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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 Strober‡*

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 pTcr 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) 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 bipotential 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⁺CD117⁺CD3⁻ committed T progenitors, 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 in the fetal and newborn thymus capable of generating T cells in vivo and in vitro. Thy1.2⁺Lin⁻CD2⁰ 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⁻CD2⁰ 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 Thy1.2⁺Lin⁻CD2⁰ CTP phenotype cells in the marrow of both adult WT and nu/nu mice. However, Thy1.2⁺Lin⁻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 Thy1.2⁺Lin⁻CD2⁻ compartment of bone marrow in nu/nu mice as compared with WT mice. Furthermore, the Thy1.2⁺Lin⁻CD2⁻ cells in nu/nu mice were deficient in pTcr 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 Thy1.1⁺Lin⁻CD2⁻ cells in the marrow within 2 wk after transplant. These HSC-derived Thy1.1⁺Lin⁻CD2⁻ cells from both euthymic and thymectomized hosts were able to reconstitute the T cell compartment of secondary hosts, whereas HSC-derived Thy1.1⁺Lin⁻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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2 Address correspondence and reprint requests to Dr. Samuel Strober, CCSR Building, Room 2215-C, 269 Campus Drive (Federal Express only), 300 Pasteur Drive (U.S. mail only), Stanford University, Stanford, CA 94305-5166. E-mail address: strober@stanford.edu
3 Abbreviations used in this paper: HSC, hemopoietic stem cell; Lin, lineage; WT, wild type; CTP, committed T cell progenitor; BrdU, 5-bromo-2'-deoxyuridine; RAG, recombination-activating gene.
Materials and Methods

Experimental mice

Congenic strains of WT C57BL/6 Ly5.2 and Ly5.1 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 were used at 7–12 wk of age. C57BL/6 RAG-2−/− mice were purchased either from Taconic Farms (Germantown, NY) or from our colonies within the Stanford Animal Facility. C57BL/6 Ly5.1+nu/nu mice were purchased from Taconic Farms. BA (Thy1.1/Ly5.1) and HZ (Thy1.1/Ly5.2) 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. TCRA−/− C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Immunofluorescent staining and progenitor isolation from 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 separation columns (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions. Enriched cells were stained and sorted thereafter as described elsewhere (11, 12). Staining was performed in the presence of unconjugated anti-TCR mAbs and anti-FITC antibody as a carrier protein to block FcRγIIA/B receptor. Dead 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) FACSVantage (BD Biosciences, Mountain View, CA). Data were acquired with FlowJo software (version 3.0; TreeStar, San Carlos, CA). PE-anti-CD4 (GL3), PE-anti-Mac-1 (M1/70.15), PE-anti-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 FACSVantage without propidium iodide. For annexin staining, cells were analyzed with the addition of FITC-conjugated annexin V (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 (RA3-6B2), PE-anti-NK1.1 (PK136), PE-anti-TCRαβ (H57-97), 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 (RT2.5) 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 FACSVantage without propidium iodide. For annexin staining, cells were analyzed with the addition of FITC-conjugated annexin V (BD Pharmingen).

Adoptive transfer of progenitor cells and monitoring of transplanted mice

C57BL/6 Ly5.2 host mice were given a single, lethal dose of total body irradiation (950 cGy) using a 200-kV (20 mA) source (Philips Medical Systems). The Thy1.2+Ly5.1+ progenitor cells from WT or RAG-2−/−Ly5.2 donors 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 Ly5.2 host mice were given a split lethal dose of total body irradiation (950 cGy) using a 200-kV (20 mA) source (Philips Medical System) and 1000 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+Ly5.1+ HSC-derived progenitors from WT euthymic or WT thymectomized recipients were mixed with 1 × 107 whole bone marrow cells from either Thy1.2+Ly5.1+ WT or nu/nu hosts that were given a single lethal dose of 950 cGy irradiation. HSC-derived CTPs were injected along with 1 × 106 RAG-1−/− whole bone marrow into the lateral tail vein of WT euthymic, lethally irradiated Thy1.2+Ly5.2+ 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.

RT-PCR

Total RNA was extracted from sorted progenitor cells, sorted spleen T cells, and sorted CD4−CD8− thymocytes from C57BL/6 Ly5.1 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 TCRα3 and β2 TCRα2 amplification were designed based on sequences found in GenBank accession number U16958 and had the following sequences: nested forward, 5′-GGGCTCCCAGGATCACACACTCGC-3′; internal forward, 5′-TGCCTGTTGGTGTCCGTGTC-3′; internal reverse, 5′-GGGAGCAGTGTTCCAGCATC-3′; and nested reverse, 5′-CATTACAAAGGGAGATCAC-3′.

Genomic PCR

Single-cell suspensions of sorted Thy1.2+nu/nuLin CD2+ progenitors and Thy1.2+nu/nuLin 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 protease K, and the aqueous phase extracted with phenol-chloroform. The DNA was precipitated with 3 M sodium acetate and 100% ethanol, dried and resuspended in TE buffer, and analyzed by PCR for TCR Vβ gene rearrangement. TCR Vβ gene rearrangements were detected using a nested PCR technique. Primers specific for the β2-β3 intronic region were (first round, 5′-TCTCGGCTTGGAGGAGCAGG-3′ and second round, 5′-TGAGGAGCTCTTCTCTACTACTC-3′) previously described. Primers specific for consensus Vβ8 exon regions were designed based on sequences found in GenBank accession number AE000522 and had the following sequences: first round, 5′-CACAGTGAGGGCTGCAGTCA-3′; second round, 5′-CATGACTCTGAGTCCAGG-3′; and second reverse, 5′-CATGACTCTGAGTCCAGG-3′. Co primers were designed based on sequences in GenBank accession number M64239 and had the following sequences: first round forward, 5′-AGGGGCTTCTGATTCCTAATTGCTG-3′; second round forward, 5′-CTCTTACATACAGTCCT-3′; first round reverse, 5′-CATCCCTCTCTCTTATCGTACG-3′; and second round reverse, 5′-GACCCGTTAAGCTTATGATGAG-3′. 

Results

Thy1.2+nu/nuLin 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 Thy 1.2 vs TCRβ markers after immunofluorescent staining (Fig. 1A). Whereas 47.6% of Thy 1.2-enriched cells from the WT mice were TCRβ+, only 0.01–0.02% from RAG-2−/−, TCRβ−/−, and nu/nu mice were Thy1.2+ TCRβ+ (Fig. 1B). Analysis of Thy1.2 vs Lin markers showed that 4–7.46% of the enriched cells of all of these strains were Thy1.2+Lin+ (Fig. 1B). These cells were gated and analyzed for surface expression of CD2 (Fig. 1C). In WT marrow, 62.0% of the Thy1.2+Lin+ cells from WT marrow were CD2+ and 38.9% were CD2−, and in TCRβ−/− mice, CD2− cells were 47.5% and

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1208

nu/nu BONE MARROW T PROGENITORS
CD2⁺ cells were 38.2%. In contrast, 98.6 and 99.4% of Thy1.2highLin−CD2− cells from RAG-2−/− and nu/nu mice, respectively, were CD2⁻ and <1% were CD2⁺. The Thy1.2highLin−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 × 10³ and 17.7 × 10³, respectively, and from TCRα−/− mice were 72.5 × 10³ and 50 × 10³, respectively (Table I). The mean numbers of CD2⁻ and CD2⁺ cells from RAG-2−/− mice were 64.5 × 10³ and 0.72 × 10³, respectively. The CD2⁻ and CD2⁺ cell populations isolated from nu/nu mice yielded 66 × 10³ and 2.75 × 10³ 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).

Thy1.2highLin−CD2⁺ cells, but not Thy1.2highLin−CD2⁻ cells, in the WT bone marrow have a rearranged TCRβ locus

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 Jβ2 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 Tκ−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).

Table I. Mean percentages (± SE) and absolute numbers (± SE) of CD2− and CD2⁺ cells in the Thy1Lin− fraction of bone marrow cells of C57BL/6 mice.

<table>
<thead>
<tr>
<th>Mice (n = 4)</th>
<th>CD2−</th>
<th>CD2⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>Absolute number</td>
</tr>
<tr>
<td>WT</td>
<td>68 ± 5</td>
<td>36.7 ± 0.8 × 10³</td>
</tr>
<tr>
<td>TCRα−/−</td>
<td>59 ± 2</td>
<td>72.5 ± 0.3 × 10³</td>
</tr>
<tr>
<td>RAG-2−/−</td>
<td>98.4 ± 0.3</td>
<td>64.5 ± 0.7 × 10³</td>
</tr>
<tr>
<td>nu/nu</td>
<td>96 ± 1</td>
<td>66 ± 1 × 10³</td>
</tr>
</tbody>
</table>

* Two femurs and two tibias were harvested from each animal.
Further indicated that nu/nu T cell progenitors in the bone marrow are abnormal; they lacked Thy1.2highLinCD2− intermediate cells with TCR Vβ rearrangements and were also deficient in pTα gene expression.

Reduction in cycling cells and increased apoptotic cells in Thy1.2highLinCD2− 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 Thy1.2highLinCD2− 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 Thy1.2highLinCD2− 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 Thy1.2highLinCD2− progenitors fail to reconstitute the T cell compartment of congenic adoptive hosts

We sorted Thy1.2highLinCD2− 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 x 10⁶ 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 Thy1.2highLinCD2− 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 x 10⁶ 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 x 10⁴ sorted marrow TCRαβ+ T cells (data not shown).
Comparisons of WT hosts injected with 500 sorted Thy1.2 high Lin CD2- cells from WT but not nu/nu mice provide T cell Lin-specific reconstitution of congenic hosts following adoptive transfer. Thy1.2 high Lin CD2- 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 high Lin CD2- cells and 1 x 10^6 Ly5.2 RAG-2-/- 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 TCRαβ+ 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 of 500 sorted WT Thy1.2 high Lin CD2- cells (H), 500 sorted nu/nu Thy1.2 high Lin CD2- cells (I), 10,000 sorted nu/nu Thy1.2 high Lin CD2- cells (J), and 10,000 sorted HSC-enriched nu/nu Thy1.2 low Lin cells (K) 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 Thy1.2 Lin CD2- cells

<table>
<thead>
<tr>
<th>Animals (n = 5)</th>
<th>Spleen</th>
<th>Lymph Nodes</th>
<th>Thymus</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Thy1.2 Lin CD2-</td>
<td>21 ± 10 x 10^4</td>
<td>14 ± 7 x 10^4</td>
<td>144 ± 99 x 10^4</td>
</tr>
<tr>
<td>nu/nu Thy1.2 Lin CD2-</td>
<td>7 ± 6 x 10^2</td>
<td>2.2 ± 0.5 x 10^2</td>
<td>3 ± 2 x 10^2</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>
</tbody>
</table>

To establish that the defect in the T cell progenitors occurred at the level of the nu/nu Thy1.2 high Lin CD2- T progenitor cell population and not at the level of the HSC in nu/nu mice, we injected...
10,000 HSC-enriched Thy\textsuperscript{low}\textsuperscript{Lin} 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).

**Thy.1.1\textsuperscript{high}\textsuperscript{Lin} - CD2\textsuperscript{+} 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 Thy.1.1\textsuperscript{low}\textsuperscript{cKit}\textsuperscript{+}Sca1\textsuperscript{+}Lin\textsuperscript{−} cells to 99.9% purity as previously described (21) and modified (17). We injected 1000 Thy.1.1/Ly5.1 HSCs into either WT euthymic or WT thymectomized Thy.1.2/Ly5.2 hosts. Since only Thy.1.2/Ly5.1 nu/nu mice were available, we performed a parallel set of experiments where 1000 Thy.1.1/Ly5.2 HSCs were sorted and injected into either Thy.1.2/Ly5.1 nu/nu hosts or WT hosts as shown in the experimental scheme in Fig. 5.

HSC-derived Thy.1.1\textsuperscript{high}\textsuperscript{Lin} 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% Thy.1.1\textsuperscript{high}\textsuperscript{Lin} cells while thymectomized and nu/nu hosts had 5.8 and 1.2% Thy.1.1\textsuperscript{high}\textsuperscript{Lin} cells, respectively. Gated Thy.1.1\textsuperscript{high}\textsuperscript{Lin} cells from all three hosts were further analyzed for CD2 surface expression. WT euthymic hosts had 89% CD2\textsuperscript{+} cells within the bone marrow Thy.1.1\textsuperscript{high}\textsuperscript{Lin} subpopulation while thymectomized and nu/nu hosts had 95.4 and 95.3% CD2\textsuperscript{+} cells within the Thy.1.1\textsuperscript{high}\textsuperscript{Lin} subpopulation, respectively (Fig. 5, B, F, and J). The Thy.1.1\textsuperscript{high}\textsuperscript{Lin} CD2\textsuperscript{+} progenitors were sorted to purity, and 500 of these cells were injected along with 1 × 10\textsuperscript{6} RAG\textsuperscript{−/−} whole bone marrow into groups of lethally irradiated congenic hosts (D. Chatterjea-Matthes, unpublished observations) further establishing that these cells are intermediates along the path of mature T cell development. Surprisingly, these Thy.1.1\textsuperscript{high}\textsuperscript{Lin} CD2\textsuperscript{+} cells were markedly reduced in the marrow of nu/nu mice that fail to develop the thymus due to a deficiency in the \textit{wtm} 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\textsuperscript{+} and CD2\textsuperscript{−} populations were present in abundance in the marrow of T cell-deficient TCR\alpha\textsuperscript{−/−} mice. Therefore, it is unlikely that mature T cells derived from the thymus play an important role in facilitating the transition between the CD2\textsuperscript{+} and CD2\textsuperscript{−} T cell progenitors.

**Discussion**

We identified intermediate cells with the Thy.1.2\textsuperscript{high}\textsuperscript{Lin} - CD2\textsuperscript{−} phenotype in the pathway of early T cell development in the bone marrow of WT mice. The latter cells had rearranged the TCR\beta chain gene segments but did not express TCR\beta on the cell surface. Thy.1.2\textsuperscript{high}\textsuperscript{Lin} - CD2\textsuperscript{−} cell development appeared to be dependent on TCR\beta gene rearrangement, since cells with this phenotype were found in the marrow of TCR\alpha\textsuperscript{−/−} mice, but were not in the marrow of RAG-2\textsuperscript{−/−} mice. Sorted Thy.1.2\textsuperscript{high}\textsuperscript{Lin} CD2\textsuperscript{−} cells were able to give rise to mature TCR\alpha\textsuperscript{+} 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-Matthes, unpublished observations) further establishing that these cells are intermediates along the path of mature T cell development. Unfortunately, these Thy.1.2\textsuperscript{high}\textsuperscript{Lin} CD2\textsuperscript{−} cells were markedly reduced in the marrow of nu/nu mice that fail to develop the thymus due to a deficiency in the \textit{wtm} 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\textsuperscript{+} and CD2\textsuperscript{−} populations were present in abundance in the marrow of T cell-deficient TCR\alpha\textsuperscript{−/−} mice. Therefore, it is unlikely that mature T cells derived from the thymus play an important role in facilitating the transition between the CD2\textsuperscript{+} and CD2\textsuperscript{−} T cell progenitors.

Earlier progenitor cells in the T cell development pathway in the bone marrow expressing the Thy.1.2\textsuperscript{high}\textsuperscript{Lin} - CD2\textsuperscript{−} phenotype were present in abundance in WT, RAG-2\textsuperscript{−/−}, 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 p\textalpha\textsuperscript{+} gene in WT mice, they expressed the RAG-1 and RAG-2 but not the p\textalpha\textsuperscript{+} gene in the nu/nu mice. Since the Thy.1.2\textsuperscript{high}\textsuperscript{Lin} CD2\textsuperscript{−} cells had been shown previously to rapidly generate Thy.1.2\textsuperscript{high}\textsuperscript{Lin} CD2\textsuperscript{−} cells in vitro (11), the current results suggested a maturation arrest at the Thy.1.2\textsuperscript{Lin} CD2\textsuperscript{−} stage associated with the deficiency in the expression of the p\textalpha\textsuperscript{+} gene. The p\textalpha\textsuperscript{+} 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 p\textalpha, may have contributed to the deficiencies in early T cell maturation and progression to TCR\alpha\textsuperscript{+} 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 Thy.1.2\textsuperscript{high}\textsuperscript{Lin} - CD2\textsuperscript{−} progenitor cells from the nu/nu mice were defective as compared with Thy.1.2\textsuperscript{high}\textsuperscript{Lin} - CD2\textsuperscript{−} progenitors from WT mice was obtained in adoptive transfer studies. Whereas the WT progenitors generated CD4\textsuperscript{+} and CD8\textsuperscript{+} TCR\alpha\textsuperscript{+} T cells in the spleen, lymph nodes, and thymus of irradiated WT Ly5 congenic hosts, the nu/nu progenitors generated 1000-fold fewer TCR\alpha\textsuperscript{+} 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 TCRαβ 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).
Thy1.2lowLin HSC was shown by experiments where 10,000 HSC-enriched however, these cells failed to give rise to mature TCR progenitors does not require the activity of the 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 wth gene and fail to deliver a variety of normal differentiation signals to the progenitors, including that which leads to expression of αβ TCR gene in 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 T lymphopoiesis (31) and thus a defect in epithelial-mesenchymal signaling caused by a defective wth gene could also lead to the disrupted early T cell development in the bone marrow. In conclusion, the experimental results show that the defective wth 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.

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References


