Early Defect Prethymic in Bone Marrow T Cell Progenitors in Athymic \textit{nu/nu} Mice

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Early Defect Prethymic in Bone Marrow T Cell Progenitors in
Athymic nu/nu Mice

Devavani Chatterjea-Matthes,* Marcos E. García-Ojeda,* Sussan Dejbakhsh-Jones,* Libuse Jerabek,† Markus G. Manz,† Irving L. Weissman,† and Samuel Strober²*°

nu/nu mice fail to develop a thymus and mature T cells due to a defect in the whn gene encoding a transcription factor necessary for terminal epithelial cell differentiation. We investigated whether early T cell progenitor development in the nu/nu bone marrow is also defective. We demonstrated a maturation arrest of nu/nu marrow T cell progenitors associated with a lack of pTα gene expression and a failure to give rise to mature T cells in adoptive euthymic hosts. Wild-type hemopoietic stem cells rapidly matured into functional T cell progenitors in the marrow of euthymic or thymectomized but not nu/nu hosts. We show that defects in bone marrow prethymic T cell development can also contribute to T cell deficiency in nu/nu mice. The Journal of Immunology, 2003, 171: 1207–1215.

The commitment of pluripotent hemopoietic stem cells (HSCs)⁵ to lymphoid lineages (Lin) (1) and the maturation of the progeny of stem cells into developmentally restricted progenitors is beginning to be well understood (2). Restricted progenitors that give rise to the T cell Lin develop in the mouse fetal blood, liver, or spleen before they migrate to the thymus for further maturation (3–5). The presence of multipotent or bipotent early progenitors in the fetal and newborn thymus capable of generating T as well as non-T cells suggest that commitment only to the T cell Lin may occur within the thymus (6–8). CD90⁺CD117lowCD3⁺ committed T cell progenitors (CTPs) that first described in the fetal blood and spleen as committed T cell progenitors, also express NK1.1 (9). More recently, the latter cells have been identified as bipotent progenitors of both T and NK cells that commit to the T cell Lin only after they migrate to the thymus and subsequently rearrange TCRβ chain genes (10).

We recently identified rare (~0.05%) Thy1.2highCD2⁻CD16⁻CD44⁺ committed T cell progenitors lacking mature Lin markers for T, B, NK, and myeloid/monocytic cells (TCRαβ, TCRγδ, B220, NK1.1, Mac-1) in the bone marrow of adult wild-type (WT) C57BL/6 mice that are able to rapidly generate mature T cells in vitro (11) and reconstitute only the T cell compartment of lethally irradiated congenic hosts (12). Maturation of TCRαβ T cells from the Thy1.2⁻Lin⁻CD2⁻ committed T cell progenitors (CTPs) that have not yet rearranged their TCRβ chain genes takes place both in vivo and in vitro via Thy1.2⁺Lin⁻CD2⁺ intermediate cells that are also found in freshly isolated bone marrow. Limiting dilution experiments and kinetics of reconstitution indicate that maturation can take place via both thymic and extrathymic pathways (12).

A defect in the whn gene that encodes a winged-helix transcription factor has been identified as the cause of the athymic and hairless phenotype of nu/nu mice (13, 14). The marked T cell deficiency in nu/nu mice is thought to be primarily due to the lack of a thymus (15). In this study, we investigated whether the lack of a functional whn gene also has an effect on the maturation, T cell gene expression, and function of Thy1.2⁻Lin⁻CD2⁻ progenitors isolated from the bone marrow of adult nu/nu mice. The CD90⁺CD3⁺CD117⁺ cells first described in fetal blood and spleen as T cell progenitors were identified in both WT and nu/nu mice: although the WT CD90⁺CD3⁺CD117⁺ cells generated mature T cells, the ability of CD90⁻CD3⁻CD117⁻ cells from nu/nu mice to generate mature T cells was not tested (9).

In this study, we identified Thy1.2highLin⁻CD2⁻ CTP phenotype cells in the marrow of both adult WT and nu/nu mice. However, Thy1.2highLin⁻CD2⁻ cells, abundant in the marrow of WT mice and shown to be intermediates in the T Lin by virtue of TCRβ chain gene rearrangement, were markedly reduced in the marrow of nu/nu mice. This reduction was associated with a decrease in the percentage of actively cycling cells that incorporated 5-bromo-2'-deoxyuridine (BrdU) following in vivo labeling and an increase in apoptotic cells within the Thy1.2highLin⁻CD2⁻ compartment of bone marrow in nu/nu mice as compared with WT mice.

Furthermore, the Thy1.2highLin⁻CD2⁻ cells in nu/nu mice were deficient in pTα gene expression as compared with their WT counterparts and were not able to reconstitute the T cell compartment of lethally irradiated hosts. WT HSCs injected into WT euthymic, WT thymectomized, or nu/nu irradiated adoptive hosts gave rise to Thy1.1highLin⁻CD2⁻ cells in the marrow within 2 wk after transplant. These HSC-derived Thy1.1highLin⁻CD2⁻ cells from both euthymic and thymectomized hosts were able to reconstitute the T cell compartment of secondary hosts, whereas HSC-derived Thy1.1highLin⁻CD2⁻ cells from nu/nu hosts were not. This suggested that the nu/nu bone marrow microenvironment is incapable of supporting the development of functional CTPs from WT HSCs. On the other hand, nu/nu HSCs reconstituted the T cell compartment of irradiated WT euthymic hosts.

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Materials and Methods
Experimental mice
Congenic strains of WT C57BL/6 Lys-5.2 and Lys-5.1 mice were bred and maintained in the Research Animal Facility at Stanford University School of Medicine in accordance with National Institutes of Health guidelines. Male mice aged 7–12 wk of age were purchased either from Taconic Farms (Germantown, NY) or from our colonies within the Stanford Animal Facility. C57BL/6 Lys-5.1 "nu/nu" mice were purchased from Taconic Farms. BA (Thy1.1/Lys-5.1) and HZ (Thy1.1/Lys-5.2) mice were obtained from our colonies at the Research Animal Facility at Stanford University. C57BL/6 Ly5.2 mice 6–8 wk of age were thymectomized as previously described (16). Completeness of thymectomy was confirmed by visual inspection at the time of sacrifice of thymectomized animals. TCR Vβ was confirmed by visual inspection at the time of sacrifice of thymectomized animals. TCR Vβ was confirmed by visual inspection at the time of sacrifice of thymectomized animals. TCR Vβ was confirmed by visual inspection at the time of sacrifice of thymectomized animals.

Adoptive transfer of progenitor cells and monitoring of bone marrow
Bone marrow cells were harvested from the femurs and tibias of C57BL/6 mice of various genetic backgrounds. For analysis and sorting of candidate T progenitor cells in the bone marrow, cells were first enriched by incubation with biotinylated anti-Thy1.2 mAb (5a-8; Caltag Laboratories, South San Francisco, CA), further incubation with streptavidin-conjugated immunomagnetic beads, and positive selection on MACS-MS magnetic beads. Total body irradiation (950 cGy) using 1% FCS. Immunomagnetic beads were used to enrich freshly isolated bone marrow cells from C57BL/6 mice for Thy1.2 expression and stained as described above. Following surface staining, cells were analyzed by FACS for Thy1.2 expression and stained as described above. Above sorting surface staining, cells were stained and sorted thereafter as described elsewhere (11, 12). Staining was performed in the presence of 1% FCS, anti-D10.2/Ab, and anti-CD45.2/Ab to block FeR γIIIB/γIIIB receptor. Thy1.2highLin− cells were excluded by propidium iodide staining. Light scatter gates were used to exclude red blood cells. Four-color FACS analysis and sorting were performed using a highly modified dual laser (488-nm argon and 599-nm dye lasers) FACS Vantage (BD Biosciences, Mountain View, CA). Data were analyzed with FlowJo software (version 3.0; Treestar, San Carlos, CA). PE-anti-B220 (RA3-6B2), PE-anti-NK-1.1 (PK136), PE-anti-TCRβ (H57-95), PE-anti-TCRγδ (GL3), PE-anti-Mac-1 (M1/70.15), PE-anti-CD4 (CT-CD4), FITC-anti-CD2 (RM2-5), and FITC-CD5.1/CD45.2 (RT7.2) mAbs were purchased from BD PharMingen (San Jose, CA). Streptavidin-Texas Red and biotin-CD8 (CT-CD8a) were purchased from Caltag Laboratories. Staining and analysis of the spleen, lymph nodes, bone marrow, and thymus for mature T cells and non-T cells were performed using the mAbs described above (11). For BrDU incorporation studies, mice were injected i.p. with 1 mg of BrDU (BD PharMingen) dissolved in PBS. After 4 h, animals were sacrificed and bone marrow cells were enriched for Thy1.2 expression and stained as described above. Following surface staining, cells were fixed, permeabilized, DNase treated, and stained with FITC-conjugated α-BrDU mAb according to the instructions in the BrDU Flow kit (BD PharMingen). Cells were then analyzed on a FACS Vantage without propidium iodide. For annexin staining, cells were analyzed with the annexin V-FITC/PI kit (BD PharMingen).

Purified populations of HSCs were sorted from hind limb bone marrow cells of mice after enrichment for ckit expression using MACS immunomagnetic microbeads as previously described (17). The following Abs were used for the HSC sort: biotin-anti-ckit (3C11), PE-anti-CD3 (KT31.1), PE-anti-CD4 (GK1.5), PE-anti-CD5 (53-7.3), PE-anti-CD8 (53-6.7), PE-anti-B220 (6B2), PE-anti-TER119 (F/P-4), PE-anti-Mac-1 (M1/70), PE-anti-Gr-1 (8C5), FITC-anti-Thy-1.1 (19X E5), TR-anti-CD11b (M1/70), and anti-lymphocytic-anti-ckit (2B8). In some experiments, bone marrow cells from Lys-5.2 "nu/nu" mice were enriched for HSCs by sorting Thy1.2+Lin− cells using the same mixture of Abs against cells of mature Lin described in the T cell progenitor isolation above. All staining was conducted in PBS (Life Technologies, Rockville, MD) supplemented with 1% FCS.

Adaptive transfer of progenitor cells and monitoring of transplanted mice
C57BL/6 Lys-5.2 host mice were given a single, lethal dose of total body irradiation (950 cGy) using a 200-kV (20 mA) source (Philips Medical Systems) to ablate any Lys-5.1 progenitor cells from WT or TCRβ−/−Ly5.2 donors. These hosts were injected into the lateral tail vein within 24 h of irradiation. Lymphoid tissues of recipient mice were harvested after 4 wk and monitored for their content of donor-type (Ly5.1+) cells by immunofluorescent staining. For HSC transplants, euthymic control or thymectomized C57BL/6 Lys-5.2 host mice were given a split lethal dose of total body irradiation (950 cGy) using a 200-kV (20 mA) source (Philips Medical Systems) and 1000

Thy1.1+Lys-5.1− sorted HSCs were injected i.v. HSC-transplanted hosts were sacrificed and the bone marrow was enriched for Thy1.1 and analyzed for donor HSC-derived T cell progenitors. At 8 wk posttransplant, Thy1.1+Lys-5.1− HSC-derived progenitors from WT euthymic or WT thymectomized recipients were mixed with 1 × 10^7 whole bone marrow cells from Thy1.2+Lys-5.2− WT or "nu/nu" hosts that were given a split lethal dose of 950 cGy irradiation. HSC-derived progenitors were injected along with 1 × 10^7 whole bone marrow into the lateral tail vein of WT euthymic, lethally irradiated Thy1.2+Lys-5.2− hosts. Lymphoid tissues of progenitor-recipient mice were harvested after 4 wk and monitored for their content of donor-type (Ly5.1+) cells by immunofluorescent staining. For HSC transfer into Thy1.2+Lys-5.1− "nu/nu" mice, Thy1.1+Lys-5.2− C57BL/6 donors were used as a HSC source and 1000 HSCs were injected into either Thy1.2+Lys-5.1− WT or "nu/nu" hosts that were given a split lethal dose of 950 cGy irradiation. HSC-derived progenitors were injected along with 1 × 10^7 whole bone marrow into the lateral tail vein of WT euthymic, lethally irradiated Thy1.2+Lys-5.1− hosts. Lymphoid tissues of recipient mice were harvested after 4 wk and monitored for their content of donor-type (Ly5.1+) cells by immunofluorescent staining.

RT-PCR
Total RNA was extracted from sorted progenitor cells, sorted spleen T cells, and sorted CD4+CD8+ thymocytes from C57BL/6 Lys-5.1 mice using the RNeasy Mini kit (Qiagen, Santa Clarita, CA). RNA was then reverse transcribed using random hexamer primers followed by PCR amplification. Primers for recombination-activating gene (RAG)-1 and RAG-2 and conditions for the PCR amplification have been previously described (18). Primers for TCRβ gene amplification were designed based on the GenBank accession number U16958 and had the following sequences: nested forward, 5'-GGCTCCACCCACACATCACTGC3'; internal forward, 5'-GTGCTGGTTGTTGTCGCTG3'; reverse, 5'-GGGAGGAC AGTTCACAGCATC3'; and nested reverse, 5'-CACATTACAAAGGGG AGATCAC3'.

Genomic PCR
Single-cell suspensions of sorted Thy1.2+Lin−CD2+ progenitors and Thy1.2+Lin−CD2+ intermediate cells from WT and "nu/nu" bone marrow as well as sorted WT spleen T cells were centrifuged, lysed in buffer containing 9.5 M urea, 0.71 M 2-ME, and 2% Triton X-100. Complete lysis was ensured by successive freeze-thaw cycles, after which the lysate was centrifuged, digested with proteinase K, and the aqueous phase extracted with phenol-chloroform. The RNA was precipitated with 3 M sodium acetate and 100% ethanol, dried and resuspended in TE buffer, and analyzed by PCR for TCR βγ gene rearrangement. TCR βγ gene rearrangements were detected using a nested PCR technique. Primers specific for the β2-Jβ-32 intronic region were (first round, 5'-TCCTGGCTTGGCAGAG AGCCG-3' and second round, 5'-TGAGACAGCTCTCTTCTACTAC-3') previously described. Primers specific for consensus Vβ8 exon regions were designed based on sequences found in GenBank accession number AE000622 and had the following sequences: first round, 5'-CACATG GAAGGCTCAGCTA-3' and second round, 5'-CATGACTGTGTTACG GCAGG-3'. Co primers were designed based on sequences in GenBank accession number M64239 and had the following sequences: first round forward, 5'-AGGAGGACAGCATTCTCATGATGG-3' and second round forward, 5'-CTTCTAACATCTGACTC3'; first round reverse, 5'-CACCTCCCT CTTCCTCTCAT3'; and second round reverse, 5'-GACCCGTAAGACT CCTTATTG-3'.

Results
Thy1.2+Lin−CD2+ intermediate cells are markedly reduced in the bone marrow of athymic nu/nu mice
We enriched freshly isolated bone marrow cells from C57BL/6 WT, recombination-defective RAG-2−/−, T cell-deficient TCRβ−/−, and T cell-deficient athymic nu/nu mice for Thy1.2+ cells using MACS immunomagnetic microbeads. The enriched cells were analyzed for Thy1.2 vs TCRβ−/−, T cell-deficient TCRβ−/−, and T cell-deficient athymic nu/nu mice for Thy1.2+ cells using MACS immunomagnetic microbeads. The enriched cells were analyzed for Thy1.2 vs TCRβ−/−, T cell-deficient TCRβ−/−, and T cell-deficient athymic nu/nu mice for Thy1.2+ cells using MACS immunomagnetic microbeads. The enriched cells were analyzed for Thy1.2 vs TCRβ−/−, T cell-deficient TCRβ−/−, and T cell-deficient athymic nu/nu mice for Thy1.2+ cells using MACS immunomagnetic microbeads. The enriched cells were analyzed for Thy1.2 vs TCRβ−/−, T cell-deficient TCRβ−/−, and T cell-deficient athymic nu/nu mice for Thy1.2+ cells using MACS immunomagnetic microbeads.
CD2− cells were 38.2%. In contrast, 98.6 and 99.4% of Thy1.2highLin− cells from RAG-2−/− and nu/nu marrow, respectively, were CD2− and <1% were CD2+. The Thy1.2high Lin−CD2− cells from WT, RAG-2−/−, and nu/nu mice were further analyzed for CD16, CD44, and Sca1 expression (Fig. 1, D–F). The CD2− cells were almost all CD44high and expressed intermediate and high levels of CD16 and Sca1. Thus, phenotypic differences were clearly observed in the WT, RAG-2−/−, and nu/nu mice among the Thy1.2highLin− cells in terms of CD2 surface expression. However, the Thy1.2highLin−CD2− cells showed similar profiles of CD44, CD16, and Sca1 expression.

The mean percentage of the Thy1.2highLin− subsets as well as the absolute numbers in the hind limb marrow are shown in Table I. The mean absolute numbers of Thy1.2highLin−CD2− and Thy1.2highLin−CD2− cells from WT mice were 36.7 × 10^3 and 17.7 × 10^3, respectively, and from TCRα−/− mice were 72.5 × 10^3 and 50 × 10^3, respectively (Table I). The mean numbers of CD2− and CD2+ cells from RAG-2−/− mice were 64.5 × 10^3 and 0.72 × 10^3, respectively. The CD2− and CD2+ cell populations isolated from nu/nu mice yielded 66 × 10^3 and 2.75 × 10^3 cells, respectively. The mean absolute numbers of Thy1.2highLin−CD2− cells were reduced ~7- to 20-fold in RAG-2−/− and nu/nu mice (Table I).

**FIGURE 1.** Thy1.2highLin− CD2− cells are reduced in nu/nu bone marrow. Immunomagnetic bead-enriched Thy1.2+ BM cells from C57BL/6 WT, RAG-2−/−, TCRα−/−, and nu/nu mice were stained for Thy1.2 vs TCRαβ markers (A) and for Thy1.2 vs mature Lin markers (TCRαβ, TCRγδ, B220, NK1.1, and Mac-1; B). Thy1.2highLin− cells were gated and analyzed for Thy1.2 vs CD2 (C). Thy1.2highLin− cells from C57BL/6 WT, RAG-2−/−, and nu/nu mice were gated and analyzed for CD2 vs CD44 (D), CD16 (E) or Sca1 (F), respectively.

### Table I. Mean percentages (± SE) and absolute numbers (± SE) of CD2− and CD2+ cells in the Thy1.2Lin− fraction of bone marrow cells of C57BL/6 mice.

<table>
<thead>
<tr>
<th>Mice (n = 4)</th>
<th>CD2−</th>
<th>CD2+</th>
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<tr>
<td></td>
<td>%</td>
<td>Absolute number</td>
</tr>
<tr>
<td>WT</td>
<td>68 ± 5</td>
<td>36.7 ± 0.8 × 10^3</td>
</tr>
<tr>
<td>TCRα−/−</td>
<td>59 ± 2</td>
<td>72.5 ± 0.3 × 10^3</td>
</tr>
<tr>
<td>RAG-2−/−</td>
<td>98.4 ± 0.3</td>
<td>64.5 ± 0.7 × 10^3</td>
</tr>
<tr>
<td>nu/nu</td>
<td>96 ± 1</td>
<td>66 ± 1 × 10^3</td>
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*Two femurs and two tibias were harvested from each animal.*

Since the expression of CD2 on developing T cell progenitors during thymic maturation is associated with the rearrangement of the TCRβ chain, we assayed the Thy1.2highLin−CD2−CTPs and the Thy1.2highLin−CD2− intermediate cells for TCRβ gene rearrangement. Vβ8 was chosen for analysis because it is the most abundant Vβ gene in the C57BL/6 and BALB/c strains of mice. The sorted Thy1.2highLin−CD2− CTPs failed to show gene rearrangement as determined by a PCR analysis of amplified genomic DNA (Fig. 2A) using primers chosen to hybridize with the Vβ8 gene segment and with an intron located between the β2 gene cluster and the Cβ2 exon. In contrast, the Thy1.2highLin−CD2− intermediate cells showed a clear band of amplified DNA of appropriate size. Control WT sorted spleen T cells also showed a band of appropriate size indicative of Vβ8 rearrangement. Genomic DNA from control Tk+L cells showed no evidence of Vβ8 rearrangement. Sorted CD2− CTPs from nu/nu bone marrow failed to show evidence of Vβ8 rearrangement as well (Fig. 2B). All samples had adequate DNA template as shown by abundant amplification of the Cα exon 1 gene segment.

nu/nu bone marrow Thy1.2highLin−CD2− progenitors do not express pTα mRNA

We examined the sorted Thy1.2highLin−CD2− CTPs from WT bone marrow for mRNA expression of T cell developmental genes by RT-PCR analysis using specific primers for the RAG-1, RAG-2, and pTα genes, all of which are expressed during early T cell maturation in the thymus (19, 20). Sorted TCRαβ+ T cells in the WT spleen expressed none of these genes, whereas all three were expressed in sorted CD4−CD8− cells from the WT thymus (Fig. 2C). The sorted Thy1.2highLin−CD2− CTPs in the WT marrow expressed all three genes (Fig. 2C), including the gene encoding pTα. In contrast, Thy1.2highLin−CD2− CTP phenotype cells sorted from the marrow of nu/nu mice showed RAG-1 and RAG-2 expression but did not express pTα mRNA (Fig. 2C). These results...
Further indicated that nu/nu T cell progenitors in the bone marrow are abnormal: they lacked Thy1.2highLin CD2− intermediate cells with TCR Vβ rearrangements and were also deficient in pTα gene expression.

Reduction in cycling cells and increased apoptotic cells in Thy1.2highLin CD2− progenitors in nu/nu compared with the WT marrow

Further analysis of the CTPs derived from the bone marrow of nu/nu mice revealed a difference in the BrdU uptake profile of these progenitor cells. Whereas in the WT mice, the Thy1.2highLin CD2− CTPs showed ~34% BrdU uptake after 4 h of labeling following i.p. injection of 1 mg BrdU, only 9% of nu/nu CTPs showed BrdU uptake after a similar labeling regimen (Fig. 3, A and B).

Since the CD2− intermediate cells are markedly reduced in the bone marrow of nu/nu mice but the Thy1.2highLin CD2− CTPs are present at levels comparable to the WT, we examined whether these CTPs were apoptotic, i.e., were undergoing programmed cell death in the absence of necessary signals for maturation and survival. Of nu/nu CTPs, 25.9% were PI− annexin−, indicating that these cells were apoptotic (Fig. 3C). In contrast, only 7% of WT CTP were PI− annexin− (Fig. 3D).

nu/nu Thy1.2highLin CD2− progenitors fail to reconstitute the T cell compartment of congenic adoptive hosts

We sorted Thy1.2highLin CD2− CTP phenotype cells from C57BL/6 WT or nu/nu mice, with the Ly5.1 congenic marker, to >99% purity, and injected 500 of these cells along with 1 × 10^6 congenic C57BL/6 Ly5.2 RAG-2−/− unfractionated bone marrow cells i.v. into two groups of lethally irradiated (950 cGy) WT C57BL/6 Ly5.2 hosts. Four weeks after the injection of WT Thy1.2− Lin− CD2− cells, hosts were sacrificed and cells from spleen, thymus, and axillary and mesenteric lymph nodes were stained and analyzed for individual Lin vs Ly5.1 markers. The lymph nodes of these hosts contained donor-derived cells that were almost all CD2+ (Fig. 4B). The latter cells contained CD2+ TCRαβ+ T cells and CD2− TCRαβ− intermediate phenotype cells (Fig. 4A), but few or no B220+, NK1.1+, Gr-1+, or Mac-1+ cells characteristic of non-T cell Lin were observed among donor Ly5.1+ cells (Fig. 4, C–F). The gated Ly5.1+ TCRαβ+ axillary lymph node cells were almost all CD4+ or CD8+ single-positive T cells (Fig. 4G). The donor-derived intermediate phenotype cells were also found in the spleen and bone marrow of the adoptive hosts along with Ly5.1+ TCRαβ+CD2+ T cells with little or no expression of non-T cell markers (data not shown). The TCRαβ+ cells were unlikely to have derived from TCR αβ+ contaminants as previous studies showed that 10,000 sorted TCRαβ+ T cells from the WT bone marrow injected into lethally irradiated congenic WT hosts along with 1 × 10^6 RAG-2−/− marrow cells were barely detectable in the lymph nodes, spleen, and bone marrow of these hosts after 4 wk (12). We also confirmed this in the current study and <1% of donor-type TCRαβ+ T cells were observed after injecting 500 or 1 × 10^4 sorted marrow TCRαβ+ T cells (data not shown).
Comparisons of WT hosts injected with 500 sorted Thy1.2<sup>high</sup>Lin<sup>−</sup>CD2<sup>−</sup> cells from WT but not nu/nu mice provide T cell Lin-specific reconstitution of congenic hosts following adoptive transfer. Thy1.2<sup>high</sup>Lin<sup>−</sup>CD2<sup>−</sup> cells were sorted to >97% purity from Thy1.2-enriched BM cells from Ly5.1 C57BL/6 WT and nu/nu mice. Lymph node cells from Ly5.2 hosts injected with 500 Ly5.1 WT C57BL/6 Thy1.2<sup>high</sup>Lin<sup>−</sup>CD2<sup>−</sup> cells and 1 × 10<sup>6</sup> Ly5.2 RAG-2<sup>−/−</sup> BM cells were stained for Ly5.1 vs single Lin markers including TCRαβ (A), CD2 (B), B220 (C), Gr-1 (D), Mac-1 (E), and NK1.1 (F) to analyze Lin specificity of T cell reconstitution. Gated Ly5.1<sup>+</sup>TCRαβ<sup>+</sup> cells from the lymph nodes were analyzed for CD4 vs CD8 (G). Cells from the spleen, mesenteric lymph nodes, and thymus of lethally irradiated Ly5.2 hosts 4 wk after adoptive transfer were analyzed for Ly5.1 vs TCRαβ markers.

Table II. Mean absolute numbers (±SE) of donor-derived T cells in the spleen, axillary lymph nodes, and thymus 4 wk after injection of 500 congenic WT and nu/nu Thy<sup>−</sup>1<sup>Lin</sup> CD2<sup>−</sup> cells

<table>
<thead>
<tr>
<th>Animals (n = 5)</th>
<th>Spleen</th>
<th>Lymph Nodes</th>
<th>Thymus</th>
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<tr>
<td>WT Thy&lt;sup&gt;−&lt;/sup&gt;1&lt;sup&gt;Lin&lt;/sup&gt; CD2&lt;sup&gt;−&lt;/sup&gt;</td>
<td>21 ± 10 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>14 ± 7 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>144 ± 99 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>nu/nu Thy&lt;sup&gt;−&lt;/sup&gt;1&lt;sup&gt;Lin&lt;/sup&gt; CD2&lt;sup&gt;−&lt;/sup&gt;</td>
<td>7 ± 6 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>2.2 ± 0.5 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>3 ± 2 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ratio of WT:nu/nu T cells</td>
<td>300:1</td>
<td>700:1</td>
<td>4800:1</td>
</tr>
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</table>

In contrast, the mean absolute numbers in the spleen, lymph nodes, and thymus from animals reconstituted with 500 Thy1.2<sup>high</sup>Lin<sup>−</sup>CD2<sup>−</sup> cells were 7 (±6) × 10<sup>4</sup>, 2.2 (±0.5) × 10<sup>2</sup>, and 3 (±2) × 10<sup>2</sup> progenitor-derived TCRαβ<sup>+</sup> cells, respectively (Table II).

To establish that the defect in T cell progenitors occurred at the level of the nu/nu Thy1.2<sup>high</sup>Lin<sup>−</sup>CD2<sup>−</sup> T progenitor cell population and not at the level of the HSC in nu/nu mice, we injected...
10,000 HSC-enriched Thy<sup>low</sup>Lin<sup>−</sup> cells from nu/nu marrow into lethally irradiated WT congenic adoptive hosts. Four weeks later, these cells generated 4% T cells in the spleen, 7.3% T cells in the mesenteric lymph node, and 17.8% T cells in the thymus (Fig. 4K). In addition to reconstituting the T cell Lin, these nu/nu HSCs also gave rise to cells of all other mature blood Lin (data not shown).

**Thy1.1<sup>high</sup>Lin<sup>−</sup> CD2<sup>−</sup> functional CTPs develop from injected HSCs in WT euthymic or WT thymectomized mice, but only nonfunctional CTP phenotype cells develop from HSC injected into nu/nu hosts**

To determine whether HSCs can give rise to CTPs in WT euthymic, WT thymectomized, or nu/nu hosts, we double-sorted Thy1.1<sup>high</sup> cKit<sup>+</sup> Sca1<sup>−</sup> Lin<sup>−</sup> cells to 99.9% purity as previously described (21) and modified (17). We injected 1000 Thy1.1/Ly5.1 HSCs into either WT euthymic or WT thymectomized Thy1.2<sup>−</sup>Ly5.2<sup>−</sup> hosts. Since only Thy1.2/Ly5.1 nu/nu mice were available, we performed a parallel set of experiments where 1000 Thy1.1/Ly5.2 HSCs were sorted and injected into either Thy1.2/Ly5.1 nu/nu hosts or WT hosts as shown in the experimental scheme in Fig. 5.

HSC-derived Thy1.1<sup>high</sup>Lin<sup>−</sup> cells were detected in the bone marrow of WT euthymic, WT thymectomized, and nu/nu primary hosts 8 wk later as shown in Fig. 5, A, E, and I, respectively. WT hosts had 3.5% Thy1.1<sup>high</sup>Lin<sup>−</sup> cells while thymectomized and nu/nu hosts had 5.8 and 1.2% Thy1.1<sup>high</sup>Lin<sup>−</sup> cells, respectively. Gated Thy1.1<sup>high</sup>Lin<sup>−</sup> cells from all three hosts were further analyzed for CD2 surface expression. WT euthymic hosts had 89% CD2<sup>−</sup> cells within the bone marrow Thy1.1<sup>high</sup>Lin<sup>−</sup> subpopulation while thymectomized and nu/nu hosts had 95.4 and 95.3% CD2<sup>−</sup> cells within the Thy1.1<sup>high</sup>Lin<sup>−</sup> subpopulation, respectively (Fig. 5, B, F, and J). The Thy1.1<sup>high</sup>Lin<sup>−</sup>CD2<sup>−</sup> progenitors were sorted to purity, and 500 of these cells were injected along with 1 × 10<sup>6</sup> RAG<sup>−/−</sup> whole bone marrow into groups of lethally irradiated WT euthymic secondary hosts (see experimental scheme, Fig. 5). Lymphoid tissues of secondary hosts from all three groups were harvested 4 wk later and analyzed for progenitor (HSC)-derived TCRαβ T cell outgrowth.

Analysis of spleen cells of secondary hosts reconstituted with 500 HSC-derived Thy1.1<sup>−</sup>Lin<sup>−</sup>CD2<sup>−</sup> CTP from WT euthymic primary hosts showed 4.8% progenitor-derived TCRαβ<sup>+</sup> T cells 4 wk following transfer (Fig. 5C). These T cells consisted of both CD4 and CD8 single-positive T cells (Fig. 5D). Analysis of spleens of secondary hosts reconstituted with 500 progenitors from WT thymectomized primary hosts showed 6.8% (Fig. 5G) progenitor-derived TCRαβ<sup>+</sup> T cells consisting of CD4 and CD8 single-positive cells (Fig. 5H). In both of these groups of secondary hosts, robust reconstitution by the CTPs was seen in the axillary lymph nodes also (Table III). Secondary hosts injected with Thy1.1<sup>high</sup>Lin<sup>−</sup>CD2<sup>−</sup> CTPs derived from nu/nu primary hosts had fewer than 0.1% progenitor-derived T cells in the spleen (Fig. 5K). Little or no progenitor-derived T cell reconstitution was observed either in the bone marrow, lymph nodes, or thymus of these secondary hosts at this time point or in all tissues analyzed as late as 8–10 wk posttransplant (data not shown).

Table III shows the mean absolute numbers of progenitor-derived T cells in the spleens and lymph nodes of secondary hosts. The spleens of secondary hosts reconstituted with progenitors derived from WT euthymic primary hosts had 700 × 10<sup>3</sup> CTP-derived T cells after 4 wk while secondary hosts reconstituted with progenitors from WT thymectomized primary hosts had 385 × 10<sup>4</sup> CTP-derived T cells. The latter mice had 17 × 10<sup>5</sup> CTP-derived T cells in the lymph nodes while the former had 31 × 10<sup>4</sup> T cells (Table III). Secondary hosts reconstituted with progenitors derived from nu/nu primary hosts had markedly reduced T cell reconstitution with 0.05 × 10<sup>4</sup> CTP-derived T cells in the spleen and 0.01 × 10<sup>4</sup> CTP-derived T cells in the lymph nodes (Table III).

**Discussion**

We identified intermediate cells with the Thy1.2<sup>high</sup>Lin<sup>−</sup>CD2<sup>−</sup> phenotype in the pathway of early T cell development in the bone marrow of WT mice. The latter cells had rearranged the TCRβ chain gene segments but did not express TCRαβ on the cell surface. Thy1.2<sup>high</sup>Lin<sup>−</sup>CD2<sup>−</sup> cell development appeared to be dependent on TCRβ gene rearrangement, since cells with this phenotype were found in the marrow of RAG<sup>−/−</sup> mice, but not in the marrow of RAG<sup>2−/−</sup> mice. Sorted Thy1.2<sup>high</sup>Lin<sup>−</sup>CD2<sup>−</sup> cells were able to give rise to mature TCRαβ<sup>+</sup> T cells but not other Lin in vitro (11) and were able to reconstitute the T cell compartment of lethally irradiated congenic hosts (D. Chatterjea-Mathes, unpublished observations) further establishing that these cells are intermediates along the path of mature T cell development. Surprisingly, these Thy1.2<sup>high</sup>Lin<sup>−</sup>CD2<sup>−</sup> cells were markedly reduced in the marrow of nu/nu mice that fail to develop the thymus due to a deficiency in the wth gene, a transcription factor that is critical to the late stage maturation of epithelial cells (22, 23). However, this maturation arrest is not caused by the absence of mature T cells in the marrow per se because both CD2<sup>−</sup> and CD2<sup>−</sup> T cell populations were present in abundance in the marrow of T cell-deficient TCRα<sup>−/−</sup> mice. Therefore, it is unlikely that mature T cells derived from the thymus play an important role in facilitating the transition between the CD2<sup>−</sup> and CD2<sup>−</sup> T cell progenitors.

Earlier progenitor cells in the T cell development pathway in the bone marrow expressing the Thy1.2<sup>high</sup>Lin<sup>−</sup>CD2<sup>−</sup> phenotype were present in abundance in WT, RAG<sup>2−/−</sup>, and nu/nu mice and were phenotypically identical based on their expression of CD44, CD16, and Sca1 surface Ags. Although these earlier progenitors expressed the RAG-1, RAG-2, and pTα genes in WT mice, they expressed the RAG-1 and RAG-2 but not the pTα gene in the nu/nu mice. Since the Thy1.2<sup>high</sup>Lin<sup>−</sup>CD2<sup>−</sup> cells had been shown previously to rapidly generate Thy1.2<sup>high</sup>Lin<sup>−</sup>CD2<sup>−</sup> T cells in vitro (11), the current results suggested a maturation arrest at the Thy1.2<sup>−</sup>Lin<sup>−</sup>CD2<sup>−</sup> stage associated with the deficiency in the expression of the pTα gene. The pTα gene deficiency may reflect a more general deficiency in the marrow T cell progenitors due to an altered microenvironment. However, we did not examine the expression of a wide panel of T cell Lin-specific genes for deficiencies in the current report, since this is the subject of continuing studies. Abnormalities in signals from the microenvironment, as well as intracellular signaling via pTα, may have contributed to the deficiencies in early T cell maturation and progression to TCRαβ<sup>+</sup> T cells (24). We also observed that after 4 h of labeling by i.p. injection, only ~10% of nu/nu CTPs were positive for BrdU compared with ~30% in the WT, indicating that the proportion of nu/nu CTP in the active cell cycle was reduced by one-third compared with the WT CTPs. nu/nu CTPs also appeared to contain a larger percentage of apoptotic cells as measured by annexin V staining compared with the WT CTP population. This suggests the lack of an appropriate survival/proliferation signal delivered by the marrow microenvironment or the lack of an ability to respond to such signals.

Further evidence that the Thy1.2<sup>high</sup>Lin<sup>−</sup>CD2<sup>−</sup> progenitor cells from the nu/nu mice were defective as compared with Thy1.2<sup>high</sup>Lin<sup>−</sup>CD2<sup>−</sup> progenitors from WT mice was obtained in adoptive transfer studies. Whereas the WT progenitors generated CD4<sup>+</sup> and CD8<sup>+</sup> TCRαβ<sup>+</sup> T cells in the spleen, lymph nodes, and thymus of irradiated WT Ly5 congenic hosts, the nu/nu progenitors generated 1000-fold fewer TCRαβ<sup>+</sup> T cells. The markedly
reduced number of Thy1.2highLin−CD2− cells, absence of pTα expression, and lack of function in the adoptive transfer experiments indicated that the Thy1.2Lin−CD2− progenitor cells in the nu/nu marrow were defective. These results also corroborated an observation in our laboratory that only WT TCRβ+ T cells were produced within 48 h in a mixed culture of T cell-depleted marrow from WT and nu/nu mice in vitro (S. Dejbakhsh-Jones, unpublished observations). Our previous studies showed that mature T cells added to Thy1.2highLin−CD2− progenitors markedly inhibit progenitor maturation in vitro as well as in adoptive irradiated hosts (11, 12). The latter progenitors have similar robust maturation in both irradiated WT and T cell-deficient scid hosts (data not shown).
Table III. Mean absolute numbers (± SE) of T cells generated in spleen and axillary lymph nodes of lethally irradiated secondary hosts

<table>
<thead>
<tr>
<th>Donor Cell Source</th>
<th>Spleen&lt;sup&gt;a&lt;/sup&gt; (donor T × 10&lt;sup&gt;5&lt;/sup&gt;)</th>
<th>Lymph Nodes&lt;sup&gt;a&lt;/sup&gt; (donor T × 10&lt;sup&gt;5&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTP (HSC/euthymic 1° host)</td>
<td>700 ± 191</td>
<td>31 ± 8</td>
</tr>
<tr>
<td>CTP (HSC/thymex. 1° host)</td>
<td>385 ± 110</td>
<td>17 ± 6</td>
</tr>
<tr>
<td>CTP (HSC/nu/nu 1° host)</td>
<td>0.05 ± 0.02</td>
<td>0.01 ± 0.05</td>
</tr>
</tbody>
</table>

<sup>a</sup> Three animals were used for each tissue at 4 wk after transplant. 
<sup>b</sup> Host, primary host. 'Thy1.2highLin' bone marrow progenitors in WT mice are derived from earlier uncommitted progenitors that must migrate to the thymus for commitment and then return to the marrow for further maturation. This is in agreement with our previous studies which indicated that WT HSCs transplanted in thymectomized irradiated WT mice are impaired in the process of development into functional CTPs, resulting in a block in their maturation into T lymphocytes thereafter. There may be a markedly slowed maturation of CTP without a complete block since we observed higher levels of CD2<sup>−</sup> intermediate cells in nu/nu mice ages 18 mo to 2 years (data not shown).

To test the above hypothesis, we performed adoptive transfer experiments and found that sorted WT HSCs transplanted into lethally irradiated WT euthymic or WT thymectomized secondary hosts gave rise to cells bearing the Thy1.1<sup>highLin</sup> CD2<sup>−</sup> phenotype. The latter cells harvested 8 wk later generated mature TCRαβ<sup>+</sup> cells in the lymphoid tissues of secondary adoptive hosts. WT HSC were also transplanted into lethally irradiated nu/nu primary host mice; Thy1.1<sup>highLin</sup> CD2<sup>−</sup> cells also developed; however, these cells failed to give rise to mature TCRαβ<sup>+</sup> T cells in secondary hosts. That HSCs could give rise to functional CTPs in thymectomized mice rules out the notion that the Thy1.2<sup>highLin</sup> CD2<sup>−</sup> progenitors in WT mice are derived from earlier uncommitted progenitors that must migrate to the thymus for commitment and then return to the marrow for further maturation. This is in agreement with our previous studies which indicated that WT HSCs transplanted in thymectomized irradiated WT mice differentiated within the bone marrow into competent T cell progenitors that rapidly matured into TCRαβ<sup>+</sup> T cells in vitro (16). HSCs transplanted into both WT euthymic and thymectomized mice gave rise to T cells in the bone marrow, lymph nodes, and spleen, but HSCs transplanted into nu/nu animals failed to give rise to detectable levels of T cells in the lymphoid compartment (D. Chatterjea-Matthes, unpublished observations). This observation also supports our finding that the nu/nu bone marrow microenvironment is not permissive for the outgrowth of extrathymic T cells from WT HSCs.

The \( \text{w} \text{h} \text{n} \) gene has been shown to be expressed in epithelial cells in mice (22, 23) and there is no report of \( \text{w} \text{h} \text{n} \) expression in lymphoid cells. Therefore, it is unlikely that the defect in the nu/nu Thy1.2<sup>highLin</sup> CD2<sup>−</sup> progenitors is due to the expression of the abnormal \( \text{w} \text{h} \text{n} \) gene in the progenitors themselves. The failure of epithelial cell development in the thymic anlage during embryogenesis of nu/nu mice results in the absence of lymphoid cell development in the gland (15). It has been shown that formation of the thymic epithelial primordium before the entry of lymphocyte progenitors does not require the activity of the \( \text{w} \text{h} \text{n} \) gene product. However, subsequent differentiation of primitive precursor cells into medullary, cortical, and subcapsular epithelium did require activity of the \( \text{w} \text{h} \text{n} \) gene (27). The development of a functional thymus has been shown to be divided into genetically separable epithelial-mesenchymal and subsequent lympho-stromal interactions (27). Cortical and medullary epithelial cells that respectively support progressive stages of maturation of the developing murine thymocytes have been identified (28, 29) and long been known to play critical roles in the development, acquisition of tolerance, MHC restriction, and immune function of these developing T cells (30). Therefore, a likely explanation of the progenitor defect seen in the bone marrow of nu/nu mice is that epithelial cells in the bone marrow microenvironment of nu/nu mice express the abnormal \( \text{w} \text{h} \text{n} \) gene and fail to deliver a variety of normal differentiation signals to the progenitors, including that which leads to expression of pTα on the cell surface. We are currently characterizing the marrow epithelial cells of normal and nu/nu mice and this may enable further investigation of the role of such cells in signaling in maturation of bone marrow-derived T cell progenitors. A role for mesenchymal-epithelial interaction in the thymus has also been shown to be important for \( \text{T} \) lymphopoiesis (31) and thus a defect in epithelial-mesenchymal signaling caused by a defective \( \text{w} \text{h} \text{n} \) gene could also lead to the disrupted early T cell development in the bone marrow. In conclusion, the experimental results show that the defective \( \text{w} \text{h} \text{n} \) gene in nu/nu mice not only causes the failure of thymic development but also causes defective early prethymic T cell development in the bone marrow.

Acknowledgments

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References


