Bone marrow transplantation restores immune system function and prevents lymphoma in Atm deficient mice.

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ABSTRACT
Ataxia-telangiectasia (A-T) is a human autosomal recessive disease caused by mutations in the gene encoding ataxia-telangiectasia mutated (ATM). A-T is characterized by progressive cerebellar degeneration, variable immunodeficiency, and a high incidence of leukemia and lymphoma. Recurrent sino-pulmonary infections secondary to immunodeficiency and hematopoietic malignancies are major causes of morbidity and mortality in A-T patients. In mice, an introduced mutation in Atm leads to a phenotype that recapitulates many of the symptoms of A-T including immune system abnormalities and susceptibility to malignancy. Here we show that the replacement of the bone marrow compartment in Atm knockout mice (Atm\(-/-\)) using a clinically relevant, non-myeloablative host conditioning regimen can be used to overcome the immune deficiencies and prevent the malignancies observed in these mice. Therefore, bone marrow transplantation may prove to be of therapeutic benefit in A-T patients.
INTRODUCTION

Ataxia telangiectasia (A-T) is a human autosomal recessive disease which affects between 1:40,000 and 1:100,000 individuals worldwide and is characterized by a wide variety of clinical manifestations \(^1\,^2\). A-T is caused by mutations in a single gene, encoding ataxia-telangiectasia-mutated (ATM). The symptoms of A-T include progressive cerebellar degeneration manifested mainly as ataxia, oculocutaneous telangiectasias, recurrent pulmonary infections due to immunodeficiency, lymphoreticular malignancies, growth retardation, incomplete sexual maturation, and premature aging of the skin and hair \(^3\). The disease is progressive and death generally occurs by the second or third decade of life. Hematologic malignancies, such as leukemia and lymphoma, can occur in as many as 40% of patients \(^4\) and together with bronchial infections are the major cause of mortality in A-T patients. Defects in the immune system include decreased IgA, IgE and IgG\(_2\) production, marked thymic hypoplasia and defects in T cell mediated responses \(^3\). Patients with A-T exhibit extreme radiation sensitivity and a decreased tolerance to chemotherapeutic agents, which prevents the use of standard therapies to treat malignancy \(^5\,^7\). Presently there is no cure for A-T, and therefore treatments are directed toward alleviating symptoms.

\( Atm^{-/-} \) mice, created by gene targeting, display many of the hallmarks of A-T in humans, including growth retardation, infertility, defects in T lymphocyte maturation, extreme sensitivity to \( \gamma \)-irradiation and a high incidence of hematologic malignancy \(^8\,^11\). Most Atm-deficient mouse strains develop malignant thymic lymphomas between 2 and 4 months of age, and generally die before 30 weeks of age \(^9\). \( Atm^{+/} \) mice also exhibit
aberrant T cell development characterized by a decrease in absolute numbers of thymocytes. In the thymus of Atm−/− mice the frequency of CD4+CD8+ double-positive and CD4+CD8− double negative thymocytes is increased, while the frequency of CD4 and CD8 single-positive mature thymocytes is decreased when compared with normal mice 9–11, suggesting that Atm may be required for transition of immature CD4+8+ double-positive thymocytes to the mature single positive stage. It has been suggested that this apparent block in T cell development may also result in a marked reduction in the number of mature CD4 and CD8 T cells in the periphery 10. In A-T patients, it has been reported that while total T cell numbers in the blood are similar to that observed in normal individuals, the frequency of naïve T cells is reduced, while the frequency of memory marker positive T cells is increased 12–14.

A-T patients exhibit both thymic hypoplasia, resulting in decreased T cell production and immunodeficiency, and hematological malignancy. These abnormalities may result from defects intrinsic to hematopoietic stem cells (HSCs), or may reflect developmental defects in the thymic microenvironment in which the progeny of these cells mature. Defects in thymic function, such as those observed in DiGeorge syndrome, are known to result in immunodeficiency (reviewed in 15). It has also been suggested that fetal thymus transplantation may reverse immunodeficiency observed in A-T by overcoming thymic hypotrophy (reviewed in 16). In addition, while thymic development of T cells is impaired in A-T patients, the function of mature T cells has been reported to be normal 12,17, suggesting that either a development-specific defect exists in T cell progenitors, or that the thymic microenvironment is unable to mediate efficient T cell maturation. We
hypothesized that if there were intrinsic defects in the HSCs of *Atm*<sup>–/–</sup> mice, the replacement of the hematopoietic compartment in these mice by bone marrow transplantation would overcome the observed hematologic abnormalities. Our results indicate that full donor-type hematopoiesis can be achieved in *Atm*<sup>–/–</sup> mice using clinically relevant host conditioning, resulting in restoration of normal immune system function. In addition, replacement of the Atm-deficient hematopoietic compartment prevents the occurrence of hematological malignancies in Atm-deficient mice. Therefore, bone marrow transplantation may prove to be of significant therapeutic benefit in A-T patients.
METHODS

Animals

Atm<sup>−/−</sup> knockout mice used as bone marrow donors for reconstitution of C3H recipients were the kind gift of Dr. Fred Alt (Children’s Hospital, Boston, MA). Mice were obtained as heterozygotes and intercrossed to obtain homozygous progeny that were genotyped by PCR according to the protocol described by Dr. Alt<sup>8</sup>. Heterozygous 129S6/SvEvTac-Atm<sup>tm1-Awb</sup> mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and used in all other experiments. Mice were genotyped by PCR as described by Jackson (Jackson Laboratories, Bar Harbour, ME). C3H mice were obtained from a colony at Massachusetts General Hospital. C3H mice are of the H-<i>2</i><sup>k</sup> haplotype and are completely MHC mismatched with 129S6/SvEvTac-Atm<sup>tm1-Awb</sup> mice, which are H-<i>2</i><sup>b</sup>. B6.CH-2<sup>bml1</sup> skin graft donors were obtained from Jackson Laboratory. All mice were housed under micro-isolator conditions in autoclaved cages and maintained on irradiated feed and autoclaved acidified drinking water. All sentinel mice housed in the same colony were viral antibody-free. Four to six week old mice were used in all experiments.

Bone marrow transplant: Conditioning by lethal irradiation was performed as described<sup>18</sup>. Mice undergoing non-myeloablative conditioning received 0.5mg anti-CD4 antibody (GK1.5<sup>19</sup>) and 1 mg anti-CD8 antibody (2.43<sup>20</sup>) seven days prior to bone marrow transplantation followed by a second dose of each antibody together with 200 mg/kg cyclophosphamide (Cytoxan, Bristol Myers Squibb, Princeton NJ) one day prior to bone marrow transplantation. Bone marrow cells were harvested from untreated donors on the
day of bone marrow transplantation, and injected intravenously into conditioned recipients.

*Skin grafts:* Tail skin grafting was performed as previously described 21.

*Flow cytometry:* Flow cytometry was performed after gating on live cells as previously described 22. Cy-chrome conjugated anti-CD4 (RM4-5), PE conjugated anti-CD8 (53-6.7), FITC conjugated anti-H-2Kb (AF6-88.5), anti-H-2Kk (36-7-5), anti-Ly6C (AL-21), anti-CD44, PE conjugated anti-B220 (RA3-6B2), anti-CD122 (TM-B1), anti-CD3 and anti-CD11b were obtained from PharMingen (San Diego, CA).

*Statistics:* All statistical calculations were performed using GraphPad Prism 2.01 software (GraphPad Software Inc., San Diego CA). The Kaplan and Meier method with a 95% confidence interval was used for the calculation of survival curves. Comparison of survival curves was performed using the log rank test. Two tailed T tests were used for all other statistics.
RESULTS

Defects in lymphocyte development observed in Atm<sup>−/−</sup> mice are stem cell intrinsic.

To determine if defects in T cell development observed in Atm<sup>−/−</sup> mice were due to defects in the ability of the thymic environment to support T cell maturation, we monitored the development of Atm<sup>−/−</sup> mutant-derived T cells in wild-type mice with a normal thymus. Wild-type C3H (H-2<sup>b</sup>) mice were lethally irradiated and reconstituted with either 10<sup>7</sup> Atm<sup>−/−</sup> (H-2<sup>b</sup>) or wild-type littermate (Atm<sup>+/+</sup>) control bone marrow cells. Both Atm<sup>−/−</sup> and Atm<sup>+/+</sup> bone marrow cells efficiently engrafted in lethally irradiated C3H recipients resulting in greater than 99% donor type cells in the blood at six weeks after bone marrow transplantation (Fig. 1). Engraftment of donor bone marrow was stable, and multi-hematopoietic lineage chimerism was maintained long-term (Fig. 1).

Analysis of T cell development in the thymus of bone marrow transplant recipients revealed defects in the ability of T cell progenitors derived from Atm<sup>−/−</sup> HSCs to develop from the double positive to the mature single positive stage. Eight weeks after bone marrow transplantation, C3H mice reconstituted with Atm<sup>−/−</sup> bone marrow exhibited a block in T cell development resulting in an increase in the frequency of CD4<sup>+</sup>CD8<sup>+</sup> double positive thymocytes (79±6%, n=8) when compared to the frequency observed in recipients of Atm<sup>+/+</sup> bone marrow (58±12%, n=8, P<0.001) (Fig. 2A). In addition, a significant decrease in the frequency of CD4 single positive cells was observed in recipients of Atm<sup>−/−</sup> bone marrow (12±3%, n=8 P<0.001) when compared with recipients of Atm<sup>+/+</sup> bone marrow (26±6%, n=8). The absolute number of CD4 T cells was also significantly decreased (0.6±0.4x10<sup>7</sup>) when compared with recipients of Atm<sup>+/+</sup> bone
marrow \((2.2\pm0.8\times10^7, P<0.001)\) (Fig. 2B). Similarly, the frequency \((4\pm1\% \text{ vs. } 13\pm5\%, n=8, P<0.001)\) (Fig. 2A) and absolute number \((0.2\pm0.1\times10^7 \text{ vs. } 1.1\pm0.6\times10^7, P<0.001)\) of CD8 single positive T cells was significantly decreased in recipients of \(Atm^{-/-}\) bone marrow when compared with recipients of \(Atm^{+/+}\) bone marrow (Fig. 2B). An increase in the frequency of CD4\(^+\)CD8\(^+\) double positive thymocytes was also observed in recipients of \(Atm^{-/-}\) bone marrow \((70\pm2\% \text{ } n=4)\) 22 weeks after bone marrow transplantation when compared to the frequency observed in recipients of \(Atm^{+/+}\) bone marrow \((66\pm2\%, n=4, P=0.03)\) (Fig. 2A). In addition, at 22 weeks, a significant decrease in the frequency of CD4 single positive cells was observed in recipients of \(Atm^{-/-}\) bone marrow \((14\pm1\%, n=4, P<0.001)\) when compared with recipients of \(Atm^{+/+}\) bone marrow \((20\pm1\%, n=4)\). The absolute number of CD4 T cells was also significantly decreased \((0.17\pm0.06\times10^7)\) when compared with recipients of \(Atm^{+/+}\) bone marrow \((0.5\pm0.1\times10^7, P<0.01)\) (Fig. 2B). Similarly, the frequency \((4\pm1\% \text{ vs. } 7\pm1\%, n=4, P<0.01)\) (Fig. 2A) and absolute number \((0.04\pm0.2 \times 10^7 \text{ vs. } 0.18\pm0.06\times10^7, P<0.01)\) of CD8 single positive T cells was significantly decreased in recipients of \(Atm^{-/-}\) bone marrow when compared with recipients of \(Atm^{+/+}\) bone marrow (Fig. 2B). These data suggest that the phenotypic differences in thymopoiesis observed in recipients of \(Atm^{-/-}\) bone marrow were not due to differences in engraftment kinetics, and that wild-type recipients of ATM deficient bone marrow cells display defects in T cell development which are similar to those observed in \(Atm^{-/-}\) mice.

\textit{ATM deficiency decreases host resistance to bone marrow engraftment, and obviates the need for irradiation.} To further analyze the ability of \(Atm^{-/-}\) mice to support engraftment
and development of wild-type HSCs and their progeny, we analyzed engraftment of $Atm^{+/+}$ bone marrow in Atm knockout mice. Because $Atm^{-/-}$ mice are extremely sensitive to irradiation\(^9\), we first set out to develop a host preparative regimen that does not require irradiation to achieve engraftment of wild-type donor bone marrow. $Atm^{-/-}$ and wild-type littermate mice were treated with a depleting dose of anti-CD4 and anti-CD8 antibodies (described in Materials and Methods) and 200 mg/kg cyclophosphamide before reconstitution with $10^8$ C3H bone marrow cells. Ten weeks after bone marrow transplantation, seven of nine $Atm^{-/-}$ recipients of C3H bone marrow exhibited full donor-type multi-hematopoietic cell lineage chimerism (Fig. 3A). In contrast, none of the wild-type littermates receiving the same preparative regimen became engrafted with C3H derived bone marrow cells (Fig. 3A). Treatment of $Atm^{-/-}$ mice with a depleting dose of anti-CD4 and anti-CD8 antibodies alone was not sufficient to establish engraftment of C3H bone marrow (data not shown). Analysis of donor-derived peripheral blood mononuclear cells (PBMC) 52 weeks after transplantation indicated that chimerism in $Atm^{-/-}$ recipients was stable (Fig. 3B), demonstrating that the $Atm^{-/-}$ hematopoietic compartment was completely replaced with C3H derived cells. No symptoms of graft-vs.-host-disease were observed. Similar results were obtained using lower bone marrow doses ($10^7$ to $5\times10^8$, data not shown).

**ATM deficient thymic microenvironment is able to support normal development of wild-type T cells.** Analysis of T cell development in $Atm^{-/-}$ mice that were engrafted with C3H bone marrow revealed that the frequency of CD4$^+$CD8$^+$ double positive thymocytes ($72\pm10\%, P=0.006, n=7$) was reduced compared to that observed in $Atm^{-/-}$ mice receiving
conditioning alone (88±6%, \( P=0.006, n=6 \)) (Fig. 4A). The frequency of CD4\(^+\)CD8\(^+\) double positive thymocytes was indistinguishable from the frequency of CD4\(^+\)CD8\(^+\) double positive thymocytes observed in C3H mice (77±2%, \( n=8, P=0.17 \)). Furthermore, we observed a significantly higher frequency of CD4\(^+\) (18±7%, \( n=7, P=0.003 \)) and CD8\(^+\) (6±2%, \( n=7, P=0.02 \)) single positive thymocytes in \( Atm^{-/-} \) mice reconstituted with C3H bone marrow when compared with \( Atm^{-/-} \) mice that received conditioning alone (6±3% and 4±2% respectively, \( n=6 \)). The frequency of single positive CD4 T cells in the thymus of \( Atm^{-/-} \) mice engrafted with C3H bone marrow was the same as that observed in untreated C3H controls (13±4%, \( n=8, P=0.15 \)). The frequency of CD8\(^+\) thymocytes (6±2%, \( n=7 \)) in \( Atm^{-/-} \) mice engrafted with C3H bone marrow was higher than the frequency of CD8\(^+\) thymocytes in C3H control mice (3±1%, \( n=8, P<0.001 \)). When total cell numbers were analyzed, \( Atm^{-/-} \) mice engrafted with C3H bone marrow had significantly more CD4 (8.0±6.5x10\(^6\) \( P=0.03 \)) and CD8 (2.6±1.4x10\(^6\) \( P=0.03 \)) single positive thymocytes than did conditioned \( Atm^{-/-} \) controls (1.7±0.8x10\(^6\) and 1.0±0.6x10\(^6\) respectively) (Fig. 4B). These data suggest that replacement of the bone marrow compartment of \( Atm \) deficient mice through transplantation overcomes abnormalities in thymocyte subset frequencies observed in \( Atm^{-/-} \) mice. In addition, these data support the hypothesis that deficiencies in T cell development caused by mutations in \( Atm \) are the result of HSC intrinsic defects rather than defects in the microenvironment in which progeny of these cells mature.

**Improved T cell development in the thymus of \( Atm^{-/-} \) mice reconstituted with C3H bone marrow transplant results in increased frequency of T cells in peripheral blood.** The
The frequency of CD4 T cells in the blood of Atm$^{-/-}$ mutant mice (15±4%, n=5) is significantly lower than in wild-type mice (30±3%, n=5, P<0.001 Fig. 5), most likely due to poor thymic output in Atm$^{-/-}$ mice, as suggested previously. In contrast, the frequency of CD4 T cells in the blood of Atm$^{-/-}$ mutant mice reconstituted with C3H bone marrow (31±5%, n=5) was the same as the frequency of CD4 T cells in the blood of Atm$^{+/+}$ controls (29±5%, n=5, P=0.5) that were treated with anti-T cell antibodies, cyclophosphamide and injected with $10^8$ C3H bone marrow cells as described above (mock BMT controls), conditioning that does not allow engraftment of donor derived cells. When compared to unmanipulated controls, the frequency of CD4 T cells in the blood of Atm$^{-/-}$ mutant mice that received C3H bone marrow was significantly higher than the frequency of CD4 T cells in the blood of untreated Atm$^{-/-}$ mutant mice (P=0.0005), but was not significantly different than the frequency of CD4 T cells found in the blood of untreated wild-type littermates (P=0.9). Similar results were observed for the frequency of CD8 T cells in the blood (Fig. 5A). The frequency of CD8 T cells in the blood of Atm$^{-/-}$ mutant mice (5±1%, n=5) is significantly lower than in wild-type mice (12±1%, n=5, P<0.001 Fig. 5). In contrast, the frequency of CD8 T cells in the blood of Atm$^{-/-}$ mutant mice reconstituted with C3H bone marrow (10±5%, n=5) was the same as the frequency of CD8 T cells in the blood of mock BMT Atm$^{+/+}$ controls (12±3%, n=5, P=0.4). When compared to unmanipulated controls, the frequency of CD8 T cells in the blood of Atm$^{-/-}$ mutant mice that received C3H bone marrow was significantly higher than the frequency of CD4 T cells in the blood of untreated Atm$^{-/-}$ mutant mice (P=0.04), but was not significantly different than the frequency of CD4 T cells found in the blood of untreated wild-type littermates (P=0.4). Thus, replacement of the Atm$^{-/-}$ hematopoietic
compartment by transplantation of wild-type bone marrow overcomes deficiencies in thymocyte development and overcomes the decrease in peripheral T cell numbers observed in \textit{Atm}^{-/-} mice.

\textit{Transplantation of wild-type bone marrow into Atm}^{-/-} mice results in normal memory T cell frequencies. A-T patients have been reported to have an increased frequency of memory T cells in their blood, and a reduced number of naïve T cells \(^{14}\). In order to determine if this was also the case in \textit{Atm}^{-/-} mutant mice, PBMC from 4-6 week old \textit{Atm}^{-/-} and wild-type littermates were analyzed by cell surface staining and flow cytometry for expression of markers expressed on memory T cells. Memory CD8 T cells are characterized by cell surface expression of CD122, CD44 and Ly6C \(^{23}\). As observed in A-T patients, the frequency of CD122\(^+\), Ly6C\(^+\) CD8 T cells was significantly higher in \textit{Atm}^{-/-} mutant mice (41±11\%, \(n=5\)) than in wild-type littermate controls (5±1\%, \(n=5\), \(P<0.001\), Table 1). Similarly, the frequency of CD44\(^{hi}\) CD8\(^+\) T cells was significantly higher in \textit{Atm}^{-/-} mice (65±8\%, \(n=5\)) than wild-type controls (24±2\%, \(n=5\), \(P<0.001\), Table 1). The frequency of CD44\(^{hi}\) CD4\(^+\) T cells in \textit{Atm}^{-/-} mice was also significantly higher (24±5\%, \(n=5\)) than in \textit{Atm}^{-/+} mice (7±2\%, \(n=5\), \(P<0.001\)). Thus, the frequency of T cells expressing memory markers is increased in \textit{Atm}^{-/-} mice when compared with normal controls, as is observed in A-T patients.

To determine if the altered memory-like T cell phenotype is overcome in mice that receive wild-type bone marrow transplants, \textit{Atm}^{-/-} mutant mice or wild-type littermate mock BMT controls were reconstituted as described with C3H bone marrow. 25 weeks
after transplantation, the frequency of memory marker positive T cells in the blood of

\( \text{Atm}^{-/-} \) mice receiving bone marrow transplants was compared to the frequency observed

in 4-6 week old \( \text{Atm}^{-/-} \) mice as well as \( \text{Atm}^{+/+} \) and mock BMT \( \text{Atm}^{+/+} \) control mice. It was

not possible to use age-matched \( \text{Atm}^{-/-} \) mutant mice, since these mice die relatively early

of thymic lymphoma\(^9\). \( \text{Atm}^{-/-} \) mice reconstituted with wild-type C3H bone marrow had a

significantly lower frequency of CD44\(^{hi}\) CD8 T cells (39±10%, \( n=5 \)) than unmanipulated

\( \text{Atm}^{-/-} \) mice (65±8%, \( n=5, P=0.0004 \)). The frequency of CD44\(^{hi}\) CD8 T cells in \( \text{Atm}^{-/-} \)

mice reconstituted with C3H bone marrow did not differ from mock BMT \( \text{Atm}^{+/+} \) control

mice (34±6%, \( n=5, P=0.4 \)). Similarly, \( \text{Atm}^{-/-} \) mice reconstituted with C3H bone marrow

had a significantly lower frequency of CD122/Ly6C double positive CD8 T cells

(10±5%, \( n=5, P<0.001 \)) than \( \text{Atm}^{-/-} \) controls (41±11%, \( n=5 \)), and the frequency of these

cells did not differ significantly from wild-type littermates that received mock BMT

(5±2%, \( n=5, P=0.06 \)). The frequency of CD44\(^{hi}\) CD4 T cells in the blood of \( \text{Atm}^{-/-} \) mice

that received C3H bone marrow transplants was also significantly lower (11±1%, \( n=5 \))

than the frequency observed in \( \text{Atm}^{-/-} \) controls (24±5%, \( n=5, P<0.001 \)). The frequency of

CD44\(^{hi}\) CD4 T cells in \( \text{Atm}^{-/-} \) mice reconstituted with C3H bone marrow did not differ

significantly from wild-type littermate mice that received mock BMT (16±6%, \( n=5, P=0.055 \)). These data suggest that frequency of CD8 and CD4 memory T cells in \( \text{Atm}^{-/-} \)

mutant mice was restored to normal following replacement of the hematopoietic

compartment by transplantation of wild-type bone marrow.

\textbf{Restoration of immune function in \( \text{Atm}^{-/-} \) mice following bone marrow transplantation.}

In order to determine whether replacement of the hematopoietic compartment in \( \text{Atm}^{-/-} \)
mice can overcome immunoincompetence, we compared the ability of \textit{Atm}^{-/-} mice reconstituted with wild-type C3H bone marrow and \textit{Atm}^{+/+} controls to reject skin allografts. Unmanipulated \textit{Atm}^{-/-} mice (\textit{H}-2\textsuperscript{b}) exhibited delayed rejection of skin allografts from allogeneic B6.CH-2\textit{bml} mice (Median survival time (MST) = 17 days, \textit{n} = 7) when compared to normal littermate \textit{Atm}^{+/+} controls (MST= 11 days, \textit{n}=5, \textit{P}=0.002, Fig. 5B). Therefore, as observed in humans, Atm deficiency leads to hyporesponsiveness to alloantigen \textsuperscript{3}. In contrast, \textit{Atm}^{-/-} mice reconstituted with wild-type C3H bone marrow were able to reject B6.CH-2\textit{bml} skin allografts with the same kinetics (MST=13, \textit{n}=5, \textit{P}=0.52) observed for \textit{Atm}^{+/+} mice receiving mock BMT (MST=12 days, \textit{n}=6, Fig. 5B). The median survival time of B6.CH-2\textit{bml} on \textit{Atm}^{-/-} mice reconstituted with wild-type C3H bone marrow was the same as that observed for unmanipulated normal littermate \textit{Atm}^{+/+} controls (\textit{P}>0.05). These data suggest that replacement of the \textit{Atm}^{-/-} hematopoietic compartment by bone marrow transplantation can overcome the immunodeficiency observed in \textit{Atm}^{-/-} mice.

\textit{Replacement of the bone marrow compartment in \textit{Atm}^{-/-} mice prevents the generation of thymic lymphoma.} It has previously been demonstrated that \textit{Atm}^{-/-} mutant mice develop fatal thymic malignancies as early as 9 weeks of age, and that by 20 weeks of age, essentially all \textit{Atm}^{-/-} mutant mice develop thymic lymphomas which prove uniformly fatal by 30 weeks of age \textsuperscript{9}. To determine if replacement of the bone marrow compartment in \textit{Atm}^{-/-} mice by bone marrow transplantation could delay or prevent the development of thymic lymphomas, 4-6 week old \textit{Atm}^{-/-} mutant mice were conditioned and reconstituted as described with \textit{10}\textsuperscript{8} C3H bone marrow cells. Animals were
monitored long-term for survival. As expected, *Atm*<sup>−/−</sup> mice that underwent conditioning alone developed thymic lymphoma and were sacrificed (MST = 11.5 weeks, range 6-22 weeks after transplant, *n*=12). In contrast, *Atm*<sup>−/−</sup> mice reconstituted with C3H bone marrow displayed prolonged survival (MST >60 weeks, *n*=21, *P*<0.001, Fig. 6). In this group, only one mouse was confirmed to have died of thymic lymphoma 13 weeks after transplantation based on post-mortem examination. We were unable to detect thymic lymphoma in the two mice in this group that died at 30 and 43 weeks after bone marrow transplantation. The remaining mice survived greater than 52 weeks after bone marrow transplantation, or were sacrificed at earlier time points without evidence of thymic lymphoma. We did not inject bone marrow cells from untreated *Atm*<sup>−/−</sup> animals into control mice, since it was possible that the transfer of malignant cells from the untreated *Atm*<sup>−/−</sup> donors could artificially accelerate deaths in the control population. These data suggest that replacement of the *Atm*<sup>−/−</sup> hematopoietic compartment by bone marrow transplantation prevents the development of thymic lymphoma.
DISCUSSION

Immunodeficiencies can arise either from defects in hematopoietic stem cells which give rise to the cells of the immune system, or to defects in the microenvironment in which immune system cells mature, such as the thymus. Our data demonstrate that $Atm^{-/-}$ bone marrow contains T cell progenitors that give rise to thymic precursors that are unable to develop normally into mature single positive T cells in a normal thymic environment. Wild-type mice reconstituted with $Atm^{-/-}$ bone marrow exhibit a block in thymocyte development similar to that observed in $Atm^{-/-}$ mice, indicating that the defects in T cell development observed in these mice are not solely the result of an abnormal thymic microenvironment. Despite the reported abnormalities in T cell development observed in the thymus of $Atm^{-/-}$ mice, progeny of wild-type bone marrow cells were able to develop normally, and restore normal T cell development in the thymus of $Atm^{-/-}$ mice. Together, these data suggest that immunodeficiencies observed in $Atm^{-/-}$ mice are attributable to intrinsic defects in progeny of bone marrow derived cells rather than the microenvironment in which these cells develop.

A-T patients have been reported to have an increased frequency of memory T cells in their blood\textsuperscript{14}. We were able to demonstrate a similar defect in $Atm^{-/-}$ mice. Bone marrow transplantation was able to restore the frequency of memory T cells in the periphery of $Atm^{-/-}$ mice to levels observed in normal controls. Furthermore, replacement of the hematopoietic compartment in $Atm^{-/-}$ mice by transplantation of wild-type bone marrow restored the frequency of mature CD4 and CD8 T cells in the peripheral blood to normal. Replacement of the $Atm^{-/-}$ hematopoietic compartment by bone marrow
transplantation was also able to overcome the functional immunodeficiency observed in
\( Atm^{-/-} \) mice, resulting in normal responses to alloantigen based on rejection of skin
allografts. These data suggest that bone marrow transplantation can be used to overcome
defects in T cell development that lead to immunodeficiency in Atm deficient mice.

In \( Atm^{-/-} \) mice, non-myeloablative conditioning consisting of T cell depletion and
administration of cyclophosphamide was sufficient to induce full donor-type chimerism.
However, the same preparative regimen failed to induce donor-type chimerism in wild-
type mice. These data suggest that barriers to bone marrow engraftment are significantly
reduced in \( Atm^{-/-} \) mice \(^{24}\). It is possible that reduced barriers to engraftment of donor-type
bone marrow may reflect a competitive disadvantage of \( Atm^{-/-} \) bone marrow as a result of
cell intrinsic defects, and as a result these cells may be unable to compete with wild-type
cells for bone marrow niches. Alternately, Atm deficiency may increase sensitivity to the
immunosuppressive effects of cyclophosphamide which in turn may allow donor bone
marrow to engraft more efficiently by reducing anti-donor immune responses more
effectively than in wild-type mice. A-T patients appear to be more susceptible to side-
effects from agents such as cyclophosphamide \(^{5}\). Previous work has suggested that host
T cell depletion is critical for efficient bone marrow engraftment \(^{22,25,26}\). In so far as \( Atm^{-/-} \)
mice exhibit reduced numbers of mature T cells, it is also possible that
immunodeficiency observed in \( Atm^{-/-} \) mice may reduce the requirement for rigorous
myeloablation to achieve donor bone marrow engraftment. Regardless of the mechanism
that allows for full replacement of the hematopoietic compartment in \( Atm^{-/-} \) mice using
relatively mild host conditioning, our results suggest that similar defects in humans may
make it possible to achieve full donor-type chimerism with minimal conditioning. Although it remains to be determined how the use of cytoreductive drugs will be tolerated in A-T patients, cyclophosphamide is routinely used in patients receiving bone marrow transplants.\textsuperscript{27-31} In addition, several clinical protocols using human-specific T cell depletion and cyclophosphamide have been shown to be tolerated in humans \textsuperscript{32}, and therefore we suggest that it may be possible to develop similar conditioning regimens that will be clinically relevant. The ability to achieve full donor chimerism using a relatively non-toxic host conditioning regimen would make bone marrow transplantation a clinically acceptable means to address hematological defects associated with A-T.

Thymic lymphomas have been shown to occur at a high frequency in Atm deficient mice, resulting in death by 30 weeks of age. The replacement of the hematopoietic system in \textit{Atm}\textsuperscript{+/−} mutant mice by bone marrow transplantation prevented the occurrence of thymic lymphomas resulting in a significantly prolonged life span that was identical to that observed for normal controls. While the role of antigen receptor gene rearrangement in the generation of lymphoma in \textit{Atm}\textsuperscript{+/−} is controversial \textsuperscript{33-35}, our results strongly suggest that in \textit{Atm}\textsuperscript{−/−} mice essentially all malignancies observed are hematological in origin, and that replacement of the Atm deficient bone marrow compartment prevents their occurrence. While the occurrence of malignancy in \textit{Atm}\textsuperscript{−/−} mice was prevented by inducing full donor-type chimerism, it is presently unclear whether full donor type chimerism is necessary in order to reduce the occurrence of lymphoma. We are currently investigating the level of donor-type chimerism needed achieve significant protection from lymphoma, and determining if solid tumors develop in these mice as they age.
A significant proportion of A-T patients suffer from recurrent pulmonary infections due to immunodeficiency. Hematologic malignancies occur in as many as 40% of patients and together with bronchial infection are the major causes of mortality in A-T patients. The demonstration that bone marrow transplantation may overcome immune system defects and the occurrence of hematological malignancy in Atm−/− mice opens up the possibility that similar therapies may eventually be able to alleviate these major causes of morbidity and mortality in A-T patients.
ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Engraftment of either Atm⁻/⁻ or Atm⁺/+ donor bone marrow in conditioned recipients results in stable multi-lineage chimerism. Lethally irradiated C3H mice were reconstituted with 10⁷ bone marrow cells from either Atm⁻/⁻ (solid line, n=6) mutant mice, or wild-type littermate controls (dashed line n=6). 6 weeks after bone marrow transplantation, PBMC were stained with donor specific anti-H-2Kb antibodies and analyzed by flow cytometry. 22 weeks after transplantation, blood cells were stained with donor specific anti-H-2Kb and lineage specific antibodies and analyzed by flow cytometry for the presence of donor-derived CD3⁺, B220⁺ or CD11b⁺ after gating. In all experiments PBMC from untreated C3H mice were used as negative controls (dotted line).

Figure 2. Defects in lymphocyte development observed in Atm⁻/⁻ mice are stem cell intrinsic. Panel A: At eight and twenty-two weeks after bone marrow transplantation, the thymi of C3H mice that had received either ATM⁻/⁻ (ATM⁻/⁻ ò C3H) or wild-type littermate control bone marrow cells (ATM⁺/+ ò C3H) were stained with anti-CD4 and CD8 antibodies and analyzed by flow cytometry. Shown is the frequency of each thymocyte subset in representative mice. Panel B: Eight weeks and twenty-two weeks after bone marrow transplantation, the total number of cells in the thymi of C3H mice that had received either Atm⁻/⁻ (white bars) or wild-type littermate control bone marrow cells (black bars) were counted, and the absolute number of each population was calculated based on the frequency of subsets as determined by flow cytometry. Shown are the combined results of three experiments. Lethally irradiated wild-type mice
reconstituted with $Atm^{+/+}$ bone marrow showed a decreased number of CD4$^+$CD8$^+$ double positive and increased number of single positive thymocytes in comparison with untreated controls, which based on our experience is most likely a result of damage caused by the radiation used to condition these animals, as has been observed in previous studies $^{36-38}$.

Figure 3. $Atm^{-/-}$ mice are more sensitive to conditioning than wild-type littermate controls. $Atm^{-/-}$ and $Atm^{+/+}$ mice were conditioned with cyclophosphamide, anti-CD4 and anti-CD8 monoclonal antibodies, and injected with $10^8$ C3H bone marrow cells. Sixteen weeks after bone marrow transplantation, PBMC were analyzed for the presence of C3H-derived $H-2K^k$ or host derived $H-2^{b}$ positive cells by flow cytometry. Panel A: Shown are representative examples of mice 16 weeks after bone marrow transplantation from one of three independent experiments. Wild-type $Atm^{+/+}$ mice did not show the presence of donor derived cells in PBMC 16 weeks after transplantation. While the majority of $Atm^{-/-}$ animals (7/9) became fully chimeric with >99% of PBMC donor derived (Chimeric), a minority (2/9) showed no donor derived cells (Non-chimeric). Panel B: Shown are representative examples of mice 52 weeks after bone marrow transplantation. Note that none of the $Atm^{-/-}$ mice which failed to become chimeric survived to 52 weeks.

Figure 4. T cell development is normal in $Atm^{-/-}$ mutant mice that receive C3H bone marrow cells. Panel A: $Atm^{-/-}$ mice were treated with anti-CD4 and anti-CD8 antibodies and cyclophosphamide, prior to receiving $10^8$ C3H bone marrow cells. 12 weeks after bone marrow transplantation, mice were sacrificed and thymi were analyzed by flow
cytometry following cell surface staining. Shown is a flow cytometry profile from representative mice. Panel B: The total number of cells in each thymus was counted, and the absolute number of each thymocyte subset was calculated based on the frequency of each subset as determined by flow cytometry. Shown are the absolute number of cells in each thymocyte subset in $Atm^{-/-}$ mice that were engrafted with C3H bone marrow (black bar) and conditioned control $Atm^{-/-}$ mice (white bar). Shown are the combined results of three experiments.

Figure 5. Restoration of lymphocyte numbers and immune function in $Atm^{-/-}$ mutant mice reconstituted with C3H bone marrow cells. Panel A: Shown are the frequency of CD4 T cells (left panel) in PBMC of $Atm^{-/-}$ mice (open squares), $Atm^{+/+}$ mice (open triangles), $Atm^{-/-}$ mice reconstituted with C3H bone marrow (open inverted triangles), and wild-type littermate controls receiving the bone marrow transplantation regimen (mock BMT, open diamonds). The frequency of CD8 T cells in PBMC of $Atm^{-/-}$ mice (solid squares), $Atm^{+/+}$ mice (solid triangles), $Atm^{-/-}$ mice receiving C3H bone marrow (solid inverted triangles), and wild-type littermate controls receiving the bone marrow transplantation regimen (mock BMT, solid diamonds) is shown in the right panel. Panel B: Left, Rejection of B6.CH-2$^{bm1}$ skin grafts by unmodified $Atm^{-/-}$ recipients (solid triangles) and $Atm^{+/+}$ littermates (solid squares). Right panel, Rejection of B6.CH-2$^{bm1}$ skin graft by $Atm^{-/-}$ mice that received C3H bone marrow transplants (open triangles) and mock BMT $Atm^{+/+}$ controls (open squares). Shown is one experiment of two.
Figure 6. *Replacement of the Atm−/− hematopoietic compartment by bone marrow transplantation prevents lymphoma.* Atm−/− mice were conditioned with cyclophosphamide and anti-CD4 and CD8 monoclonal antibodies. Mice that received C3H bone marrow (triangles) had a significantly longer lifespan than Atm−/− control mice that did not receive C3H bone marrow (squares). Shown are the combined results of 5 experiments.
**Table 1.** Expression of memory phenotype markers in peripheral blood mononuclear cells.

<table>
<thead>
<tr>
<th></th>
<th>Memory CD4 T cells</th>
<th>Memory CD8 T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% CD44+ CD4+</td>
<td>% CD44+ CD8+1</td>
</tr>
<tr>
<td>ATM-/- mutant</td>
<td>24±5</td>
<td>65±8</td>
</tr>
<tr>
<td>Wild-type littermate</td>
<td>7±2</td>
<td>24±2</td>
</tr>
<tr>
<td>C3H → ATM-/-</td>
<td>11±1</td>
<td>39±10</td>
</tr>
<tr>
<td>Mock BMT → ATM +/+</td>
<td>16±6</td>
<td>34±6</td>
</tr>
</tbody>
</table>

1Shown are the percentages of CD4 and CD8 T cells that express CD44
2Shown are the percentages of CD8 T cells that express both CD122 and Ly6C
One experiment of two is shown, n=5 for all groups.
FIGURE 1

[Graph showing the distribution of cell number and intensity for different cell populations (PBMC, CD3+, B220+, CD11b+).]
FIGURE 2

A

ATM +/+ → C3H

8 Weeks

19% 71%

2% 88%

ATM -/- → C3H

49% 86%

9% 22%

ATM +/+ → C3H

19% 68%

6% 6%

ATM -/- → C3H

13% 73%

12% 2%

B

8 weeks

22 weeks

$\times 10^7$ cells

CD4+CD8-

CD4-CD8+

CD4

CD8

$\times 10^7$ cells

CD4+CD8-

CD4-CD8+

CD4

CD8
FIGURE 3

**A**

<table>
<thead>
<tr>
<th></th>
<th>ATM&lt;sup&gt;+/−&lt;/sup&gt;</th>
<th>ATM&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAH control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chimera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-chimera</td>
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</tbody>
</table>

**B**

Cell number vs. log K<sub>6</sub>
FIGURE 4

A

Conditioned ATM -/-

C3H → ATM -/-

C3H

CD4

CD8

B

\( \times 10^7 \) cells

CD4+CD8-

CD4

CD8
FIGURE 5

A

B
FIGURE 6
Bone marrow transplantation restores immune system function and prevents lymphoma in Atm deficient mice

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