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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References

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Ataxia-telangiectasia (A-T) is a rare neurodegenerative immunodeficiency disorder caused by mutations in the ataxia telangiectasia mutated (ATM) gene, which encodes a protein kinase that has a major role in the cellular response to DNA damage. 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 homoeostasis 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 α-chain (CD127), which dimerizes with the γ-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.

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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 homoeostasis 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 α-chain (CD127), which dimerizes with the γ-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).

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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 for the controls was 19–30 y, and the mean age was 8 y 6.4 mo (102.4 mo). Both A-T patient and control groups contained samples from the 1–30 y age range with similar mean and median ages so were suitably age matched for the study. Additional blood samples were collected from four A-T patients and three healthy laboratory donors for use in apoptosis assays. The samples were age matched, with an age range of 19–28 y. Samples were collected into heparinized syringes, and PBMCs were isolated using Lymphoprep (Nycomed, Solna, Sweden). Samples intended for phenotyping analysis were frozen in liquid nitrogen until required; apoptosis assays were carried out on fresh PBMCs.

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 CD56+ and CD56- NK cells) and analysis of CD95, FasL, and CD127 expression were carried out using an 11-color panel consisting of 10 conjugated Abs (CD3-Amcyan [clone SK7; BD Biosciences, San Jose, CA], CD45-Fluoro 450 [clone HIB19; eBiosciences, San Diego, CA], CD56-PE-Cy7 [clone NCAM16.2; BD Biosciences, San Diego, CA], CD4-allophycocyanin [clone RPA-T4; BD Biosciences, San Diego, CA], CD8-Q dot 655 [clone 3B3; Invitrogen], CD45 RA-AF700 [clone HI100; BioLegend, San Diego, CA], CD27-allophycocyanin-eFluor 780 [clone OX23; eBiosciences], CD95-PE [clone DX2; Dako, Glostrop, 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 (Sigma) with 10% FCS. They were washed in cold MACS buffer (PBS, 2% BSA, 0.5% EDTA) to preserve viability and prior to staining with an Ab mixture (conjugated Abs without PI) for 20 min on ice, washed, resuspended in cold MACS buffer, and transferred to FACS tubes. PI was added to each tube immediately before analysis on an LSRII flow cytometer (BD Biosciences), according to the manufacturer’s instructions.

Apoptosis assays

PBMCs (1 × 10^6/ml) were cultured overnight in RPMI 1640 with 10% FCS. For apoptosis assays on activated T cells, PBMCs were incubated with 25 ng/ml anti-CD3 for 2 d and 100 U/ml IL-2 for an additional 3 d. The cells were washed, resuspended in RPMI 1640 medium, and incubated with 1 μg/ml anti-CD95 mAb (CH11; Upstate, Lake Placid, NY) for 24 h to induce apoptosis.

For TUNEL assays, the PBMCs were washed and incubated with an Ab mixture (CD3, CD4, CD8, CD27, CD45RA, and CD95) to label T cell subsets. TUNEL staining was carried out using the In Situ Cell Death Detection Kit, Fluorescein (Roche, Basel, Switzerland), according to the manufacturer’s instructions, and the cells were analyzed by flow cytometry.

For caspase 3- and caspase 8-activation assays, cells were harvested, and staining of activated caspases was carried out using the FAM-FMKEFMK caspase 3 and FAM-LETD-FMK caspase 8 detection kits (Cell Technology, Mountain View, CA), according to the manufacturer’s instructions. The cells were incubated with an Ab mixture (CD3, CD4, CD8, CD27, CD45RA, and CD95) to label T cell subsets prior to flow cytometry analysis.

Statistical analysis

Data were analyzed using GraphPad Prism software (GraphPad Software, San Diego, CA). The significance of differences between populations of data was analyzed using the Student t test (Mann–Whitney), with a level of significance of p < 0.05. The Spearman test (nonparametric) was used for analysis of correlations and linear regression for drawing trend lines. Correlations were considered significant if r < 0.05.

Results

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

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).

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) 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).

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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>
<thead>
<tr>
<th>Patient Designation</th>
<th>Age at Bleed</th>
<th>Sex</th>
<th>Residual ATM Protein</th>
<th>ATM Mutations</th>
<th>Mutation 1</th>
<th>Mutation 2</th>
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<tr>
<td>AT 1</td>
<td>28 y, 8 mo</td>
<td>Male</td>
<td>0</td>
<td>c.2639-7_2838+?del; p.(Gly880fs)</td>
<td>c.8206_8207delAA; p.(Asn2736fs)</td>
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<td>AT 4</td>
<td>10 y, 4 mo</td>
<td>Male</td>
<td>0</td>
<td>c.5623C &gt; T; p.(Arg1857X)</td>
<td>c.8305insA; p.(Tyr2769X)</td>
<td></td>
</tr>
<tr>
<td>AT 6</td>
<td>14 y, 11 mo</td>
<td>Male</td>
<td>0</td>
<td>c.691delAG; p.2306fs</td>
<td>c.4850T &gt; C; p.(Leu1617Pro)</td>
<td></td>
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<tr>
<td>AT 7</td>
<td>12 y, 1 mo</td>
<td>Male</td>
<td>0</td>
<td>c.5515C &gt; T; p.(Gln1839X)</td>
<td>IVS16-1G &gt; C</td>
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<tr>
<td>AT 8</td>
<td>7 y, 4 mo</td>
<td>Female</td>
<td>0</td>
<td>c.7013T &gt; C; p.(Leu2338Pro)</td>
<td>c.6056A &gt; G; p.(Tyr2019Cys)</td>
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<td>AT 9</td>
<td>2 y, 7 mo</td>
<td>Male</td>
<td>0</td>
<td>c.216_217delAG; p.(Glu736)</td>
<td>c.8300T &gt; C; p.(Leu2767Pro)</td>
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<tr>
<td>AT 10</td>
<td>4 y, 8 mo</td>
<td>Male</td>
<td>0</td>
<td>c.1402_1403delAA; p.(Lys468fs)</td>
<td>Homozygous</td>
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</tr>
<tr>
<td>AT 11</td>
<td>2 y, 11 mo</td>
<td>Male</td>
<td>Trace</td>
<td>c.7638_7646del19; p.(Arg2547_Ser2549del)</td>
<td></td>
<td></td>
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<tr>
<td>AT 12</td>
<td>16 y, 1 mo</td>
<td>Male</td>
<td>Trace</td>
<td>c.2T &gt; C; p.(Met1Thr)</td>
<td>c.9139C &gt; T; p.(Arg3047X)</td>
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<tr>
<td>AT 13</td>
<td>10 y, 6 mo</td>
<td>Male</td>
<td>10</td>
<td>c.2932T &gt; C; p.(Ser978Pro)</td>
<td>c.8395-8404del10</td>
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<tr>
<td>AT 14</td>
<td>7 y, 5 mo</td>
<td>Female</td>
<td>5</td>
<td>c.8520G &gt; C; p.(Leu2840Phe)</td>
<td>Homozygous</td>
<td></td>
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<td>AT 15</td>
<td>3 y, 11 mo</td>
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<td>c.5825C &gt; T; p.(Ala1942Val)</td>
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<tr>
<td>AT 16</td>
<td>1 y, 7 mo</td>
<td>Male</td>
<td>5</td>
<td>c.7638_7646del19; p.(Arg2547_Ser2549del)</td>
<td>c.5825C &gt; T; p.(Ala1942Val)</td>
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<td>AT 17</td>
<td>6 y, 6 mo</td>
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<td>100</td>
<td>c.1441/2delT</td>
<td>c.9022C &gt; T; p.(Arg3008Cys)</td>
<td></td>
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<tr>
<td>AT 18</td>
<td>11 y, 1 mo</td>
<td>Male</td>
<td>100</td>
<td>c.1441/2delT</td>
<td>c.9022C &gt; T; p.(Arg3008Cys)</td>
<td></td>
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<td>Apoptosis assay</td>
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<td>AT-19</td>
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<td>Female</td>
<td>0</td>
<td>c.4388delT</td>
<td>7928del83 (deletion of exon 56)</td>
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<td>0</td>
<td>c.4373delG; p.Gly1458Glut fsX15</td>
<td>c.497-10_497-4del1p.p.Glu166Asp fsX9</td>
<td></td>
</tr>
<tr>
<td>AT-21</td>
<td>20 y, 0 mo</td>
<td>Male</td>
<td>50</td>
<td>c.8096C &gt; T; p.(Pro2699eu)</td>
<td>c.2125_2250del</td>
<td></td>
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<tr>
<td>AT-22</td>
<td>26 y, 5 mo</td>
<td>Male</td>
<td>10</td>
<td>c.8098A &gt; T; p.(Lys2700X)</td>
<td>c.8506A &gt; G; p.(Met2836Val)</td>
<td></td>
</tr>
</tbody>
</table>

*Measured by Western blot.

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 CD95+ T, B, and NKT cells (Fig. 5C). This increase in CD95 expression on lymphocytes with age over the 1–30-y age range was consistent with published literature (9).
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 CD56<sup>dim</sup> and CD56<sup>bright</sup> NK cell subsets in A-T patients and controls (as a percentage of total NK cells). (E) CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets and B cell subsets in controls and A-T samples (as percentage of total CD4<sup>+</sup> or CD8<sup>+</sup> T cells, or B cells). Mean values of all samples analyzed.

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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 central and effector memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells in A-T patients compared with controls (as a percentage of total CD4<sup>+</sup> or CD8<sup>+</sup> T cells). (E) CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets and B cell subsets in controls and A-T samples (as percentage of total CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, or B cells). Mean values of all samples analyzed.

**FIGURE 3.** A-T patients have a naive T cell and B cell deficiency. The percentage of naive CD4<sup>+</sup> (A) and CD8<sup>+</sup> (B) T cells was significantly lower, and the percentages of central and effector memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells were significantly higher in A-T patients than in controls (as a percentage of total CD4<sup>+</sup> or CD8<sup>+</sup> T cells). (C) The percentage of naive B cells was reduced and the percentage of memory B cells was increased in A-T patients compared with controls (as a percentage of total B cells). (D) There was no significant difference in the percentages of CD56<sup>dim</sup> and CD56<sup>bright</sup> NK cell subsets in A-T patients and controls (as a percentage of total NK cells). (E) CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets and B cell subsets in controls and A-T samples (as percentage of total CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, or B cells). Mean values of all samples analyzed.

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**Plasma IL-7 concentration is not increased in A-T patients**

The concentration of IL-7 in the plasma samples collected during blood preparation was measured by ELISA. Despite the lymphopenia of the A-T patients, plasma IL-7 concentration was not increased compared with controls (Fig. 6C). However, because the total cell number was significantly reduced in A-T patients (p < 0.0001; Fig. 1C), the amount of IL-7 available per cell was significantly higher than in the controls (p = 0.0037; Fig. 6D). Interestingly, there was a significant negative correlation between plasma IL-7 concentration and age in A-T patients (r = -0.7464, p = 0.0014) but no correlation in the controls (Fig. 6E).

There were no significant correlations between plasma IL-7 concentration and percentage of any T cell lymphocyte subset in either A-T patients or controls (Fig. 6F, 6G).

**Plasma IL-7 concentration correlates positively with CD95 expression on A-T CD4<sup>+</sup> T cells**

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 further to the lymphopenia (12, 13). This could explain the lack of correlation between IL-7 concentration and the percentage of naive T cells in A-T patients (Fig. 6F, 6G). Therefore, correlations between IL-7 concentration and CD95 expression on T cell subsets in A-T patients and controls were analyzed.

In the controls, there were positive correlations between IL-7 concentration and the percentages of CD4<sup>+</sup>CD95<sup>+</sup> naive and central memory T cells. The A-T patients showed positive correlations between IL-7 concentration and the percentages of CD4<sup>+</sup>CD95<sup>+</sup> naive, central memory, and effector memory T cells (Fig. 7A).

The effect of increasing IL-7 concentration on CD95 expression on CD8<sup>+</sup> T cell subsets was not as clear as for CD4<sup>+</sup> T cell subsets. There was a positive correlation between IL-7 concentration and the percentages of CD4<sup>+</sup>CD95<sup>+</sup> naive and central memory CD8<sup>+</sup> T cells in the four A-T patients analyzed was increased, a significant positive correlation would be seen.

**Activated T cells from A-T patients show an increased sensitivity to CD95-mediated apoptosis**

The sensitivities of A-T and normal T cell subsets to CD95-mediated apoptosis were measured by TUNEL assay and caspase 3- and caspase 8-activation assays. PBMCs were stimulated with CD3 and IL-2 to activate the T cells prior to incubation with the apoptosis-caspase 8-activation assays. PBMCs were stimulated with CD3 and IL-2 to activate the T cells prior to incubation with the apoptosis-caspase 8-activation assays. PBMCs were stimulated with CD3 and IL-2 to activate the T cells prior to incubation with the apoptosis-caspase 8-activation assays. PBMCs were stimulated with CD3 and IL-2 to activate the T cells prior to incubation with the apoptosis-caspase 8-activation assays. PBMCs were stimulated with CD3 and IL-2 to activate the T cells prior to incubation with the apoptosis-caspase 8-activation assays. PBMCs were stimulated with CD3 and IL-2 to activate the T cells prior to incubation with the apoptosis-caspase 8-activation assays.
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](http://www.jimmunol.org/)

**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](http://www.jimmunol.org/)

**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 aging (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.
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-7R 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. (A) Plasma IL-7 concentration correlated positively with the percentages of CD4+CD95+ naive and central memory T cells in controls and CD4+CD95+ naive, central memory, and effector memory T cells in A-T patients. (B) IL-7 plasma concentration did not correlate with the percentage of CD8+CD95+ T cells of any subset in A-T patients, but did correlate positively with the percentage of CD8+CD95+ naive T cells in the controls.

**FIGURE 7.** Correlations between plasma IL-7 concentration and CD95 expression on T cells. (A) Plasma IL-7 concentration correlated positively with the percentages of CD4+CD95+ naive and central memory T cells in controls and CD4+CD95+ naive, central memory, and effector memory T cells in A-T patients. (B) IL-7 plasma concentration did not correlate with the percentage of CD8+CD95+ T cells of any subset in A-T patients, but did correlate positively with the percentage of CD8+CD95+ naive T cells in the controls.

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 IL2. 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.

**FIGURE 8.** The sensitivity of CD3- and IL-2–activated naive and central memory T cells to CD95-mediated apoptosis is increased in A-T patients. (A) There was no difference in CD95 expression on CD4+ and CD8+ T cell subsets in A-T patients and controls following activation with CD3 and IL2. 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.
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