Classical Ataxia Telangiectasia Patients Have a Congenitally Aged Immune System with High Expression of CD95

Ellen F. Carney, Venkataramanan Srinivasan, Paul A. Moss and A. Malcolm Taylor

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Ataxia-telangiectasia (A-T) is a rare neurodegenerative immunodeficiency disorder caused by mutations in the \textit{ataxia telangiectasia mutated} (\textit{ATM}) gene, which encodes a protein kinase that has a major role in the cellular response to DNA damage. \textit{ATM} mutations lead to the inability to repair some DNA double-strand breaks (1), aberrant chromosomal translocations (2), and defective VDJ recombination (3). Characteristic immunological features of A-T include lymphopenia and Ig deficiencies. Patients also show progressive neurodegeneration with unsteady gait, abnormal vascular development, increased radiation sensitivity, and increased incidence of cancer.

The majority of A-T patients are lymphopenic due to reduced numbers of T and B cells (4). They have reduced proportions of naive T and naive B cells and increased proportions of memory T cells, memory B cells, and NK cells compared with normal healthy individuals (5). The cause of the T cell lymphopenia in A-T patients is thought to be reduced output from the thymus (6), although increased spontaneous apoptosis of naive cells was suggested as a contributing factor (7). Interestingly, an increase in both spontaneous and CD95-mediated apoptosis of lymphocytes, as well as reduced numbers of naive cells, has been associated with aging of the immune system in the normal population (8–10). A similarly high CD95 expression and consequent sensitivity to CD95-mediated apoptosis in A-T patients could contribute to their naive cell deficiency and lymphopenic phenotype.

The cytokine IL-7 is important in the regulation of T cell homeostasis and proliferation in response to lymphopenia. Under normal conditions, its production from the stromal cells of the thymus and bone marrow is relatively stable, and T cell responses are regulated through modulation of the IL-7R \( \alpha \)-chain (CD127), which dimerizes with the \( \gamma \)-chain to form the IL-7R complex. 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; if the signal is strong enough, it can lead to lymphopenia-induced proliferation of naive cells (11).

Alternatively, increased availability of IL-7 as a response to T cell lymphopenia may also contribute to the lymphopenia by upregulating CD95 on T cells and consequently increasing their sensitivity to CD95-mediated apoptosis (12, 13). Peripheral T cell depletion associated with increased serum IL-7 concentration and consequent CD95 upregulation in lymphopenic individuals has been reported in HIV patients (12) and bone marrow transplant recipients (13). Significantly higher CD95 expression on A-T patient T cells compared with controls has also been described, although IL-7 was not investigated (5, 7).

We used 11-color flow cytometry and IL-7 ELISA to investigate the possible association of CD95 expression and IL-7 availability with lymphopenia in A-T patients, as well as the effect of age on lymphocyte phenotype and CD95, Fas ligand (FasL), and CD127 expression in healthy controls and A-T patients. We also analyzed the sensitivity of naive and memory subsets of A-T and normal T cells to CD95-mediated apoptosis using TUNEL and caspase-activation assays. Our findings reveal a congenitally aged immune system and an increased sensitivity of activated naive and central memory T cells to CD95-mediated apoptosis in A-T patients, which suggests a possible contributory role of CD95 up-regulation and CD95-mediated apoptosis to lymphopenia in A-T.
Materials and Methods

Blood samples

Blood samples for phenotyping analysis were obtained from 15 A-T patients (Table I) with an age range of 1 y 7 mo to 28 y 8 mo and a mean age of 9 y 4 mo (112.1 mo). ATM mutations were confirmed by sequencing. No A-T patient had residual ATM activity as measured by Western blot, using the method described by Barone et al. (14).

The control group consisted of 3 healthy laboratory donor samples and 28 surplus diagnostic bloods from patients with nonhematological disorders obtained from the Regional Genetics Laboratory, Birmingham Women’s Hospital (Edgbaston, Birmingham, U.K.) with appropriate ethical approval. The laboratory donor samples were a useful control for checking the normality of the surplus diagnostic samples; multicolor flow cytometry analysis confirmed that all controls had normal immune system phenotypes. The age range of the donors was 19–30 y.

Flow cytometry

Phenotyping of lymphocyte subsets (T cells; B cells; NK cells; NKT cells; CD4+ T cells; CD8+ T cells; CD4+ and CD8+ naive, central memory, effector memory, and T cell effector memory CD45RA+ [TEMRA] T cell subsets; naive and memory B cells; and CD56bright and CD56dim NK cells) and the dead cell-exclusion dye propidium iodide (PI; Sigma) and the dead cell-exclusion dye FITC (clone SB93a; Abcam, Cambridge, U.K.) and CD127–PerCP–Cy5.5 (clone 0323; eBiosciences), CD95-PE (clone DX2; Dako, Glostrup, Denmark), FasL-Legend, San Diego, CA, CD27–allophycocyanin–eFluor 780 [clone 0323; eBiosciences], CD95-PE [clone DX2; Dako, Glostrup, Denmark], FasL-FITC [clone SB93a; Abcam, Cambridge, U.K.], and CD127–PerCP-Cy5.5 [clone hIL-7R-M21; BD Biosciences] and the dead cell-exclusion dye propidium iodide (PI; Sigma).

Frozen PBMC samples were thawed at 37°C and washed in warm RPMI 1640 (10% FCS) with 10% PFCs. They were washed in cold MACS buffer (PBS [pH 7.2], 0.5% BSA, 2 mM EDTA) prior to staining with an Ab mixture (CD3, CD4, CD8, CD27, CD45RA and CD95) to label T cell subsets. PBMC samples from 15 A-T patients with no residual ATM activity (Table I) had a significantly lower cell count than did samples from age-matched controls (p < 0.0001; Fig. 1A), consistent with the reported lymphopenia of A-T patients. There was no correlation between cell count and age in either group (Fig. 1B).

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A-T patients showed a significantly lower percentage of T cells (p < 0.0001) but higher percentages of NK (p < 0.0001) and NKT cells (p = 0.0093) than did controls (Fig. 2A). There was no significant difference in the percentage of B cells.

The percentages of lymphocytes in different subsets changed significantly with age in both groups. In the controls, there were significant increases in the percentages of NK and NKT cells with age, whereas in the A-T patients, the percentage of T cells increased and NK cells decreased significantly with age (Fig. 2B). This suggested differential maturation of the immune system in A-T patients and controls.

A-T patients have a naive T and B cell deficiency

To investigate lymphocyte subsets in more detail, T cells were subdivided into CD4+ and CD8+ naive (CD27−CD45RA−), central memory (CD27−CD45RA−), effector memory (CD27+CD45RA−), and TEMRA (CD27−CD45RA−) subsets; B cells were subdivided into naive (CD19−CD27+) and memory (CD19+CD27−) subsets; and NK cells were subdivided into CD56dim (cytotoxic) and CD56bright (cytokine-producing) subsets (Supplemental Fig. 1).

There were very obvious differences between the proportions of T and B cells in each subset in A-T patients and controls. A-T patients had a significantly lower percentage of CD4+ and CD8+ naive T cells (CD4+, p < 0.0001; CD8+, p < 0.0001) and significantly higher percentages of CD4+ and CD8+ central memory (CD4+, p < 0.0001; CD8+, p < 0.0001; CD8+, p < 0.0001; CD8+, p < 0.0001) and effector memory (CD4+, p < 0.0001; CD8+, p = 0.0002) T cells than the controls (Fig. 3A, 3B). They also had a significantly lower percentage of naive B cells (p < 0.0001) and a significantly higher percentage of memory B cells (p < 0.0001) than the controls (Fig. 3C).

There was no significant difference in the percentage of CD56dim or CD56bright NK cells between the groups (Fig. 3D). The mean proportions of T and B cell subsets in A-T patients and controls are summarized in Fig. 3E.

The proportions of naive and memory T and B cells in A-T patients do not change significantly with age

In the controls, the percentages of both CD4+ (Fig. 4A) and CD8+ (Fig. 4B) naive T cells decreased significantly with age, whereas the percentages of central memory, effector memory, and TEMRA T cells increased significantly. However, the A-T patients showed no significant correlation between the percentage of T cells in each subset and age.

Similarly to T cell subsets, the percentage of naive B cells decreased and memory B cells increased significantly with age in the controls (Fig. 4C). In contrast, there was no correlation between the percentage of naive or memory B cells and age in the A-T patients (Fig. 4C).

Results

A-T patients are lymphopenic with a reduced proportion of T cells and increased NK cells

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There was no significant difference in the percentage of CD56dim or CD56bright NK cells between the groups (Fig. 3D). The mean proportions of T and B cell subsets in A-T patients and controls are summarized in Fig. 3E.

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Similarly to T cell subsets, the percentage of naive B cells decreased and memory B cells increased significantly with age in the controls (Fig. 4C). In contrast, there was no correlation between the percentage of naive or memory B cells and age in the A-T patients (Fig. 4C).
Unstimulated A-T lymphocytes have increased CD95 expression but normal FasL and CD127 expression

Both the percentage (Fig. 5A) and median fluorescence intensity (data not shown) of CD95+ T cells (p = 0.0001), B cells (p = 0.0001), and NKT cells (p = 0.0019) in unstimulated PBMC samples were significantly higher in A-T patients than in controls; there was no significant difference in CD95 expression on NK cells. Interestingly, the increase in CD95 expression in A-T patients was particularly dramatic on naive T (CD4+, p = 0.0001; CD8+, p = 0.0001) and naive B cells (p = 0.0001) (Fig. 5B).

The controls showed significant positive correlations between age and the percentages of NK and NKT cells. A-T patients showed a positive correlation between age and percentage of T cells and a negative correlation between age and percentage of NK cells.

Table I. ATM mutations, residual ATM protein expression (as percentage of normal levels), and ATM activity in A-T patients

<table>
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<tr>
<th>Patient Designation</th>
<th>Age at Bleed</th>
<th>Sex</th>
<th>Amount Expressed (%)</th>
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<th>Mutation 2</th>
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<tr>
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<td></td>
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<td>Trace</td>
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<tr>
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<td>Male</td>
<td>5</td>
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<tr>
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*Measured by Western blot.

**FIGURE 1.** A-T patients are lymphopenic. (A) A-T patients had a significantly lower cell count (PBMC × 10^6/ml) than did controls (p < 0.0001). (B) There was no significant change in PBMC cell count with age in A-T patients or controls.

**FIGURE 2.** Lymphocyte subsets in A-T patients and controls. (A) Proportions of T cells (CD3+CD19-CD56-), B cells (CD19+CD56-), NK cells (CD56+CD3-), and NKT cells (CD56+CD3+) in the live lymphocyte population (PI-) of A-T patients and controls. (B) Controls showed positive correlations between age and the percentages of NK and NKT cells. A-T patients showed a positive correlation between age and percentage of T cells and a negative correlation between age and percentage of NK cells.
There was no increase in CD95 expression with age in the A-T patients, possibly because expression was already very high.

CD95-mediated apoptosis can be induced by the binding of FasL expressed on activated T or NK cells to CD95; therefore, FasL expression on lymphocytes was also analyzed. There was no significant difference in the percentages of CD56dim and CD56bright NK cell subsets in A-T patients and controls (as a percentage of total NK cells). (E) CD4+ and CD8+ T cell subsets and B cell subsets in controls and A-T samples (as percentage of total CD4+ T cells, CD8+ T cells, or B cells). Mean values of all samples analyzed.

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and TEMRA T cell subsets was similar in A-T patients and controls. TUNEL and caspase-activation assays on unstimulated A-T patient and control PBMCs showed very little T cell apoptosis (<4%), and there was no difference in apoptotic sensitivity between the two groups (data not shown).

**Discussion**

The immune system phenotype of classical A-T patients illustrates the effect of complete loss of ATM protein kinase activity on the immune system. Analysis of 15 A-T patients showed that they were lymphopenic, with deficiencies in naive T and naive B cells and increased percentages of memory T cell subsets, memory B cells, NK cells, and NKT cells compared with controls. CD95 expression was increased on all lymphocyte subsets, with the exception of NK cells. 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 were reported previously (15). Therefore, the lymphopenia in A-T patients appears to be due to a deficiency in naive cells.

There were clear differences in the way that the lymphocyte phenotypes of controls and A-T patients changed with age over the 1–30-y age range. In the controls, the proportions of T and B cells did not change; however, the proportions of memory T and B cells, NK cells, and NKT cells increased and naive T and B cells decreased over the age range. 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 significant 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 those of the oldest controls than to controls of comparable age. This suggested a differential aging profile of the immune system in A-T patients compared with controls.

The change in proportion of lymphocyte subsets with age in the controls was unsurprising, because thymic involution begins in early childhood, leading to a decrease in the production of naive T cells, whereas increasing numbers of Ag encounters over time lead to oligoclonal expansion of memory T cells (5). Similarly, because the volume of hematopoietic tissue in the bone marrow decreases with age, it is likely that B cell lymphopoiesis also decreases (16), leading to a proportional reduction in naive B cells.

Despite the young age range, the stability of lymphocyte subsets over time in the A-T patients was similar to an elderly group of normal individuals (17), with a low stable output of naive T cells from the thymus and naive B cells from the bone marrow, as well as a high proportion of memory cells resulting from oligoclonal expansions in response to previously encountered Ags. In contrast to normal individuals, thymic output in A-T patients is low, even in infants (6). Therefore, it is possible that the effect of thymic involution on T cell repertoire in A-T patients is small, because it starts from a low baseline. Although there was no significant percentage increase in any individual T cell subset with age in A-T patients, it is likely that the increase in total T cells was due to

**FIGURE 4.** Lymphocyte subsets in A-T patients do not change significantly with age. Controls showed significant correlations between age and increasing memory/ decreasing naive CD4+ (A) and CD8+ (B) T cells. A-T patients showed no correlations. (C) The percentage of naive B cells decreased and memory B cells increased significantly with age in the controls. There was no correlation in A-T patients.

**FIGURE 5.** A-T patients have increased CD95 expression on all cell types, with the exception of NK cells. (A) The percentages of CD95+ T, B, and NKT cells were increased in A-T patients; there was no significant difference in the percentage of CD95+ NK cells. (B) CD95 expression was significantly increased on naive T and B cells in A-T patients. (C) In the controls, the percentages of CD95+ T, B, and NKT cells showed a positive correlation with age; however, there was no significant change with age in A-T patients.
oligoclonal expansions of memory cells in response to antigenic stimulation. However, analysis of absolute numbers of A-T T cell subsets with age, as well as TCR spectrotyping, would be required to confirm this hypothesis. Interestingly, oligoclonal expansions of memory T cells have been reported in both A-T patients (5) and the elderly (17).

Our analysis of lymphocyte subsets in A-T patients showed an immune system phenotype with striking similarities to that of healthy elderly individuals. Both groups have a naive T and B cell deficiency and increased NK cells compared with healthy young individuals (5, 10, 18), and CD95 expression on lymphocytes in vivo is increased in both A-T patients and the elderly and increases with normal aging (8, 9). Interestingly, both A-T patients (19) and the elderly normal population (20) have low thymic output and elevated levels of oxidative stress, and both normal elderly (21, 22) and some A-T patients show decreased production of specific Abs and decreased Ab responses to vaccination (4, 23). These similarities suggest an aged immune system phenotype in A-T. However, the finding of no significant change in the proportions of B and T cell subsets in A-T patients of different ages allows us to describe more precisely the classical A-T immune system as being congenitally aged rather than showing premature aging. This differs from the immune system in mild variant A-T patients, which may undergo premature aging (24).

Although there was no significant correlation, it could be argued that linear-regression analysis did suggest an age-related decrease in the proportion of naive CD8+ T cells in A-T patients (Fig. 4B). However, analysis of a larger group of A-T patients would be required to determine whether this is a genuine effect or an artifact resulting from small sample size. If the effect were genuine, it would not invalidate the hypothesis of a congenitally aged immune system in A-T, because the proportion of naive CD8+ T cells in the youngest patients is clearly reduced compared with age-matched controls. However, it may suggest that CD4+ T cells in A-T patients are more severely affected than CD8+ T cells, as their phenotype in the youngest patients is immunologically older (lower proportion of naive T cells that does not decrease with age). A-T patients and the elderly are similar in terms of CD95 expression. As CD95 is upregulated on T and B cells in response to activation (25, 26), CD95+ T cells decrease as a direct result of ageing (18). Naive CD95+ T cells are important for defense against new pathogens, so their age-related decline weakens the immune system. Similarly to the elderly, A-T patients with a CD95+ naive T cell deficiency might have an impaired ability to respond to new Ags compared with normal young individuals. However, systemic bacterial, severe viral, and opportunistic infections are rare in A-T patients (4), possibly because of their intact T cell responses (27) and the increase in NK and NKT cells.

FIGURE 6. CD127 expression and plasma IL-7 concentration is not increased in A-T patients. (A) There was no significant difference in the percentages of CD127+ T cells, B cells, or NKT cells between A-T patients and controls. However, the percentage of CD127+ NK cells was slightly reduced in A-T patients. (B) There was a positive correlation between the percentage of CD127+ T cells and age in controls, but A-T patients did not show any correlations. (C) There was no significant difference in plasma IL-7 concentration between A-T patients and controls. (D) The amount of IL-7 available per lymphocyte was significantly higher in A-T patients than in controls. (E) There was a negative correlation between plasma IL-7 concentration and age in A-T patients but no correlation in controls. IL-7 plasma concentration did not correlate with the percentages of CD4+ (F) or CD8+ (G) T cells of any subset in either A-T patients or controls.
which might compensate for the naive T cell deficiency. Although recurrent bacterial sinopulmonary infections are common in A-T (28), these may be related to neurologic decline, leading to difficulties in chewing and swallowing, increased pulmonary aspiration, and poor nutrition (29), rather than immunodeficiency.

Our analysis suggested that the lymphopenia and naive T cell deficiency of A-T patients was not due to an IL-7 or CD127 deficiency, because the expression levels of both the cytokine and its receptor were normal. However, because samples were frozen prior to phenotyping, CD127 expression could be artificially low due to the lability of IL-7Rα on cryopreserved PBMCs (30). A differential lability of the receptor on control and A-T samples cannot be ruled out but seems unlikely. Analysis of IL-7–signaling modifiers, such as Bcl-2 expression and stat-5 phosphorylation, would be required to determine whether IL-7 signal transduction is normal in A-T patients.

Interestingly, there was a correlation between IL-7 plasma concentration and CD95 expression on T cells in A-T patients. This suggests that IL-7 could be involved in the upregulation of CD95 on these cells, although further work is required to determine whether this is the case. If IL-7 does upregulate CD95 on A-T T cells, attempting to increase naive T cell proliferation using IL-7 therapy could be detrimental, because increased CD95 expression could potentially result in increased CD95-mediated apoptosis of T cells and consequent worsening of lymphopenia.

It is not possible to determine from our study whether increased CD95 expression on A-T lymphocytes in vivo 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 (31) resulting from the inability to repair DNA damage caused by reactive oxygen species, 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 (8). However, oxidative stress is also increased in the elderly (20), and this could contribute to CD95 upregulation. We found that in vitro activation with CD3 and IL-2 upregulated CD95 to a similar level of expression on T cells from four A-T patients and three age-matched young controls (19–28 y). Interestingly, Gupta and Gollapudi (10) found no difference in the CD95 expression of T cells from elderly and young donors following incubation with CD3 and IL-2. The percentages of CD3+ and IL-2–activated TUNEL+ (B), caspase 3+ (C), and caspase 8+ (D) CD4+ and CD8+ naive and central memory T cells following 24 h of incubation with CH11 were increased in A-T patients compared with controls. There was no difference in the apoptotic sensitivity of effector memory or TEMRA T cell subsets.

The primary cause of immune deficiency in A-T and in immune senescence during aging of the normal population is probably the naive cell deficiency. In A-T, this deficiency is primarily the result of a failure in the DNA damage response due to ATM loss.
resulting in defective V(D)J recombination (3) and low thymic output of naïve cells (6), which has been shown both by flow cytometry (24) and by analysis of TCR rearrangement excision circles (5). Interestingly, a reduction in DNA double-strand break repair capacity was reported in PBMCs from elderly people (32). 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. 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 the 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. Low thymic output undoubtedly has an important role in shaping the immune system of A-T patients. However, our findings of increased in vivo CD95 expression on all lymphocyte subsets, with the exception of NK cells, and the increased sensitivity to CD95-mediated apoptosis of activated naïve and central memory T cells in A-T suggest that increased CD95-mediated apoptosis may also contribute to the immune deficiency.

Disclosures

The authors have no financial conflicts of interest.

References

Supplementary Figure 1. Gating strategy. An 11 color antibody panel was developed to analyze lymphocyte subsets and CD95, FasL and CD127 expression. Example plots from a 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- 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 color antibody panel and the bottom row shows
the corresponding ‘all minus one plus isotype’ (CD95 and FasL) or ‘all minus one’ controls (CD127).

**Supplementary Figure 2.** FasL expression on A-T cells is normal. There was no significant difference in the percentage of FasL+ T cells or NK cells in A-T patients and controls.
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
% of FasL+ cells

- T cells
- NK cells

- control
- A-T