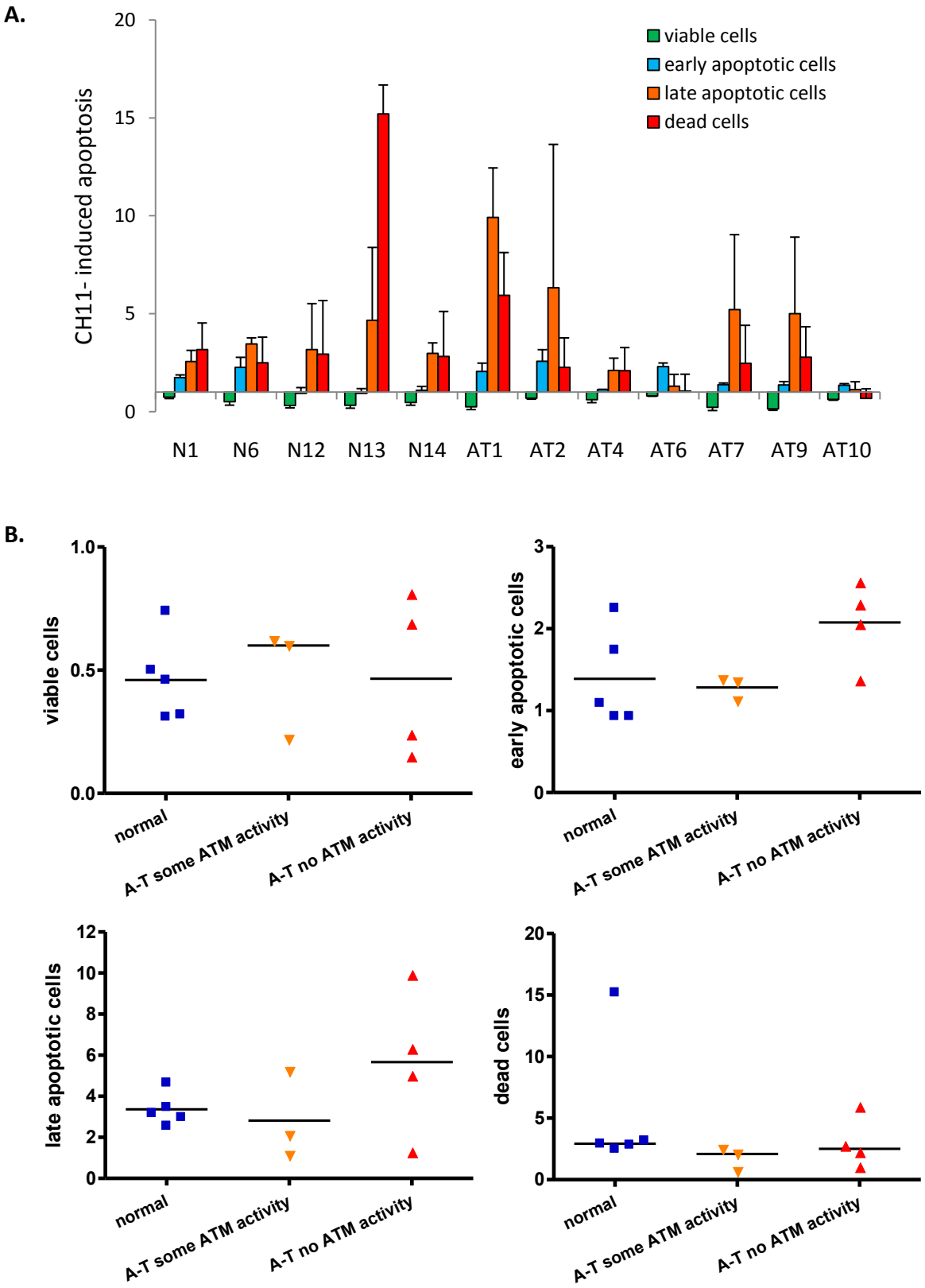


Fig 4:2:4: LCLs were treated with 500ng/ml CH11 for 15h then harvested and analysed by Annexin V/PI staining. Untreated cells of each LCL were used as a control. Gating was carried out as in Fig 4:2:3C.

A. Apoptosis of LCLs in response to CH11 treatment (mean of 3 experiments). CH11-induced apoptosis was quantified as the % of CH11-treated cells at each stage of apoptosis divided by the % of untreated cells at each stage of apoptosis.

B. Comparison of level of apoptosis of normal and A-T LCLs in response to CH11 treatment.

4:2:5: Apoptosis assays using T cell clones.

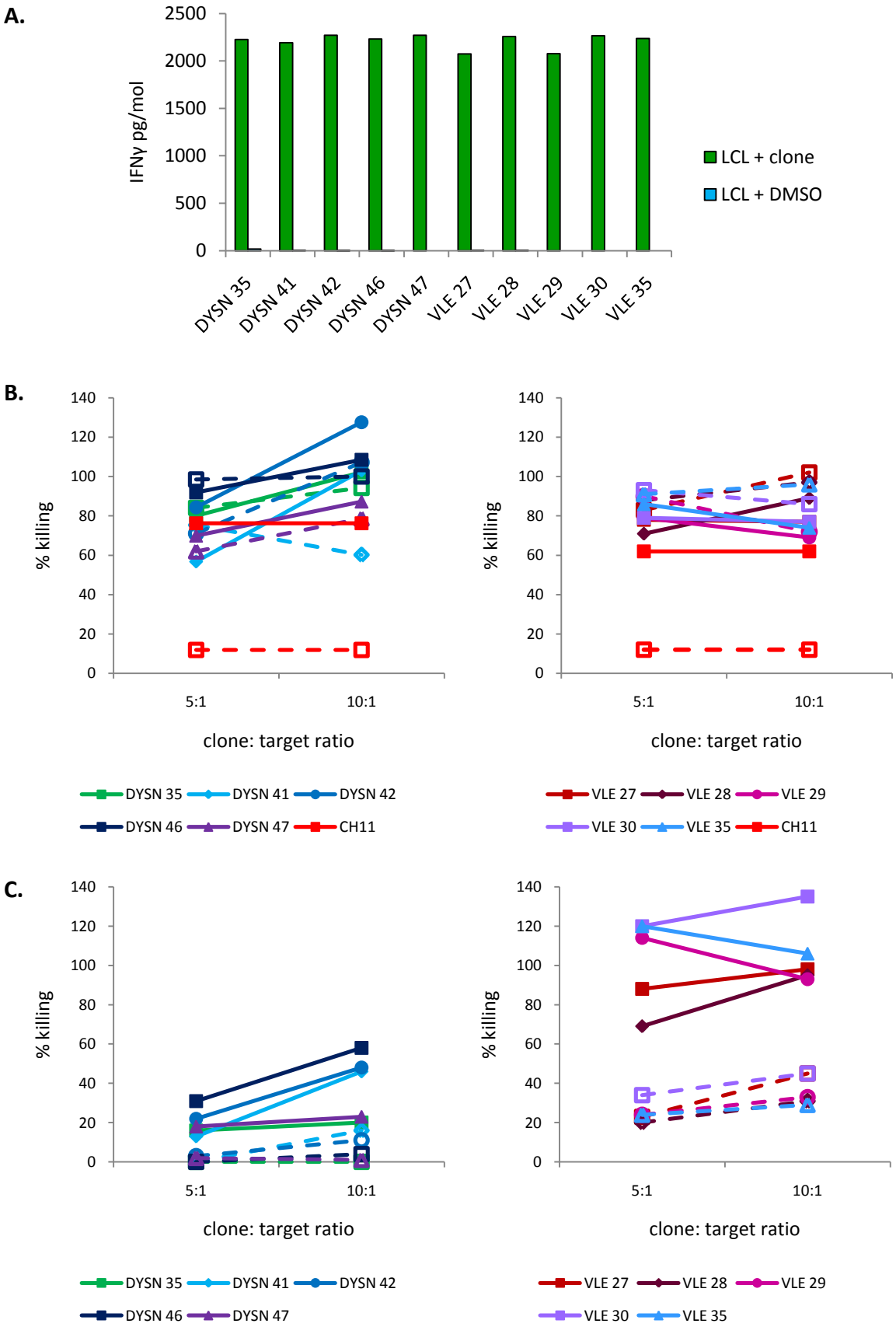
CH11 treatment is a very convenient and effective method of activating the CD95-mediated pathway of apoptosis, however it is not as physiologically relevant as using T cell clones for killing. Therefore an assay was developed to allow the sensitivity of different LCLs to T cell cytotoxicity to be compared. CMV peptide (VLE and DYSN) specific T cells were cloned from HLA A2 lab donor PBMCs using IFNγ capture selection and limiting dilution. DYSN is a CD4 restricted peptide and VLE is restricted through CD8. Clones were selected and tested for peptide specificity using an IFNγ ELISA. This assay measures the amount of IFNγ produced by the T cell clone in response to antigen. An autologous LCL was loaded with either VLE or DYSN peptide and used as antigen presenting cells. The ten clones which showed the greatest peptide specificity (high IFNγ production in response to peptide loaded LCL and low background in the absence of peptide) were selected for use in further experiments. The IFNγ production of the selected clones is shown in Fig 4:2:5A.

The next step was to determine the mechanism of cytotoxicity of the T cell clones. The effect of specifically blocking either the CD95-mediated or perforin/granzme pathway of apoptosis on T cell clone cytotoxicity against peptide loaded autologous LCL was analysed by chromium release. Blocking of the CD95-mediated apoptotic pathway using the blocking antibody ZB4 had no significant effect on the cytotoxicity of either VLE or DYSN clones (Fig 4:2:5B). However the cytotoxicity of both VLE and DYSN clones was effectively blocked by the perforin/granzyme inhibitor concanamycin A (Fig 4:2:5C). This indicated that both the CD4+ (DYSN) and CD8+ (VLE) T cell clones killed their targets primarily through perforin and granzyme rather than CD95-mediated apoptosis. Therefore they could not be used in assays aimed at analysing the sensitivity of LCLs to CD95-mediated apoptosis.

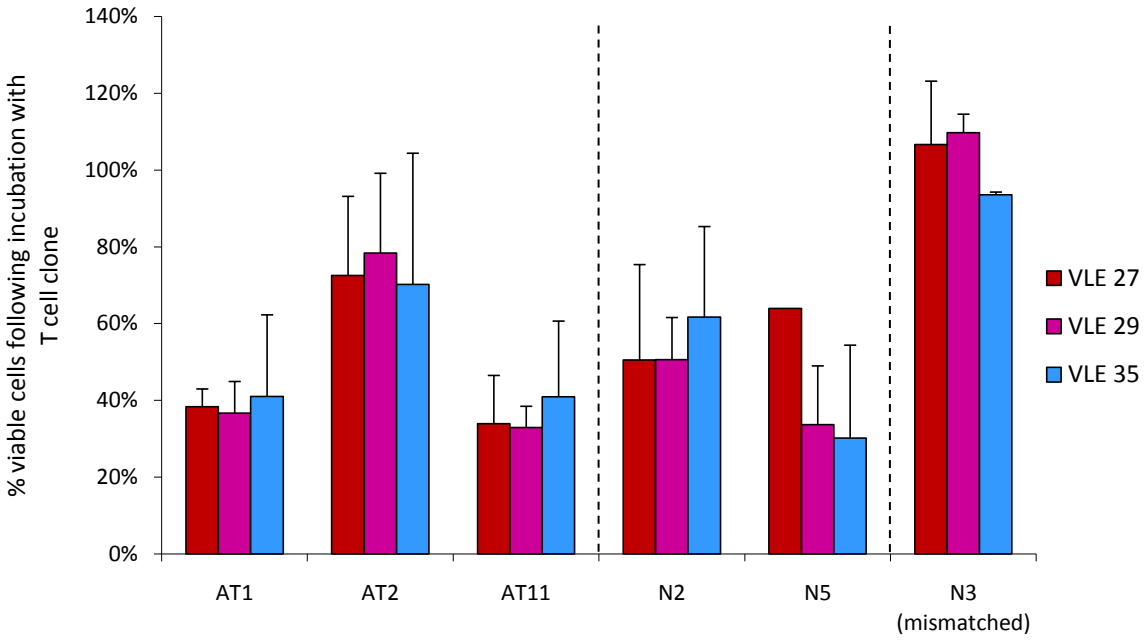
However, the sensitivity of A-T and normal LCLs to perforin and granzyme-mediated apoptosis was analysed using the T cell clones. As the CD8+ VLE T cell clones showed the greastest cytotoxicity they were used in the assay. A-T and normal LCL targets with the appropriate HLA restriction (A2) were peptide loaded and labelled with the fluorescent dye CFSE. They were then incubated with unlabelled, VLE-specific T cell clones for 6 hours, harvested and analysed using flow cytometry. As CFSE is a cytoplasmic dye apoptotic cells lose their fluorescence and the percentage of surviving CFSE+ve cells of each LCL can be calculated (Fig 4:2:5D). As the HLA types of the LCL targets could not be perfectly matched, each LCL was tested against 3 different VLE-specific T cell clones, the results for each clone were comparable. There was no evidence of a difference in sensitivity of A-T and normal LCLs to perforin/granzyme-mediated apoptosis, however analysis of a larger panel of LCLs would be required to confirm this result (Fig 4:2:5D&E).

The CFSE cytotoxicity assay has several advantages. The use of T cell clones rather than CH11 to activate CD95-mediated apoptosis is undoubtedly more physiologically relevant than using artificial activators of apoptotic pathways such as CH11, and the labelling of A-T and normal LCLs with CFSE rather than chromium avoids the use of radiation. As A-T cells are radiation sensitive the use of chromium could potentially affect assay results. However, the requirement for HLA matching of T cell clones and LCL targets severely restricts the number of LCLs that can be used in the assay. Therefore assays using artificial activators such as CH11 to induce apoptosis are more appropriate for comparing the sensitivity to apoptosis of a large number of different LCLs.

Fig 4:2:5: A-T LCLs do not show increased sensitivity to perforin/granzyme cytotoxic T cell killing.



D.



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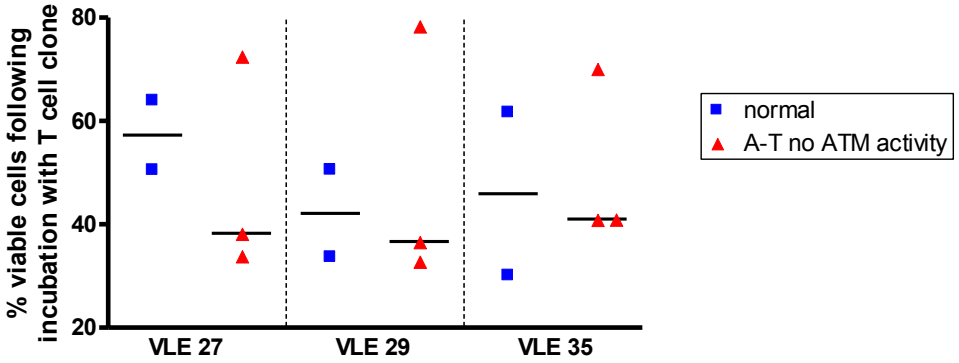


Fig 4:2:5: A-T LCLs do not show increased sensitivity to perforin/granzyme-induced apoptosis.

A. VLE and DYSN-specific T cell clones produced high amounts of IFNγ (analysed by ELISA) in response to incubation with peptide loaded autologous LCL (5:1 ratio of LCL:T cell). Background IFNγ production in the absence of peptide was very low.

B. Blocking of CD95-mediated apoptosis had little effect on the cytotoxicity of DYSN (CD4+ve) or VLE (CD8+ve) specific T cell clones as measured by chromium release. T cells were incubated with peptide loaded autologous LCL for 15 hours at clone:target ratios of 5:1 and 10:1. The CD95-mediated apoptotic pathway was blocked by incubating LCLs with ZB4

for one hour prior to addition of T cell clones. Incubation of LCL with CH11 was used as a control. Dashed lines = ZB4 blocked LCLs, solid lines = unblocked controls.

C. VLE and DYSN-specific T cells killed LCL targets through a perforin/granzyme-dependent mechanism. Incubation of T cell clones with the perforin/granzyme inhibitor concanamycin A for 2 hours prior to addition of LCL target cells significantly reduced their cytotoxicity as measured by chromium release. Dashed lines = perforin/granzyme-blocked T cell clones, solid lines = unblocked controls.

D. The sensitivity of normal and A-T LCLs to perforin/granzyme-mediated apoptosis induced by cytotoxic VLE-specific T cell clones. CFSE loaded A-T and normal LCLs were incubated with T cell clones at a ratio of 5:1 for 6 hours then harvested and analysed by flow cytometry. The percentage of viable cells following incubation with T cell clones was calculated using the formula (absolute number of CFSE+ve cells following incubation with T cell clone/absolute number of CFSE+ve cells in the no clone control) x 100/1. A HLA mismatched (A1) normal LCL (N3) was included as a negative control. (Mean of 3 experiments).

E. There was no significant difference in the sensitivity of normal and A-T LCLs with no ATM activity to perforin/granzyme-induced apoptosis initiated by 3 different VLE-specific (CD8+ve) T cell clones. (Mean of 3 different experiments).

4:3: Investigation of the effect of ATM mutations on CD95 expression.

4:3:1: CD95 expression on normal and A-T LCLs.

Preliminary results suggested an increased sensitivity to CH11-induced apoptosis of A-T LCLs with no ATM activity compared to normal LCLs. Therefore the level of expression of CD95 was compared on normal and A-T LCLs. As CH11 mimics Fas ligand by binding to CD95 it was hypothesised that the more sensitive A-T LCLs would express higher levels of CD95.

Normal and A-T LCLs were stained with an antibody to CD95, and analysed by flow cytometry. FS/SS gating was used to select viable cells and PI was used for dead cell exclusion. The gating strategy is shown in Fig 4:3:1A. In both normal and A-T LCLs almost 100% of live cells in the FS/SS gate expressed CD95 (median of 95.08% in the normal LCLs and 94.1% in the AT LCLs with no ATM activity). However the percentage of CD95+ve cells in the A-T LCL with some ATM activity was reduced (73.05%) (Fig 4:3:1B).

Median fluorescence intensity (MFI) was used to compare the level of CD95 expression on CD95+PI- cells. Surprisingly given the findings reported in Chapter 3 which showed significantly higher expression of CD95 on A-T compared to normal B cells, the CD95 MFI of the normal LCLs (median MFI 9.09) were significantly higher than that of A-T LCLs with no ATM activity (median MFI 4.11) ($p<0.001$) (Fig 4:3:1C). The A-T LCL with some ATM kinase activity (AT21) had an intermediate level of CD95 expression (MFI 6.99) between that of the normal LCLs and the A-T LCLs with no ATM activity. If ATM has a role in regulating CD95 expression it is possible that this intermediate level of expression on AT21 could be due to its intermediate level of ATM activity.

Fig 4:3:1: Expression of CD95 on A-T LCLs with no ATM activity is significantly lower than on normal LCLs.

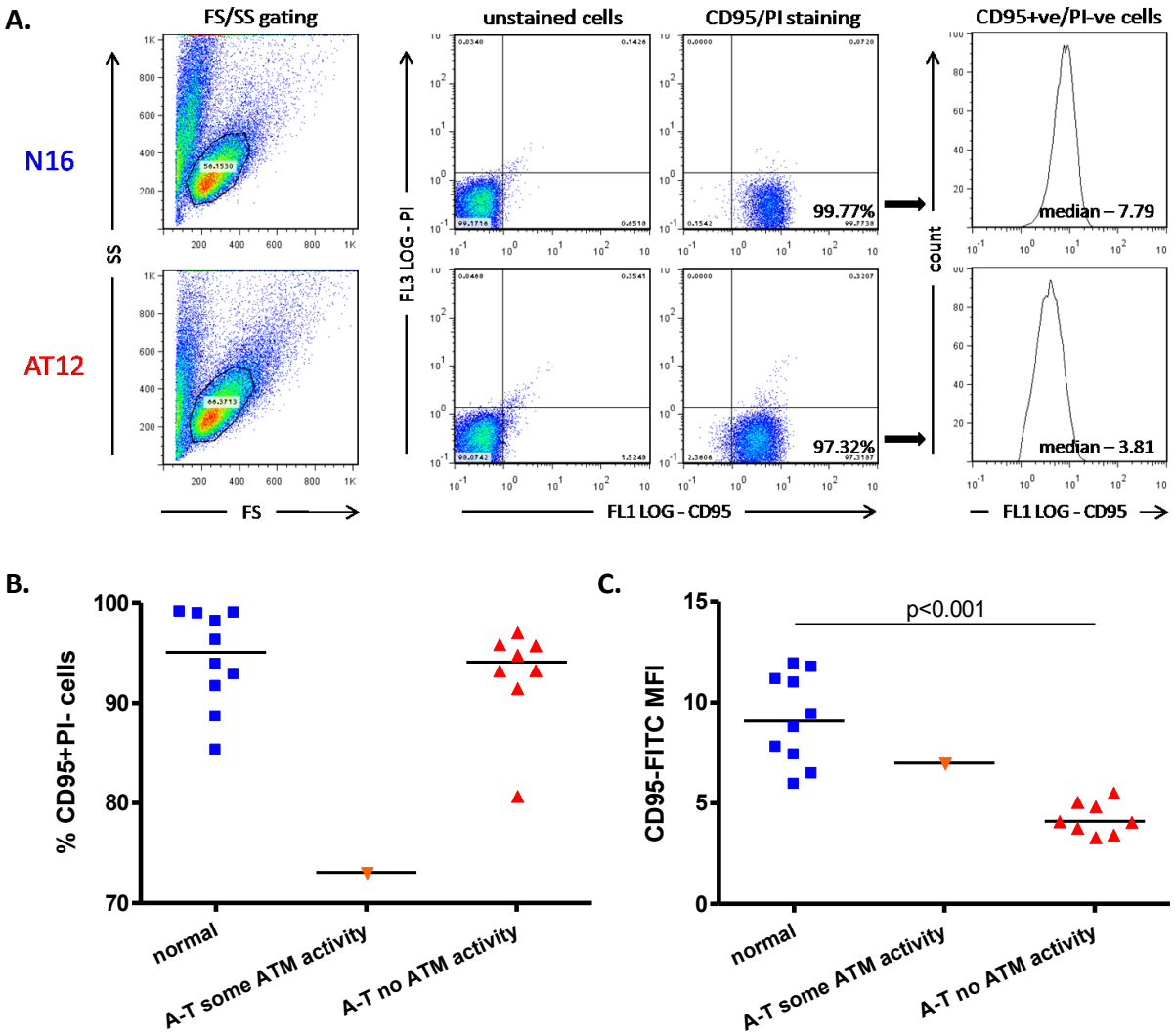


Fig 4:3:1: CD95 expression on normal and A-T LCLs was analysed by flow cytometry.

A. Example FACS plots and gating strategy for one normal (N16) and one A-T LCL with no ATM activity (AT12). LCLs were stained with CD95-FITC for analysis of CD95 expression and PI to allow exclusion of dead cells. FS/SS plots were used to gate on non-apoptotic cells and unstained samples of each LCL were used for the setting of quadrants (x axis = CD95-FITC, y axis = PI). Histograms show the CD95-FITC median fluorescence intensity of CD95+PI- cells.

B. There is no significant difference in the percentage of live cells which express CD95 (CD95+PI-) in normal and A-T LCLs.

C. A-T LCLs which have no ATM activity express significantly lower levels of CD95 than normal LCLs ($p<0.001$). (Median fluorescence intensity of CD95+PI- cells).

4:3:2: The effect of inhibition of ATM activity on constitutive CD95 expression.

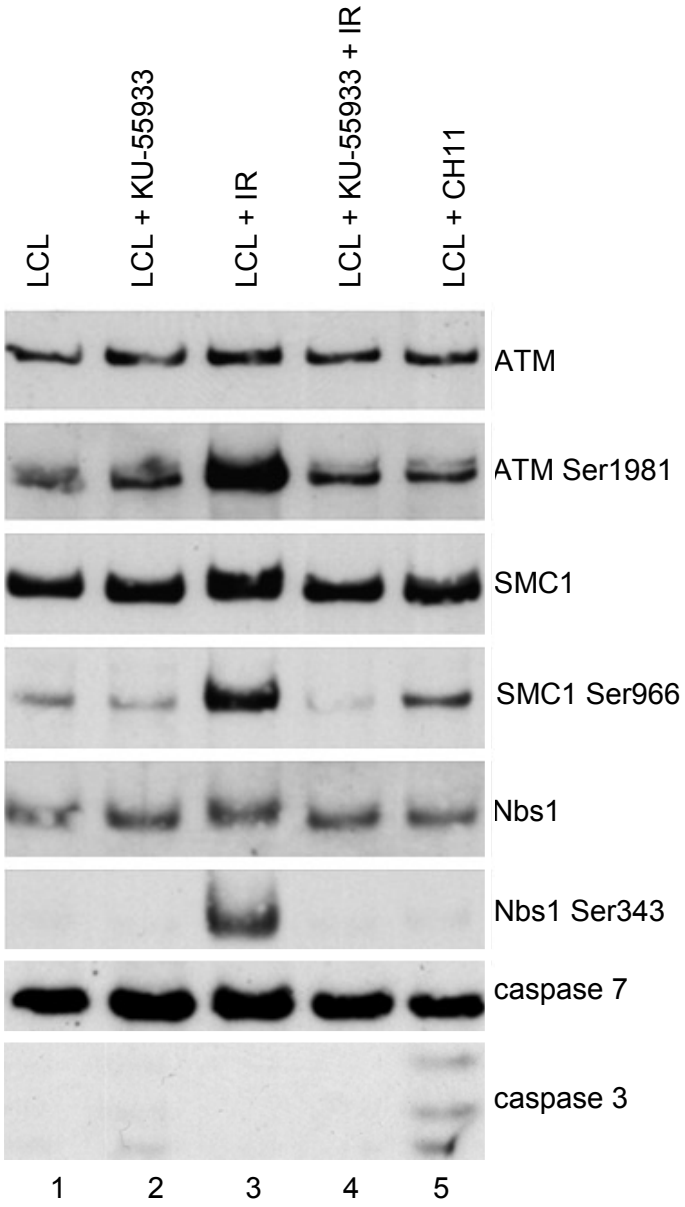
A-T LCLs with no ATM activity had significantly lower expression of CD95 than normal LCLs (Fig 4:3:1C). In order to investigate a possible role for ATM kinase activity in determining the level of expression of CD95 on LCLs, the effect of the ATM inhibitor KU-55933 on CD95 expression was analysed.

Firstly, the ability of KU-55933 to inhibit irradiation-induced activation of ATM protein kinase in a normal LCL was analysed by western blotting (Fig 4:3:2A). Incubation with 10μM KU-55933 for 90min prior to 10Gy of irradiation completely prevented irradiation-induced activation of ATM as measured by phosphorylation of ATM on Ser1981 and its downstream substrates Nbs1 on Ser343 and SMC1 on Ser966. No cleavage of caspases 7 or 3 in response to KU-55933 treatment occurred. This indicated that treatment with the ATM inhibitor did not induce apoptosis of the LCL. A CH11-treated LCL was used as a positive control for caspase cleavage.

To analyse the effect of KU-55933 induced inhibition of ATM activity on CD95 expression on LCLs normal and A-T LCLs were incubated with the inhibitor over a 24 hour timecourse and CD95 expression analysed by flow cytometry. Inhibition of ATM activity had no effect on the CD95 expression of the LCLs (Fig 4:3:2B&C). As there was no significant downregulation of CD95 on the cell surface of the normal LCLs in response to inhibition of ATM activity it seems unlikely that lack of ATM activity is directly responsible for the low levels of CD95 expression on the cell surface of A-T LCLs.

Fig 4:3:2: Inhibition of ATM activity had no effect on constitutive CD95 expression of normal or A-T LCLs.

A.



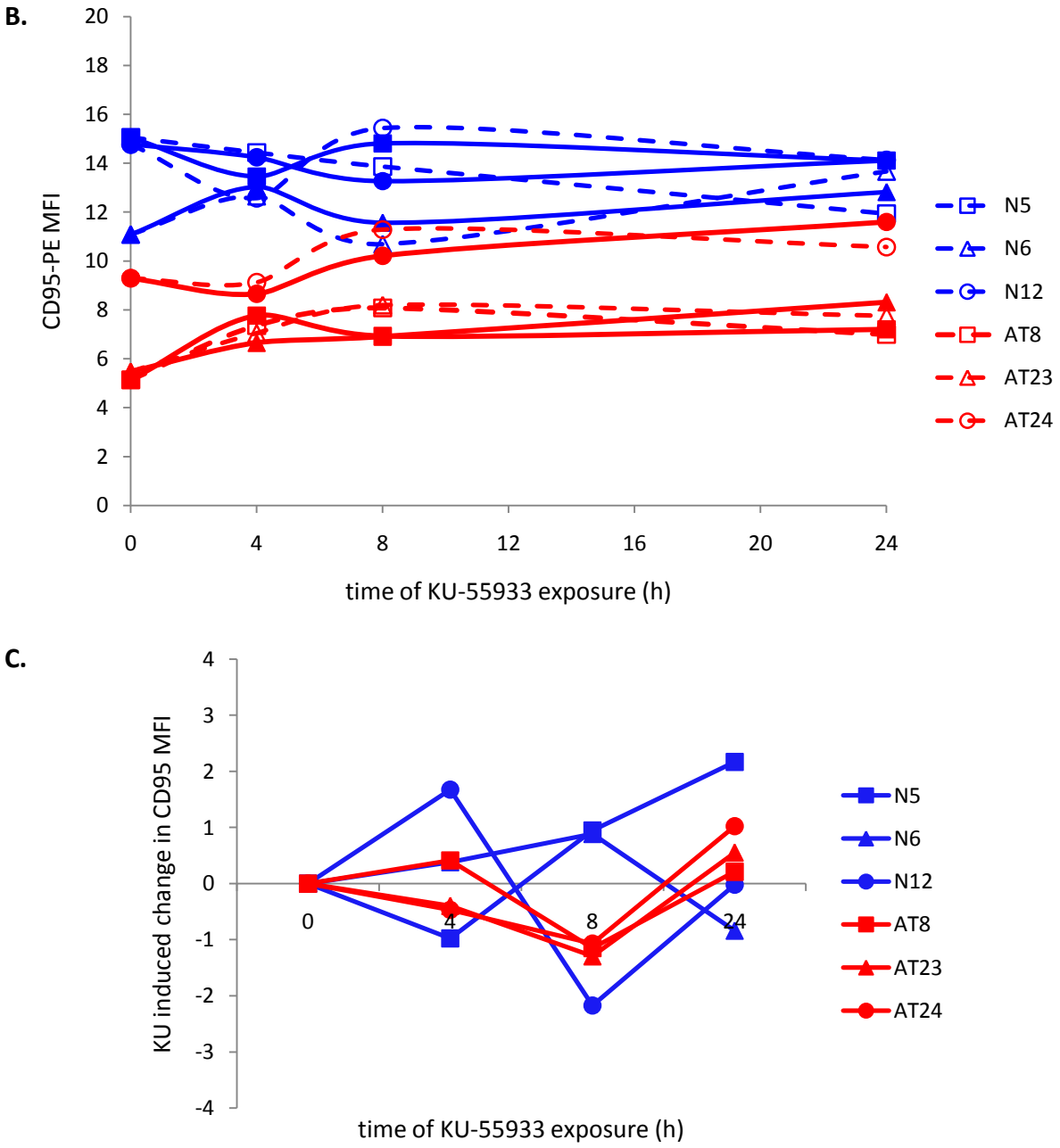


Fig 4:3:2: Inhibition of ATM activity has no effect on constitutive CD95 expression of normal or A-T LCLs.

A. KU-55933 inhibits ATM activation in response to irradiation. A normal LCL (N8) was resuspended in RPMI media at 1x10^{6}cells/ml, 2x10^6 cells were used per test. Samples were treated as follows – lane 1 - LCL: no treatment (120min incubation in RPMI media), lane 2 - LCL + KU-55933: 120min incubation with 10µM KU-55933, lane 3 - LCL + IR: 90min incubation in complete RPMI followed by 5Gy IR and a further 30min incubation, lane 4 - LCL + KU-

55933 + IR: 90min incubation with 10μM KU-55933 followed by 5Gy IR and a further 30min incubation, lane 5 - LCL + CH11: 120 min incubation with 500ng/ml CH11. All incubations were at 37°C. Cells were harvested and lysates made for western blotting. Samples were run on a biphasic gel (6%/10%) to allow simultaneous separation of very large (ATM) and very small proteins (caspases).

B. Three normal LCLs and 3 A-T LCLs with no ATM activity were seeded in tissue culture plates at a concentration of $0.5x10^6$/ml. The specific ATM inhibitor KU-55399 (10μM) was added to individual wells. At 0, 4, 8 and 24h following addition of the inhibitor treated and untreated cells were harvested and stained with CD95-PE and PI for detection of CD95 receptor levels on viable cells. Gating was carried out as in Fig 2:2.1A. Median florescence intensity of KU-55933 treated (solid lines) and untreated (dashed lines) CD95+PI- cells is shown (mean of two experiments).

C. Change in MFI of KU-55933 treated cells compared to untreated controls at the same timepoint. (CD95-MFI of KU-55933 treated cells at time A – CD95-MFI of untreated controls at time A).

4:3:3: The effect of ATM activation on cell surface expression of CD95.

The effect of ATM activation on CD95 expression on LCLs was also analysed. Normal and A-T LCLs were irradiated with a dose of 10Gy to create DNA double strand breaks and activate ATM, and expression of CD95 was then analysed by flow cytometry over a 24h timecourse following IR. Non-irradiated LCLs were used as a control for each timepoint (Fig 4:3:3A). Irradiation led to a statistically significant increase in CD95 expression on the LCLs (Fig 4:3:3B). However, as this increase occurred in both normal and A-T LCLs, including AT9 which has no ATM activity, it cannot be ATM-dependent.

Although constitutive expression of CD95 is significantly lower on A-T LCLs with no ATM activity than normal LCLs, this result, and the result of the ATM inhibitor assay suggest that ATM does not have a direct role in determining its expression. ATM activity could have an indirect effect on CD95 expression by influencing other factors such as sensitivity to CD95-mediated apoptosis and cell viability.

Fig 4:3:3: Surface expression of CD95 increases following activation of ATM.

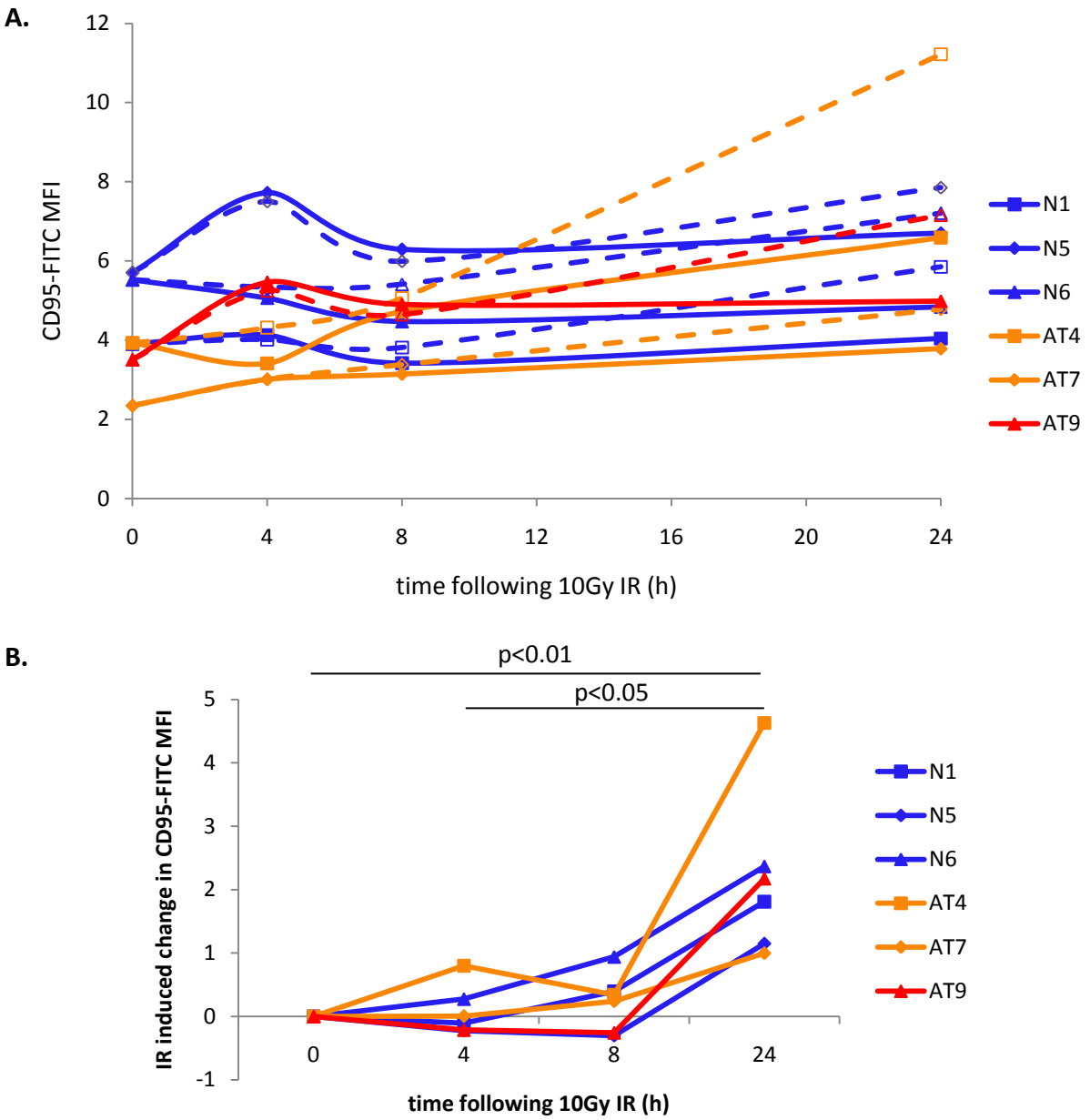


Fig 4:3:3: LCLs were harvested and resuspended in RPMI media at a concentration of $0.5x10^6$cells/ml. Each LCL was divided into an irradiated and non-irradiated culture. Following irradiation (10Gy) of appropriate samples LCLs were transferred to tissue culture plates. At each time point following IR irradiated and non-irradiated cells of each LCL were harvested and stained with CD95-FITC and PI for flow cytometry analysis.

A. CD95 MFI of irradiated (dashed lines) and non-irradiated control samples (solid lines) of each LCL following irradiation.

B. There was a significant increase in CD95 expression between 0 and 24 hours ($p<0.01$) and 4 and 24 hours ($p<0.05$) following irradiation. (Irradiation-induced change in CD95 MFI = CD95 MFI of irradiated sample - CD95 MFI of corresponding non-irradiated control).

4:4: The effect of ATM mutation on the sensitivity of A-T LCLs to CH11-induced apoptosis.

4:4:1: Comparing the sensitivity of A-T and normal LCLs to CH11-induced apoptosis.

In order to investigate the effect of CD95 expression on CH11 sensitivity of normal and A-T LCLs the Annexin V/PI apoptosis assay was modified to include the CD95 antibody. CD95 staining was carried out prior to Annexin V staining during the assay. This allowed the effect of CH11 treatment on CD95 expression on LCLs to be investigated and the effect of CD95 expression on CH11 sensitivity to be analysed. The assay was carried out using a large panel of 16 normal LCLs, 13 A-T LCLs with no ATM activity and 3 A-T LCLs with some ATM activity.

The percentage of viable cells which expressed CD95 was slightly higher in normal LCLs (median 99.1%) than in A-T LCLs with no ATM activity (median 95.4%) and the A-T LCLs with some ATM activity were very similar to the normal LCLs (median 99.4%) (Fig 4:4:1A). The median fluorescence intensity of CD95 expression was significantly lower on A-T LCLs with no ATM activity than normal LCLs (Fig 4:4:1B). There was no significant difference in MFI between A-T LCLs with some ATM activity and normal LCLs.

CH11-induced apoptosis of CD95+ cells led to a reduction in the level of CD95 expression (MFI) on viable cells of both normal and A-T LCLs (Fig 4:4:1B). However, even after 15 hours of CH11 treatment the CD95 MFI of normal LCLs (median MFI of 2.2) was still significantly higher ($p<0.001$) than that of A-T LCLs with no ATM activity median MFI of 0.66I).

The A-T LCLs with no ATM activity were significantly more sensitive to CH11 treatment than both normal LCLs and A-T LCLs with some ATM activity (Fig 4:4:1C). The apoptotic sensitivity

of normal and A-T LCLs were analysed in terms of the change in the proportion of cells at each stage of apoptosis following CH11 treatment (% of cells in stage of apoptosis following CH11 treatment/% of untreated cells at same stage of apoptosis). Values greater than 1 represent an increase and less than 1 a decrease in the proportion of cells at an individual stage of apoptosis.

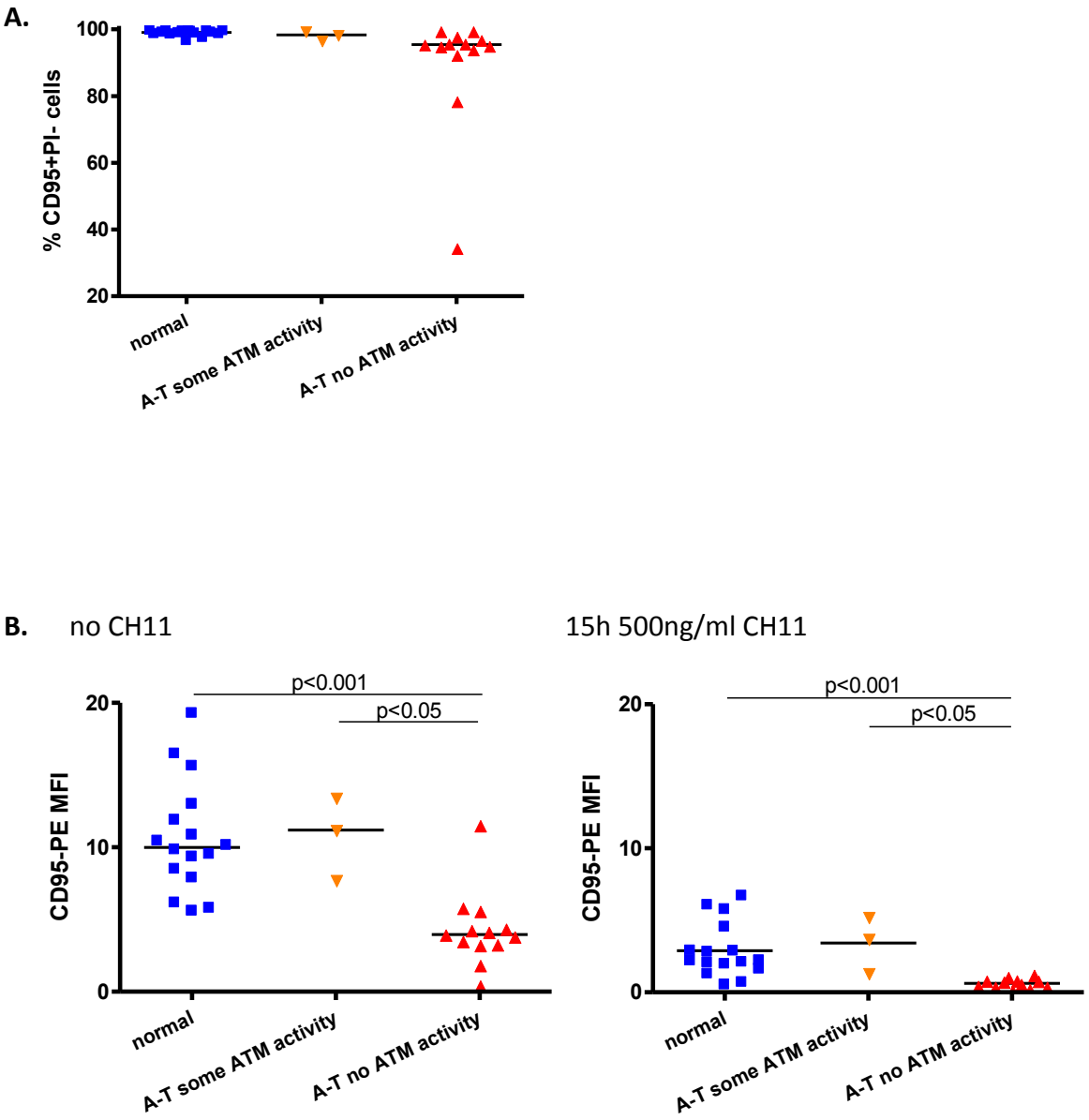
Unsurprisingly the proportions of viable cells in both A-T and normal LCLs decreased and the proportions of apoptotic and dead cells increased in response to CH11 treatment (Fig 4:4:1C). There was a significantly larger change (decrease) in the proportion of viable cells after CH11 treatment in A-T LCLs with no ATM activity than in normal LCLs ($p<0.05$). There was also a significantly larger change (increase) in the proportion of cells in the early stage of apoptosis in response to CH11 treatment in A-T LCLs with no ATM activity compared to both normal LCLs ($p<0.01$) and A-T LCLs with some ATM activity ($p<0.05$). There were no significant differences in the change in proportions (increase) of cells in the late stage of apoptosis or dead cells between A-T LCLs with no ATM activity and normal LCLs. However, there was a significant difference in both cases between A-T LCLs with no ATM activity and A-T LCLs with some ATM activity (late apoptosis $p<0.05$, dead cells $p<0.05$). These results indicate an increased sensitivity of A-T LCLs with no ATM activity to CD95-mediated apoptosis compared to normal LCLs. The A-T LCLs with some ATM activity were more similar to normal LCLs than A-T LCLs with no ATM activity in terms of their apoptotic sensitivity.

The modified apoptosis assay allowed the relationship between the sensitivity of LCLs to CH11 and their CD95 expression to be analysed (Fig 4:4:1D). There were significant correlations between increasing CD95 expression (MFI) on A-T LCLs with no ATM activity and decreasing viability/increasing apoptosis in response to CH11 (viable cells – $r=-0.6364$, $p=0.0299$; late apoptotic cells - $r=0.6455$, $p=0.0368$). AT15 was classed as an outlier as it

expressed unusually high levels of CD95 for an A-T LCL with no ATM activity (more than two standard deviations above the mean) so it was excluded from the correlation analysis. Interestingly AT15 did express a trace of ATM protein and in terms of apoptotic sensitivity and CD95 expression it appeared to belong to the group of A-T LCLs with some ATM activity; however its ATM mutations (Table 4:2:1) and western blot analysis indicate that this is not the case.

Normal LCLs, which expressed significantly higher levels of CD95 than A-T LCLs with no ATM activity, did not show a statistically significant correlation between CD95 expression and apoptosis (Fig 4:4:1D). This suggests that above a certain threshold concentration of CD95 receptor expression increasing CD95 expression does not increase sensitivity to apoptosis.

Fig 4:4:1: A-T LCLs with no ATM activity are more sensitive to CH11-induced apoptosis than normal LCLs despite their lower level of CD95 expression.



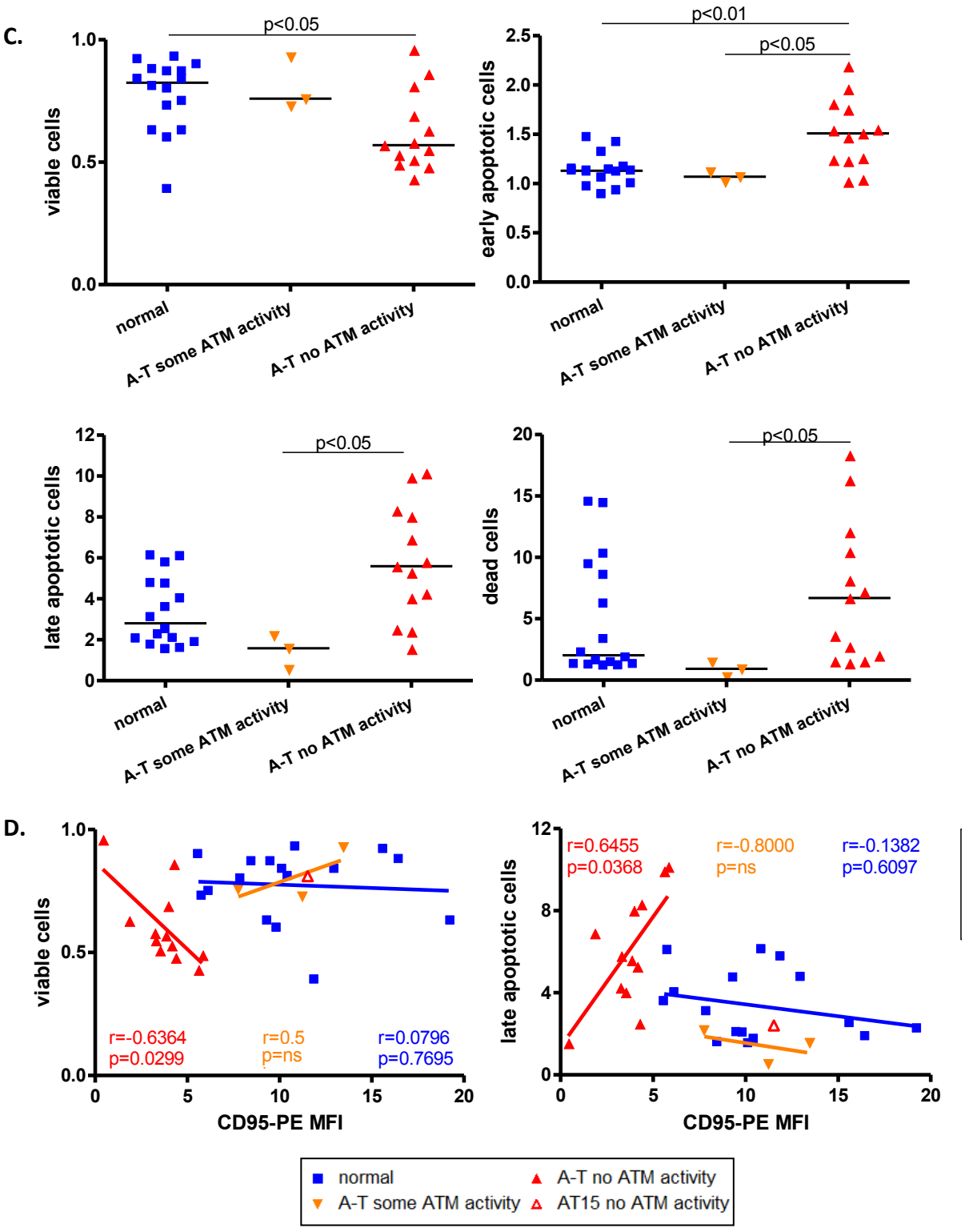


Fig 4:4:1: LCLs in the exponential phase of growth were seeded in tissue culture plates at a concentration of 0.5x10^{6}cells/ml and cultured for 24 hours in RPMI media. 500ng/ml CH11 was added to selected wells and the cells were incubated for a further 15 hours at 37°C. Treated and untreated cells were harvested and stained with CD95-PE followed by Annexin V-FITC and PI for detection of CD95 expression and apoptosis.

A. The percentage of live cells which express CD95 (CD95+PI-) is slightly higher in normal LCLs than in A-T LCLs with no ATM activity, this difference is significant ($p<0.001$). Gating was carried out as in Fig 4:3:1A.

B. CD95-PE MFI of viable (Annexin V-PI-) cells of LCLs with (right) and without (left) 15h of CH11 treatment. Gating was carried out as in Fig 4:3:1A.

C. Apoptosis of normal and A-T LCLs in response to CH11 treatment. Cells were gated as in Fig 4:2:3C. Apoptosis was quantified as the % of CH11-treated cells at each stage of apoptosis divided by the % of untreated cells at each stage of apoptosis.

D. Correlation between CD95 MFI of untreated viable cells and sensitivity to CH11-induced apoptosis (left - % viable cells following CH11 treatment/% viable cells without treatment against CD95 expression, right - % cells in late apoptosis following CH11 treatment/%cells in late apoptosis without treatment against CD95 expression).

4:4:2: The effect of CD95 expression on the viability in culture of A-T and normal LCLs.

The level of CD95 at the cell surface is a potent regulator of cell viability. Overexpression of CD95 can cause spontaneous apoptosis whereas decreasing its expression has been shown to reduce levels of spontaneous apoptosis in LCLs (Le Clorennec et al., 2008). The data from untreated LCLs shown in Fig 4:4:1B was used to analyse the effect of constitutive CD95 expression on the viability of normal and A-T LCLs under normal culture conditions.

Consistent with the finding that CD95 expression on A-T LCLs with no ATM activity was low compared to normal LCLs we found that the A-T LCLs had a significantly higher percentage of viable cells (Annexin V-, PI-) in culture (Fig 4:4:2A). This suggests that they underwent lower levels of spontaneous apoptosis than the normal LCLs. The CD95 expression of A-T LCLs with some ATM activity was similar to that of normal LCLs and significantly higher than A-T LCLs with no ATM activity ($p<0.05$) (Fig 4:4:1B). Taking both normal and A-T LCLs into consideration there was a significant negative correlation between increasing CD95 expression on LCLs and decreasing viability in culture ($r=-0.4274$, $p=0.0185$) (Fig 4:4:2B).

The low level of CD95 expression on the CH11 sensitive A-T LCLs with no ATM activity may be the result of downregulation of the receptor in order to reduce spontaneous apoptosis of the CD95-sensitive cells rather than a direct result of lack of ATM activity.

Fig 4:4:2: A-T LCLs with no ATM activity and CD95 expression are more viable in culture than normal LCLs.

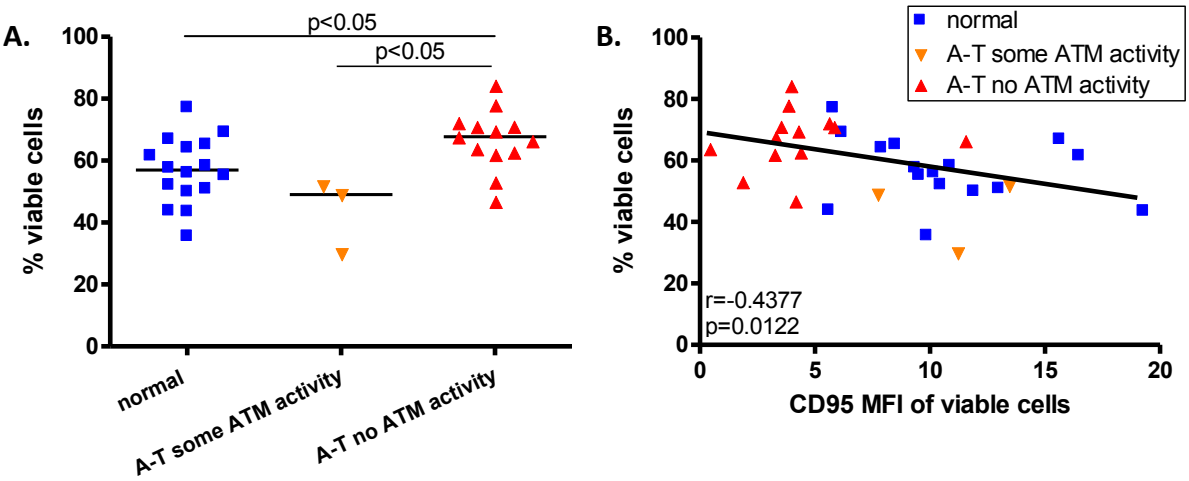


Fig 4:4:2: Untreated control data from Fig 4:4:1. LCLs in the exponential phase of growth were seeded in tissue culture plates at a concentration of $0.5x10^6$cells/ml and cultured for 39 hours in complete RPMI. Cells were stained with CD95-PE followed by Annexin V-FITC and PI for detection of CD95 expression and apoptosis.

A. Viability of normal and A-T LCLs in culture (% of Annexin V-PI- cells).

B. Correlation between CD95 cell surface expression on viable cells and the percentage of viable cells in culture (in the absence of CH11 treatment).

4:4:3: Comparing the sensitivity of normal and A-T LCLs to apoptosis induced by very low concentrations of CH11.

The finding that A-T LCLs with no ATM activity are more sensitive to CH11-induced apoptosis than normal LCLs despite their low CD95 expression seems counter-intuitive. *In vivo* cells are exposed to both soluble and membrane bound forms of Fas ligand, however a 500ng/ml concentration of CH11 may be too high to be physiologically relevant. It is possible that at very low concentrations of CH11 the high CD95 expression of normal LCLs could make them more sensitive to CH11 treatment than A-T LCLs with no ATM activity.

A titration experiment was carried out to compare the apoptotic effect of very low concentrations of CH11 on normal LCLs and A-T LCLs with no ATM activity (Fig 4:4:3). At the lowest CH11 concentration (0.05ng/ml) there was very little apoptosis and no obvious difference in CH11 sensitivity between the normal and A-T LCLs. However at CH11 concentrations of 0.5ng/ml and above the A-T LCLs underwent higher levels of apoptosis in response to CH11 than the normal LCLs. The exception to this was the A-T LCL AT9 which expressed exceptionally low levels of CD95 (MFI of 0.22) and was therefore highly resistant to CH11 treatment over the entire concentration range.

A-T LCLs are more sensitive than normal LCLs to apoptosis induced by CH11 treatment even at very low concentrations of CH11. Therefore the high CD95 expression of normal LCLs does not make them more sensitive to CH11 treatment than A-T LCLs with low CD95 expression even when the amount of CH11 antibody available for binding to CD95 is limited as is likely to be the case with Fas ligand *in vivo*.

Fig 4:4:3: A-T LCLs with no ATM activity are more sensitive to CH11-induced apoptosis than normal LCLs over a range of CH11 concentrations.

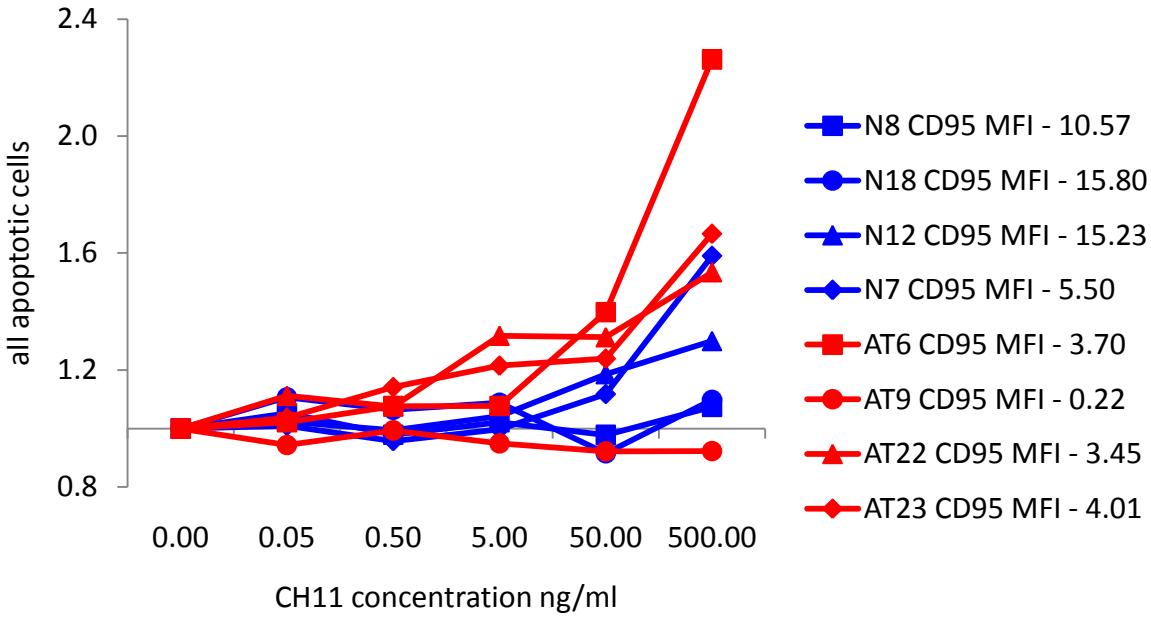


Fig 4:4:3: A-T and normal LCLs were incubated with increasing concentrations of CH11 (0-500ng/mL) and apoptosis measured using Annexin V/PI staining. Apoptosis was calculated as the % of all Annexin V+ cells following treatment divided by the % of all Annexin V+ cells without treatment. CD95 MFI of individual LCLs (viable cells prior to treatment) is indicated in the figure key.

4:4:4: Comparing the rate of CH11-induced apoptosis in normal and A-T LCLs.

The results shown in Fig 4:4:1 strongly suggest an increased sensitivity of A-T LCLs to CH11-induced apoptosis compared to normal LCLs despite their comparatively low CD95 expression. However, this finding is surprising as normal LCLs express significantly higher levels of CD95 than A-T LCLs with no ATM activity so would be expected to be the more sensitive group.

It is probable that the Annexin V/PI apoptosis assay underestimates the amount of cell death that occurs over the 15h assay period as dead cells which have fragmented will appear as debris and are excluded from the flow cytometry analysis. If normal LCLs undergo apoptosis at a faster rate than A-T LCLs with no ATM activity they will have a lower proportion of cells in early and late apoptosis and a higher proportion of dead cells than A-T LCLs at the end of the assay. Therefore they will appear to be less sensitive to CH11-induced apoptosis than the A-T LCLs. However, the finding that CH11 induces a significantly greater reduction in the proportion of viable cells in A-T LCLs with no ATM activity than in normal LCLs suggests that this is not the case (Fig 4:4:1C&D).

To investigate the possibility of an increased rate of apoptosis in normal LCLs compared to A-T LCLs affecting the reliability of the Annexin V/PI apoptosis assay a CH11 timecourse experiment was carried out on 2 normal LCLs and 2 A-T LCLs with no ATM activity. Cells were incubated with CH11 and apoptosis analysed every 2 hours over a 12 hour period to allow comparisons to be made between the rates at which cells entered different stages of apoptosis (Fig 4:4:4).

The A-T LCLs underwent a continuous decrease in the proportion of viable cells over the 12 hour incubation period, however the normal LCLs reached a minimum level of viability by 6

(N6) or 8 hours (N7) after which their viability began to increase. By 12 hours of treatment the decrease in viability of the A-T LCLs was greater than that of the normal LCLs.

There was no clear pattern to the rate at which cells of different LCLs entered the different stages of apoptosis (Fig 4:4:4). The A-T LCL AT6 was most sensitive to treatment. This is in agreement with earlier findings as AT6 has no ATM activity and has higher CD95 expression than the other A-T LCL, AT22. The normal LCL N8 was the least sensitive to treatment.

AT6 showed an increase in both early and late apoptotic cells by 2h of treatment although the largest increase was in the early apoptotic cells. The proportion of early apoptotic cells began to decrease between 6 and 8 hours whilst the late apoptotic cells continued to increase. A very small increase in dead cells began between 2 and 4 hours of treatment. Surprisingly the other A-T LCL, AT22, showed no increase in early apoptotic cells over the 12 hour timecourse. However there was an increase in both late apoptotic and dead cells during the first 2 hours of treatment. The proportion of cells in late apoptosis continued to increase steadily over the timecourse. In the first 6 hours of treatment AT22 had a faster and larger increase in the proportion of dead cells than any other LCL.

The most CH11-sensitive normal LCL, N7, showed a large increase in cells in early apoptosis in the first 2 hours of treatment. The proportion of cells in late apoptosis began to increase after 4 hours but there was very little change in the proportion of dead cells over the timecourse. The most resistant LCL, N8, showed very little decrease in viable cells over the first 4 hours of CH11 treatment. There was a rapid increase in early apoptotic cells between 4 and 6 hours but this decreased rapidly as cells began to enter late apoptosis after 6 hours of treatment. N8 showed a steady increase in dead cells over the 12 hour incubation period.

With the exception of the faster recovery of viability of normal LCLs, the most obvious difference between the two groups was seen in the rate at which cells entered the late stage of apoptosis. Both A-T LCLs showed a steady increase in late apoptotic cells over the 12 hours of CH11 treatment. However, the normal LCLs, N7 and N8, did not show any significant increase in the percentage of late apoptotic cells for the first 4 or 6 hours of treatment respectively. This delay may indicate that apoptosis is proceeding at a faster rate in A-T LCLs with no ATM activity than in normal LCLs.

Although the Annexin V/PI assay does have its limitations the result of the CH11 timecourse assay indicates that the increased sensitivity to CH11-induced apoptosis of A-T LCLs with no ATM activity compared to normal LCLs is not an artefact due to underestimation of the percentage of dead cells in the assay.

Fig 4:4:4: CH11-induced apoptosis may progress at a faster rate in A-T LCLs with no ATM activity than in normal LCLs.

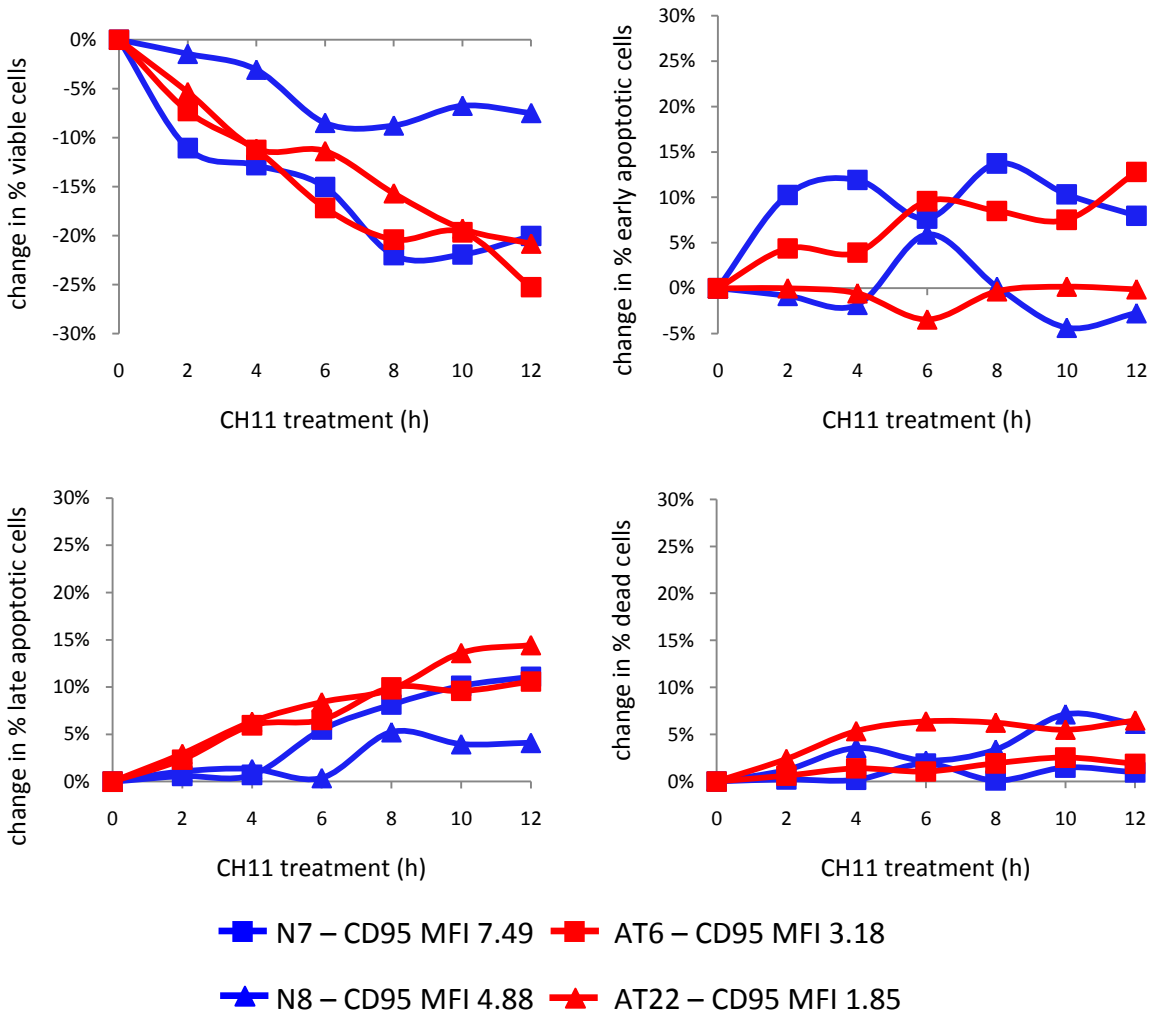


Fig 4:4:4: Timecourse of CH11-induced apoptosis of 2 normal LCLs and 2 A-T LCLs with no ATM activity. Cells were stained with Annexin V-FITC and PI for analysis of apoptosis and CD95-PE for comparison of CD95 expression. The change in the percentage of cells at each stage of apoptosis was calculated as the % of cells at stage of apoptosis following treatment - % of cells at stage of apoptosis prior to treatment. The CD95-PE MFI of each LCL prior to treatment is indicated in the figure legend.

4:4:5: Analysis of the effect of ATM inhibition on the sensitivity of LCLs to CH11-induced apoptosis.

A-T LCLs with no ATM activity are more sensitive to CH11-induced apoptosis than normal LCLs. Therefore inhibition of ATM activity may sensitise normal LCLs to CH11-induced apoptosis. However, as activation of ATM is not required for initiation of CH11-induced apoptosis (Fig 4:2:3) it seems unlikely that ATM kinase activity has a direct role in sensitivity to CH11. The ATM inhibitor KU-55933 was used to prevent activation of ATM activity and the effect on the sensitivity of LCLs to CH11-induced apoptosis was analysed.

LCLs were incubated with KU-55933 for 1.5 or 8 hours to inhibit ATM activation prior to incubation with CH11 (15h). To minimise the risk of any cytotoxic effects of the ATM inhibitor influencing the result cells incubated with KU-55933 for 1.5+15 or 8+15 hours without CH11 were used as controls for each individual LCL. Treatment of LCLs with KU-55933 alone had very little effect on the percentage of viable or early apoptotic cells, however there was a small increase in PI+ve cells compared to untreated controls suggesting that some necrotic cell death may have occurred (result not shown).

Fig 4:4:5A shows the effect of ATM inhibition on the CH11 sensitivity of one normal LCL (N8), one A-T LCL with some ATM activity (AT21) and one A-T LCL with no ATM activity (AT18). In all three LCLs the loss of viability in response to CH11 treatment and the increase in apoptotic cells were slightly increased in KU-55933 treated cells compared to those treated with CH11 alone. However this effect was most pronounced in AT18 which expresses no ATM (Table 4:2:1) so cannot be affected by inhibition of ATM activity. This suggests that the apparent slight sensitisation to CH11 in the presence of KU-55933 may be due to its cytotoxicity rather than its inhibitory effect on ATM activation. The relatively large increase

in dead cells in the presence of KU-55933 treated cells supports this idea as these cells may have died through necrosis.

Finally the effect of ATM inhibition and CH11 treatment on 3 normal LCLs was analysed (Fig 4:4:5B). The sensitivity of N8 appeared to be slightly decreased in response to ATM inhibition, whereas the sensitivity of N9 was slightly increased and there was no real effect on N11. These results indicate that short term inhibition of ATM activity does not have any significant effect on the CH11 sensitivity of LCLs.

Fig 4:4:5: Inhibition of ATM activity does not sensitise LCLs to CH11-induced apoptosis.

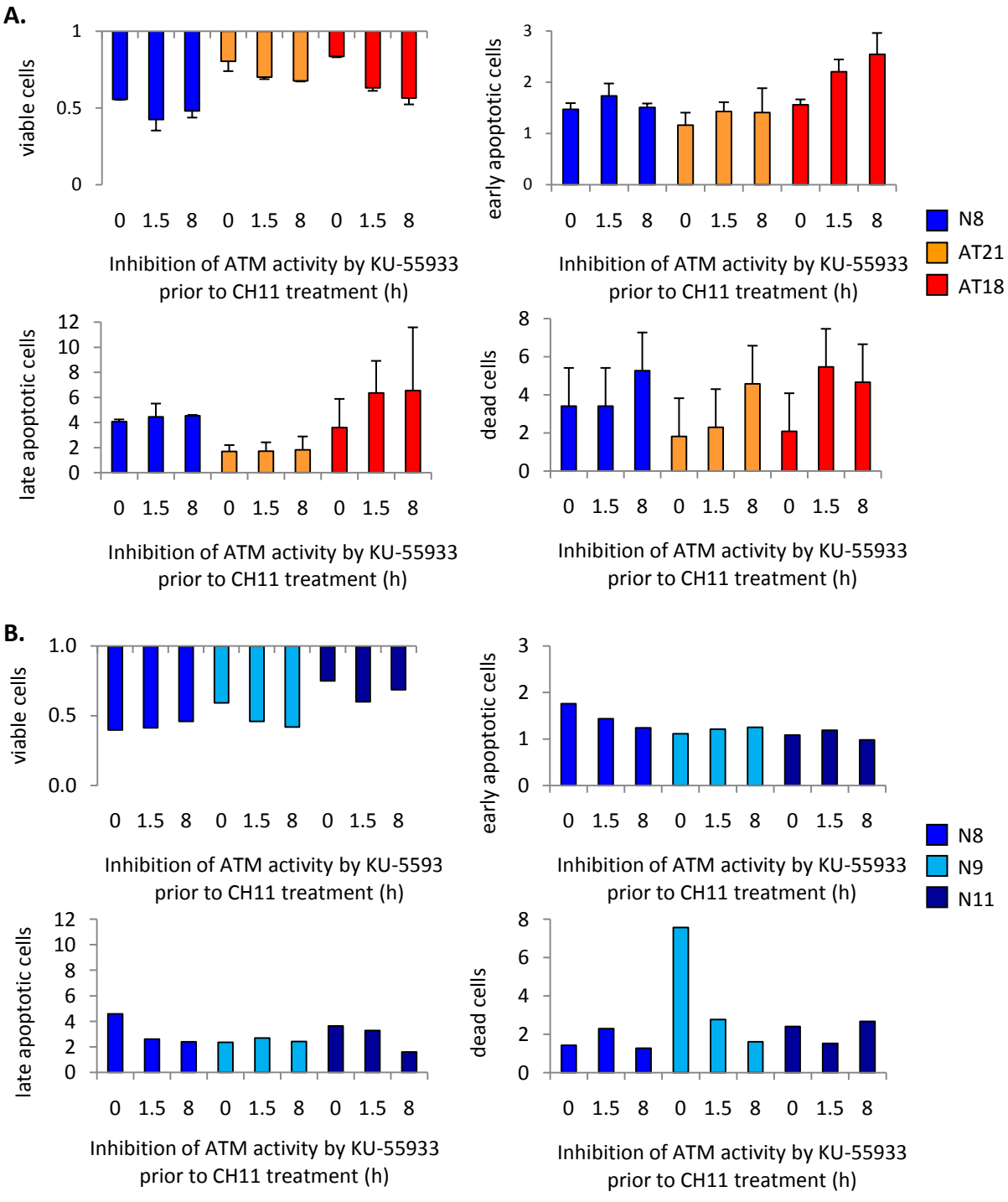


Fig 4:4:5: Inhibition of ATM activity by KU-55933 does not sensitise LCLs to CH11 treatment. LCLs were incubated with 10µM of the ATM inhibitor KU-55933 for 1.5 or 8 hours before addition of 500ng/ml CH11. Following 15h of CH11 incubation cells were harvested and

analysed using the Annexin V/PI apoptosis assay. Untreated cells and cells treated with KU-55933 without CH11 were used as controls for each LCL. Apoptosis was quantified as the % of KU-55933 treated or untreated cells at each stage of apoptosis following CH11 treatment divided by the % of KU-55933 treated or untreated cells at each stage of apoptosis without CH11 treatment.

A. KU-55933 and CH11-induced apoptosis of 1 normal LCL (N8), one A-T LCL with some ATM activity (AT21) and one A-T LCL with no ATM activity. (Mean of 3 experiments).

B. KU-55933 and CH11-induced apoptosis of 3 normal LCLs. Inhibition of ATM activity by KU-55933 had no significant effect on the sensitivity of normal LCLs to CH11-induced apoptosis.

4:5: cFLIP expression in normal and A-T LCLs.

4:5:1: Analysis of cFLIPL and cFLIPS expression by western blot.

The increased sensitivity of A-T LCLs to CH11 treatment, despite their reduced CD95 expression, suggests that there must be another factor influencing their sensitivity to CD95-mediated apoptosis. The caspase 8 inhibitor cFLIP (cellular FLICE-like inhibitory protein) has an important role in regulation of CD95-mediated apoptosis as it competes with pro-caspase 8 for binding to the DISC (death inducing signalling complex). DISC formation occurs in response to the binding of FasL ligand (or CH11) to CD95. Pro-caspase 8 is then recruited to the DISC and cleaved to generate active caspase 8. This in turn cleaves caspase 3 to initiate the caspase cascade leading to apoptosis. Binding of cFLIP to the DISC prevents the binding of caspase 8 and activation of the caspase cascade so inhibits apoptosis (Krueger et al., 2001).

In humans there are two main isoforms of cFLIP which arise from alternative splicing, a 28kDa short form (cFLIPS) and a 55kDa long form (cFLIPL). The protein is structurally similar to procaspase-8 and can compete with it for binding to the DISC, preventing caspase 8 activation and apoptosis.

Western blot was chosen as the method of measuring cFLIP protein levels in LCLs. As I had difficulty finding a reliable antibody for detecting cFLIP a total of four antibodies were tested; H202 (Santa Cruz) (Stagni et al., 2008), #3210 (Cell Signalling Technology) (Dutton et al., 2004), ab8421 (Abcam) and NF6 (Alexis Biochemicals) (Ivanov et al., 2009). All antibodies were described as recognising both the long and short forms of cFLIP.

Initially 3 antibodies were tested; H202, #3210 and ab8421. These are all rabbit polyclonal antibodies and should recognise both human and mouse cFLIP. Lysates of one normal (N8)

and one A-T LCL with no ATM activity (AT18) were run on a gel along with lysates from HeLa cells (human cervical cancer cell line which expresses cFLIPL) and NIH3T3 cells (mouse fibroblasts which express both isoforms) as positive controls. Blotting with all three antibodies using the manufacturer's recommended dilution conditions produced multiple bands suggesting a high degree of non-specific binding (Fig 4:5:1A). However none of the antibodies showed clear bands of the appropriate size for cFLIPL (55-58kDa) or cFLIPS (28-30kDa). A second blot of HeLa and NIH3T3 lysates was carried out and the membranes incubated under different dilution conditions in an attempt to increase sensitivity and minimise non-specific binding of #3210 and H202 (Fig 4:5:1B). However changing the diluents and antibody concentrations did not significantly improve the blots.

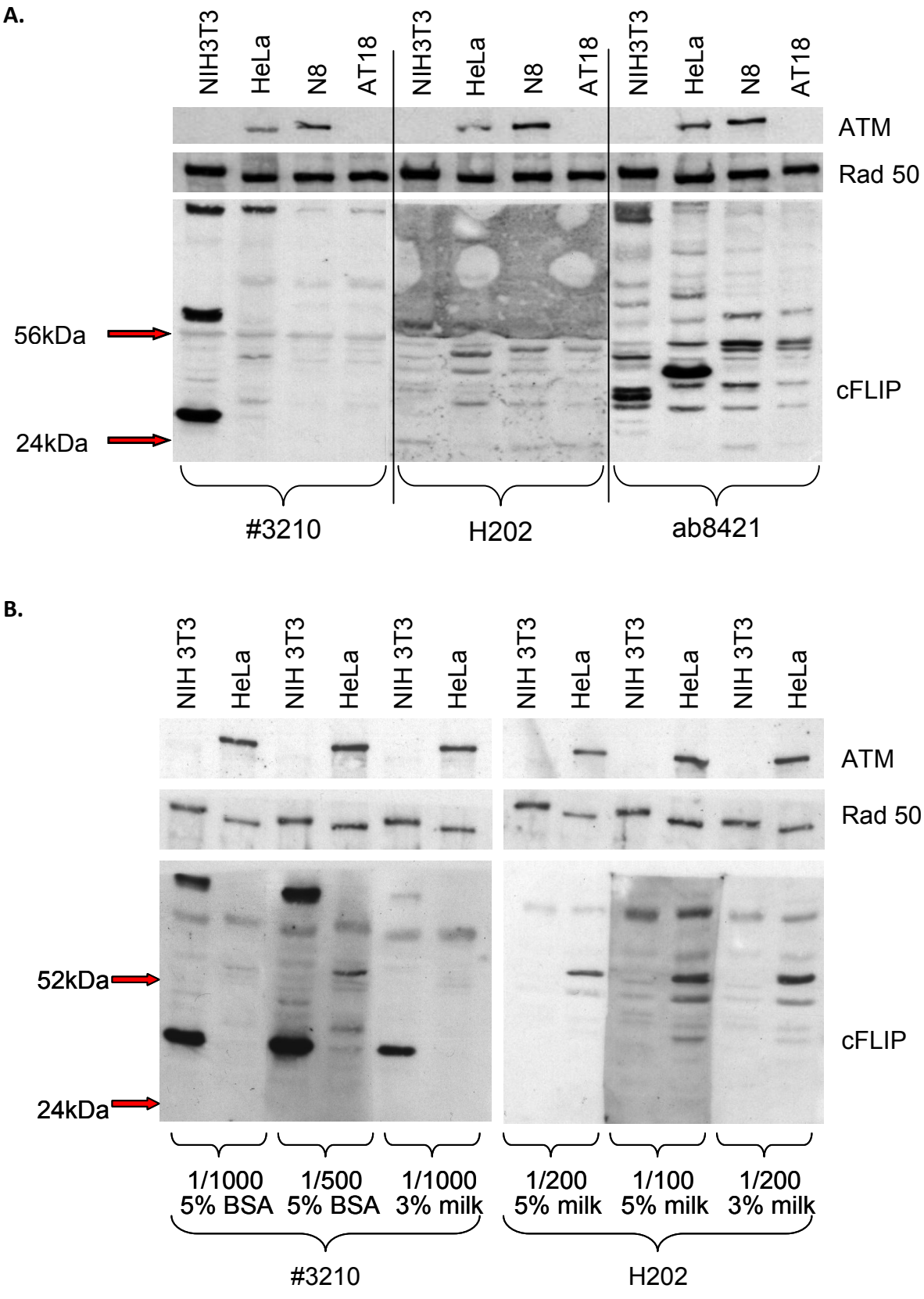
As the antibodies were clearly binding multiple proteins I attempted to determine which bands (if any) were cFLIP and which were the result of non-specific binding. An siRNA knockdown of cFLIP was carried out in HeLa cells in order to produce a negative control which would express no or very little cFLIPL. Lysate from the knockdown was run on a gel alongside HeLa and NIH3T3 lysates and the membrane probed with #3210 and H202 antibodies (Fig 4:5:1C). The membrane stained with #3210 did show a slight reduction in a band of approximately the correct size to be cFLIPL in the cFLIP siRNA transfected lysate. However, the effect was so slight that it could have been a loading effect. The cFLIP siRNA transfection had no effect on the band pattern of the membrane stained with H202. Therefore I was unable to conclusively determine which bands were cFLIPL or if the knockdown had been successful.

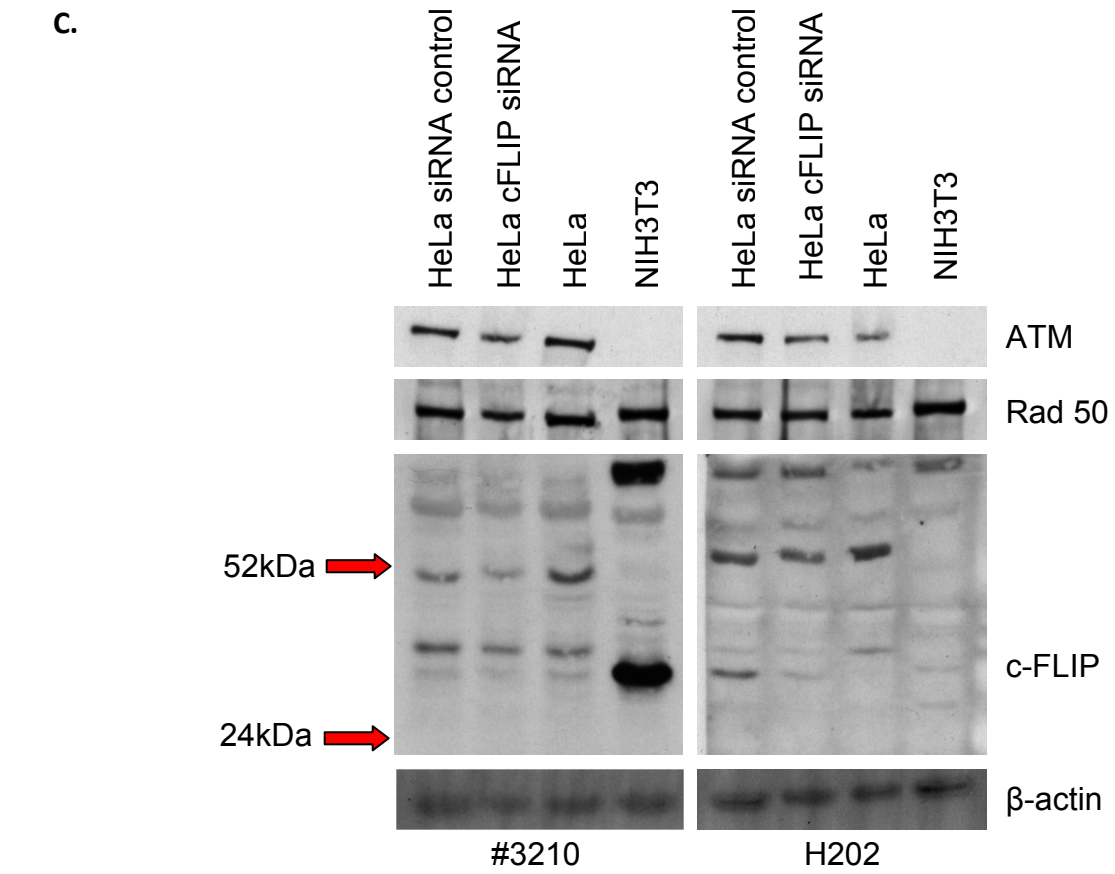
A further blot was carried out to try and identify bands of cFLIP using the opposite strategy – by selectively blocking specific binding to cFLIP protein so that only bands produced by non-specific antibody binding would be visible. As the FLIP peptide to which the ab8421 antibody

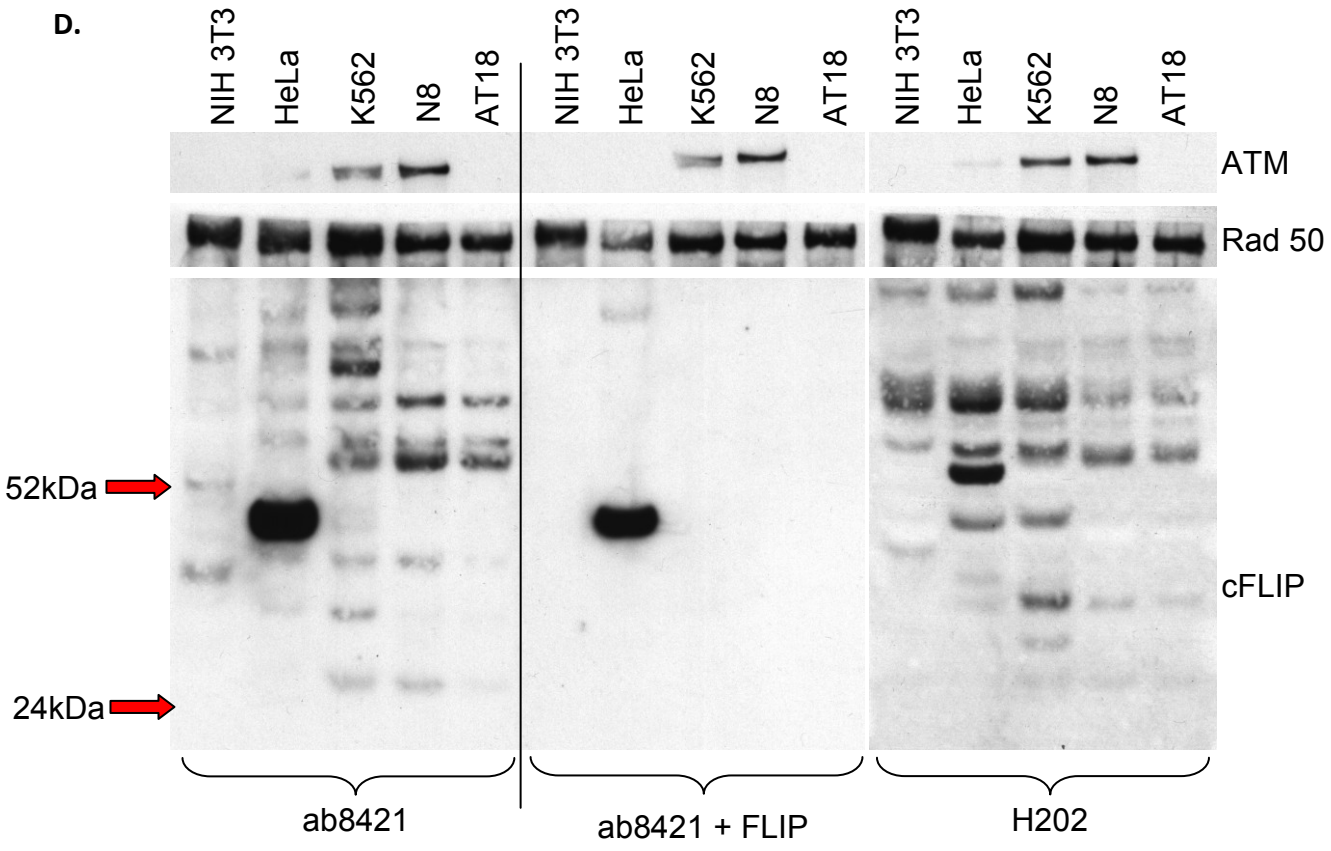
had been raised (ab8457) was commercially available we incubated the antibody with the peptide to try and selectively block specific binding to cFLIP protein. I then compared the band pattern achieved using blocked and non-blocked antibody (Fig 4:5:1D). Unsurprisingly incubation with the peptide blocked both specific and non-specific binding of the antibody so the experiment was unsuccessful.

Finally as #3210, H202 and ab8421 did not work as required, a fourth antibody, NF6, was tested. This is a mouse monoclonal antibody raised against recombinant human cFLIP (aa1-194) which recognises both short and long isoforms of the human protein. Normal LCL (N8) and A-T LCL (AT18) lysates were run in triplicate on a gel along with lysates of NIH3T3 and K562 (a human myeloid leukaemia cell line which expresses both cFLIPL and cFLIPS). Following transfer the membrane was divided in three and sections incubated with NF6, H202 or ab8421 to allow comparison of the banding patterns (Fig 4:5:1E). Unlike the other antibodies NF6 revealed the presence of strong bands of the appropriate size for cFLIPL and cFLIPS and very little non-specific binding. There was a relatively faint band of cFLIPL and no cFLIPS visible in the NIH3T3 lysate, however this is unsurprising as NIH3T3 is a mouse cell line and NF6 was raised against human cFLIP. NF6 was the most effective cFLIP antibody of those tested and showed the least non-specific binding so was chosen for use in further experiments.

Fig 4:5:1: Analysis of cFLIPL and cFLIPS expression by western blot.




C.
HeLa siRNA control
HeLa cFLIP siRNA
HeLa
NIH3T3
HeLa siRNA control
HeLa cFLIP siRNA
HeLa
NIH3T3
ATM
Rad 50
52kDa
24kDa
c-FLIP
β-actin
#3210
H202


D.
NIH 3T3
HeLa
K562
N8
AT18
NIH 3T3
HeLa
K562
N8
AT18
NIH 3T3
HeLa
K562
N8
AT18
ATM
Rad 50
52kDa
24kDa
cFLIP
ab8421
ab8421 + FLIP
H202

E.

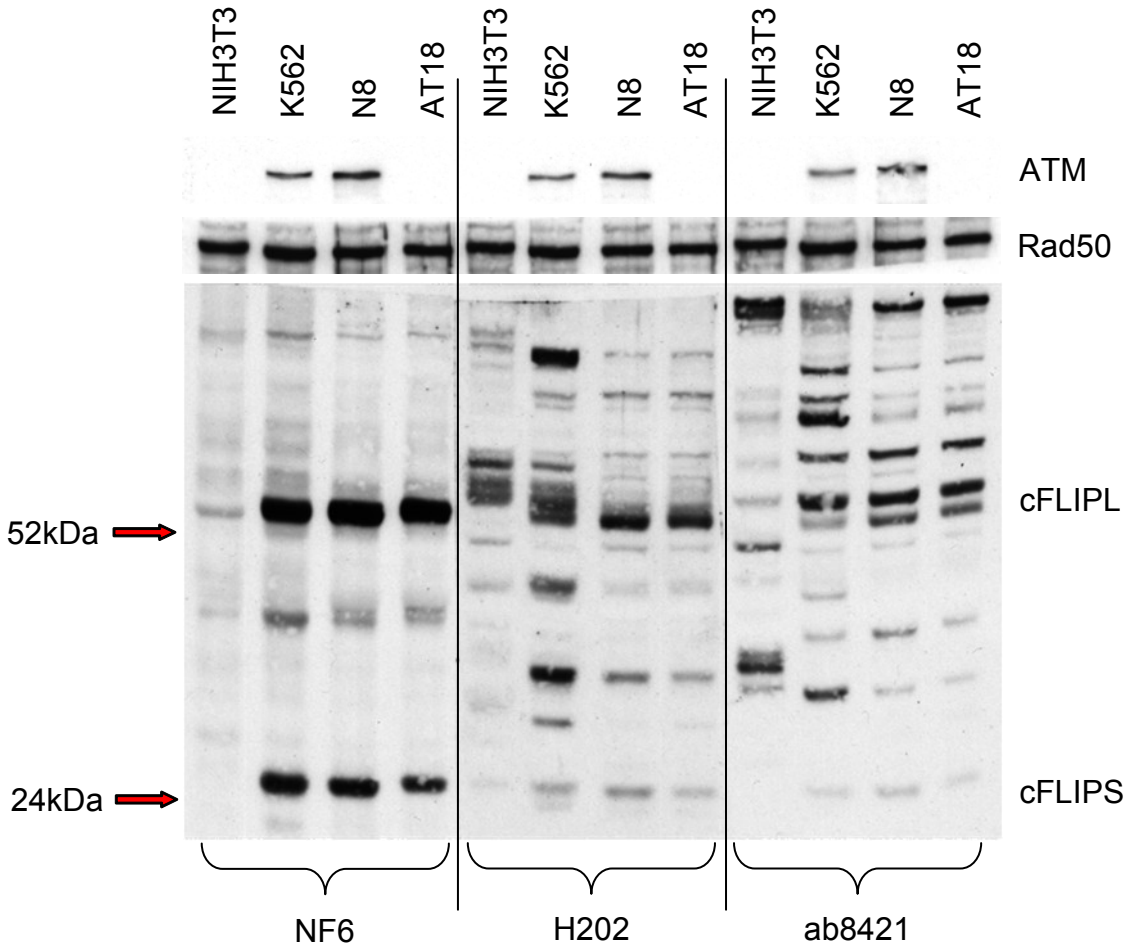


Fig 4:5:1: Analysis of cFLIP protein expression by western blot. For each experiment lysates from some or all of the following cell lines were run in duplicate or triplicate on single gels to allow direct comparison of banding patterns; N8 (normal LCL), AT18 (A-T LCL with no ATM activity), NIH3T3 (mouse fibroblast), K562 (human myeloid leukaemia), HeLa (human cervical cancer). Following transfer the membranes were divided into sections and incubated with the indicated antibodies. Membranes were also blotted for ATM and Rad 50 was used as a loading control.

A. Comparison of cFLIP blotting results obtained using #3210, H202 and ab8421 antibodies (manufacturers recommended dilution conditions).

B. Optimisation of dilution conditions for #3210 and H202 antibodies.

C. Comparison of cFLIP banding patterns obtained using #3210 and H202 antibodies on lysates from untransfected, cFLIP siRNA transfected and control siRNA transfected HeLa cells and NIH3T3 cells.

D. Blocking of ab8421 with cFLIP peptide (ab8457). To block ab8421 binding the undiluted antibody was incubated with an equal volume of peptide for 30min at 37°C prior to dilution in 5% TBST milk and membrane incubation.

E. Comparison of cFLIP blotting results achieved using N6, H202, and ab8421 cFLIP antibodies.

4:5:2: cFLIP protein expression in normal and A-T LCLs

cFLIP expression in normal and A-T LCLs was analysed by western blot and protein levels determined by densitometry. To confirm the ATM expression and ATM activity status of each LCL irradiated and non-irradiated lysates were made. These were run on 8% Tris-Bicine gels and activity assayed by blotting for total and phosphorylated forms of ATM, SMC1 and Nbs1. As cFLIP functions by competing with caspase 8 the expression of caspase 8 in each LCL was also analysed. Total SMC1 was used as a loading control for densitometry. The ATM activity analysis confirmed the known ATM status of each LCL (Fig 4:5:2A).

As A-T LCLs with no ATM activity were more sensitive to CH11-induced apoptosis than normal LCLs, I hypothesised that they would either express lower levels of cFLIP or would have a higher caspase 8:cFLIP ratio than the normal LCLs. LCLs with a higher caspase 8:cFLIP ratio will have proportionally less cFLIP available to compete with caspase 8 for binding to the DISC complex. Therefore the efficiency of inhibition of apoptosis by cFLIP may be less than in a LCL with a low caspase 8:cFLIP ratio, leading to increased sensitivity to CH11-induced apoptosis.

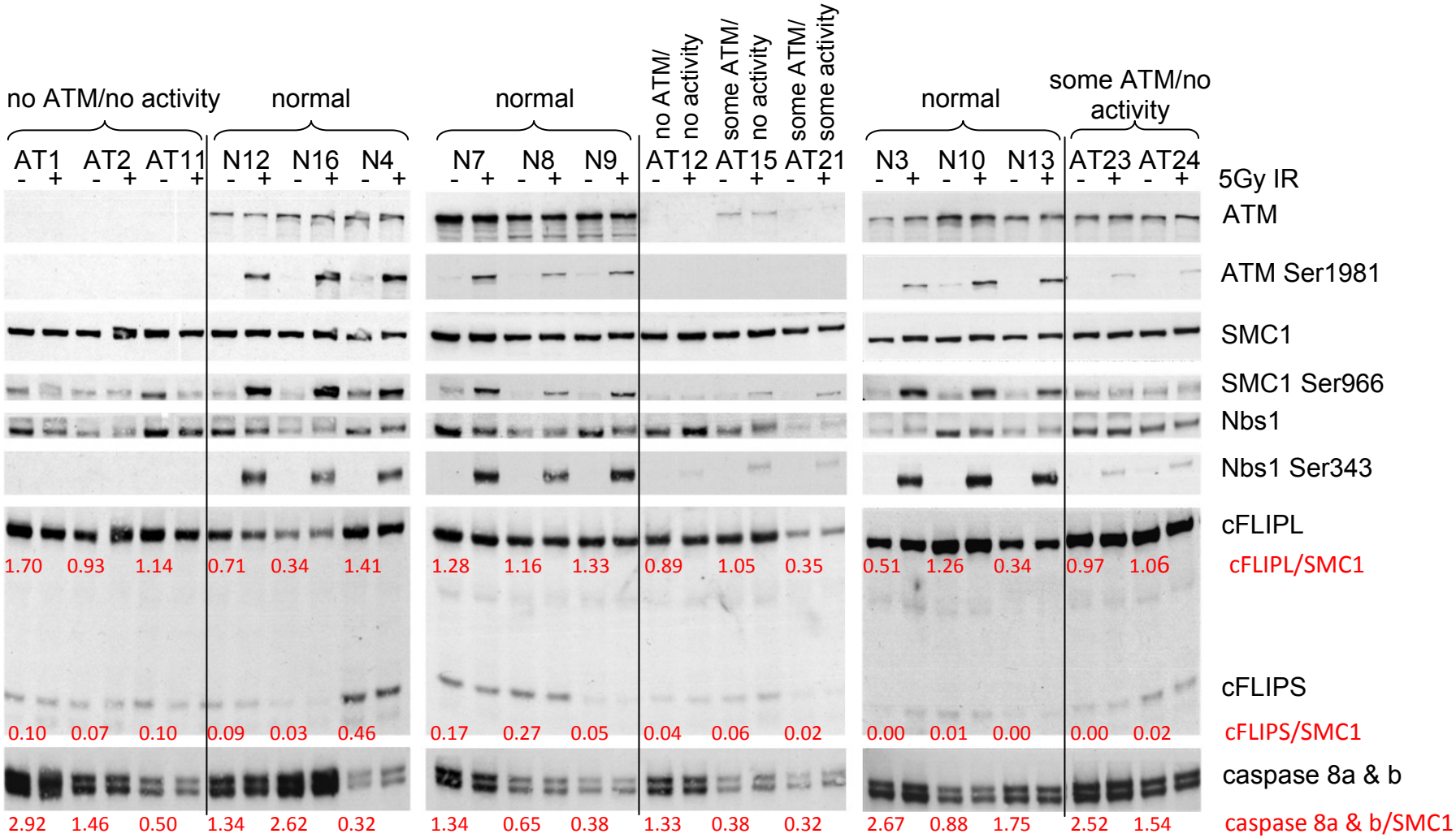
Although there was some variation in cFLIP expression between different LCLs there was no significant difference in expression of either cFLIPL, cFLIPS or total cFLIP between normal and A-T LCLs (Fig 4:5:2A&B). There was also no significant difference in caspase 8 expression or the caspase 8:cFLIP ratio of normal and A-T LCLs (Fig 4:5:2C). However, in normal LCLs there was a significant negative correlation between total cFLIP expression and caspase 8 expression ($r=-0.7866$, $p=0.0172$) (Fig 4:5:2D). This is counterintuitive as I expected that an LCL with high caspase 8 expression would also have high cFLIP expression in order to regulate its apoptosis. A-T LCLs with no ATM activity showed no correlation between total

cFLIP and caspase 8 expression which may indicate a lack of regulation of cFLIP or caspase 8 expression in A-T LCLs.

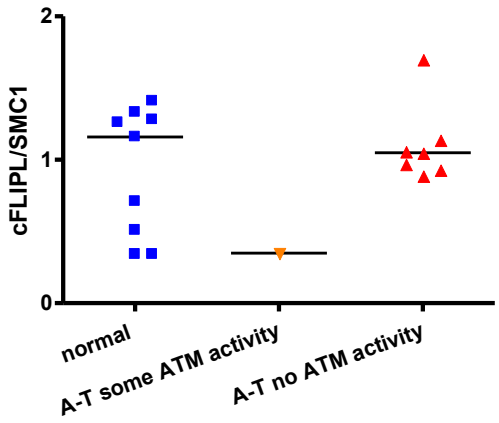
An Annexin V/PI apoptosis assay was carried out on the LCLs to test their sensitivity to CH11-induced apoptosis (Fig 4:5:2E). The group of normal LCLs showed a significant correlation between increasing cFLIPL expression and decreasing loss of viability in response to CH11 treatment ($r=0.7950$, $p=0.0138$), however the A-T LCLs with no ATM activity did not show a similar correlation (Fig 4:5:2F). There was also no correlation between caspase8:cFLIP ratio and change in viability in either group (Fig 4:5:2F), this suggests that the caspase 8:cFLIP ratio of LCLs is not important in regulation of their apoptotic sensitivity.

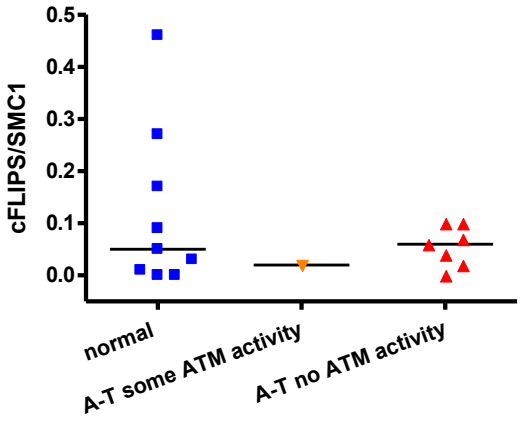
Fig 4:5:2: There was no significant difference in cFLIPL expression between normal and A-T LCLs.

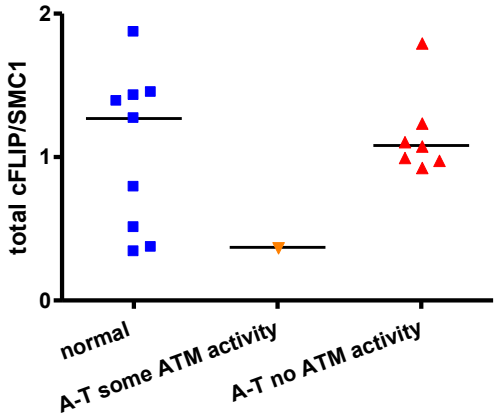
A.



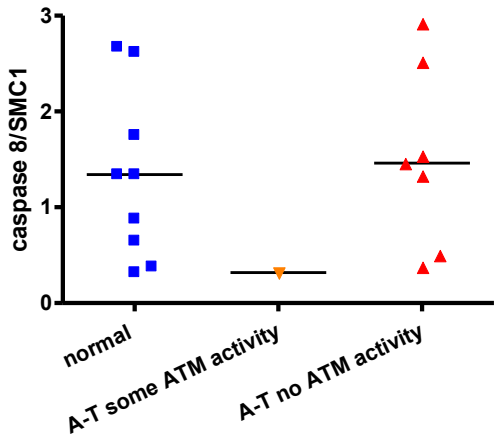
B.

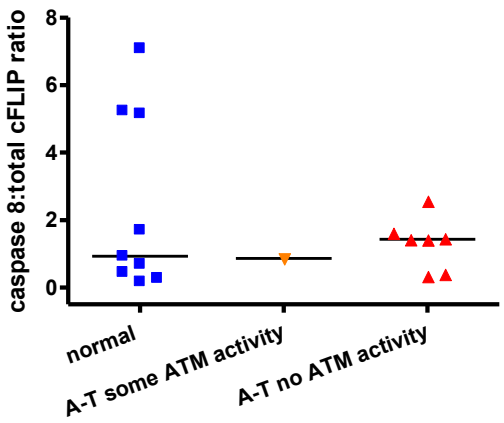

cFLIPL/SMC1
2
1
0
normal
A-T some ATM activity
A-T no ATM activity


cFLIPS/SMC1
0.5
0.4
0.3
0.2
0.1
0.0
normal
A-T some ATM activity
A-T no ATM activity

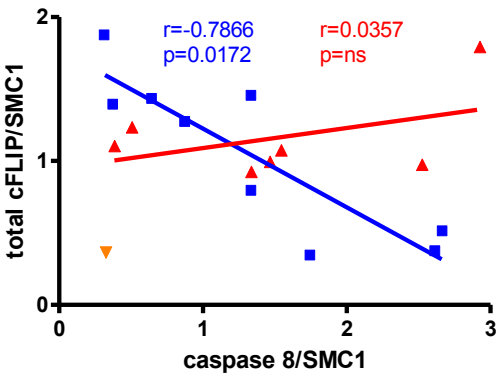

total cFLIP/SMC1
2
1
0
normal
A-T some ATM activity
A-T no ATM activity

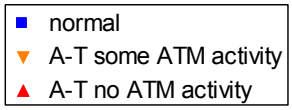
C.


caspase 8/SMC1
3
2
1
0
normal
A-T some ATM activity
A-T no ATM activity


caspase 8:total cFLIP ratio
8
6
4
2
0
normal
A-T some ATM activity
A-T no ATM activity

D.


total cFLIP/SMC1
caspase 8/SMC1
r=-0.7866
p=0.0172
r=0.0357
p=ns
2
1
0
0
1
2
3


normal
A-T some ATM activity
A-T no ATM activity

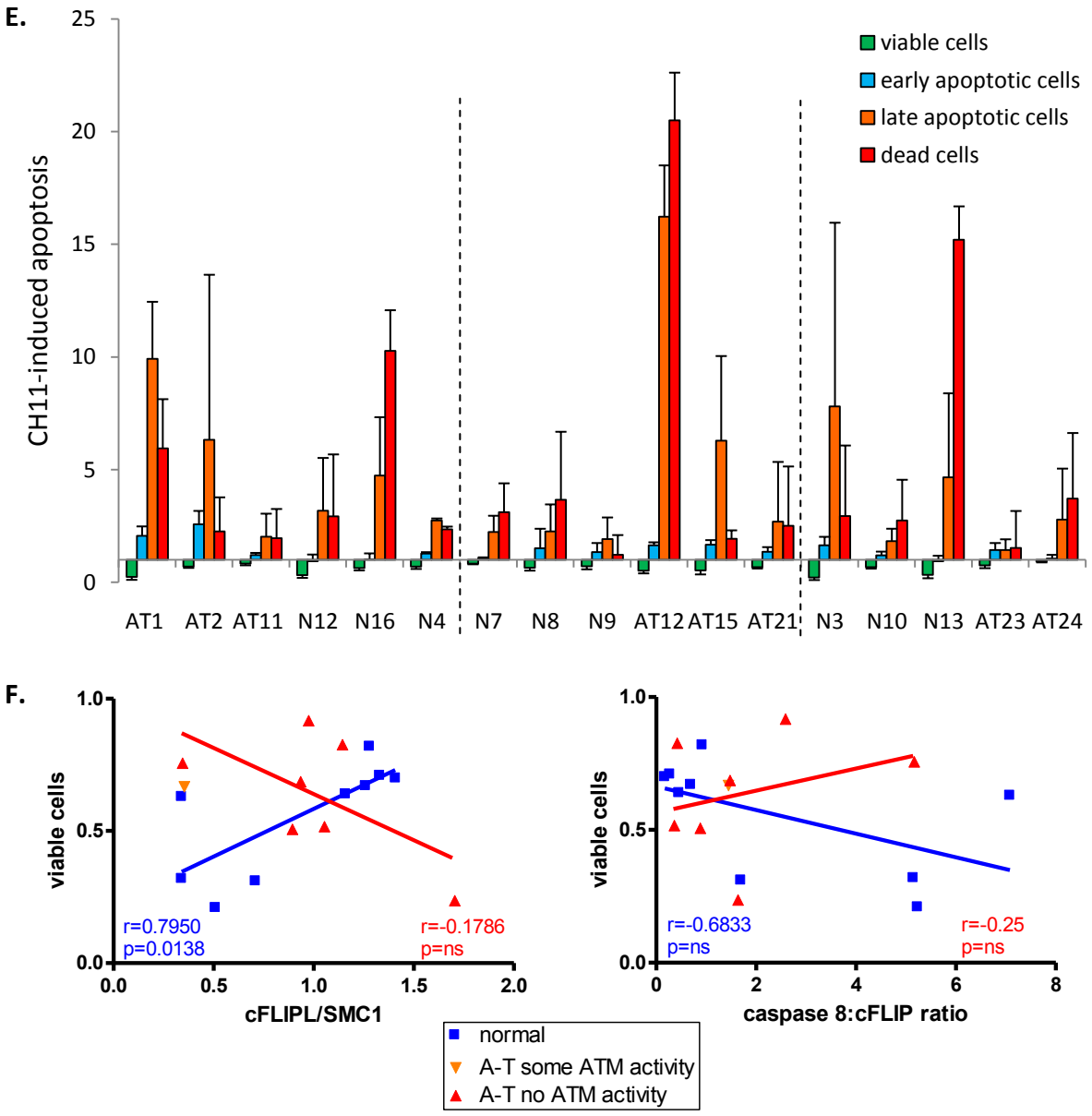


Fig 4:5:3: There was no significant difference in cFLIP protein expression between normal and A-T LCLs.

A. Western blots of irradiated and non-irradiated lysates from normal and A-T LCLs. 30ug protein lysate was run on an 8% acrylamide gel. Blots were probed for ATM, ATM Ser1981, Nbs1, Nbs1 Ser343, SMC1 and SMC1 Ser966 for analysis of ATM expression and activity in each LCL, as well as cFLIP and caspase 8 expression. The relative levels of cFLIP and caspase 8 expression in non-irradiated lysates were measured by densitometery (indicated in red), total SMC1 was used as the loading control.

B. There was no significant difference between the expression of cFLIPL, cFLIPS or total cFLIP (cFLIPL + cFLIPS) in normal and A-T LCLs.

C. There was no significant difference in caspase 8 expression or caspase 8:total cFLIP ratio between normal and A-T LCLs. The caspase 8:total cFLIP ratio was calculated using the formula – caspase 8:cFLIP ratio = ((caspase 8/SMC1)/(total cFLIP/SMC1)).

D. There was a significant negative correlation between caspase 8 expression and total cFLIP expression in normal LCLs. A-T LCLs with no ATM activity did not show any correlation.

E. Apoptosis of LCLs used in western blots in response to CH11 treatment (mean of 3 experiments). LCLs were treated with 500ng/ml CH11 for 15h then harvested and analysed by Annexin V/PI staining. Untreated cells of each LCL were used as a control. Gating was carried out as in Fig 2:1:2C. CH11-induced apoptosis was quantified as the % of CH11-treated cells at each stage of apoptosis divided by the % of untreated cells at each stage of apoptosis.

F. Normal LCLs show a significant correlation between cFLIPL expression and viability following CH11 treatment ($r=0.7950$, $p=0.0138$). A-T LCLs with no ATM activity did not show a correlation. There was no correlation between caspase 8:total cFLIP ratio and viability following CH11 treatment in either A-T or normal LCLs.

4:5:3: Caspase 8 activation and cFLIP degradation in response to CH11 treatment.

Although there was no significant difference in cFLIP expression between normal and A-T LCLs it is possible that they may react differently to CH11 treatment in terms of the rate and efficiency of cFLIP degradation and caspase 8 activation.

A western blot was carried out to analyse the effect of CH11 treatment on cFLIP expression and caspase 8 activation in normal and A-T LCLs. Lysates of one normal LCL (N8), one A-T LCL with some ATM activity (AT21) and one A-T LCL with no ATM activity (AT18) were made over a timecourse of CH11 treatment. All three LCLs showed degradation of cFLIP in response to CH11 treatment (Fig 4:5:3). This is necessary to allow caspase 8 activation and induction of apoptosis. There was no indication of abnormality in cFLIP degradation in the A-T LCLs.

Interestingly, comparisons of caspase 8 activation measured by the accumulation of the intermediate processing products p43 and p26 and the active form of caspase 8, p18, clearly showed that caspase 8 activation following 8 or 24 hours of incubation with CH11 was greatest in AT18, the LCL with no ATM activity. The lowest level of caspase 8 activation was in the normal LCL, whilst AT21, the A-T LCL with some ATM activity, showed an intermediate level of caspase 8 processing. However, these findings cannot be taken as an indication of a faster rate of apoptosis in the A-T LCLs as the lack of active caspase 8 in the normal LCL following 8 or 24 hours of incubation with CH11 was consistent with the result of the CH11 timecourse experiment (Fig 4:4:4) in which no further decrease in the viability of normal LCLs occurred after 8 hours of CH11 incubation. In contrast the viability of A-T LCLs continued to decrease after 8 hours of incubation with CH11 which is consistent with the

finding of active caspase 8 and intermediate processing products in the A-T LCLs after 8 and 24 hours of CH11 incubation.

Fig 4:5:3: cFLIP degradation in response to CH11 treatment is normal in A-T LCLs.

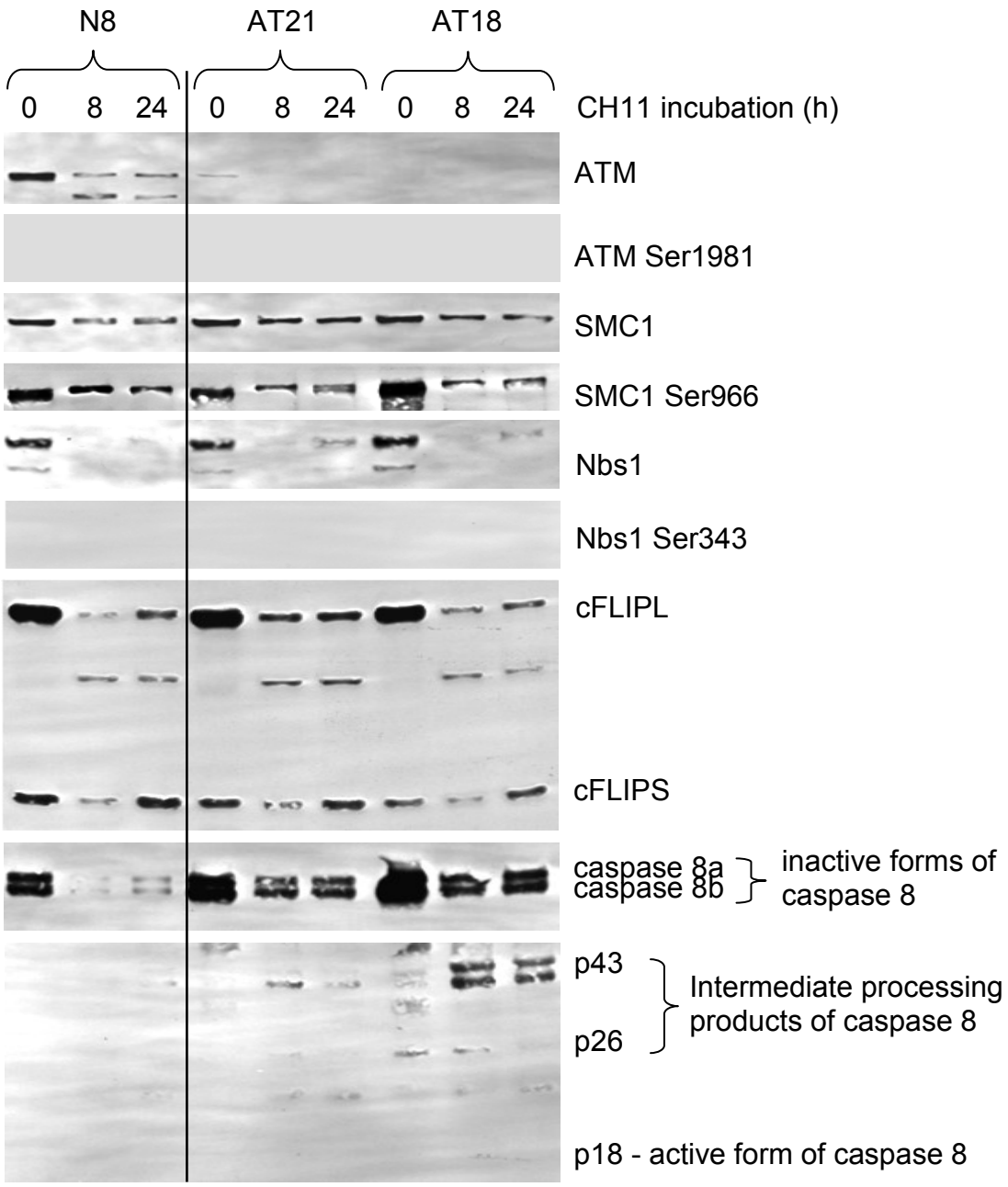


Fig 4:5:3: LCLs were seeded in tissue culture plates at a concentration of $0.5x10^6$cells/ml. 0, 8 and 24h after addition of 500ng/ml CH11 cells were harvested and protein lysates made. 50ug of lysate was run on an 8% acrylamide gel. SMC1 was used as a loading control.

4:5:4: The effect of ATM activation on cFLIP protein expression.

There was no evidence of an ATM-dependent difference in constitutive cFLIP expression between normal and A-T LCLs, however it has been suggested that cFLIP expression may be regulated by ATM activation (Ivanov et al., 2009). Therefore the effect of DNA damage (IR) induced activation of ATM on the cFLIP expression of two normal LCLs was analysed by western blot. Two A-T LCLs with no ATM activity were used as negative controls.

There was very little change in cFLIPL or cFLIPS expression in any of the LCLs 8h after irradiation, however after 24h the expression of cFLIPL and to a lesser extent cFLIPS increased in both normal and A-T LCLs (Fig 4:5:4A&B). This suggests that although cFLIP expression increases after IR this effect is not ATM-dependent as it can occur independently of ATM activity.

Fig 4:5:4: cFLIP protein expression increases following IR independently of ATM.

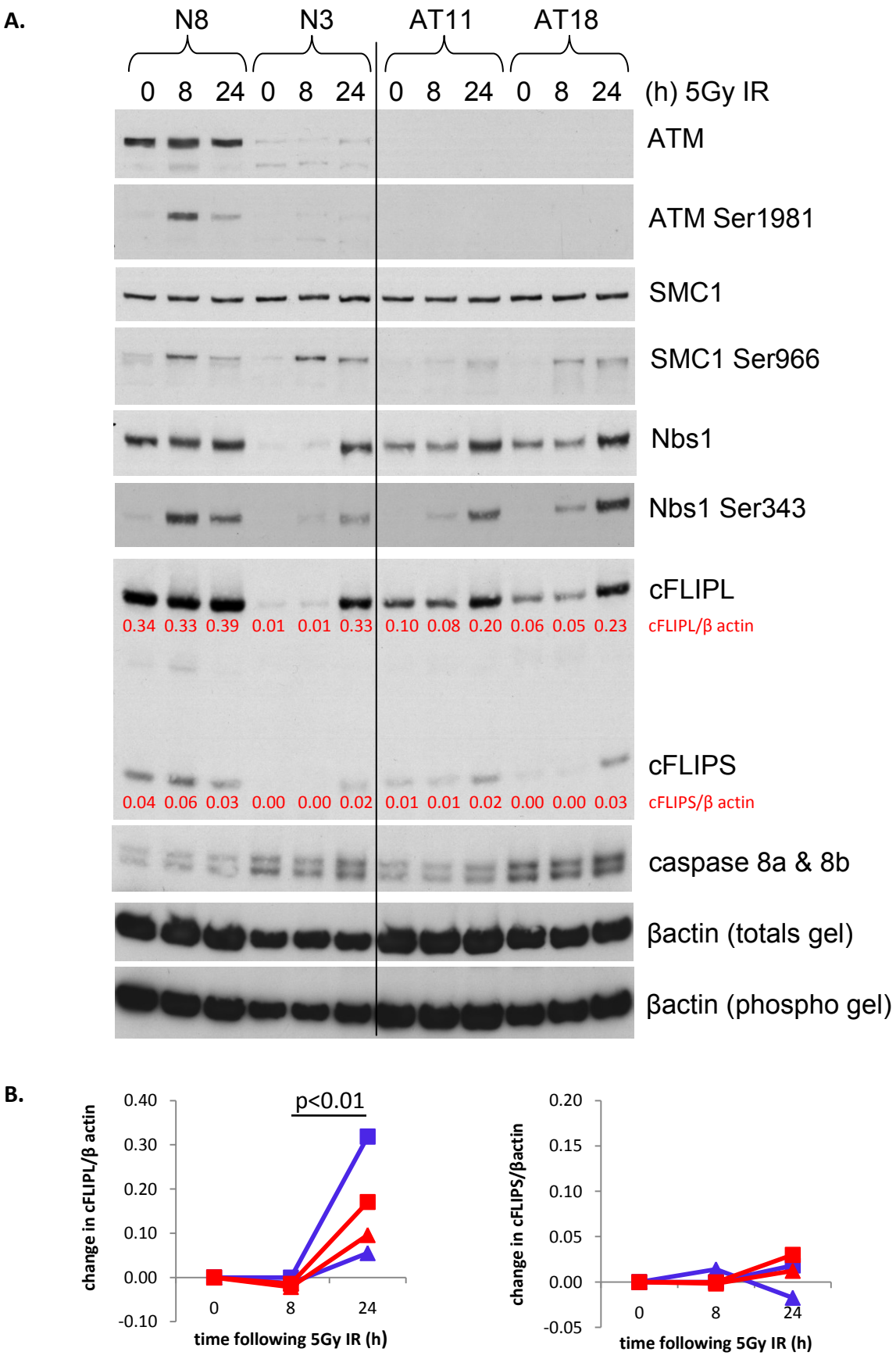


Fig 4:5:4: cFLIP protein expression increases following IR independently of ATM.

A. cFLIP protein expression in 2 normal (N8, N3) and 2 A-T LCLs with no ATM activity (AT11, AT18) following 5Gy IR. LCLs were irradiated, transferred to tissue culture plates ($2x10^6$ cells/well) and incubated at 37°C for the indicated time prior to harvest (0h = no IR). β actin was used as a loading control for densitometery (shown in red).

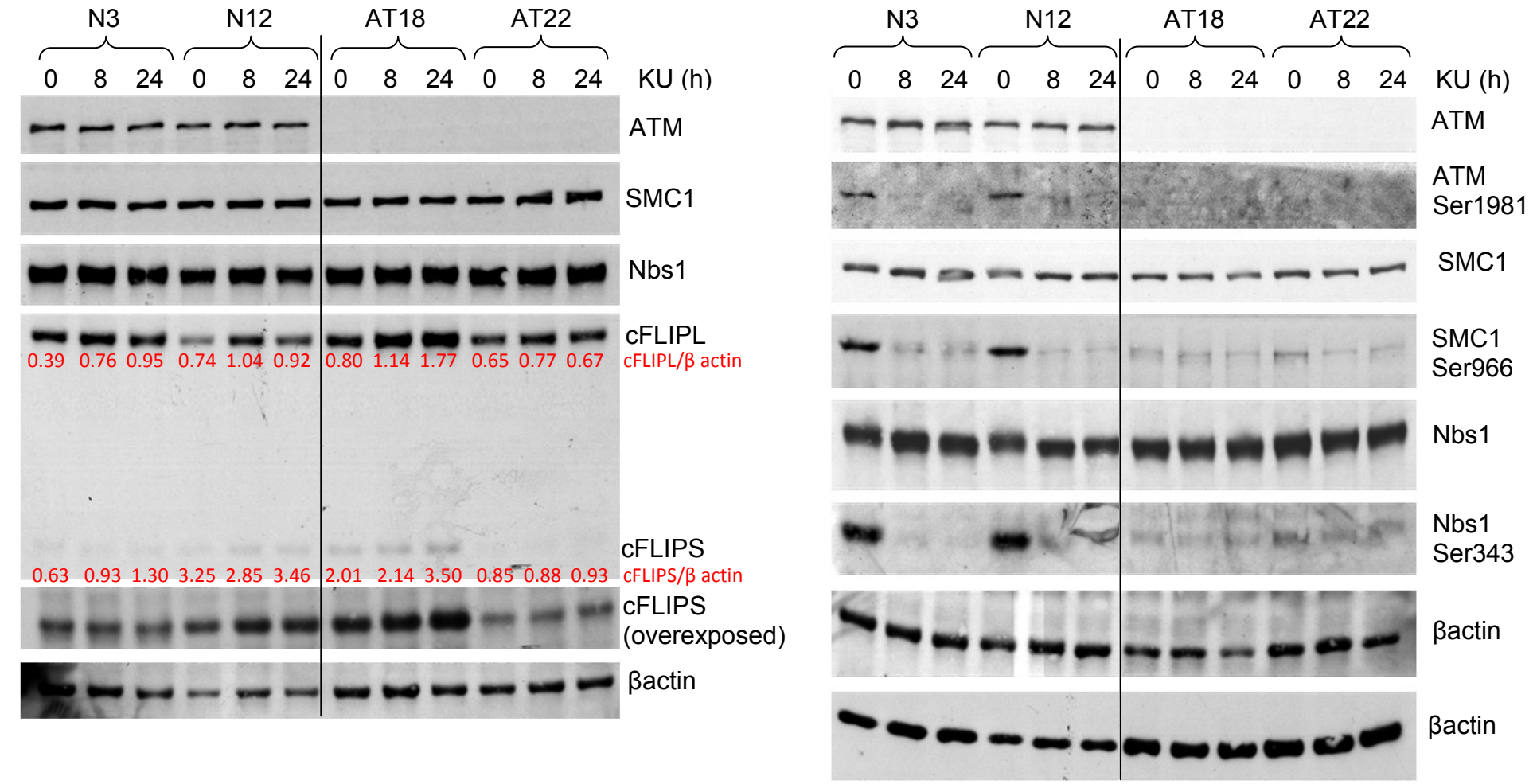
B. Change in cFLIPL and cFLIPS expression following IR.

4:5:5: The effect of ATM inhibition on cFLIP expression.

The effect of inhibition of ATM protein kinase activity on constitutive expression of cFLIP was also investigated. Normal LCLs (N3 and N12) and A-T LCLs with no ATM activity (AT18 and AT22) were treated with the specific ATM inhibitor KU-55933 and their lysates analysed by western blotting. Incubation with KU-55933 for 8 or 24h completely inhibited IR-induced ATM activation in the normal LCLs (Fig 4:5:5B) and significantly increased expression of cFLIPL and cFLIPS in both normal and A-T LCLs with the biggest increase occurring in the A-T LCL AT18 (Fig 4:5:5A&C). Therefore the increase in cFLIP in the presence of KU-55933 could not be attributed to the inhibition of ATM activity as it occurred even in the LCLs which did not express ATM.

There was no difference in cFLIP expression between normal and A-T LCLs and inhibition of ATM activity by KU-55933 did not have a differential effect on cFLIP expression in normal compared to A-T LCLs. Therefore *ATM* mutations do not affect cFLIP expression in LCLs. However, the inhibitory effect of cFLIP on CD95-mediated apoptosis may be more important for normal LCLs than A-T LCLs as CD95 expression seemed to have little effect on the sensitivity of normal LCLs to CH11-induced apoptosis.

Fig 4:5:5: Inhibition of ATM activity increases cFLIP expression in both normal and A-T LCLs.



C.

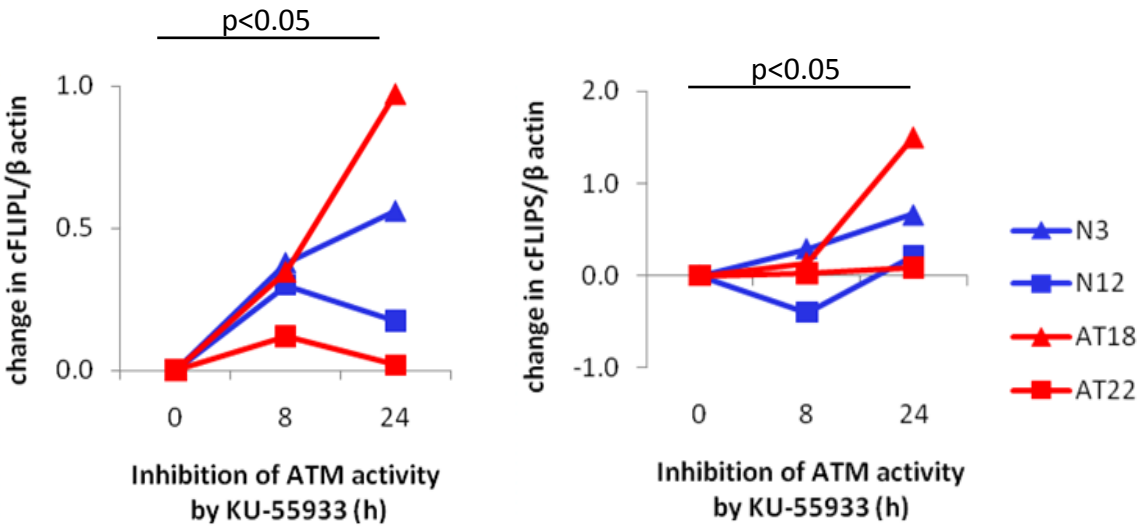


Fig 4:5:5: LCLs were treated with 10mM KU-55933 for 0, 8 or 24h. Cells were harvested and half of each sample irradiated (5Gy) and incubated at 37°C for 30min. Lysates of irradiated and non-irradiated LCLs were made.

A. cFLIP protein expression in KU-55933 treated cells (non-irradiated lysates).

B. ATM activity in ATM inhibitor (KU-55933) treated LCLs (irradiated lysates).

C. cFLIPL (left) and cFLIPS (right) expression in LCLs increases significantly in response to KU-55933 treatment.

4:6: Analysis of the role of EBV in regulation of apoptotic sensitivity.

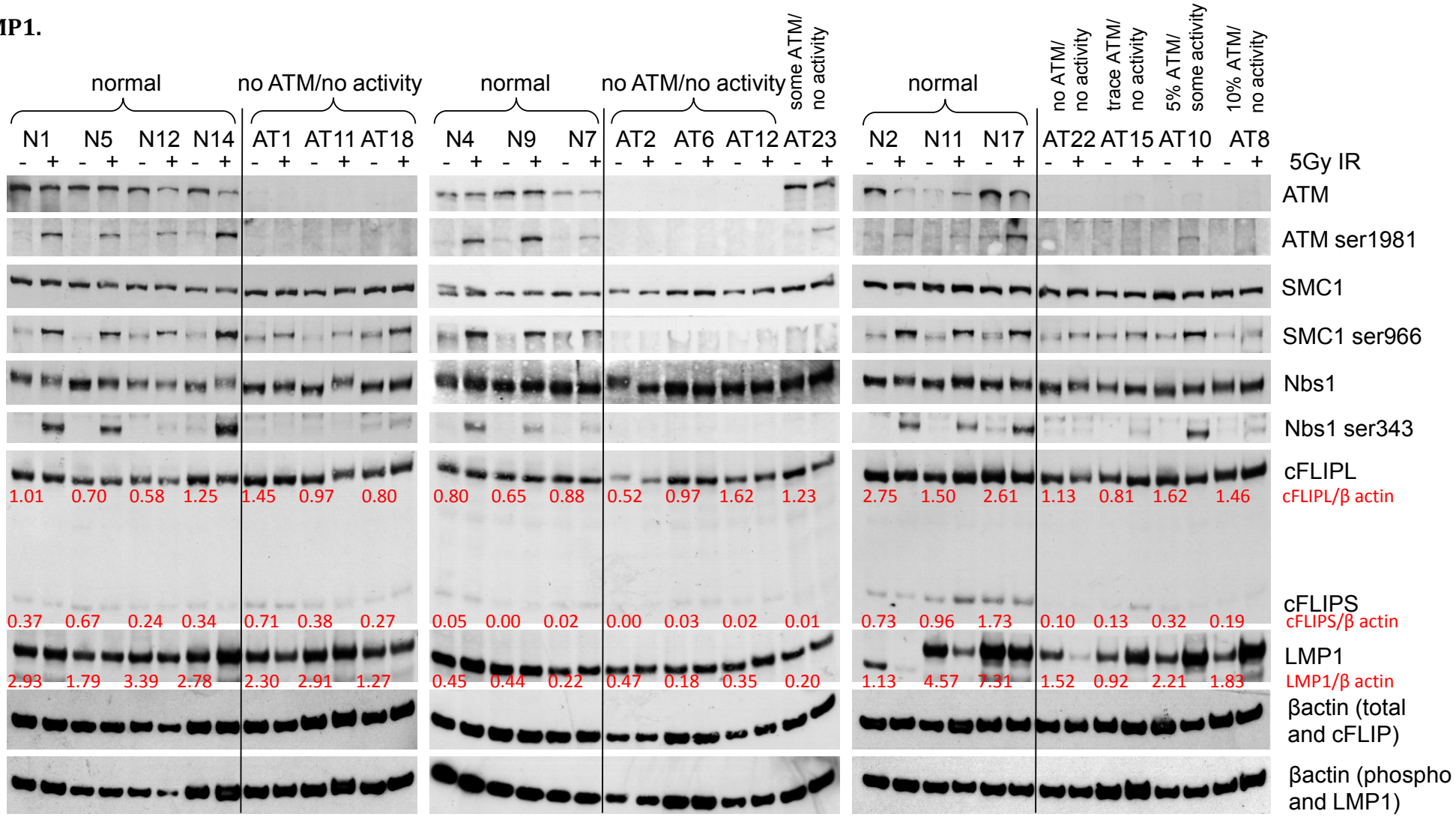
4:6:1: LMP1 expression in A-T and normal LCLs.

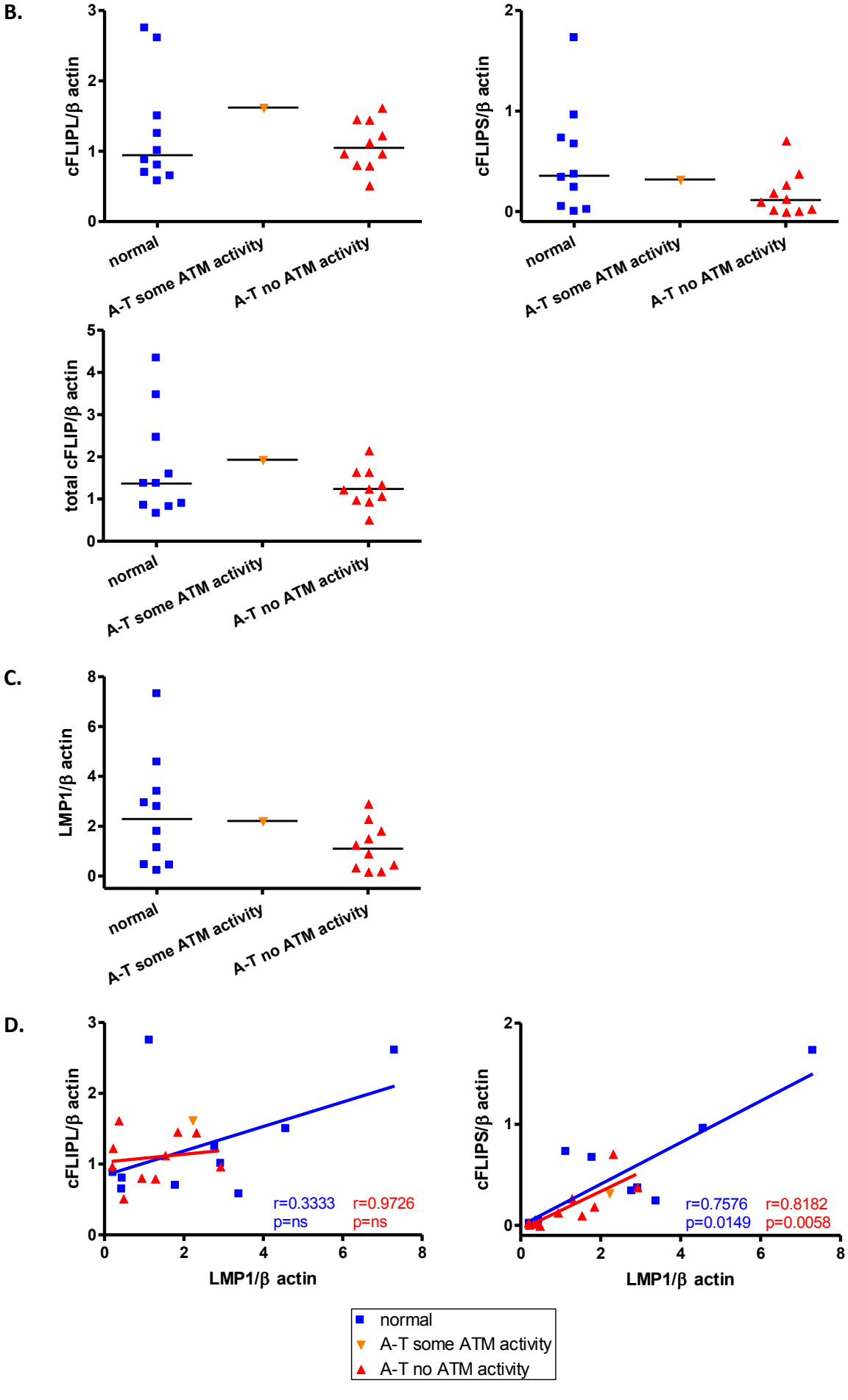
Although A-T LCLs have lower CD95 expression than normal LCLs there was no evidence of a role of ATM in regulating CD95 expression. LCLs are transformed by infection with EBV, a virus which is able to regulate both pro- and anti-apoptotic genes. Interestingly CD95 upregulation on B cells in response to EBV infection involves the EBV latent membrane protein-1 gene (LMP-1) which is also able to regulate cFLIP protein expression (Cahir-McFarland et al., 2004). It is thought that LMP1 has an important role in maintaining a balance between virally infected cells and the host immune system. Therefore the difference in CD95 expression between normal and A-T LCLs may be due to a difference in LMP1 protein expression by EBV in the presence or absence of ATM activity.

LMP1, cFLIP and CD95 expression in A-T and normal LCLs was analysed by western blot (Fig 4:6:1A) and flow cytometry (Fig 4:6:1E). Consistent with earlier findings (Fig 4:5:2) there was no significant difference in cFLIP expression between the normal and A-T LCLs (Fig 4:6:1B). LMP1 expression was slightly higher in normal than A-T LCLs (Fig 4:6:1A&C) but this was also not statistically significant. However, there was a significant correlation between LMP1 expression and cFLIPS expression in both normal and A-T LCLs. cFLIPL showed a similar trend but this was not significant (Fig 4:6:1D). These findings suggest that the presence of *ATM* mutations does not influence the regulation of cFLIP expression by LMP1. There was no correlation between LMP1 expression and CD95 expression on normal or A-T LCLs (Fig 4:6:1E)

As there was no difference in LMP1 protein expression between normal and A-T LCLs and no correlation between LMP1 and CD95 expression it seems unlikely that the difference in CD95 expression between normal and A-T LCLs is due to a difference in regulation by EBV.

Fig 4:6:1: The difference in CD95 expression of A-T and normal LCLs is not due to a difference in expression of the EBV protein LMP1.




B.
cFLIPL/β actin
cFLIPS/β actin
total cFLIP/β actin
normal
A-T some ATM activity
A-T no ATM activity
C.
LMP1/β actin
D.
cFLIPL/β actin
cFLIPS/β actin
LMP1/β actin
r=0.3333
p=ns
r=0.9726
p=ns
r=0.7576
p=0.0149
r=0.8182
p=0.0058
normal
A-T some ATM activity
A-T no ATM activity

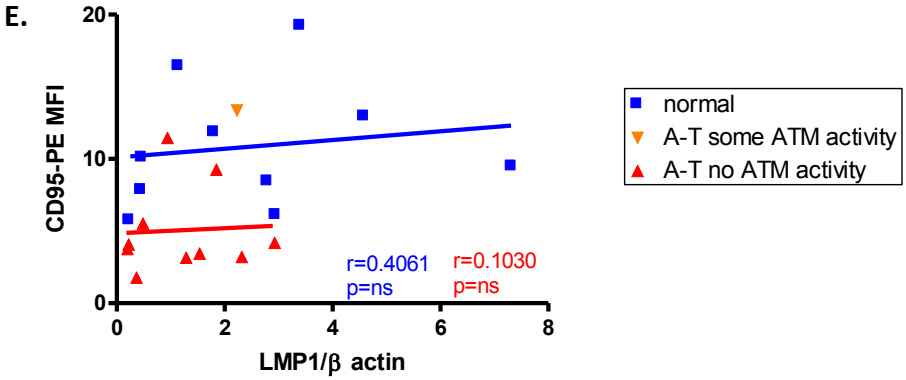


Fig 4:6:1: The difference in CD95 expression of A-T and normal LCLs is not due to a difference in expression of the EBV protein LMP1**.**

A. Western blots of irradiated and non-irradiated lysates from normal and A-T LCLs. 30ug protein lysate was run on an 8% acrylamide gel. Blots were probed for ATM, ATM ser1981, Nbs1, Nbs1 Ser966, SMC1 and SMC1 Ser344 for analysis of ATM expression and activity in each LCL as well as cFLIP and LMP1 protein expression. cFLIP and LMP1 expression in non-irradiated samples were measured by densitometery using β actin as a loading control (shown in red).

B. There was no significant difference between the expression of cFLIPL, cFLIPS or total cFLIP (cFLIPL + cFLIPS) in normal and A-T LCLs.

C. There was no significant difference in LMP1 protein expression between normal and A-T LCLs.

D. There was no correlation between LMP1 expression and cFLIPL expression in LCLs (top). However there is a significant correlation between expression of LMP1 and cFLIPS in both normal and A-T LCLs.

E. There was no correlation between LMP1 expression and CD95 MFI (measured by flow cytometry Fig 4:4:1B) in normal or A-T LCLs.

4:7: Investigating the effect of *ATM* mutation on sensitivity to CD95-mediated apoptosis using isogenic LCLs.

4:7:1: Analysis of cFLIP and CD95 expression and sensitivity to CH11-induced apoptosis of isogenic LCLs.

All experiments so far were carried out using LCLs derived from either healthy lab donors (normal LCLs) or A-T patients (A-T LCLs). The genetic background of each LCL was different and this may explain the great variation in CD95 expression, cFLIP expression and CH11 sensitivity even within the group of normal LCLs. Comparisons made using a panel of isogenic LCLs may be more informative as the LCLs will differ only in their ATM gene.

A panel of 6 isogenic LCLs was made by transfecting wildtype and mutant ATM constructs into an A-T LCL which expressed no ATM protein (ATM mutations: c.796_797insGATT, c.2921+1G>A). The panel consisted of a 'wildtype' LCL (ATMwt) produced by transfection of a wildtype ATM construct, an 'ATM –ve' LCL (MEP4) transfected with a control construct (empty vector) and four ATM mutant LCLs transfected with ATM constructs containing different mutations which had been found in A-T patients; 8189 A>C (8189), 8293 G>A (8293), 8672 G>A (8672) and 9022 C>T (9022).

ATM expression and kinase activity in the transfected LCLs was validated by western blot (Fig 4:7:1A). The ATMwt LCL expressed active ATM protein which autophosphorylated on Ser1981 (trace) and phosphorylated the downstream substrates SMC1 on Ser966 and Nbs1 on Ser343 in response to DNA damage induced by ionising radiation. In contrast no ATM expression or activity was seen in the ATM-ve LCL MEP4. The ATM LCLs (8189, 8293, 8672 and 9022) expressed varying levels of mutant ATM protein but lacked any ATM kinase activity. ATM protein expression was particularly low in the 8672 LCL, this is characteristic of

the mutation. The apparent loss of ATM expression in 8189 in response to IR appears to be a loading effect.

The result of the cFLIP expression analysis was similar to that obtained using the panel of non-isogenic LCLs in that there was some individual variation between the LCLs in terms of expression of cFLIPL, cFLIPS and total cFLIP but no difference between the median expression levels of the ATMwt LCL which had ATM activity and the ATM mutant LCLs which had no ATM activity (Fig 4:7:1B). Restoring ATM activity to produce the ATMwt LCL did lead to an increase in the expression of cFLIP compared to the ATM-ve MEP4 LCL which expressed no ATM protein. However increased cFLIP expression compared to MEP4 was also seen in the 8189, 8293, 8672 and to a lesser extent 9022 LCLs all of which had no ATM activity. This suggests that the increase in cFLIP expression in ATMwt compared to MEP4 was not due to the restoration of ATM activity as the effect was also seen in the LCLs which expressed mutant ATM protein but did not have ATM activity. There was no real difference in caspase 8 expression or caspase 8:cFLIP ratio between the ATMwt and ATMmut LCLs (Fig 4.7.1C).

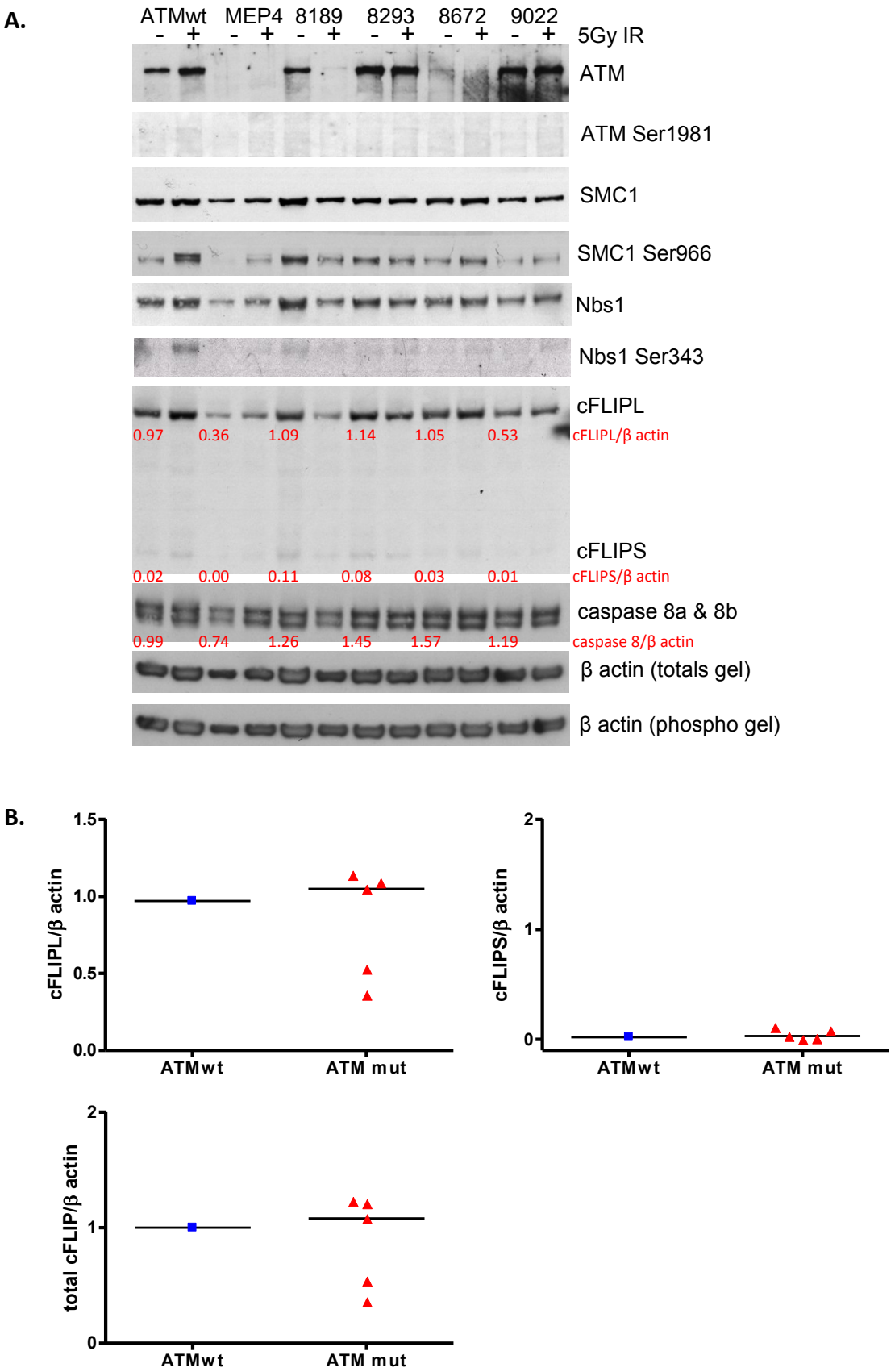
CD95 expression on the ATMmut LCLs was high (median MFI of 7.8) compared to the untransfected A-T LCLs used in previous panels (median MFI of 4 in Fig 4:4:1B). In contrast to previous findings there was no difference in CD95 expression between the ATMwt and ATMmut LCLs (Fig 4:7:1D). This confirms that ATM kinase activity *per se* does not increase constitutive CD95 expression on LCLs.

Transfection with the wildtype ATM construct greatly decreased the sensitivity of the LCL to CH11 treatment compared to MEP4 (Fig 4:7:1E). The ATMwt LCL showed a smaller decrease in viability in response to CH11 treatment than the ATMmut LCLs and a smaller increase in

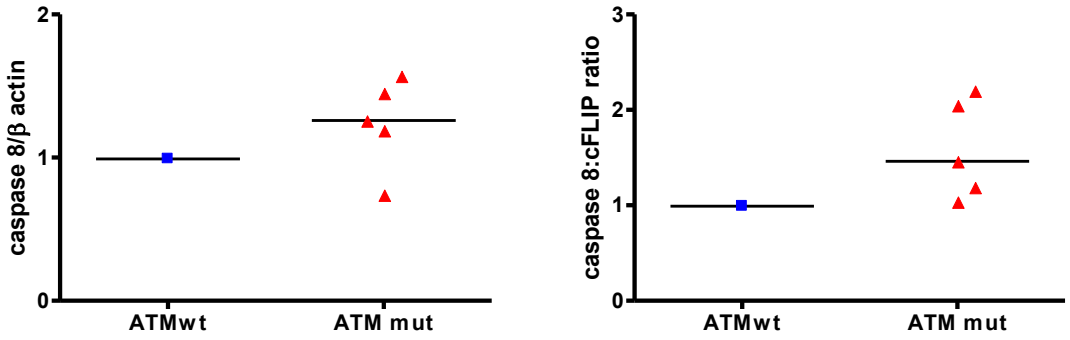
late apoptotic and dead cells in response to CH11 treatment than MEP4, 8189 and 8293 (Fig 4:7:1F). Interestingly, the median increase in cells in early apoptosis in response to CH11 was greater in the ATMwt LCL than the ATMmut LCLs. This could indicate a slower rate of apoptosis in the presence of active ATM protein kinase consistent with earlier findings.

These results support the previous findings in that there is no evidence of a direct role for ATM in regulating expression of either cFLIP or CD95 but there is an indication that the presence of functional ATM kinase may reduce the sensitivity to CH11-induced apoptosis of LCLs.

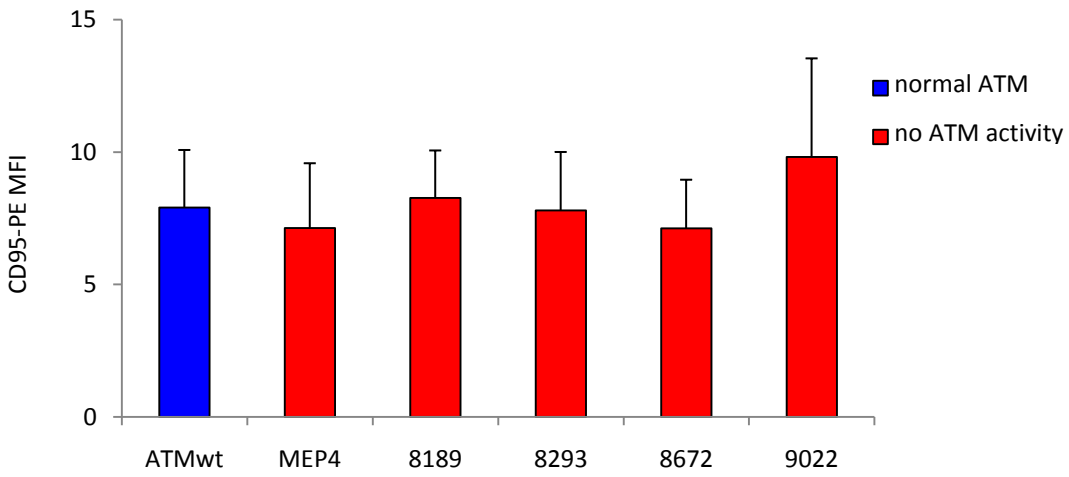
Fig 4:7:1: Transfection of an A-T LCL with an ATMwt construct reduces its sensitivity to CH11-induced apoptosis.



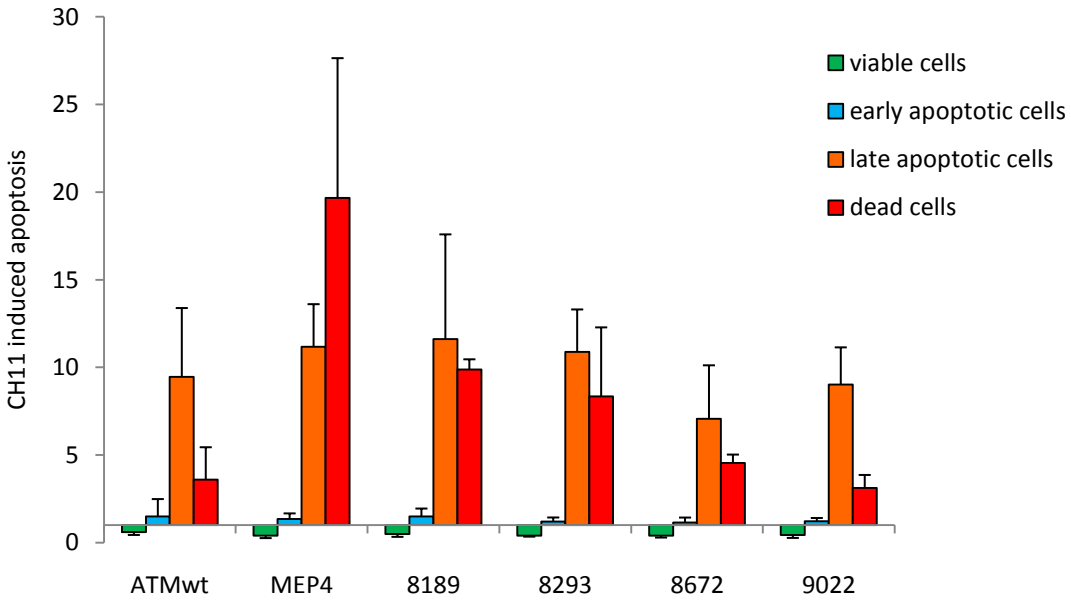
C.


caspase 8/β actin
2
1
0
ATMwt
ATM mut
caspase 8:cFLIP ratio
3
2
1
0
ATMwt
ATM mut

D.


CD95-PE MFI
15
10
5
0
ATMwt
MEP4
8189
8293
8672
9022
normal ATM
no ATM activity

E.


CH11 induced apoptosis
30
25
20
15
10
5
0
ATMwt
MEP4
8189
8293
8672
9022
viable cells
early apoptotic cells
late apoptotic cells
dead cells

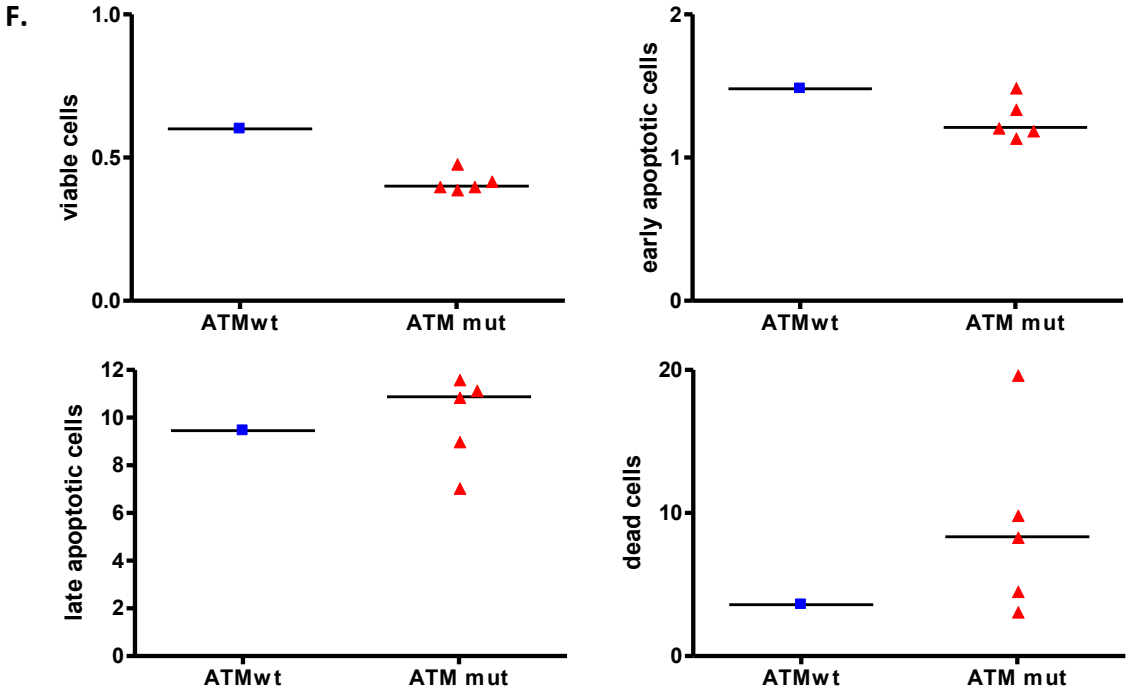


Fig 4:7:1: A panel of isogenic LCLs was produced by transfection of ATM constructs into an A-T LCL which expressed no ATM protein. The panel consisted of 6 LCLs; ATMwt (wildtype ATM), MEP4 (control construct – no ATM), 8189 (8189 A>C mutation), 8293 (8293 G>A mutation), 8672 (8672 G>A mutation) and 9022 (9022 C>T mutation).

A. Western blot showing ATM, cFLIP and caspase 8 expression and ATM kinase activity (phosphorylation of ATM at Ser1981 and downstream substrates SMC1 at Ser966 and Nbs1 at Ser343 in response to IR-induced DNA damage) of isogenic LCLs transfected with ATM constructs. cFLIP and caspase 8 expression was measured by densitometery (red text) using β actin as a loading control.

B. Comparison of cFLIPL, cFLIPS and total cFLIP levels in ATMwt and ATMmut (MEP4, 8189, 8293, 8672, 9022) LCLs.

C. Comparison of caspase 8 expression and caspase8:total cFLIP ratio ((caspase 8/β actin)/total cFLIP/β actin)) of ATMwt and ATMmut (MEP4, 8189, 8293, 8672, 9022) isogenic LCLs.

D. CD95 MFI of isogenic panel of LCLs. Cells were stained with CD95-PE and PI and gated as in Fig 2:2:1. (Mean of 3 experiments).

E. CH11-induced apoptosis of isogenic panel of LCLs. Cells were treated with 500ng/ml CH11 for 15h prior to staining with Annexin V-FITC and PI. CH11-induced apoptosis was quantified as the % of CH11-treated cells at each stage of apoptosis divided by the % of untreated cells at each stage of apoptosis (mean of 3 experiments).

F. Comparison of the sensitivity to CH11-induced apoptosis of ATMwt and ATMmut (MEP4, 8189, 8293, 8672, 9022) LCLs. CH11-induced apoptosis was quantified as the % of CH11-treated cells at each stage of apoptosis divided by the % of untreated cells at each stage of apoptosis (mean of 3 experiments).

4:8: ATM mutations and tumour resistance to CD95-mediated apoptosis.

4:8:1: Analysis of the sensitivity to CD95-mediated apoptosis of B-CLL and T-PLL tumours.

A-T patients have a predisposition to the development of lymphoid tumours (Tran et al., 2008) and, additionally, *ATM* mutations are frequently found in sporadic B-CLL tumours (Stankovic et al., 2002). As mutations in the CD95-mediated apoptotic pathway are also commonly found in lymphoid tumours, CD95 and cFLIP expression in T-PLL and B-CLL samples was analysed.

Expression of CD95 and cFLIP as well as sensitivity to CH11-induced apoptosis was analysed in B-CLL tumours with either mutant or wildtype *ATM,* and two T-PLL tumours with mutant *ATM* (Table 4:8:1). In contrast to LCLs, in which more than 90% of all viable cells (Annexin V-PI-) express CD95, fewer than 15% of viable B-CLL tumour cells expressed CD95 (Fig 4:8:1A). The mean fluorescence intensity (MFI) was similar to the median MFI of the panel of A-T LCLs (Fig 4:8:1B). Interestingly there was no significant difference in CD95 expression between B-CLL tumours with wildtype *ATM* and those with *ATM* mutations.

Both T-PLL tumours had *ATM* mutations, T-PLL2 was a tumour from an A-T patient and did not express any ATM and T-PLL1 was from a non-A-T individual but the tumour cells had acquired mutations inactivating ATM (Fig 4:8:1C). The frequency of viable T-PLL tumour cells expressing CD95 was much higher (60.64% and 38.7% of cells) than in B-CLL samples (Fig 4:8:1A) and the MFI was similar to that of the median of the panel of A-T LCLs, which have no ATM kinase activity (Fig 4:8:1B).

cFLIPL and cFLIPS levels were comparable between B-CLL and LCLs (Fig 4:8:1D). However, B-CLL cells also expressed several intermediate bands of cFLIP which may contribute to Fas resistance (Fig 4:8:1C). 43kDa and 41kDa intermediate bands of cleaved cFLIPL which bind stably to the DISC and inhibit apoptosis have previously been reported in B-CLL (MacFarlane et al., 2001). T-PLL cells did not express cFLIPS or intermediate bands of the protein and expression of cFLIPL was low compared to B-CLL and LCLs (Fig 4:8:1C & D).

As could be predicted from their low CD95 expression and the presence of cFLIP intermediates, B-CLL tumours were highly resistant to CH11-induced apoptosis (Fig 4:8:1E). T-PLL tumours were sensitive to CH11 although they were more resistant to treatment than both the normal and A-T LCLs (Fig 4:8:1E) perhaps due to the lower percentage of cells expressing CD95. CH11-induced apoptosis appeared to occur more slowly in the T-PLL tumours than in the LCLs as after 15h of CH11 treatment the majority of T-PLL cells were in the early stages of apoptosis (Annexin V+PI-) whereas the majority of LCL cells were in late apoptosis (Annexin V+PI+) or already dead (Annexin V-PI+). Interestingly the tumour from the A-T patient (T-PLL2) which expressed no ATM protein was more sensitive to CH11-induced apoptosis than the non-A-T patient tumour (T-PLL1) which did express some ATM but lacked ATM kinase activity.

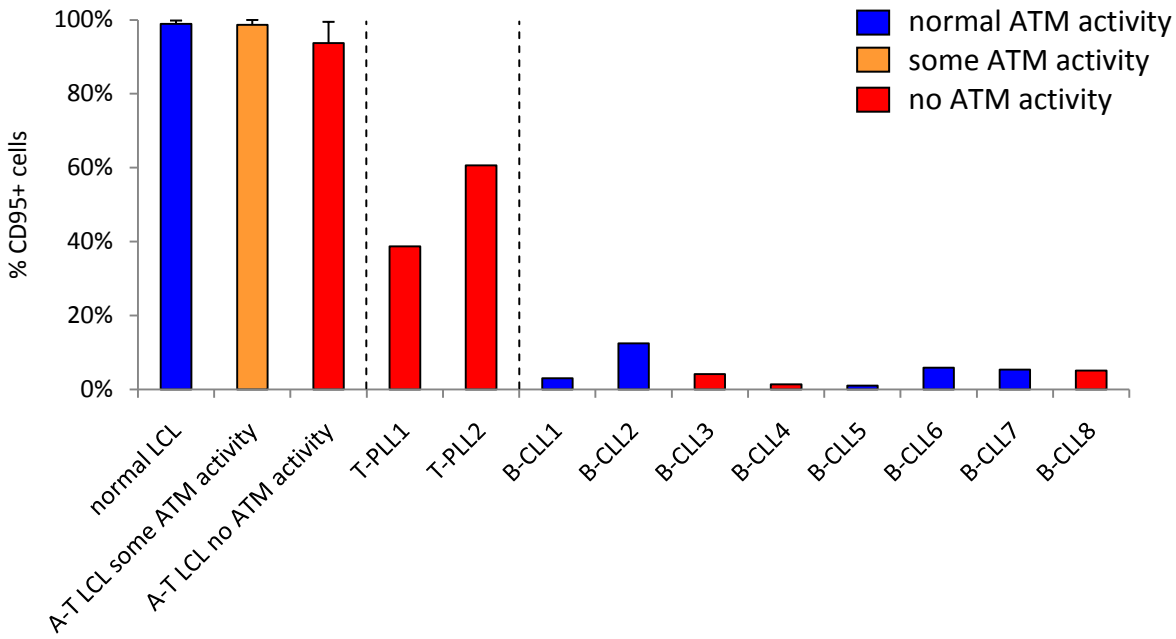
Table 4:8:1: ATM mutations in T-PLL and B-CLL tumours.

	ATM protein	ATM activity	mutation 1	mutation 2
T-PLL1	100%	none	c.5204_5205insA; pThr1735fs	not identified
T-PLL2	trace	none	c.2T>C; p.(Met1Thr)	c.6405_6406insTT; p.(Arg2136X)
B-CLL1	100%	normal		
B-CLL2	100%	normal		
B-CLL3	0%	none	1058delGT	5464G>A 1822E>Q
B-CLL4	0%	none	11q deletion	2308G>T (stop codon)
B-CLL5	100%	normal		
B-CLL6	100%	normal		
B-CLL7	100%	normal		
B-CLL8	0%	none	not identified	not identified

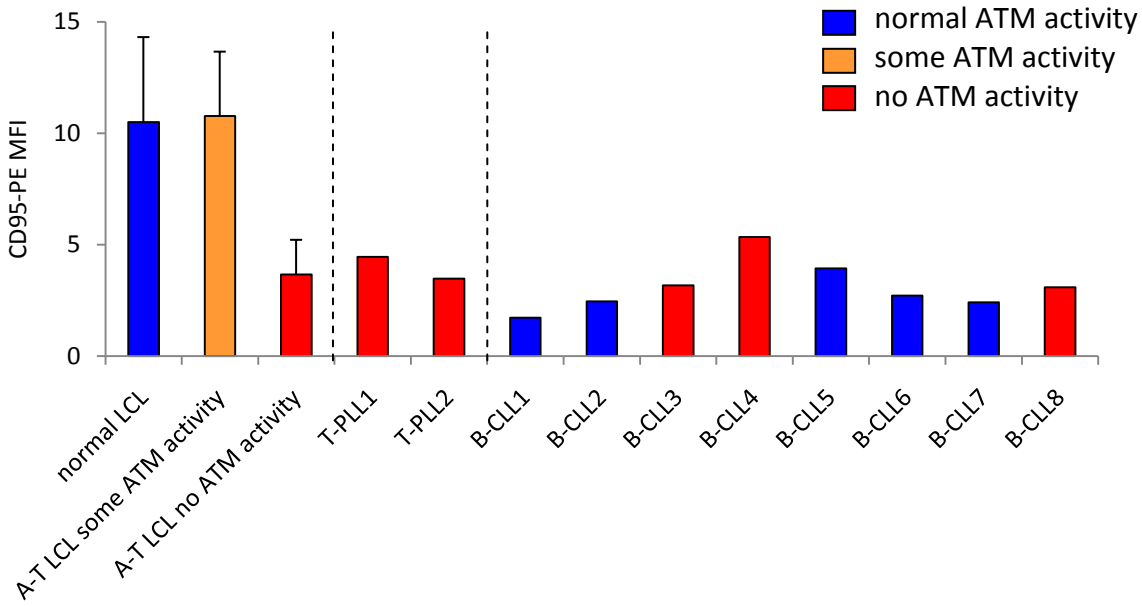
Table 4:8:1: ATM protein (as percentage of normal levels), kinase activity and *ATM* mutations in T-PLL and B-CLL tumour samples. T-PLL2 was from an A-T patient. Samples with normal ATM activity are highlighted in blue, those with no ATM activity are highlighted in red.

Fig 4:8:1: ATM mutations are not required for tumour resistance to CD95-mediated apoptosis.

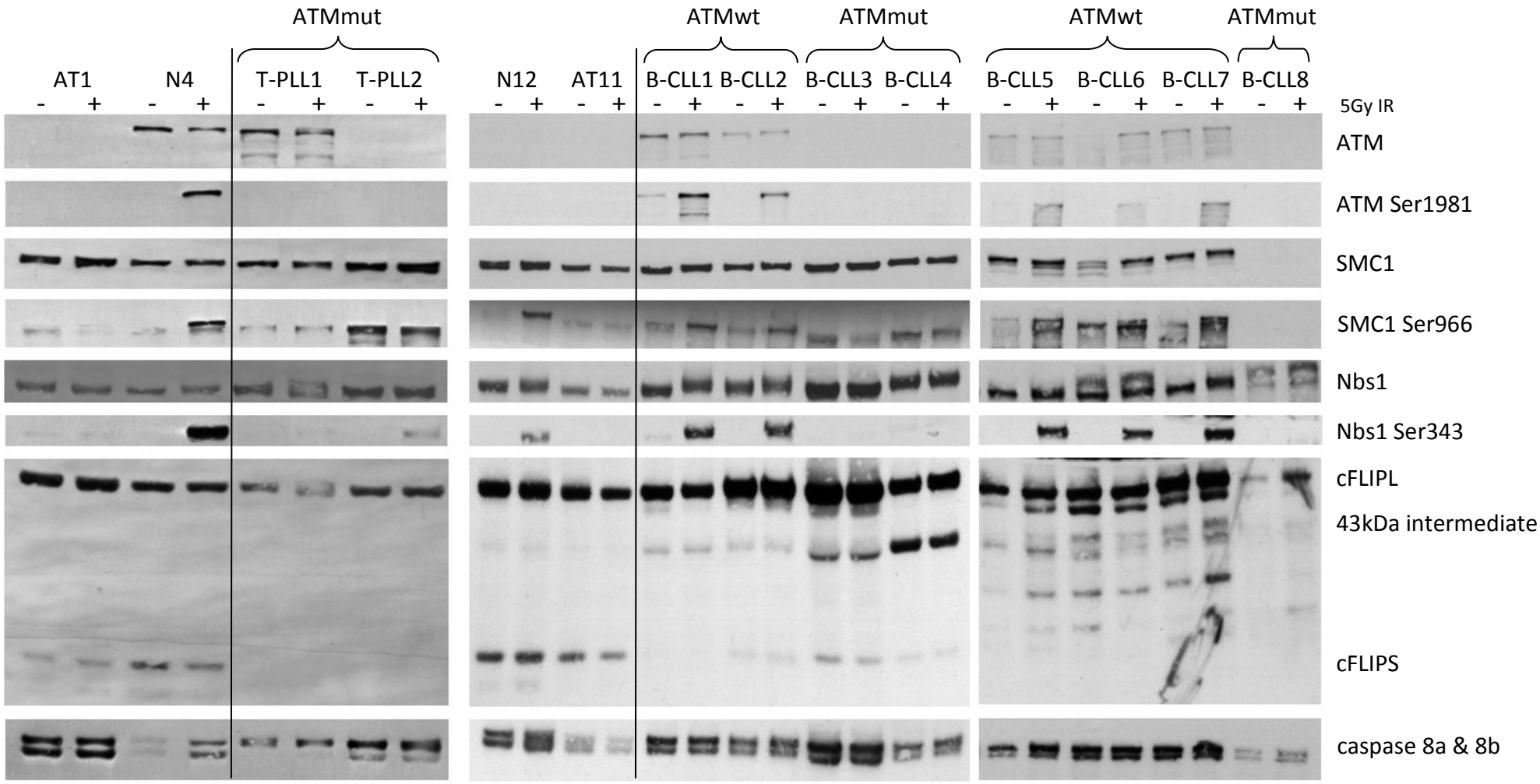
A.



B.



C.


ATMmut
ATMwt
ATMmut
ATMwt
ATMmut
AT1
N4
T-PLL1
T-PLL2
N12
AT11
B-CLL1
B-CLL2
B-CLL3
B-CLL4
B-CLL5
B-CLL6
B-CLL7
B-CLL8
- + - + - + - + - + - + - + - + - + - + - + - + - +
5Gy IR
ATM
ATM Ser1981
SMC1
SMC1 Ser966
Nbs1
Nbs1 Ser343
cFLIPL
43kDa intermediate
cFLIPS
caspase 8a & 8b

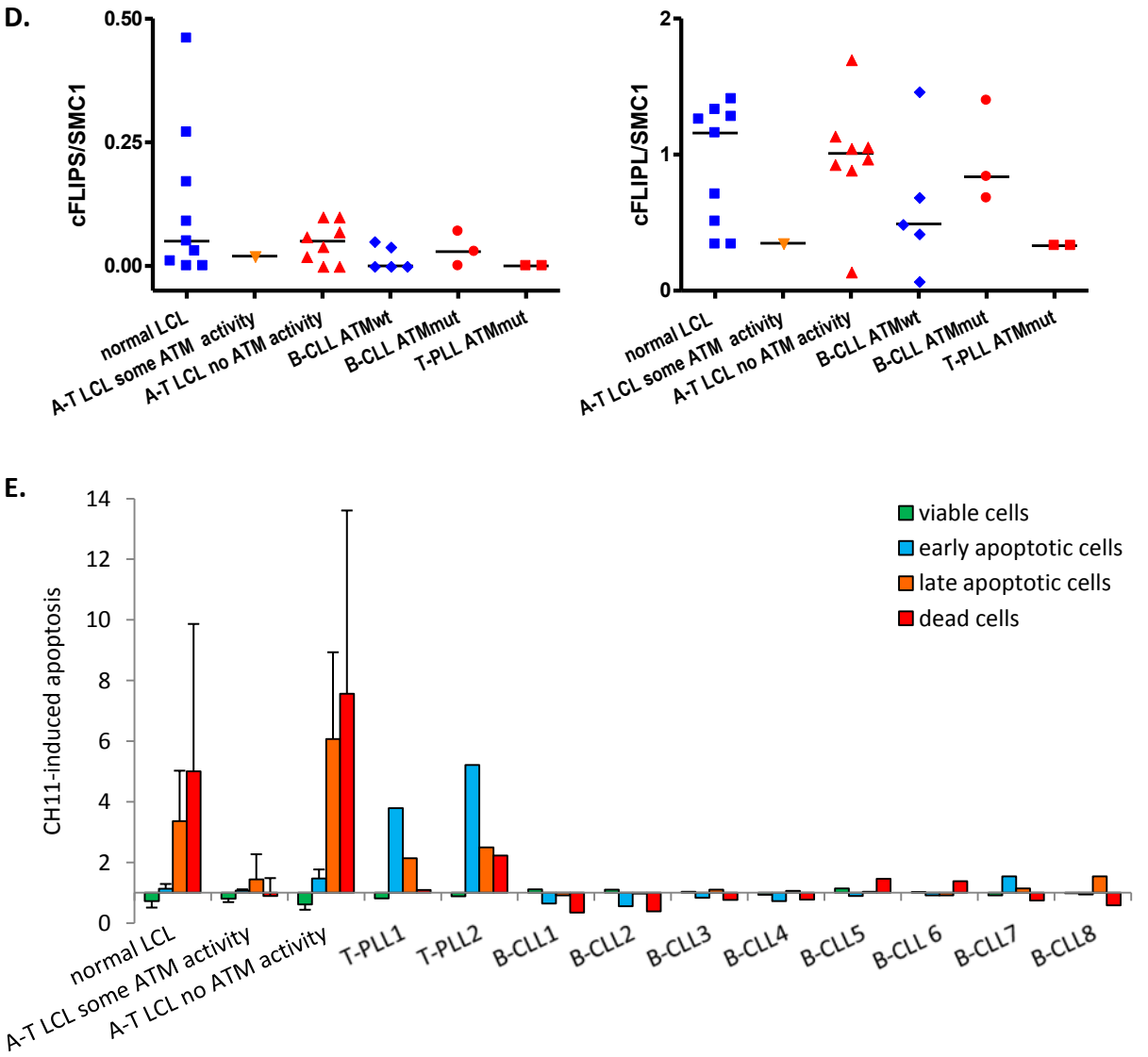


Fig 4:8:1: ATM mutations are not required for tumour resistance to CH11-induced apoptosis.

A. Frequency of CD95 expression on viable cells of normal and A-T LCLs (mean values from panel used in Fig 4:4:1), T-PLL and B-CLL tumour samples.

B. CD95 MFI of viable cells of normal and A-T LCLs (mean values from panel used in Fig 4:4:1), T-PLL and B-CLL tumour samples.

C. Western blot showing ATM activity (phosphorylation of ATM at Ser1981 and downstream substrates SMC1 at Ser966 and Nbs1 at Ser343 in response to IR-induced DNA damage) and cFLIP expression of LCLs with normal (N4) or no ATM activity (AT1), and T-PLL and B-CLL tumour samples.

D. cFLIPL and cFLIPS expression (analysed by densitometry, using SMC1 as a loading control) in B-CLL with wildtype and mutant ATM and in T-PLL. cFLIP levels of normal and A-T LCLs reproduced from Fig 4:5:2 for comparison.

E. CH11 (500ng/mL) induced apoptosis and viability of individual T-PLL and B-CLL tumour samples (analysis of cells at each individual stage of apoptosis) and mean values for the panel of normal and A-T LCLs used in Fig 4:4:1.

4:9: Discussion.

4:9:1: A-T LCLs showed increased sensitivity to CD95-mediated apoptosis.

An increased level of CD95-mediated apoptosis in A-T lymphocytes *in vivo* is important as it may contribute to the immune deficiency of A-T patients. Different studies have shown either increased (Ivanov et al., 2009) or decreased (Stagni et al., 2008) sensitivity to death receptor-induced apoptosis as a result of ATM inhibition in cultured cells. However, a major immunological feature of A-T patients is lymphopenia and increased sensitivity to CD95-mediated apoptosis, rather than resistance to CD95-mediated apoptosis, could contribute to this lymphopenia. I showed that EBV-immortalised B cell lymphoblastoid cell lines (LCLs) derived from A-T patients with no ATM kinase activity were more sensitive to CD95-mediated apoptosis activated by CH11 treatment than LCLs derived from normal individuals. This was despite a significantly reduced cell surface expression of CD95 on A-T compared with normal LCLs.

Several reports have suggested that high cell surface expression of CD95 results in a level of spontaneous apoptosis of cells (Le Clorennec et al., 2008;Roger et al., 1999). Supporting this hypothesis I found a significant negative correlation between CD95 expression and viability of LCLs in culture. My results showed that A-T LCLs with no ATM activity were significantly more viable in culture than normal LCLs. In addition specific inhibition of ATM protein kinase did not reduce CD95 expression on the cell surface of normal LCLs and conversely, expression of functional ATM protein kinase in an A-T LCL did not increase expression. This suggested that lack of ATM activity may not be directly responsible for the downregulation of CD95 on A-T compared to normal LCLs. As LCLs are infected with EBV which is able to regulate the expression of CD95 (Le Clorennec et al., 2006), the reduced expression on A-T

LCLs with no ATM activity may be virally induced in order to reduce spontaneous apoptosis of the apoptotic sensitive cells. If this is the case it could explain my finding that transfection of an ATMwt construct into an A-T LCL with no ATM activity had no effect on its CD95 expression, because the EBV infection in the LCL was already well established.

My results showed no significant difference in expression of cFLIP between normal and A-T LCLs. In addition, specific inhibition of ATM protein kinase activity did not have a differential effect on the cFLIP levels of normal and A-T LCLs. However, normal LCLs, which express much higher levels of CD95 than A-T LCLs, showed a strong negative correlation between increasing cFLIPL expression and decreasing apoptosis. This suggested that above a certain threshold concentration of CD95 receptor expression, cFLIP protein mediated inhibition of apoptosis is a more important determinant of apoptotic sensitivity than surface CD95 expression. Therefore, since normal LCLs express high levels of CD95 and their apoptotic sensitivity does not increase with increasing CD95 expression, cFLIP may have a greater inhibitory effect on their apoptosis than it does in A-T LCLs.

There was evidence of a faster rate of CH11-induced CD95-mediated apoptosis in A-T LCLs with no ATM activity than in normal LCLs as they showed faster progression through to the late stage of apoptosis. The slower rate of apoptosis in normal LCLs may be related to a requirement for inactivation of ATM by caspase-dependent cleavage in order to facilitate the progression of apoptosis (Smith et al., 1999;Wang et al., 2006).

My results suggest that *ATM* mutations lead to increased sensitivity to CD95-mediated apoptosis. A recent study (Ivanov et al., 2009) reported that inhibition of ATM activity in melanoma cells prior to irradiation enhanced TRAIL-mediated apoptosis post-irradiation by upregulation of the TRAIL receptor DR5 and downregulation of the anti-apoptotic protein

cFLIP, an important inhibitor of both TRAIL and CD95-mediated apoptosis. The findings of Ivanov et al. support the hypothesis that loss of functional ATM kinase increases sensitivity to death receptor-induced apoptosis. They differ from another report by Stagni et al. that found that inhibition of ATM activity upregulated cFLIP expression in LCLs leading to resistance to CD95-mediated apoptosis (Stagni et al., 2008).

Although both were carried out using LCLs, the findings of my study and the Stagni study differ significantly. Whereas Stagni et al. (2008) used isogenic transfected LCLs, my study mainly relied on a larger panel of non-isogenic LCLs. However, when I analysed isogenic LCLs transfected with different ATM constructs the ATM-ve and ATMmut LCLs showed increased sensitivity to CD95-mediated apoptosis compared to the ATMwt LCL. In contrast to Stagni et al. (2008) I found no difference in cFLIP expression between ATMwt and ATM-ve or ATMmut isogenic LCLs. These findings were in agreement with the results of my analysis of the non-isogenic LCL panel which also showed increased sensitivity of A-T LCLs to CD95-mediated apoptosis and no significant difference in cFLIP expression between normal and A-T LCLs.

4:9:2: The immune system phenotype of A-T is similar to that of other lymphopenic conditions associated with increased sensitivity to CD95-mediated apoptosis.

A comparison of the immune system phenotype of A-T patients with that of other conditions associated with resistance or sensitivity to CD95-mediated apoptosis supports my finding of increased sensitivity of A-T patients' cells. However, there are some similarities between the immune system abnormalities and predisposition to cancer of A-T patients and patients with autoimmune lymphoproliferative syndrome (ALPS), a disorder most commonly caused by mutations in the *Fas* gene which encodes CD95 (Straus et al., 2001). *Fas* mutations result in

resistance to CD95-mediated apoptosis, therefore ALPS predisposes to autoimmune disorders and lymphoma development (Straus et al., 2001). Similarly mice lacking functional CD95 expression also suffer from autoimmune conditions (Adachi et al., 1995) and increased incidence of B cell lymphoma (Davidson et al., 1998). Like ALPS patients and mice with *Fas* mutations, A-T patients show an increased incidence of lymphoid tumours, autoimmune disorders and possible defects in immunoregulatory processes.

However, there is a major difference between the immune systems of patients with A-T and those with ALPS: A-T patients are lymphopenic whereas *Fas* mutations resulting in resistance to CD95-mediated apoptosis cause an accumulation of lymphocytes in both humans and mice leading to enlargement of liver, spleen and lymph nodes (Bleesing et al., 2001;Lim et al., 1998;Poppema et al., 2004;Takahashi et al., 1994;Watanabe-Fukunaga et al., 1992). As shown in Chapter 3, A-T patients have deficiencies in naive B and T cells and increased proportions of memory T cells and NK cells. Absolute numbers of T cells and B cells are also reduced (Nowak-Wegrzyn et al., 2004).

In contrast to the lymphopenia of A-T patients, the resistance to CD95-mediated apoptosis seen in ALPS patients causes an accumulation of both T and B cells, especially CD3+CD4+CD8- T cells thought to derive from previously activated cells that have failed to die by activation-induced cell death (AICD) after restimulation through their TCR (Sneller et al., 1997). The proportion of naive T cells is also increased in ALPS (Lim et al., 1998), whereas the opposite is true of A-T (Reichenbach et al., 2002). ALPS patients have increased numbers of both total B cells and CD5+ B cells (Bleesing et al., 2001;Lim et al., 1998;Poppema et al., 2004) and CD95-resistant mice develop progressive lymphadenopathy and accumulate several-fold increases in conventional CD4+ and CD8+ T lymphocytes and B cells (Takahashi et al., 1994;Watanabe-Fukunaga et al., 1992).

In terms of lymphocyte number the immune system phenotype of A-T patients is similar to that of lymphopenic patients such as HIV patients and stem cell transplant recipients. Peripheral T cell depletion associated with increased levels of CD95-mediated apoptosis has been reported in both these groups of patients (Brugnoni et al., 1999;Rethi et al., 2008) and increased sensitivity to CD95-mediated apoptosis has also been described in several cases of idiopathic CD4+ T cell lymphocytopenia (Laurence et al., 1996;Roger et al., 1999). In terms of lymphocyte subsets A-T patients are similar to the elderly who have a naive cell deficiency which may also be partly due to increased sensitivity to CD95-mediated apoptosis (Gupta & Gollapudi, 2008).

The major differences between the immune system phenotype of A-T patients and patients with ALPS, together with the similarities to lymphopenic conditions with increased CD95-mediated apoptosis and the findings of Ivanov et al. (2009), support the hypothesis of increased sensitivity of A-T lymphocytes to CD95-mediated apoptosis compared to cells with normal ATM.

4:9:3: T-PLL tumours were sensitive and B-CLL tumours resistant to CD95-mediated apoptosis.

As A-T predisposes to B and T cell lymphomas my study was concluded by investigating tumour sensitivity to CD95-mediated apoptosis. *ATM* mutations are found in approximately 40% of sporadic B-CLL (Stankovic et al., 1999) and the majority of T-PLL tumours (Matutes et al., 1991;Nowak et al., 2009;Yamaguchi et al., 2003;Yuille et al., 1998). However, whilst T-PLL is relatively frequent in A-T patients, perhaps because loss of *ATM* function is important in initiation of the tumour (Stankovic et al., 2002;Stoppa-Lyonnet et al., 1998), the incidence of B-CLL is low, possibly because *ATM* mutation is important for progression but not

initiation of B-CLL tumours. Neither tumour type is common in the *Fas* mutation associated disorder ALPS.

I analysed the sensitivity to CH11-induced CD95-mediated apoptosis of B-CLL and T-PLL tumours with *ATM* mutations. It was significant that B-CLL was highly resistant to CH11-induced apoptosis regardless of *ATM* status. In B-CLL the most important factor in resistance to CH11-induced apoptosis appeared to be the complete loss of CD95 expression on the majority of the cells. I found no significant difference in CD95 expression, cFLIP expression or apoptotic resistance of ATMwt and ATMmut B-CLL tumour cells. This suggests that *ATM* mutations are not necessary for resistance to CD95-mediated apoptosis. My results showed upregulation of intermediate isoforms of cFLIP in B-CLL which may be important for apoptotic resistance, but there was no significant upregulation of expression of cFLIPL or cFLIPS compared to LCLs. In contrast, T-PLL tumours with *ATM* mutations did not express cFLIPS or intermediates and were more like A-T LCLs in showing similar CD95 expression and sensitivity to CH11-induced apoptosis.

4:9:4: Loss of ATM function increases sensitivity to CD95-mediated apoptosis.

I have shown that increased sensitivity to CH11-induced CD95-mediated apoptosis *in vitro*, seen both in lymphoid cells derived from A-T patients and probably in sporadic T-PLL, is associated with total loss of ATM function in each case. However, I have found no evidence for a direct role of ATM in regulation of CD95 or cFLIP expression. It is possible that ATM may mediate sensitivity to CD95-mediated apoptosis through regulation of other pro- or anti-apoptotic proteins such as the pro-apoptotic adaptor FADD (Fas-associated via death domain) which binds to the death domain of CD95 and recruits pro-caspase 8 to the DISC complex (reviewed in (Krammer, 2000)). Interestingly increased FADD expression in T cells

in the elderly has been suggested as a cause of the increased sensitivity of aged lymphocytes to CD95-mediated apoptosis (Gupta et al., 2004;Gupta & Gollapudi, 2008). As I believe that A-T patients have a congenitally aged immune system (Chapter 3) it would be interesting to investigate if a similar upregulation of FADD in A-T lymphocytes may contribute to their sensitivity to CD95-mediated apoptosis. The X-linked inhibitor of apoptosis (XIAP) which binds to and inhibits activation of caspases 9, 3 and 7 (Deveraux et al., 1997) would also be an interesting candidate for further investigation as it is reportedly upregulated in response to DNA damage-induced ATM activation (Bruno et al., 2008). Downregulation of XIAP in A-T lymphocytes could potentially explain their increased sensitivity to CD95-mediated apoptosis.

The increased sensitivity to CD95-mediated apoptosis of A-T patients' cells could contribute to lymphopenia, the principal feature of their immunodeficiency. In contrast, while T-PLL tumour cells retain the same characteristics *in vitro*, presumably because of early loss of ATM function in this clone, any killing of the tumour cells, due to increased sensitivity to CD95-mediated apoptosis, is outweighed by far by the considerable growth advantage of the tumour cells.

Chapter 5: Investigating the role of ATM in DNA damage-induced upregulation of NKG2D Ligands.

5:1: Introduction

Patients with Ataxia-telangiectasia have a predisposition to cancer. The cancer immunosurveillance hypothesis (Burnet, 1967) suggests that in healthy individuals cells with the potential to develop into malignant tumours are recognised and destroyed by the immune system. It is possible that a failure of this process of immunosurveillance in A-T patients could contribute to the high incidence of cancer in these patients.

Cancer cells acquire mutations due to damaged DNA. In healthy individuals ATM activation in response to DNA damage initiates DNA repair or apoptosis of the damaged cell. This prevents damaged DNA from persisting in the body and potentially leading to cancer. As A-T patients' cells have reduced or absent ATM protein kinase activity they are unable to respond effectively to DNA damage and so have an increased risk of cancer.

As well as initiation of DNA repair and apoptosis, ATM activation may also have a role in signalling the presence of potentially dangerous cells containing damaged DNA to the immune system through inducing upregulation of natural killer group 2, member D (NKG2D) ligands (Gasser et al., 2005). These ligands are frequently expressed on primary tumour cells, tumour cell lines and on cells infected with some viruses including HCMV, EBV and influenza A (reviewed in (Eagle and Trowsdale, 2007)). They are also expressed on activated T cells, mature dendritic cells, B cells, granulocytes and monocytes (Cerboni et al., 2007).

Two different types of NKG2D ligands have been discovered in humans, the MHC class 1 chain-related molecules (MICA and MICB) and members of the UL-16 binding protein family (ULBP). These ligands are all recognised by cytotoxic cells expressing the NKG2D receptor (NK cells, NKT cells, γδ T cells and some αβ T cells). Binding of the receptor to the ligand leads to activation of the perforin/granzyme mechanism of apoptosis and death of the

damaged cell. In this way ATM-dependent upregulation of NKG2D ligands in response to DNA damage may have an important role in cancer immunosurveillance, by providing a means by which potentially dangerous cells are recognised and destroyed by the immune system. Many tumours shed soluble NKG2D ligands from their surface as a mechanism of immune evasion. The presence of soluble NKG2D ligands downregulates the NKG2D receptor on the surface of cytotoxic cells and therefore allows the tumour cells to evade NK cell killing (Groh et al., 2002).

ATM-dependent NKG2D ligand upregulation may also have a role in regulation of T cell responses. Cerboni et al (2007) showed phosporylation of ATM on Serine 1981 and upregulation of MICA in response to T cell activation (Cerboni et al., 2007). Upregulation of the ligand could be prevented using inhibitors of ATM activity. As NKG2D ligand upregulation increases the sensitivity of activated T cells to NK cell mediated killing it could have a role in limiting T cell responses.

The effect of irradiation-induced DNA damage on NKG2D ligand surface expression on normal and A-T LCLs, normal B cells and normal fibroblasts was investigated using flow cytometry. However there was no evidence of NKG2D ligand upregulation in response to DNA damage and no difference in NKG2D ligand expression between normal and A-T LCLs.

5:2: NKG2D ligand expression on LCLs.

5:2:1: Detection of NKG2D ligand expression by flow cytometry.

Surface expression of NKG2D ligands is required for NKG2D receptor binding and initiation of apoptosis. Therefore surface antibody staining and flow cytometry was chosen as the method of NKG2D ligand detection. The antibodies chosen were the same as those used by Gasser et al (2005). As the ligands are frequently expressed on primary tumour cells and tumour cell lines the sarcoma cell line LB23 SARC was used as a positive control for NKG2D ligand expression. The flow cytometry method showed that this cell line expressed MICA, MICB, ULBP-1 and ULBP-3. ULBP-2 was not expressed (Fig 5:2:1).

Fig 5:2:1: NKG2D ligands can be detected by flow cytometry.

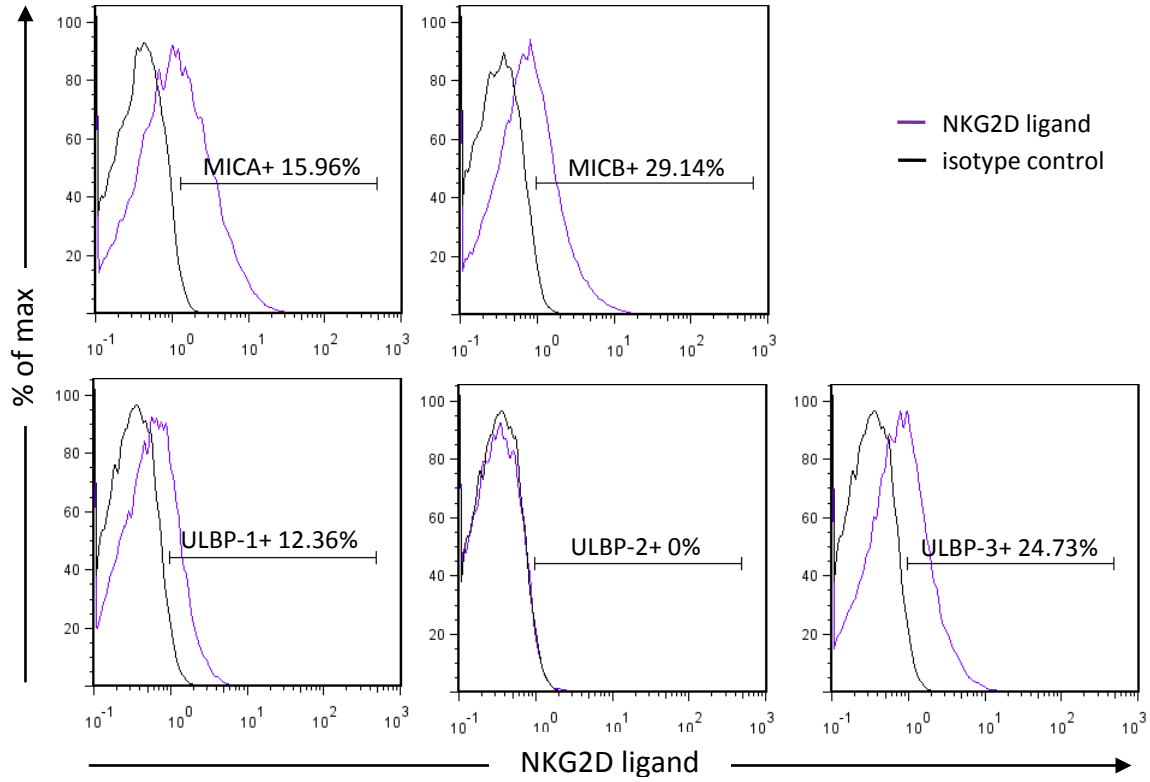


Fig 5:2:1: NKG2D ligand expression on LB23 SARC.

The NKG2D ligands MICA, MICB, ULBP-1 and ULBP-3 were expressed on LB23 SARC and could be detected by flow cytometry. ULBP-2 was not expressed. Cells were stained with single NKG2D or isotype antibodies, PI was used for dead cell exclusion. $0.5x10^6$ cells were stained per test. Histograms show NKG2D ligand staining overlaid on the appropriate isotype control (MICA-PE - IgG2b-PE, MICB unconjugated – IgG2b uncongugated, ULBP-1,2&3-PE – IgG2a-PE). The percentage of positive cells was calculated as the percentage of NKG2D ligand positive cells minus the percentage of background staining in the isotype control.

5:2:2: NKG2D ligand expression on LCLs.

Next the antibodies were tested on a normal LCL (Fig 5:2:2A). This cell line expressed all five of the NKG2D ligands, the most highly expressed being MICB (17%) followed by ULBP-3 (12.83%), ULBP-1 (8.07%), ULBP-2 (2.98%) and the lowest level of expression was of MICA (1.31%).

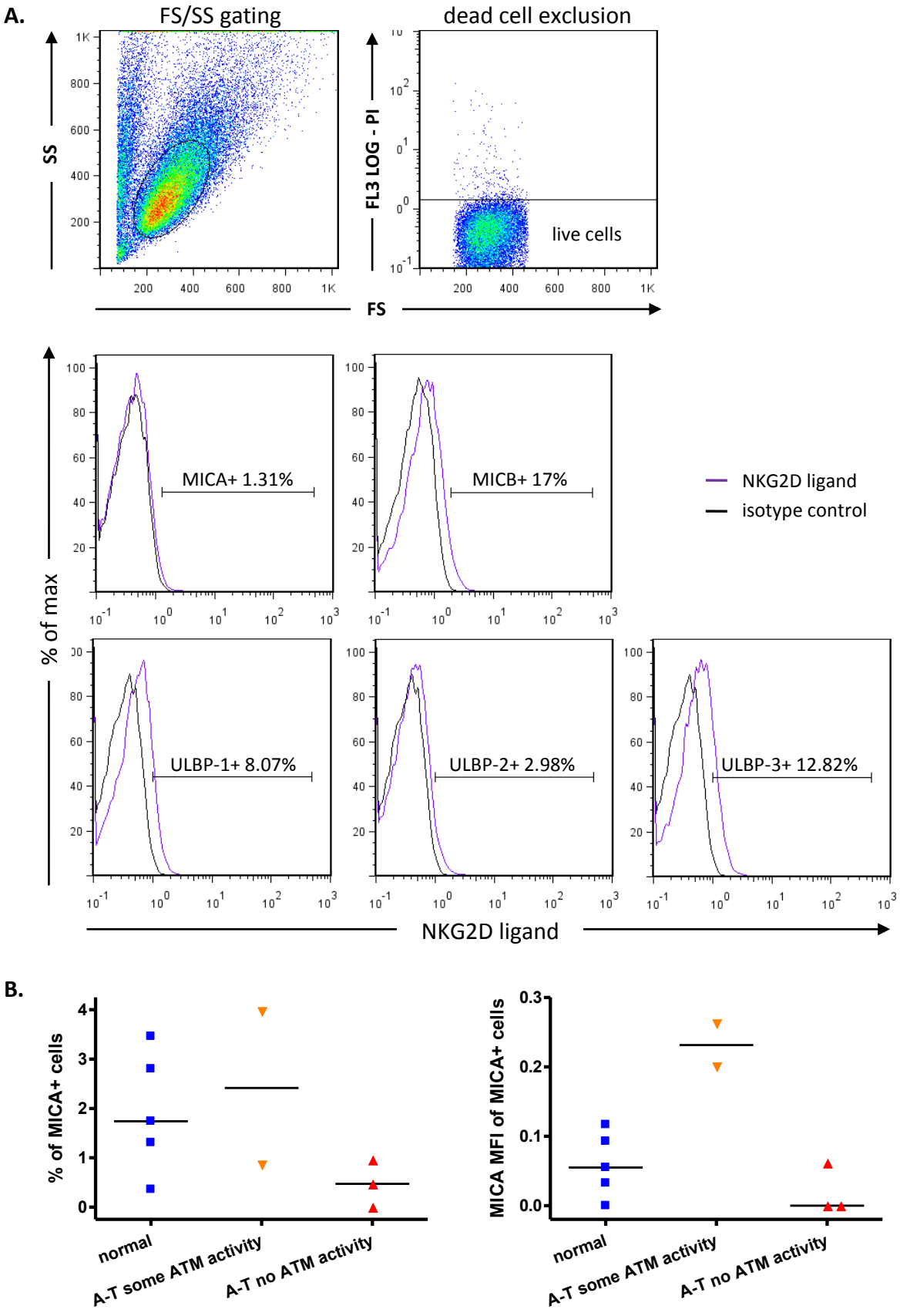
As ATM may be involved in the upregulation of NKG2D ligands I compared the basal expression levels of NKG2D ligands on five normal and five A-T LCLs, three with no ATM activity and two with some residual ATM activity (Table 5:2:2). Although there was quite a lot of variation in NKG2D ligand expression between the different LCLs there was no significant difference in either the MFI or percentage of positive cells expressing MICA (Fig 5:2:2B), MICB (Fig 5:2:2C), ULBP-1 (Fig 5:2:2D), ULBP-2 (Fig 5:2:2E) or ULBP-3 (Fig 5:2:2F).

Table 5:2:2: Normal and A-T LCLs.

	ATM protein		*ATM* mutations	
	amount expressed	**activity**	**mutation 1**	**mutation 2**
N1	100%	normal		
N2	100%	normal		
N3	100%	normal		
N5	100%	normal		
N6	100%	normal		
AT1	0%	none	c.7638_7646del9; p.(Arg2547_Ser2549del)	c.6997_6998insA p.(Thr2333X)
AT6	5% (R)	none	c.8520G>C; p (Leu2840Phe)	homozygous
AT7	20% (R)	yes	not identified	not identified
AT9	trace (R)	none	c.2T>C; p.(Met1Thr)	c.9139 C>T; p.(Arg3047X)
AT10	5% (R)	yes	c. 5623C>T; p.(1875Arg>X)	not identified

Table 5:2:2: Normal and A-T LCLs. The relative amount of ATM protein expressed (compared to normal levels, (R) = residual protein), ATM protein kinase activity and *ATM* mutations present in normal and A-T LCLs. ATM protein expression levels and kinase activity were measured by western blot and mutations detected by sequencing of the ATM gene. Normal LCLs are highlighted in blue, A-T LCLs with no ATM activity in red and A-T LCLs with some ATM activity in orange.

Fig 5:2:2: There was no significant difference in NKG2D ligand expression on A-T and normal LCLs.



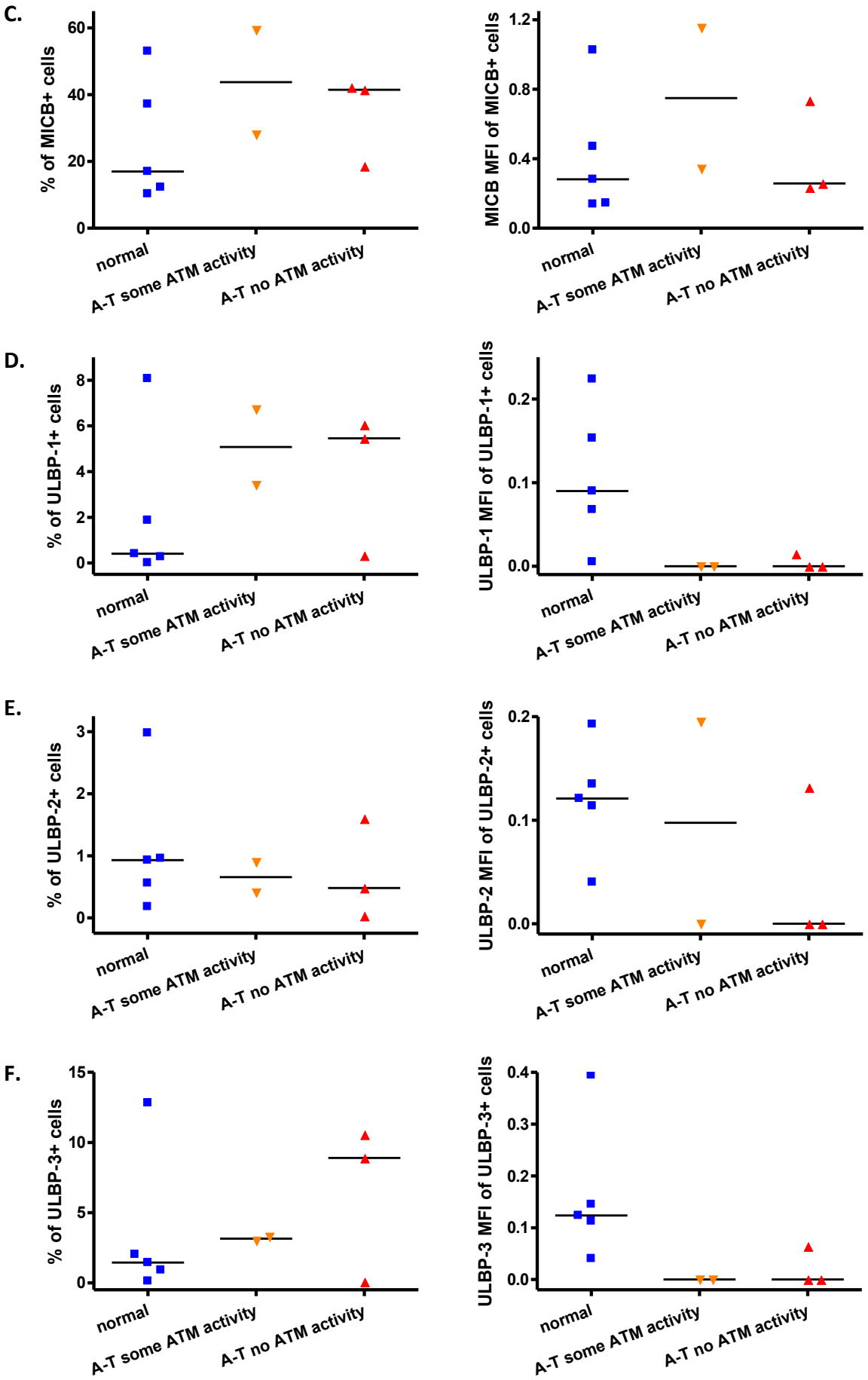

C.
% of MICB+ cells
60
40
20
0
normal
A-T some ATM activity
A-T no ATM activity
MICB MFI of MICB+ cells
1.2
0.8
0.4
0.0
normal
A-T some ATM activity
A-T no ATM activity
D.
% of ULBP-1+ cells
8
6
4
2
0
normal
A-T some ATM activity
A-T no ATM activity
ULBP-1 MFI of ULBP-1+ cells
0.2
0.1
0.0
normal
A-T some ATM activity
A-T no ATM activity
E.
% of ULBP-2+ cells
3
2
1
0
normal
A-T some ATM activity
A-T no ATM activity
ULBP-2 MFI of ULBP-2+ cells
0.2
0.1
0.0
normal
A-T some ATM activity
A-T no ATM activity
F.
% of ULBP-3+ cells
15
10
5
0
normal
A-T some ATM activity
A-T no ATM activity
ULBP-3 MFI of ULBP-3+ cells
0.4
0.3
0.2
0.1
0.0
normal
A-T some ATM activity
A-T no ATM activity

Fig 5:2:2: There was no significant difference in NKG2D ligand expression on normal and A-T LCLs. Five normal (N1, N2, N3, N5, N6) and five A-T LCLs, three of which had no ATM activity (AT1, AT6, AT9) and two with some ATM activity (AT7, AT10) were resuspended in complete RPMI and seeded in tissue culture plates at a concentration of $0.5x10^6$cells/ml. Following overnight culture cells were harvested and split into facs tubes for staining with single NKG2D ligand or isotype antibodies. The dead cell exclusion die PI was added to tubes prior to analysis on the flow cytometer.

A. Gating strategy and example NKG2D ligand staining on a normal LCL (N2). FS/SS gating was used to select the viable cell population followed by dead cell exclusion using a plot of FS against PI. The percentage and MFI of NKG2D ligand positive cells could then be calculated by subtracting any background in the appropriate isotype control from the test sample. Example histograms of MICA, MICB, ULBP-1, ULBP-2 and ULBP-3 ligand staining overlaid with relevant isotype controls are shown.

B. There was no significant difference in the percentage or MICA MFI of MICA+ cells in normal and A-T LCLs.

C. There was no significant difference in the percentage or MICB MFI of MICB+ cells in normal and A-T LCLs.

D. There was no significant difference in the percentage or ULBP-1 MFI of ULBP-1+ cells in normal and A-T LCLs.

E. There was no significant difference in the percentage or ULBP-2 MFI of ULBP-2+ cells in normal and A-T LCLs.

F. There was no significant difference in the percentage or ULBP-3 MFI of ULBP-3+ cells in normal and A-T LCLs.

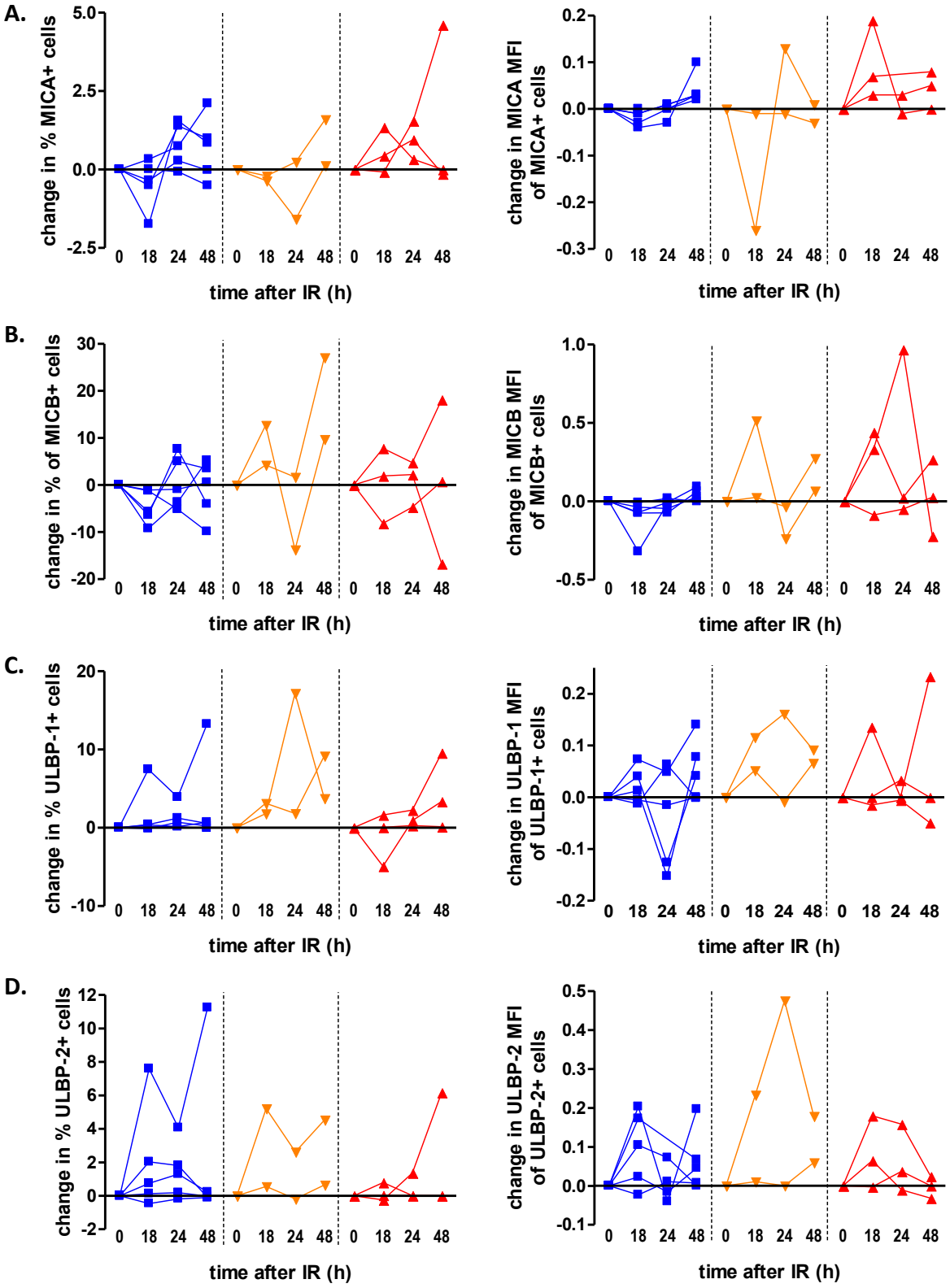
5:2:3: The effect of irradiation-induced DNA damage on NKG2D ligand expression on LCLs.

As NKG2D ligands are expressed on LCLs I tested the hypothesis that ATM is required for upregulation of NKG2D ligands on cells in response to DNA damage by irradiating normal and A-T LCLs and staining with NKG2D ligand antibodies at different timepoints up to 48 hours following irradiation. The irradiation dose (10Gy) was sufficient to cause DNA double strands breaks and to activate ATM in the normal LCLs. Therefore I expected to see NKG2D ligand upregulation in the normal LCLs but no response in the A-T LCLs which lacked ATM activity.

There was no significant upregulation of MICA (Fig 5:2:3A), MICB (Fig 5:2:3B), ULBP-1 (Fig 5:2:3C), ULBP-2 (Fig 5:2:3D) or ULBP-3 (Fig 5:2:3E) on normal LCLs or A-T LCLs with or without ATM activity over a 48 hour timecourse following irradiation-induced DNA damage. There was also no significant difference between the levels of expression of NKG2D ligands on normal and A-T LCLs following irradiation.

LCLs differ from normal B cells in that they are infected with EBV in order to immortalise the cell line. As NKG2D ligands can upregulated in response to viral infections it is possible that the virus may have evolved an immune escape mechanism to prevent NKG2D ligand upregulation and evade cytotoxic cells expressing the NKG2D receptor. Other herpes viruses including CMV are able to do this (Eagle et al., 2009;Wu et al., 2003). Interestingly it has been shown that although infection with EBV upregulates NKG2D ligands, normal latently infected LCLs are resistant to NK cell-mediated lysis (Pappworth et al., 2007).

Fig 5:2:3: NKG2D ligands are not significantly upregulated on normal or A-T LCLs in response to irradiation-induced DNA damage.



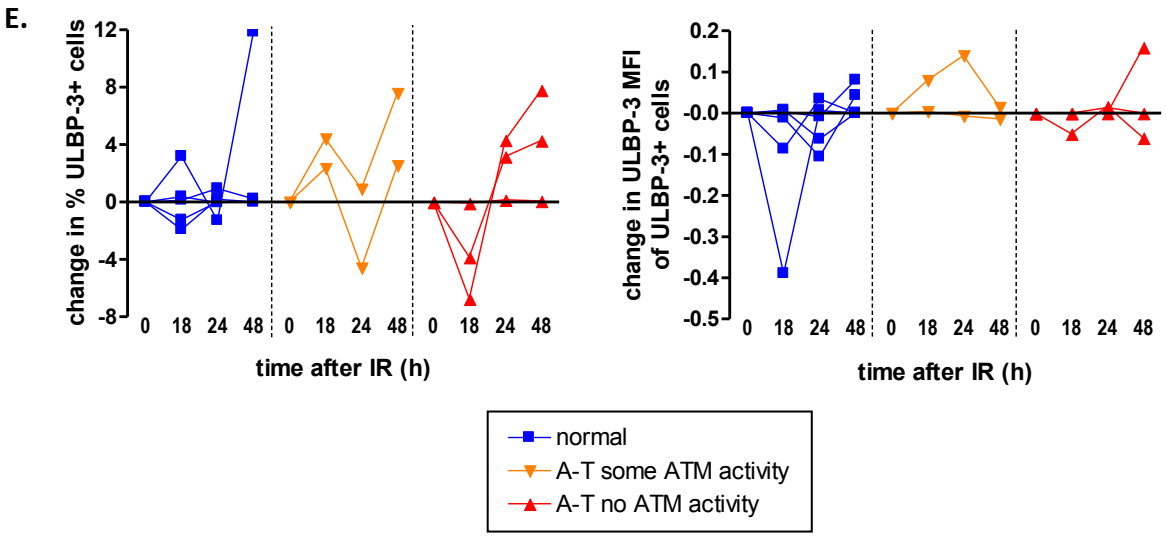


Fig 5:2:3: NKG2D ligands are not upregulated on LCLs in response to irradiation-induced DNA damage. Five normal (N1, N2, N3, N5, N6) and five A-T LCLs, three of which had no ATM activity (AT1, AT6, AT9) and two with some ATM activity (AT7, AT10) were resuspended in complete RPMI at a concentration of $0.5x10^6$cells/ml. Each LCL culture was split in two and half was irradiated with 10Gy IR. Samples were then plated out into wells and at indicated time points, irradiated and non-irradiated samples from each donor was harvested. Irradiated and non-irradiated samples from each LCL were stained with single NKG2D or isotype control antibodies (MICA, IgG2b, MICB, IgG2b (unconjugated), ULBP-1, ULBP-2, ULBP-3, IgG2a). Approximately $0.5x10^6$ cells were used per test. The dead cell exclusion die PI was added to tubes prior to running on the FACS. The percentage and NKG2DL MFI of NKG2DL+ve cells was calculated by subtracting any background in the isotype control from the percentage or NKG2DL MFI of NKG2DL+ve cells in the test sample. The change following IR was calculated as the percentage or NKG2DL MFI at a time point following IR minus the percentage or NKG2DL MFI of the corresponding non-irradiated control at the same time point

A. Irradiation-induced DNA damage had no significant effect on either the percentage or MICA MFI of MICA+ve cells in normal or A-T LCLs. There was also no significant difference in the effect of DNA damage on MICA expression of normal and A-T LCLs.

B. Irradiation-induced DNA damage had no significant effect on either the percentage or MICB MFI of MICB+ve cells in normal or A-T LCLs.

C. Irradiation-induced DNA damage had no significant effect on either the percentage or ULBP-1 MFI of ULBP-1+ve cells in normal or A-T LCLs.

D. Irradiation-induced DNA damage had no significant effect on either the percentage or ULBP-2 MFI of ULBP-2+ve cells in normal or A-T LCLs.

E. Irradiation-induced DNA damage had no significant effect on either the percentage or ULBP-3 MFI of ULBP-3+ve cells in normal or A-T LCLs.

5:3: MICA expression on B cells.

5:3:1: The effect of irradiation-induced DNA damage on MICA expression on normal B cells.

To investigate the possibility that EBV infection in the LCLs was preventing NKG2D ligand upregulation in response to DNA damage, the effect of irradiation-induced DNA damage on MICA expression on B cells from seven healthy lab donors was tested. Blood samples were collected, separated using lymphoprep and irradiated with a dose of 10Gy to produce DNA double strand breaks and activate ATM. Cells were stained with either MICA or the isotype control IgG2B as well as the B cell marker CD20.

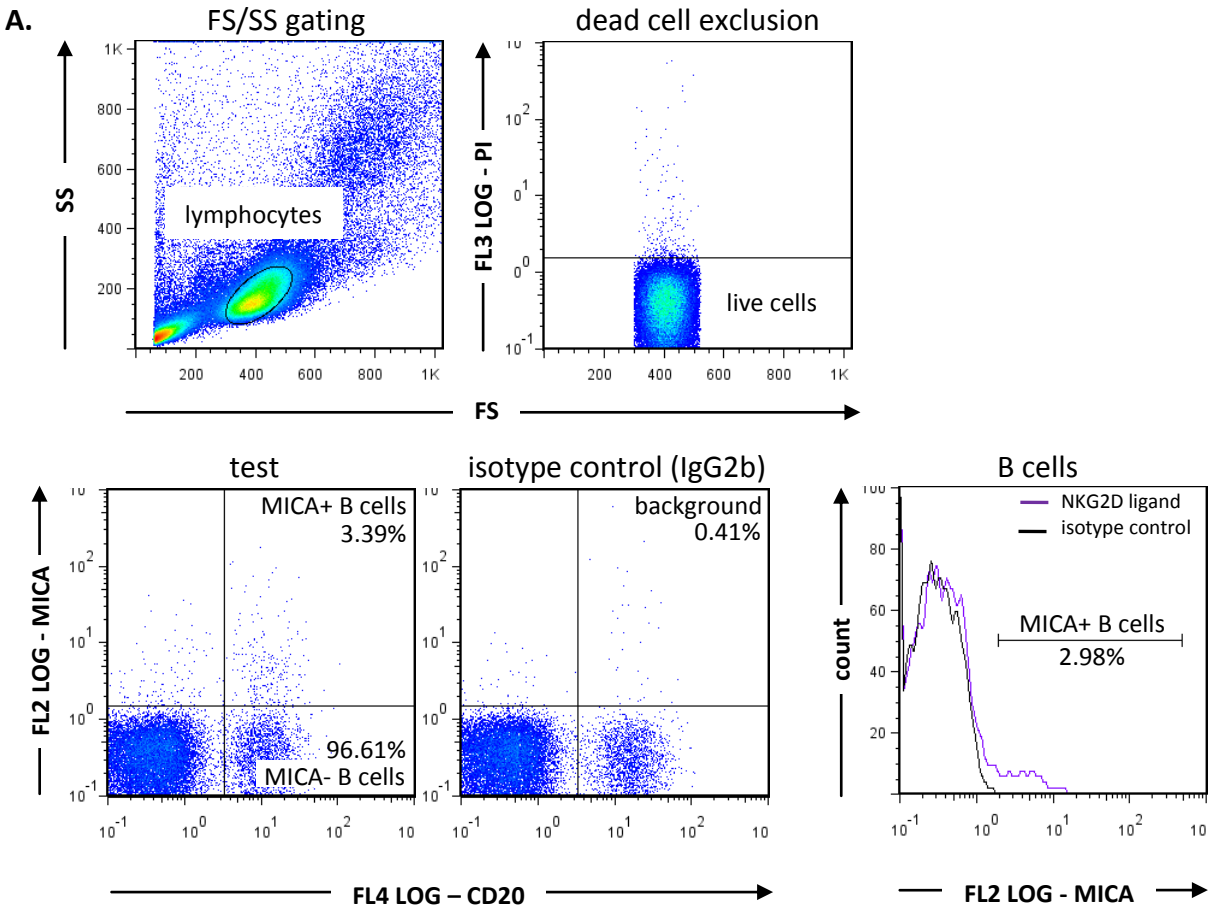
The lymphocyte population was selected using a FS/SS plot and dead cells excluded with PI. The percentage of B cells expressing MICA was calculated using a plot of CD20 against MICA and any background staining in the isotype control sample was deducted (Fig 5:3:1A).

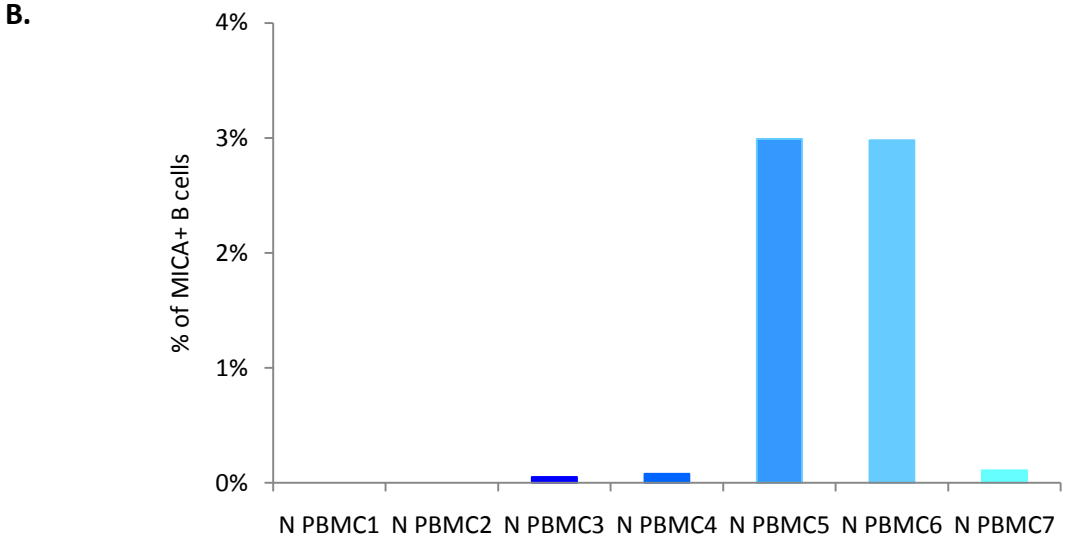
Similar to the LCLs, there was considerable variation in the basal level of expression of MICA in non-irradiated B cells at time 0 (Fig 5:3:1B). The highest level of expression was in samples N PBMC5 and N PBMC6 in which 2.98% of B cells expressed MICA. N PBMC1 and N PBMC2 did not express any MICA at time 0 and in the remaining three samples less than 0.2% of B cells expressed the ligand.

There was no significant increase in MICA expression on normal B cells in response to irradiation. The levels of expression did vary over the timecourse but this occurred in both the irradiated and non-irradiated controls. Therefore the apparent increase in the percentage of MICA+ve B cells in response to irradiation in four of the samples (N PBMC1, N PBMC2, N PBMC3 and N PBMC4, Fig 5:3:1C) may be simply due to normal fluctuations rather than a result of the DNA damage. A comparison of the MICA MFI of MICA+ve B cells before

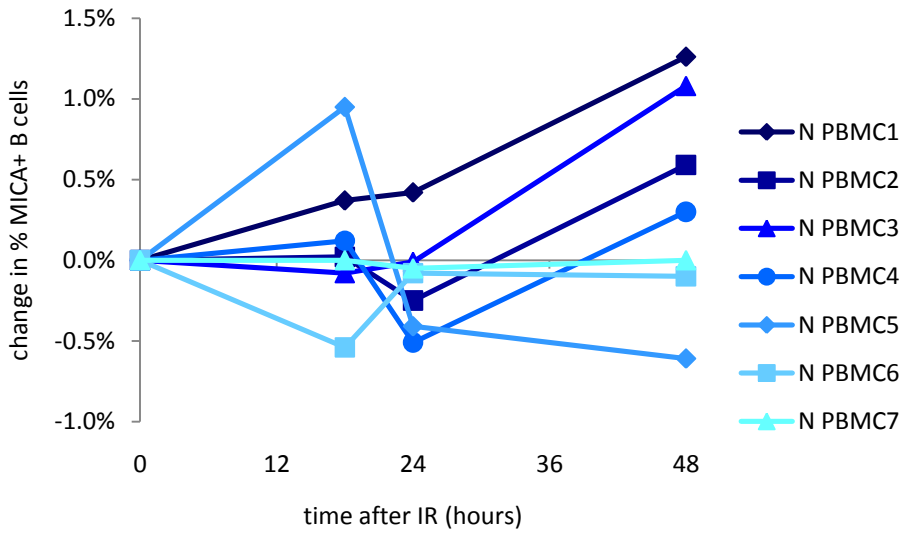
and after irradiation also showed no significant upregulation of the ligand in response to DNA damage (Fig 5:3:1D). There was also no upregulation of MICA on the CD20-ve cells (data not shown).

Fig 5:3:1: Irradiation-induced DNA damage had no effect on MICA expression on normal B cells.





C.



D.

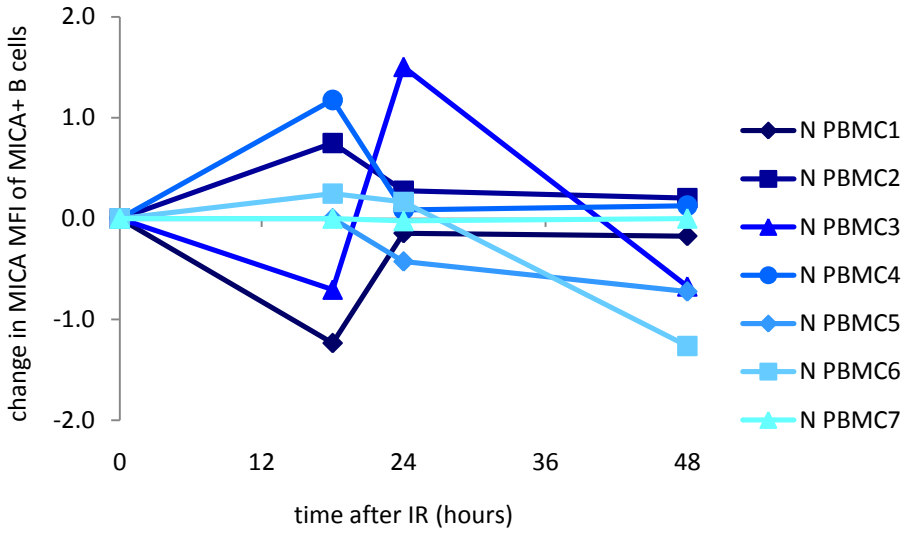


Fig 5:3:1: Blood samples from healthy lab donors were separated by lymphoprep and resuspended in complete RPMI at a concentration of $0.5x10^6$cells/ml. Each sample was split in two and half was irradiated with 10Gy IR. Samples were then plated out into wells and at indicated time points one irradiated and one non-irradiated well from each donor was harvested and again divided into two. Half was stained with antibodies for MICA and the B cell marker CD20 and half with CD20 and the isotype control IgG2b. The dead cell exclusion die PI was also added to tubes prior to running on the FACS.

A. Gating strategy and example plots of a non-irradiated sample at time 0 (N PBMC6). Gating on a FS/SS plot was used to select the lymphocyte population and dead cells (PI+)

were excluded using a plot of FS against PI. A plot of CD20 against MICA was then used to distinguish MICA+ve and MICA-ve B cells (CD20+). The percentage and MICA MFI of MICA+ve cells was calculated by subtracting any background in the isotype control from the percentage or MICA MFI of MICA+ve cells in the test sample. A histogram overlay of MICA+ve B cells and isotype control staining is shown.

B. The percentage of MICA+ve B cells in normal non-irradiated PBMCs at time 0h was very low. Two of the samples, N PBMC1 and N PBMC2, did not express any MICA at this time point. The median percentage of MICA+ve B cells at time 0h was 0.08% and the mean 0.89%.

C. Irradiation-induced DNA damage had no significant effect on the percentage of MICA+ B cells over a 48 hour timecourse following IR. The change in percentage of MICA+ B cells following IR was calculated as the percentage of MICA+ve B cells at a time point following IR minus the percentage of MICA+ve B cells in the corresponding non-irradiated control sample at the same time point.

D. Irradiation-induced DNA damage had no significant effect on the MICA MFI of MICA+ve B cells over a 48 hour timecourse following IR. The change in MICA MFI of MICA+ve B cells following IR was calculated as the MICA MFI of MICA+ve B cells at a time point following IR minus the MICA MFI of MICA+ve B cells in the corresponding non-irradiated control sample at the same time point.

5:4: NKG2D ligands expression on fibroblasts.

5:4:1: The effect of irradiation-induced DNA damage on NKG2D ligand expression on fibroblasts.

Gasser et al. (2005) showed upregulation of NKG2D ligands on normal fibroblasts in response to irradiation-induced DNA damage. As experiments using LCLs and B cells failed to show any DNA damage-induced NKG2D ligand upregulation I attempted to repeat the fibroblast experiment of Gasser et al. Gasser et al. used secondary neonatal human dermal foreskin fibroblasts, whereas I used dermal fibroblasts derived from skin biopsies taken from healthy lab donors. The experiment was designed to repeat that of Gasser et al. as closely as possible using the same dose of radiation (40Gy), incubation period (16 hours) and NKG2D ligand antibodies.

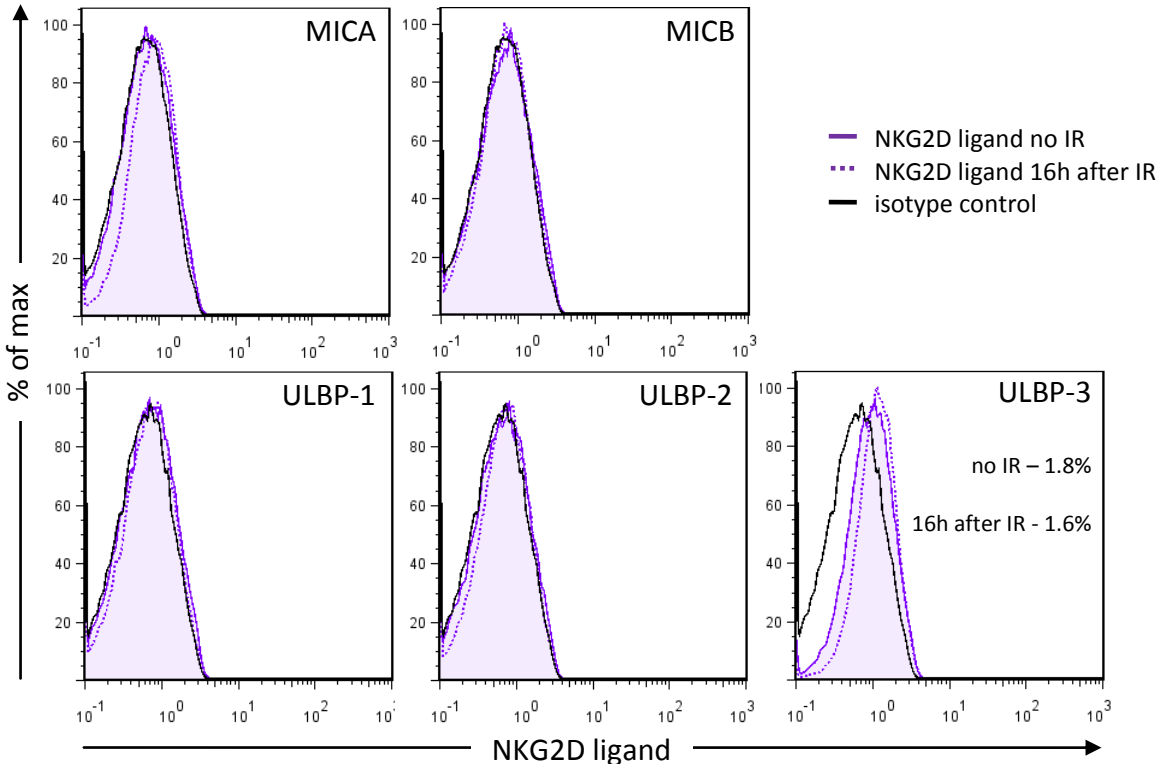
The experiment was carried out on two fibroblast cell lines. The fibroblasts expressed ULBP-3 but there was no expression of the other NKG2D ligands (Fig 5:4:1A&B). The fibroblasts used by Gasser et al. expressed ULBP-1, ULBP-2, ULBP-3 and MICA (MICB was not investigated), this difference may be due to the difference in origin of the fibroblasts or could be the result of genetic variation.

Neither fibroblast cell line showed any upregulation of NKG2D ligands in response to irradiation-induced DNA damage. In fibroblast cell line 2 (Fig 5:4:1B) the percentage of cells expressing ULBP-3 after 16 hours of incubation was higher on the non-irradiated sample than the irradiated sample (1.59% compared to 0.1%).

As I was unable to show DNA damage-induced upregulation of NKG2D ligands on LCLs, B cells or fibroblasts the project was discontinued.

Fig 5:4:1: NKG2D ligands were not upregulated on fibroblasts in response to irradiation-induced DNA damage.

A. fibroblast cell line 1



B. fibroblast cell line 2

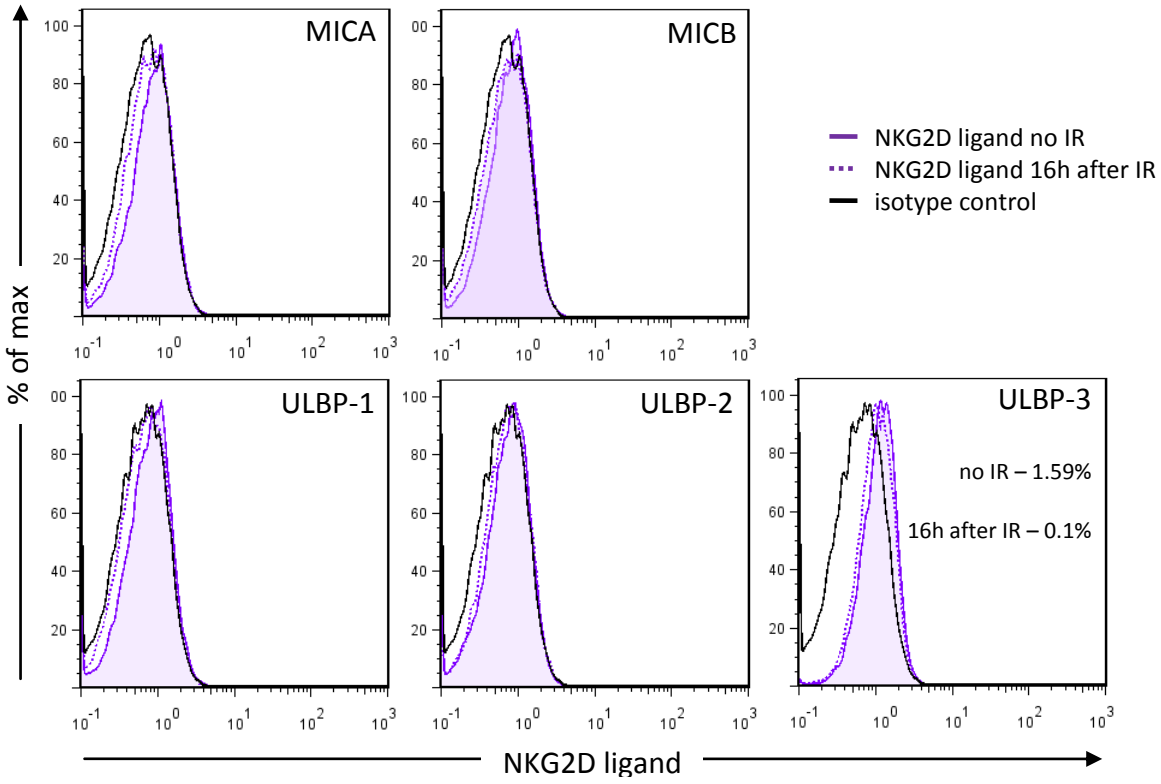


Fig 5:4:1: NKG2D ligands were not upregulated on fibroblasts in response to DNA damage induced by 40Gy irradiation. Fibroblast cell cultures derived from healthy lab donors were detached from culture flasks using trypsin/EDTA and resuspended in fresh media. A sample of each was collected and stained with individual NKG2D or isotype control antibodies ($0.5x10^6$cells/tube). The remaining cells were divided in two cultures, one of which was irradiated with 40Gy IR. The irradiated and non-irradiated cultures were then incubated at 37C for 16 hours prior to harvesting (trypsin/EDTA) and staining as for time 0. Histogram overlays show NKG2D ligand expression after 16 hours in irradiated and non-irradiated samples as well as the isotype control for the irradiated sample. NKG2D ligand expression at time 0 is not shown. The percentage of positive cells was calculated as the percentage of positive cells in the test sample minus any background staining in the corresponding irradiated or non-irradiated isotype control sample.

A. The NKG2D ligands MICA, MICB, ULBP-1 and ULBP-2 were not expressed on fibroblast cell line 1. There was a very low level of expression of ULBP-3 in both irradiated and non-irradiated cultures (1.6% and 1.8% positive cells) but no upregulation in response to irradiation-induced DNA damage.

B. The NKG2D ligands MICA, MICB, ULBP-1 and ULBP-2 were not expressed on fibroblast cell line 2. There was a very low level of expression of ULBP-3 in both irradiated and non-irradiated cultures (0.1% and 1.59% positive cells) but no upregulation in response to irradiation-induced DNA damage.

5:5: Discussion.

A role for ATM in immune surveillance through the DNA damage induced upregulation of NKG2D ligands has been reported (Gasser et al., 2005). As A-T patients have a high incidence of leukaemia and lymphoma I attempted to investigate the potential contributory role of a defect in immune surveillance through NKG2D ligand upregulation on lymphocytes, to the development of these tumours.

There was no evidence of NKG2D ligand upregulation on LCLs, normal B cells or dermal fibroblasts in response to DNA damage. Therefore it was not possible to investigate the role of ATM in NKG2D ligand upregulation.

LCLs were chosen as a model for investigating NKG2D ligand upregulation in response to DNA damage primarily because of the large number of different normal and A-T LCLs available. An alternative approach would have been to concentrate on fibroblasts and use ATM inhibitors to investigate the effect of ATM activation on NKG2D ligand upregulation in response to DNA damage. However, inhibitors may have nonspecific effects and inhibition may not be complete whereas an A-T LCL which does not express ATM protein definitely has no ATM kinase activity. The ease of culture of LCLs was also a factor. As it was not possible to obtain NKG2D ligand antibodies conjugated to a large range of different flourochromes staining with all of the antibodies in the same tube was not possible. Therefore a large number of cells of each LCL were required for each experiment so the quick doubling time of LCLs in culture was a major advantage.

It is possible that the lack of NKG2D ligand upregulation in response to DNA damage in LCLs may be due to EBV infection. However the lack of MICA upregulation on normal B cells or of any of the NKG2D ligands tested on normal fibroblasts in response to irradiation suggests

that this is not the case. It is possible that although MICA was not upregulated on B cells some or all of the other NKG2D ligands may have been upregulated. However, due to the large numbers of cells (and therefore volumes of blood) required it was not possible to investigate expression of all five ligands on PBMCs as for the LCLs. It would also not have been possible to obtain sufficient volumes of blood from A-T patients for comparison with normal donors. Therefore PBMCs were not an appropriate cell type for further experiments.

Gasser et al. (2005) showed upregulation of NKG2D ligands on fibroblasts in response to irradiation-induced DNA damage; however I was unable to reproduce this result despite using the same antibodies, dose of radiation and incubation time. I was concerned that the trypsin/EDTA method of cell detachment may be too harsh and could be stripping NKG2D ligands off the cell surface. I contacted the authors for method clarification and they suggested using 4mM EDTA instead of trypsin/EDTA for cell detachment. This method was attempted; however it killed the fibroblasts so the experiments were not continued. The difference in detachment method could explain the difference in findings between my study and that of Gasser et al.

It is possible that further experiments using fibroblasts and the 4mM EDTA detachment method may have shown upregulation of NKG2D ligands in response to radiation induced DNA damage. However, as the primary aim of the project was to investigate the role of ATM activation in NKG2D ligand upregulation and fibroblasts from A-T patients were not available, the scope of this experimental approach was limited. As no upregulation of NKG2D ligands in response to DNA damage could be detected on normal B cells, normal fibroblasts or either normal or A-T LCLs the project was discontinued.

Chapter 6: General Discussion

Since Ataxia-telangiectasia was first reported in 1926 (Syllaba & Henner, 1926) major technological advances including the development of positional cloning, DNA sequencing and flow cytometry techniques have led to a greater understanding of the function of the *ATM* gene and the cause of both the neurological and immunological symptoms (reviewed in (Lavin, 2008). However, the immune system defect in A-T is not yet fully understood.

Recent advances in multicolour flow cytometry allow a wide range of markers to be analysed simultaneously on a small sample of PBMCs. Using this technique I was able to examine T cell, B cell and NK cell subsets, NKT cells and expression of CD95, FasL and the IL-7 receptor α chain, CD127 on A-T lymphocytes. This analysis suggested a congenitally aged immune system phenotype in A-T. I also used flow cytometry assays to analyse the sensitivity of lymphoid derived cell lines (LCLs) to CD95-mediated apoptosis induced by the CD95-activating antibody CH11, and to analyse NKG2D ligand expression on LCLs, B cells and fibroblasts following DNA damage-induced ATM activation. Interestingly, A-T LCLs showed an increased sensitivity to CD95-mediated apoptosis compared to normal controls. There was no evidence of DNA damage-induced NKG2D ligand upregulation on either A-T or normal cells.

My phenotype analysis of A-T PBMCs clearly showed that the relative proportions of lymphocyte subsets in A-T are skewed with a naive T and B cell deficiency that I believe is similar to that of an aged immune system (Gupta & Gollapudi, 2008;Sansoni et al., 2008). However, unlike age-matched healthy individuals, the deficient immune system of A-T patients did not change significantly with age. Therefore A-T patients have a congenitally aged immune system from birth rather than a premature or accelerated rate of aging. This explains why, in contrast to the progressive neurological symptoms which lead to increasing loss of motor function with age and commonly result in patients becoming wheelchair

bound, the clinical consequences of the immune deficiency in A-T (immunoglobulin deficiency, lymphopenia, frequency of non-respiratory tract infections) do not worsen with age (Nowak-Wegrzyn et al., 2004).

I also found an increase in the percentages of both NK and NKT cells in A-T patients compared to normal controls. A similar increase in NK and NKT cells (both percentages and absolute numbers) has been reported in the elderly (reviewed in (Sansoni et al., 2008)). NK cells are particularly important in defence against viruses and NKT cells protect against viral, bacterial and parasitic infections. Therefore, the increase in these cells in A-T and the elderly may compensate for naive T cell deficiency and reduced antigen receptor repertoire. This could explain why A-T patients do not more frequently suffer from severe viral or opportunistic infections despite their T cell deficiency (Nowak-Wegrzyn et al., 2004). The increased proportion of NKT cells in A-T patients also suggests that glycolipid viral adjuvants which activate NKT cells may be useful in improving their vaccination responses.

In general the group of A-T patients with some ATM activity showed an intermediate phenotype between that of A-T patients with no ATM activity and normal controls. This indicates that the presence of even a small amount of residual ATM kinase activity may reduce the severity of the immune deficiency in A-T. It confirms the findings of Staples et al. who also showed evidence of a protective effect of residual ATM activity (Staples et al., 2008).

Other than the abnormalities in lymphocyte subsets, the most dramatic difference between PBMCs from A-T patients and normal controls was the significantly increased expression of the death receptor CD95 on A-T cells. Increased CD95 expression on CD4+ and CD8+ T cells in A-T patients has been reported previously (Giovannetti et al., 2002;Schubert et al., 2000)

but CD95 expression on other lymphocyte subsets was not investigated. Therefore as CD95 is a marker of activation, these studies (Giovannetti et al., 2002;Schubert et al., 2000) attributed the increased CD95 expression to the naive T cell deficiency. I found that CD95 was increased on all lymphocyte subsets with the exception of NK cells and that the increase in CD95 expression was particularly high on naive T cells and naive B cells. Therefore the increased expression is not simply a consequence of reduced output of CD95-ve naive T cells from the thymus or CD95-ve B cells from the bone marrow as CD95 expression was also increased on memory cells. My results indicate that the majority of lymphocytes in A-T patients have an activated phenotype, this is in keeping with the idea of a congenitally aged immune system as a similar predominance of activated cells and increased CD95 expression has been shown in the elderly (Aggarwal & Gupta, 1998;Potestio et al., 1999).

The high CD95 expression on A-T lymphocytes together with the increased sensitivity to CD95-mediated apoptosis of A-T LCLs suggests that increased apoptosis could contribute to the lymphopenia of A-T patients. However, patients were not deficient in memory T cells or memory B cells despite the high CD95 expression on these subsets. This apparent contradiction may be explained by a difference in sensitivity of naive and memory lymphocytes to CD95-mediated apoptosis. It would be interesting to compare the sensitivity of different subsets as it seems likely that in common with elderly individuals (Gupta & Gollapudi, 2008), A-T patients' naive T cells may be sensitive to and effector memory and TEMRA T cells, resistant to CD95-mediated apoptosis.

As well as its role in apoptosis, CD95 can provide co-stimulatory signals, which in combination with TCR signalling can induce proliferation of T cells recognising low-affinity antigens (Rethi et al., 2008). Therefore increased CD95 expression may lead to increased proliferation of activated memory T cells in A-T patients. Resistance to CD95-mediated

apoptosis of effector memory and TEMRA T cells together with increased proliferation may explain why there is no deficiency of memory T cells in A-T patients despite their high CD95 expression.

There was an indication from my results that increased availability of the cytokine IL-7 (due to lymphopenia) may be involved in CD95 upregulation on both naive and memory CD4+ T cell subsets. Therefore, treating A-T patients with IL-7 in an attempt to promote naive T cell proliferation could potentially worsen their lymphopenia.

However, it is possible that antioxidants may prove beneficial in treating lymphopenia in A-T. A role of reactive oxygen species (ROS) in CD95 upregulation on lymphocytes has been reported recently (McKallip et al., 2010) and as A-T patients' cells show evidence of increased oxidative stress (Reichenbach et al., 2002) it is possible that ROS induced upregulation of CD95 may contribute to the increased expression on A-T lymphocytes. ROS have been implicated in CD95-mediated apoptosis in neuronal cells, both by inducing upregulation of CD95 and FasL, and in signalling DISC formation following FasL binding to CD95 (Facchinetti et al., 2002). Therefore, increased CD95-mediated apoptosis of neuronal cells in A-T patients as a consequence of increased oxidative stress may contribute to their neurological decline. Supporting this hypothesis, evidence from A-T mouse models suggests that antioxidants slow the rate of progression of the neurological decline by reducing oxidative stress (Gueven et al., 2006;Reliene et al., 2008;Reliene & Schiestl, 2006). These mouse studies focussed on the anti-tumour effects of antioxidants in A-T, so the mechanism by which they slowed the neurological decline was not investigated. However, it is possible that they work by reducing CD95-mediated apoptosis of neuronal cells. Antioxidants may also be useful in treating the immune deficiency in A-T if they are able to down regulate CD95 expression on lymphocytes and consequently reduce sensitivity to both spontaneous

and FasL induced CD95-mediated apoptosis. This could potentially explain the increase in lymphocyte count reported in A-T patients undergoing treatment with the steroid bethamethosone (Broccoletti et al., 2008) which is thought to act through an antioxidant mechanism (Russo et al., 2009).

The effect of antioxidants on CD95 expression and sensitivity to CD95-mediated apoptosis of A-T lymphocytes could be investigated by culturing A-T LCLs in the presence of an antioxidant such as N-acetyl-L-cysteine (NAC) and analysing CD95 expression and sensitivity to CH11-induced CD95-mediated apoptosis. It is not clear if clinical trials are currently being carried out to analyse the effect of antioxidant treatment on the neurological decline of A-T patients. However, it would be interesting to also analyse CD95 expression on lymphocytes and look for any improvement in the lymphopenia of A-T patients during such trials.

Interestingly, in contrast to my finding of increased sensitivity, increased resistance of A-T cells to CD95-mediated apoptosis as a direct consequence of *ATM* mutation was suggested by the work of Stagni et al. (2008). It could be argued that increased CD95 expression on A-T T cells indicates increased resistance rather than increased sensitivity of both naive and memory subsets to CD95-mediated apoptosis, as CD95+ve cells which fail to die by activation-induced cell death during the down phase of an immune response persist in the bloodstream. However, this hypothesis does not fit with the lymphopenic phenotype of A-T patients. My results showed an increased sensitivity of A-T LCLs to CD95-mediated apoptosis but no evidence of a direct role for ATM in regulation of expression of CD95 or of the caspase 8 inhibitor cFLIP.

I found that CD95 expression (MFI) was reduced on A-T compared to normal LCLs. This was surprising as my PBMC phenotype analysis clearly showed increased CD95 expression on A-T

lymphocytes. This difference is difficult to explain, however it is possible that it may be related to differential regulation of CD95 expression by EBV in normal and A-T LCLs. This hypothesis is hard to prove and there was no evidence of a difference in expression of LMP1 (an EBV protein able to regulate expression of both CD95 and cFLIP (Cahir-McFarland et al., 2004)) in A-T compared to normal LCLs. However, it would explain why both Stagni et al. (2008) and I found no difference in CD95 expression between ATMwt and ATMmut transfected isogenic LCLs, as the EBV infection was already well established prior to transfection. Primary cell samples are more informative than transformed cell lines as experimental results are not influenced by viral transformation. However, LCLs are a useful tool for research, especially when obtaining large volumes of blood for analysis is not possible as was the case with my study.

An increased sensitivity of A-T lymphocytes to CD95-mediated apoptosis may seem surprising in the context of the high incidence of leukaemia and lymphoma in A-T patients as apoptotic resistance is an important characteristic of many tumours. Therefore I analysed the sensitivity of T-PLL and B-CLL tumours with *ATM* mutations to CH11-induced CD95-mediated apoptosis. My results showed that sensitivity to CD95-mediated apoptosis does not prevent tumour development, as evidenced by the apoptotic sensitivity of T-PLL tumours. Although common in A-T, T-PLL can also develop in non-lymphopenic, non-A-T patients. Therefore T cell lymphopenia and subsequent reduced efficiency of immune surveillance do not fully explain how an apoptotic sensitive tumour is able to develop and persist. It is likely that the proliferative advantage of the tumour cells outweighs the effect of increased CD95-mediated apoptosis.

Gasser et al. recently suggested a role for ATM in the upregulation of NKG2D, this may protect against tumour development by signalling the presence of potentially malignant cells

containing damaged DNA to cytotoxic cells of the immune system (Gasser et al., 2005). However, I was unable to show DNA damage-induced upregulation of NKG2D ligands on normal LCLs, B cells or fibroblasts. This suggests that ATM may not be involved in the process; therefore it is unlikely that a deficiency in the NKG2D system of immune surveillance contributes to the high incidence of cancer in A-T patients.

I can conclude that the lymphopenia in A-T is primarily caused by a combination of low thymic output of naive T cells (Giovannetti et al., 2002;Micheli et al., 2003) and possibly increased CD95-mediated apoptosis of naive T and B cells. It is not caused by a deficiency in expression of the proliferative cytokine IL-7 or its receptor. Naive T cell deficiency may account for increased sinopulmonary infections and reduced vaccination responses in A-T as patients' immune systems have a reduced capacity to respond to new antigenic challenges. The similarities of the immune system phenotype of A-T patients with that of the elderly suggest a possible role of impaired DNA damage repair in immune senescence. Interestingly a decline in ATM expression and function during normal aging has been shown in mice (Fang 2007, Panda 2007) and a defect in DNA double strand break repair has been reported in PBMCs from elderly people (Frasca et al., 1999). Regardless of the cause of the deficiency, the similarities of the aged and A-T immune systems suggest that therapies that prove beneficial in treating A-T patients (potentially including antioxidants and glycolipid viral adjuvants) may also be beneficial in improving immune system function in the elderly.

Further work is required to fully explain the potential role of ATM protein kinase in the increased sensitivity to CD95-mediated apoptosis of A-T lymphocytes and possibly in immune surveillance. However, advances in our knowledge of the cause of the immune deficiency in A-T could not only potentially lead to the development of effective new

treatments but also provide an insight into the role of ATM in the immune system, immunological function, tumour development and ageing in healthy individuals.

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Appendix.

Appendix 1: Testing the 'normality' of anonymous surplus diagnostic blood samples.

The cohort of PBMC samples used as normal controls in Chapter 3 consisted of 28 anonymous samples (surplus diagnostic bloods) obtained from the Regional Genetics Laboratory, Birmingham Women's Hospital and 3 samples obtained from healthy lab donors. In order to determine if the anonymous samples were 'normal' their immune system phenotypes were compared with that of the healthy lab donor samples.

The definition of what was normal was complicated by the difference in ages of the lab donors and patients who provided the anonymous samples, the lab donor samples were from donors with an age range of 22 years 11 months to 30 years 10 months, and the anonymous samples were from donors with an age range of 1 year 3 months to 14 years 9 months. Therefore differences in the percentages of lymphocyte subsets between lab donor and anonymous samples which fit the general trend of change in immune system phenotype with age were not considered to indicate abnormalities in the anonymous samples.

However two samples obtained from the Regional Genetics Laboratory did appear to have abnormal immune system phenotypes. N PBMC20 was taken from a donor aged 11 years 9 months with tall stature and coordination problems and N PBMC29 from a donor aged 9 years 2 months with possible Turner syndrome. These samples were not included in the normal control cohort used in Chapter 3 but are shown in Fig A1 as 'excluded samples'.

The first part of the analysis was a comparison of the percentages of T cells, B cells, NK cells and NKT cells. There were no significant differences in the percentages of T cells, NK cells or NKT cells between the lab donor and anonymous samples and no abnormalities in the excluded samples (Fig A1A). The percentage of B cells was increased in the anonymous

samples compared to the normal controls ($P<0.05$), however this was consistent with a decrease in the percentage of B cells with age (Fig A1B).

The main rational for exclusion of two samples from the study came from analysis of T cell subsets. The excluded samples had reduced percentages of CD4+ T cells and increased CD8+ T cells compared to the lab donor and remaining anonymous samples (Fig A1C), resulting in abnormal CD4:CD8 T cell ratios (Fig A1D). These differences were not age related (Fig A1E) as the percentages of CD4+ and CD8+ T cells and CD4:CD8 T cell ratios of the lab donor and anonymous samples were very similar despite their age difference.

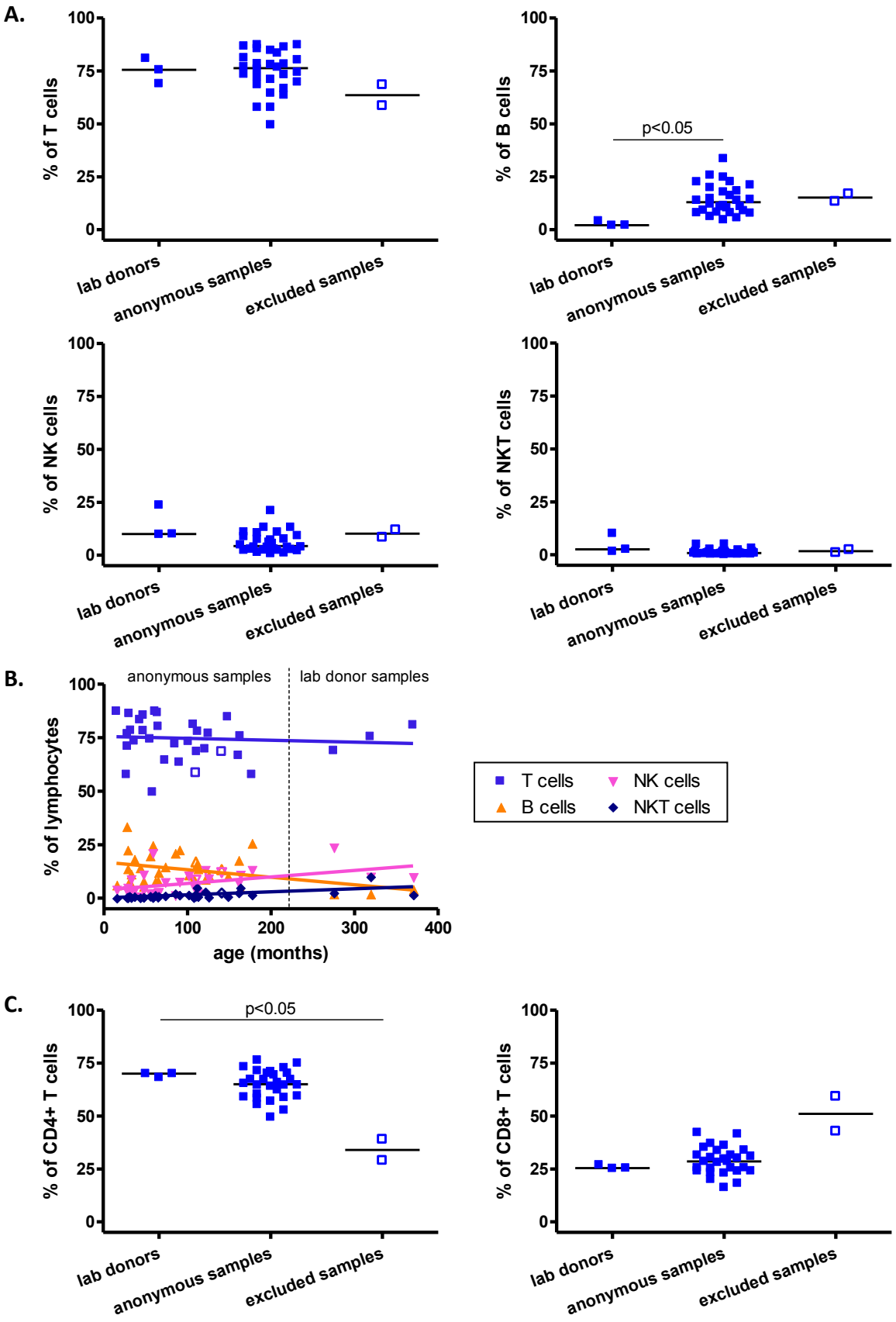
Analysis of T cell subsets showed a decrease in the percentage of naive T cells and increase in the percentage of memory T cells with age (Fig A1J). Therefore there was a higher percentage of naive T cells (Fig A1F) and lower percentage of central memory T cells (Fig A1G) in the lab donor than the anonymous samples. The two excluded samples had abnormally low percentages of naive T cells and abnormally high percentages of memory T cell subsets for their age (Fig A1F-J).

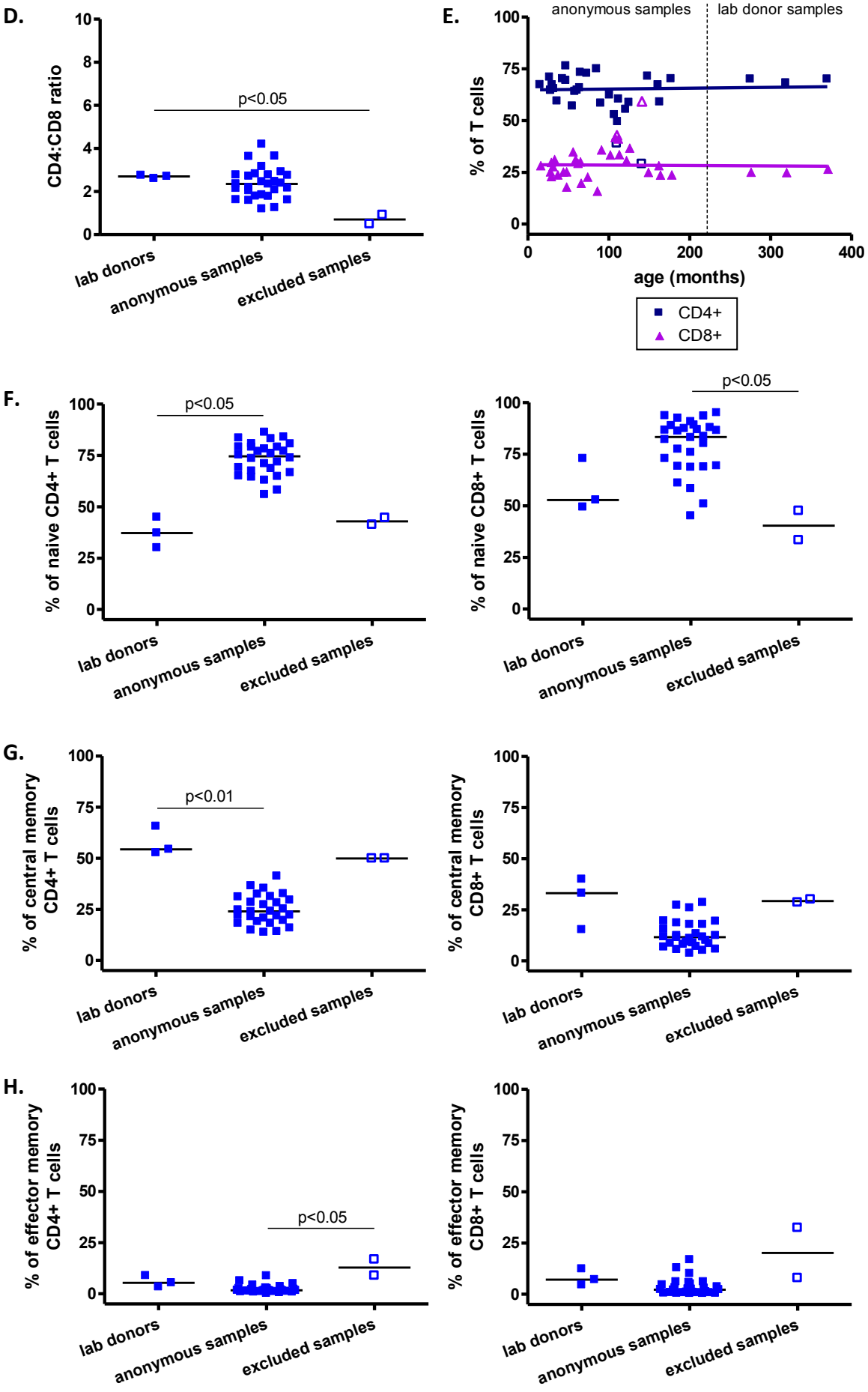
The percentages of naive B cells were high and the percentages of memory B cells were low in the lab donors compared to both the anonymous samples and the excluded samples (Fig A1K. These differences fit the trend with age (Fig A1K) so did not suggest abnormality of the anonymous samples or excluded samples although the differences between lab donor and excluded samples were statistically significant (naive B cells $p<0.05$, memory B cells <0.01).

Finally there were no significant differences in the percentages of CD56bright or CD56dim NK cells between lab donor and anonymous samples. The two excluded samples also had normal percentages of these cell types (Fig A1M&N).

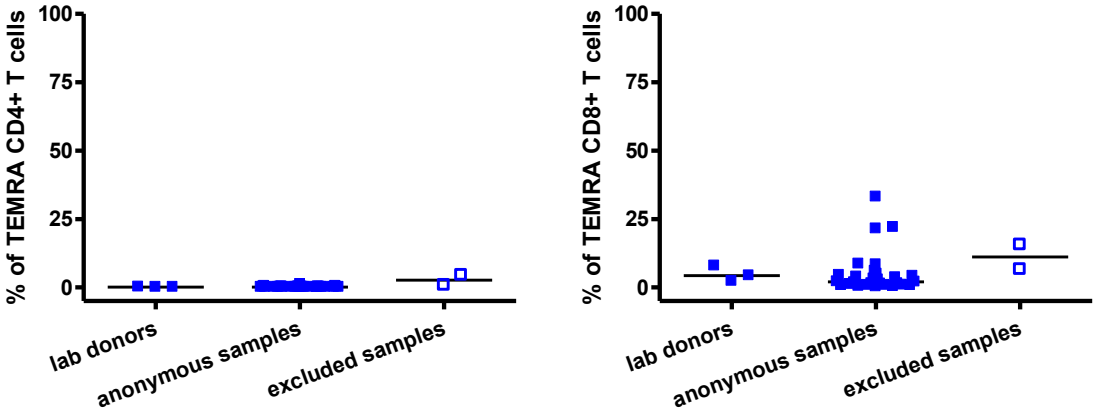
This analysis confirmed that with the exception of the two excluded samples the immune system phenotypes of the anonymous samples were normal so they could be used as part of a normal control cohort.

Fig A1: All but two of the anonymous surplus diagnostic blood samples had normal immune system phenotypes similar to healthy lab donors.

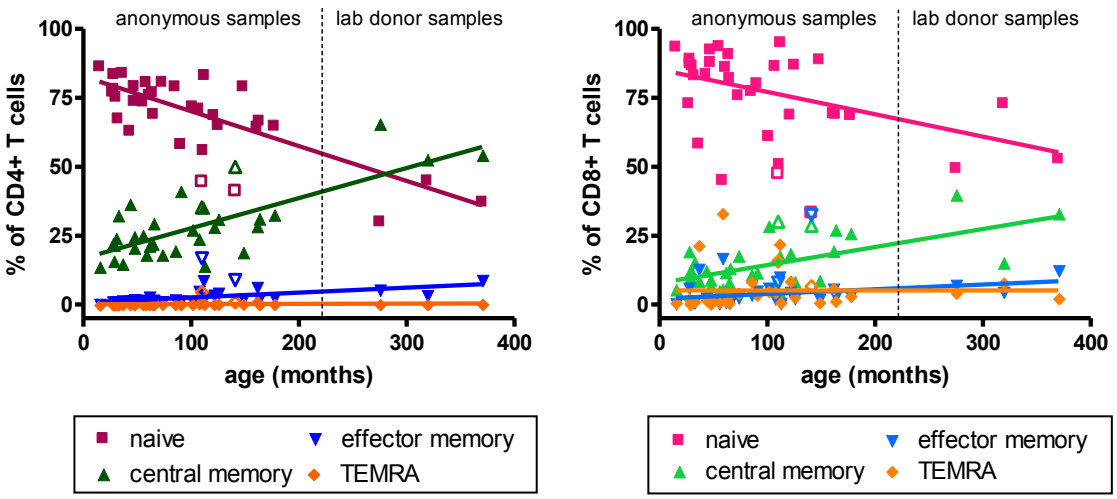



D.
CD4:CD8 ratio
p<0.05
lab donors
anonymous samples
excluded samples
E.
anonymous samples
lab donor samples
% of T cells
age (months)
CD4+
CD8+
F.
% of naive CD4+ T cells
p<0.05
% of naive CD8+ T cells
p<0.05
G.
% of central memory CD4+ T cells
p<0.01
% of central memory CD8+ T cells
H.
% of effector memory CD4+ T cells
p<0.05
% of effector memory CD8+ T cells

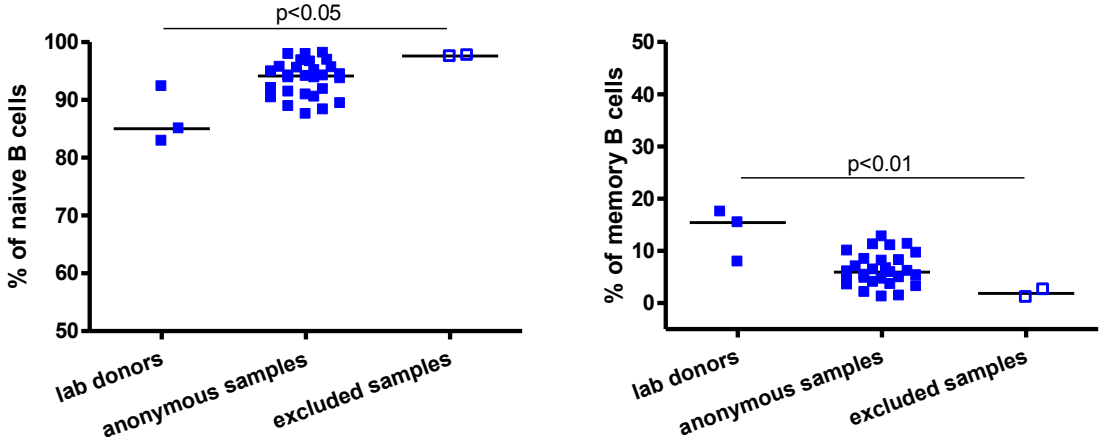
I.


% of TEMRA CD4+ T cells
100
75
50
25
0
lab donors
anonymous samples
excluded samples
% of TEMRA CD8+ T cells
100
75
50
25
0
lab donors
anonymous samples
excluded samples

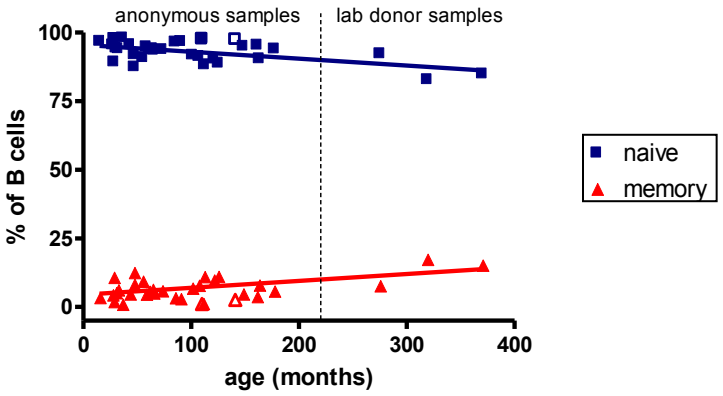
J.


anonymous samples
lab donor samples
% of CD4+ T cells
100
75
50
25
0
0
100
200
300
400
age (months)
naive
effector memory
central memory
TEMRA
anonymous samples
lab donor samples
% of CD8+ T cells
100
75
50
25
0
0
100
200
300
400
age (months)
naive
effector memory
central memory
TEMRA

K.


p<0.05
% of naive B cells
100
90
80
70
60
50
lab donors
anonymous samples
excluded samples
p<0.01
% of memory B cells
50
40
30
20
10
0
lab donors
anonymous samples
excluded samples

L.


anonymous samples
lab donor samples
% of B cells
100
75
50
25
0
0
100
200
300
400
age (months)
naive
memory

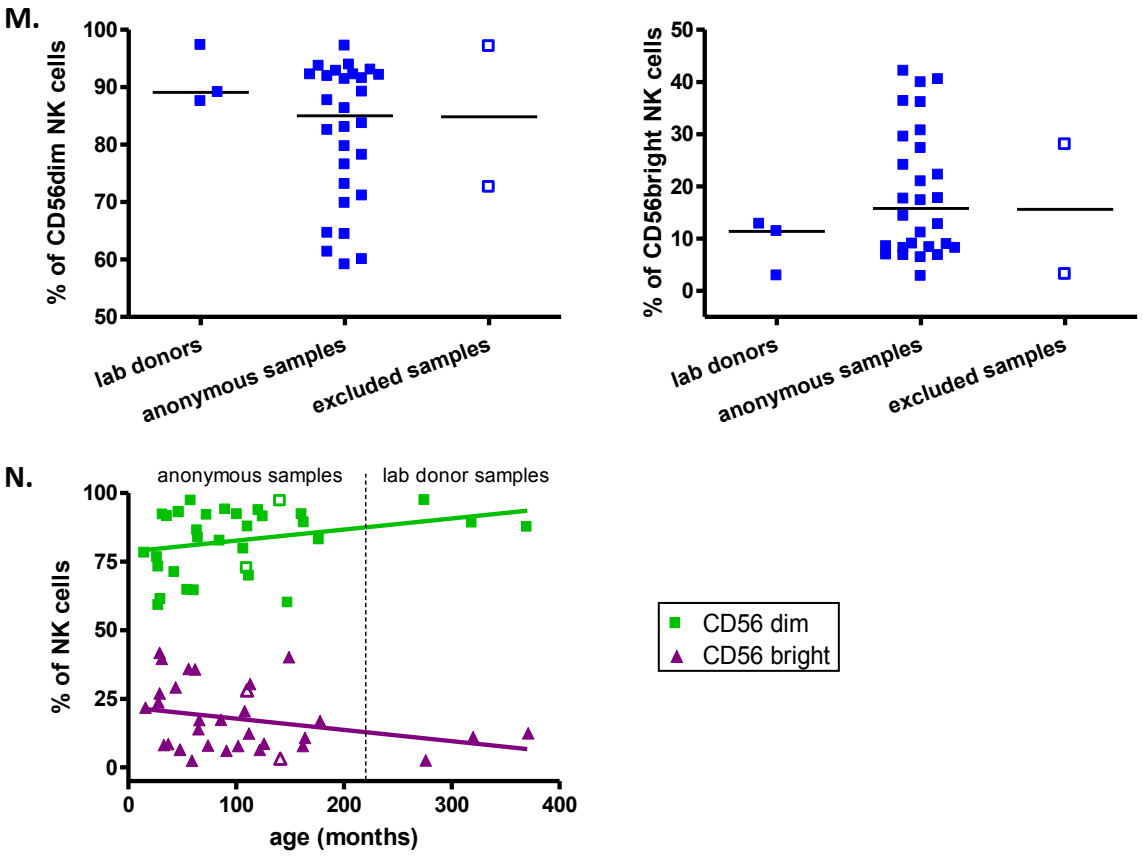


Fig A1: Comparison of immune system phenotypes of PBMC samples obtained from healthy lab donors and surplus diagnostic bloods from the Regional Genetics Laboratory, Birmingham Women's Hospital. PBMC samples were thawed then stained using the 11 colour antibody panel and analysed on a BD LSRII flow cytometer. Gating was carried out as described in Fig 3:3:1. 28 of the surplus diagnostic bloods were classed as normal 'anonymous samples' and 2 samples were classed as abnormal 'excluded samples'. The 'anonymous samples' and 'lab donor samples' were treated as a single group for linear regression analysis. The 'excluded samples' are shown as open symbols.

A. Comparison of the percentages of T cells, B cells, NK cells and NKT cells in lab donors, anonymous samples and excluded samples.

B. There was a decrease in the percentage of B cells and increase in the percentages of NK and NKT cells with age.

C. Comparison of the percentages of T cells expressing CD4 and CD8 in lab donor samples, anonymous samples and excluded samples.

D. Comparison of the CD4:CD8 T cell ratio (% of CD4+ve T cells/% of CD8+ve T cells) of lab donor samples, anonymous samples and excluded samples.

E. There was no change in the percentages of CD4+ve or CD8+ve T cells with age.

F. Comparison of the percentages of naive CD4+ve and CD8+ve T cells in lab donor samples, anonymous samples and excluded samples.

G. Comparison of the percentages of central memory CD4+ve and CD8+ve T cells in lab donor samples, anonymous samples and excluded samples.

H. Comparison of the percentages of effector memory CD4+ve and CD8+ve T cells in lab donor samples, anonymous samples and excluded samples.

I. Comparison of the percentages of TEMRA CD4+ve and CD8+ve T cells in lab donor samples, anonymous samples and excluded samples.

J. The percentages of naive T cells decreased and memory T cell subsets increased with age.

K. Comparison of the percentages of naive and memory B cells in lab donor samples, anonymous samples and excluded samples.

L. The percentages of naive B cells decreased and memory T cells increased with age.

M. Comparison of the percentages of CD56dim and CD56bright NK cells in lab donor samples, anonymous samples and excluded samples.

N. There was no change in the percentages of CD56dim and CD56bright NK cells with age.