

Thymocyte proliferation and differentiation in human granulocyte-macrophage colony-stimulating factor receptor transgenic mice

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Thymocytes display varying responses to cytokines depending on their stage of differentiation. Whether these responses are due to stage-specific cytokine receptor expression or to downstream signaling mechanisms is unknown. We examined the relationship between receptor expression and thymocyte proliferation or differentiation by using thymocytes from transgenic mice that constitutively expressed the human granulocyte-macrophage colony stimulating factor (hGM-CSF) receptor. Transgenic $CD4^-CD8^-$, $CD4^+CD8^-$, and $CD4^-CD8^+$ thymocyte populations expressing the hGM-CSF receptor proliferated when cultured with hGM-CSF, whereas $CD4^+CD8^+$ cells failed to proliferate despite expressing this receptor. We next examined the effect of hGM-CSF receptor signaling on thymocyte differentiation in fetal thymic organ culture supporting a full program of T cell development in vitro. Addition of hGM-CSF to the transgenic fetal thymic organ culture resulted in failure of $CD4^-CD8^-$ cells to differentiate into $CD4^+CD8^+$ cells. To investigate this maturational inhibition more closely, we repopulated wild-type fetal lobes with sorted pro-T, pre-T or post pre-T precursor cells from hGM-CSF receptor transgenic mice. In these cultures hGM-CSF blocked both pro-T and pre-T cell differentiation, whereas the more mature post pre-T cells differentiated normally. These results suggest that hGM-CSF receptor signaling during thymocyte differentiation causes stage-specific inhibition of precursor cell maturation. In addition, repopulation studies of transgenic fetal lobes with sorted wild-type thymocyte precursors indicated that hGM-CSF inhibited proliferation and differentiation of the wild-type precursors, suggesting a secondary effect via transgenic stromal cells. (J Allergy Clin Immunol 1997;100:S87-96.)

Key words: GM-CSF receptor, transgenic mice, receptor expression, thymocyte proliferation, thymocyte differentiation, fetal thymic organ culture

Differentiation of T cells from hematopoietic stem cells occurs mainly in the thymus. The expression of a variety of molecules, including adhesion molecules, the T cell receptor (TCR), and cytokine receptors has been implicated in the maturational process. The cytokine response profile of various thymocyte populations varies

Abbreviations used

DN:	Double-negative
DP:	Double-positive
FTOC:	Fetal thymic organ culture
hGM-CSF:	Human granulocyte-macrophage colony-stimulating factor
hGMR:	Human granulocyte-macrophage colony-stimulating factor receptor
PBS:	Phosphate-buffered saline
SP:	Single-positive
TCR:	T-cell receptor
TN:	Triple-negative

depending on the stage of differentiation, as shown in Fig. 1.¹ Immature $CD4^-CD8^-$ double-negative (DN) thymocytes respond to cytokines, including interleukin-2 (IL-2), IL-7, and stem cell factor. However, responsiveness to cytokines and production of cytokines are lost as DN cells begin to express CD4 and CD8 to enter the double-positive (DP) stage. During this DP stage, cells undergo positive and negative selection to mature into CD4 or CD8 single-positive (SP) cells, which have regained the ability to respond to and produce cytokines. The mechanisms responsible for this stage-specific cytokine responsiveness are unknown. Expression of several cytokine receptors, including the IL-2 receptor, IL-7 receptor, and stem cell factor receptor (c-kit) decreases as thymocyte precursors differentiate into DP cells,²⁻⁴ but the changes of other cytokine receptors are not clear.

We have previously generated transgenic mice that express the human granulocyte-macrophage colony-stimulating factor (GM-CSF) high affinity receptor.⁵ Expression of this receptor is under the control of the H-2L^d class I promoter, resulting in its expression on numerous hematopoietic cells, including splenocytes, thymocytes, and bone marrow cells. The functional high-affinity GM-CSF receptor (GMR) is composed of two distinct α and β subunits.⁶ The α subunit confers GM-CSF specificity and binds GM-CSF with low affinity, whereas the β subunit is shared with the IL-3 and IL-5 receptors.⁷⁻¹⁰ Previous studies have shown that the high-affinity GMR could be reconstituted by transfecting a pro-B cell line (BA/F3), a T cell line (CTLL-2), or a

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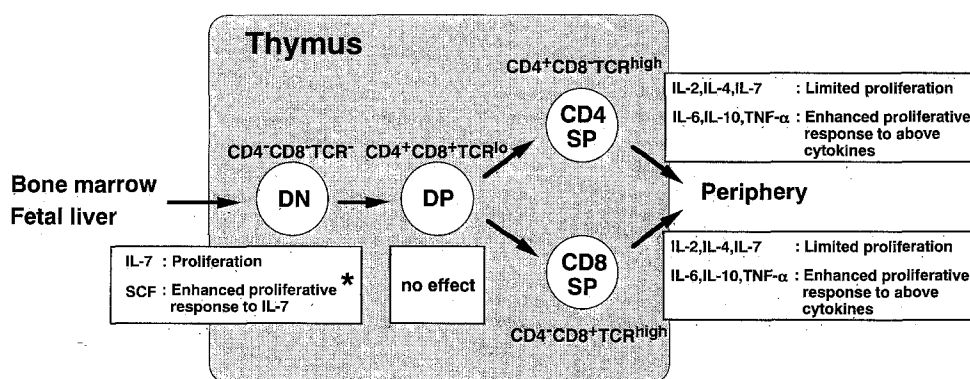


FIG. 1. Effect of cytokines on thymocyte subsets. Only well-known examples are given in this figure. *, DN cells can be divided into subpopulations, each of which show different cytokine responses. SCF, stem cell factor; TNF- α , tumor necrosis factor- α .

fibroblastic cell line (NIH 3T3) with both α and β subunit cDNAs. The reconstituted GMR was capable of transducing a proliferative signal in all three cell lines in the presence of GM-CSF,¹⁰⁻¹³ suggesting that the signal transduction components associated with this receptor are present in multiple cell lineages and that the response to GM-CSF is regulated at the level of receptor expression. This concept has received support from experiments on hematopoietic progenitors from hGMR transgenic mice. Murine GM-CSF does not bind to the human receptor, therefore hGMR transgenic mice display a normal phenotype in the absence of exogenous hGM-CSF. Addition of hGM-CSF to methylcellulose colony assays of bone marrow hematopoietic progenitors from these mice resulted in the formation of colonies of multiple lineages, including erythrocyte colonies that normally are erythropoietin-dependent. In the presence of murine GM-CSF, however, only granulocyte and macrophage colonies were generated. These data suggest that the response of myeloid progenitors to hGM-CSF is regulated at the level of receptor expression and that GM-CSF does not induce differentiation of hematopoietic progenitors along specific myeloid lineages; rather it induces the proliferation of multipotent progenitors whose differentiation may be regulated by an intrinsic mechanism.

In the present study, we investigated the effect of hGM-CSF signaling on the proliferation and differentiation of thymocytes from hGMR transgenic mice in order to evaluate the relationship between receptor expression and the proliferation and differentiation of thymocytes.

MATERIALS AND METHODS

Mice

The generation of hGMR transgenic mice has been described previously.⁵ The transgenic line H2-81 was used in all experiments reported here. Heterozygous hGMR transgenic mice or wild-type C3H/HeN mice (Simonsen Laboratories, Gilroy, Calif.), 3 to 6 weeks of age were used for sorting thymic

populations. For fetal thymic organ culture (FTOC) experiments, timed pregnant mice were generated by mating wild-type or homozygous transgenic males mice with wild-type female mice.

Culture medium and cytokines

The standard culture medium consisted of RPMI 1640 medium (JRH BioScience, Lenexa, Kan.) containing 10% fetal calf serum, 200 mmol/L L-glutamine, 5×10^{-5} mol/L 2-mercaptoethanol, MEM amino acids and vitamins, sodium bicarbonate, penicillin, streptomycin, and gentamycin. For FTOC, medium containing 20% fetal calf serum was used (FTOC-medium). Recombinant hGM-CSF was kindly provided by Dr. R. Kastelein (DNAX, Palo Alto, Calif.) and recombinant murine GM-CSF produced in yeast was kindly provided by Dr. A. Miyajima (Tokyo University, Tokyo).

Antibodies

All antibodies were purchased from PharMingen (San Diego, Calif.) unless otherwise specified. For isolation of CD4 and CD8 thymocyte populations, anti-CD4-PE (clone RM4-5) and anti-CD8-FITC (clone 53-6.7) were used. For isolation of thymocyte triple negative (TN) precursor populations, the following antibodies were used: anti-CD3-biotin (clone 144-2C11), anti-CD4-biotin, anti-CD8-biotin, anti-B220-biotin (clone RA3-6B2), anti-Mac-1-biotin (clone M1/70), anti-Gr-1-biotin (clone RB6-8C5), anti-CD25-FITC (clone 7D4), anti-CD44-PE (clone IM7), and streptavidin-TriColor (Caltag Laboratories, South San Francisco, Calif.). For analysis of in vitro repopulation of fetal lobes, the following antibodies were used: anti-CD4-PE, anti-CD8 α -biotin, anti-TCR $\alpha\beta$ -FITC (clone H57-597) or anti-TCR $\gamma\delta$ -FITC (clone GL3), and streptavidin-TriColor (Caltag). Before staining, cells were incubated with anti-CD32 (anti-Fc γ R2/III, clone 2.4G2) to reduce nonspecific antibody binding.

Sorting and multiparameter analysis

Thymocytes from hGMR transgenic heterozygous mice of wild-type controls were stained with anti-CD4-PE and anti-CD8-FITC. The four CD4/CD8 subsets were sorted with a FACStar Plus flow cytometer (Becton Dickinson, San Jose, Calif.). The identification and isolation of TN thymocyte precursor populations has been described previously.¹⁴ In brief,

thymocytes were depleted with anti-CD4 (clone RL172, used as culture supernatant) and anti-CD8 (clone AD4, Cedarlane Laboratories, Hornby, Ont.) antibodies followed by treatment with low-tox M rabbit complement (Cedarlane) and 20 mg/ml DNase I (Sigma, St. Louis, Mo.). Viable cells were isolated with Histopaque 1083 (Sigma) and then stained with a panel of lineage antibodies (all biotinylated) directed against CD3, CD4, CD8, B220, Mac-1, and Gr-1 as well as anti-CD25-FITC, and anti-CD44-PE. After washing, cells were incubated with streptavidin-TriColor. Cells were sorted with a FACStar Plus or FACS Vantage flow cytometer (Becton Dickinson), and the resulting purity was routinely greater than 95%. For analysis of T cell repopulation in FTOC, single-cell suspensions were prepared for staining. To reduce nonspecific staining, cells were incubated with anti-CD32 (clone 2.4G2) before staining for specific antigen expression. FTOC were stained with anti-TCR $\alpha\beta$ -FITC or anti-TCR $\gamma\delta$ -FITC, anti-CD8 α -biotin, and anti-CD4-PE followed by streptavidin-TriColor. Analysis was performed with a FACScan flow cytometer (Becton Dickinson) and CellQuest software (Becton Dickinson).

Proliferation analysis of thymocyte subsets

Thymocyte CD4/CD8 subsets were sorted from hGMR transgenic mice and cultured at 2×10^5 cells/ml in 96-well plates containing standard culture medium supplemented with hGM-CSF (10 ng/ml) or murine GM-CSF (5 ng/ml). After 2 days, the cells were pulsed with tritium-labeled thymidine (1 μ Ci per well) for 18 hours and then harvested.

FTOC

For conventional FTOC, fetal thymic lobes were removed on days 14 to 16 of gestation and cultured under standard conditions as described elsewhere.¹⁵ In brief, thymic lobes were removed from plug-timed pregnant mice and cultured at the air-liquid interface, resting on 0.45 μ m filters (Millipore, Bedford, Mass.) supported by small pieces (10 $\times$ 10 $\times$ 5 mm) of Gelfoam sponge (Upjohn, Kalamazoo, Mich.). Cultures were carried out in six-well plates containing 2.5 ml of FTOC medium. Selected cultures were supplemented with purified recombinant hGM-CSF. Fresh FTOC medium was supplied every 6 days. For fetal thymic lobe repopulation studies, lobes were removed on day 15 of gestation and depleted of endogenous T cell progenitors by culturing in FTOC medium containing 1.35 mmol/L deoxyguanosine for 5 days as described elsewhere.¹⁶ Depleted lobes were then individually plated with 1×10^3 pro-T or 1×10^4 pre-T or post pre-T cells in 30 μ l of FTOC medium in Terasaki plates (Nunc, Kamstrup, Denmark). Plates were then inverted to allow the lobe and cells to combine at the bottom of a hanging drop.¹⁷ After 24 to 48 hours, repopulated lobes were transferred back into FTOC, and fresh FTOC medium was supplied every 6 days. At the indicated time points, lobes were gently pressed under a glass coverslip in 100 μ l of PBS containing 2% fetal calf serum to release thymocytes. Thymocyte phenotyping was then performed as described above.

In vivo hGM-CSF administration

The injection schedule for hGM-CSF was modified from the method described previously.^{18,19} In brief, hGMR transgenic mice were injected subcutaneously with 0.2 ml of phosphate-buffered saline (PBS) containing 1% C3H/HeN serum (serum/PBS) or hGM-CSF (500 or 2500 ng) twice a day at 12-hour intervals for 7 consecutive days.

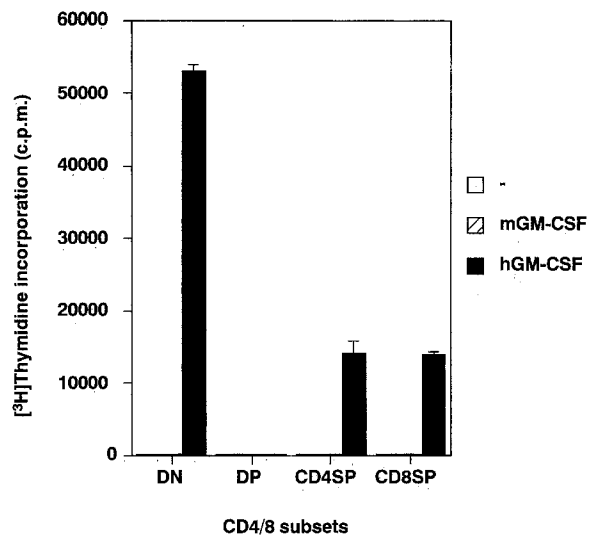


FIG. 2. Proliferation of thymocyte subpopulations expressing hGMR induced by hGM-CSF. Thymocyte subpopulations were sorted on the basis of the expression of CD4 and CD8, cultured with or without hGM-CSF or with mGM-CSF for 2 days, and then pulsed with tritiated thymidine. The results are representative of three different experiments. (Modified from Yasuda Y et al. Blood 1997;89:1349-56.)

RESULTS

Proliferation of hGMR transgenic thymocytes in response to hGM-CSF

The hGMR transgenes are under the control of the H-2L^d class I promoter, which results in broad expression of the receptor.⁵ All thymocyte populations from hGMR transgenic mice express both the α and β hGMR subunits on the cell surface, and the expression levels of these subunits are the same among the DN, CD4⁺ SP, CD8⁺ SP, and DP populations.²⁰ We previously found that cell lines expressing the hGMR acquired the ability to proliferate in response to hGM-CSF.¹⁰⁻¹³ We therefore examined whether hGMR expression on thymocytes led to hGM-CSF responsiveness. The four thymocyte subsets delineated by CD4 and CD8 expression were sorted from hGMR transgenic mice by flow cytometry and cultured for 2 days with or without hGM-CSF or murine GM-CSF, followed by pulsing with tritiated thymidine to measure proliferation (Fig. 2). DN, CD4⁺ SP, and CD8⁺ SP cells displayed a high level of proliferative activity when cultured with hGM-CSF but did not respond to murine GM-CSF. However, despite expression of the hGMR, DP cells failed to proliferate in response to hGM-CSF. Thymocytes from wild-type control mice also failed to proliferate in response to hGM-CSF (data not shown).

hGMR transgenic FTOC

We have previously shown that hGM-CSF supports the growth of hematopoietic progenitor cells from hGMR transgenic bone marrow cells without altering their ability to differentiate.⁵ To examine the effect of

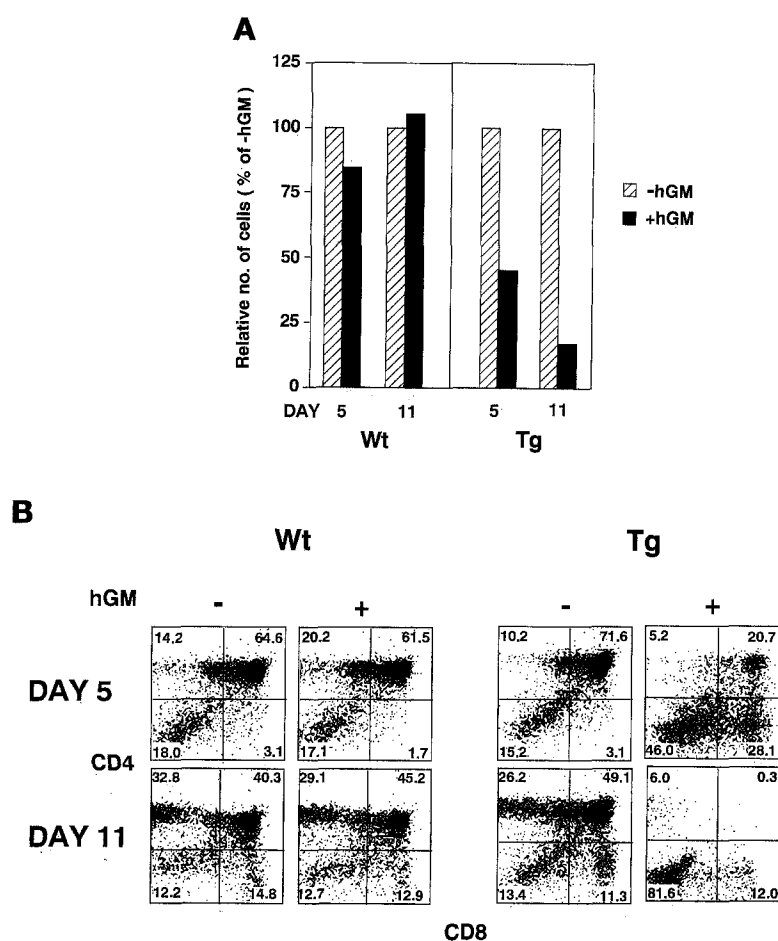


FIG. 3. Effect of hGM-CSF on FTOC development. Day 15 fetal thymic lobes were harvested from hGMR transgenic and wild-type control embryos and placed into FTOC in the presence or absence of 50 ng/ml hGM-CSF. After 5 or 11 days, the lobes were harvested and analyzed. **A**, The number of cells within the lymphoid scatter gate per lobe is presented as a percentage of the value without hGM-CSF. The ratio was calculated separately for wild-type control and transgenic FTOC at each time point. **B**, Harvested cells were stained for expression of CD4 and CD8. CD4 and CD8 expression are indicated by a dot plot, and the quadrant percentages are shown. The results are representative of three different experiments. *Tg*, transgenic; *Wt*, wild-type. (Modified from Yasuda Y et al. *Blood* 1997;89:1349-56.)

hGMR signaling on thymic T cell development, we added hGM-CSF to FTOC, which is the best system for supporting a full program of T cell development in vitro. Fetal lobes were removed from hGMR transgenic mouse embryos on day 15 of gestation and cultured with or without 50 ng/ml hGM-CSF. After 5 or 11 days, lobes were harvested and differentiation was examined by phenotyping for CD4 and CD8 expression by using flow cytometry. As shown in Fig. 3, *A*, the number of cells within the lymphoid scatter gate was decreased by addition of exogenous hGM-CSF to transgenic FTOC, whereas transgenic DN and SP cells proliferated in response to hGM-CSF in cell suspension culture (see Fig. 1). This reduction became more severe as the duration of culture increased. The frequency of DP cells was reduced, with a corresponding increase of DN cell frequency in response to hGM-CSF on day 5 of culture (Fig. 3, *B*). This decrease of DP cells and increase of DN

cells was even more pronounced by day 11 of culture, so that DP and SP cells were essentially absent from FTOC cultured with hGM-CSF. These data suggested stage-specific inhibition of the differentiation of DN cells into DP cells in response to exogenous hGM-CSF. Differentiation of DN cells into DP cells was inhibited by hGM-CSF, whereas DP cells differentiated into SP cells, which resulted in the disappearance of DP cells. This hypothesis was confirmed by using FTOC from hGMR transgenic mice on days 14, 15, and 16 of gestation.²⁰ After 7 days of culture with hGM-CSF, day 15 and day 16 transgenic fetal lobes contained fewer DP cells and showed a corresponding increase of DN cells compared to age-matched wild-type lobes. On day 14, the decrease of DP cell frequency was more marked, when compared to days 15 and 16. These data suggest that the more gestationally mature day 15 or day 16 fetal lobes contain thymocyte precursors that have passed a putative block point for hGM-CSF, whereas

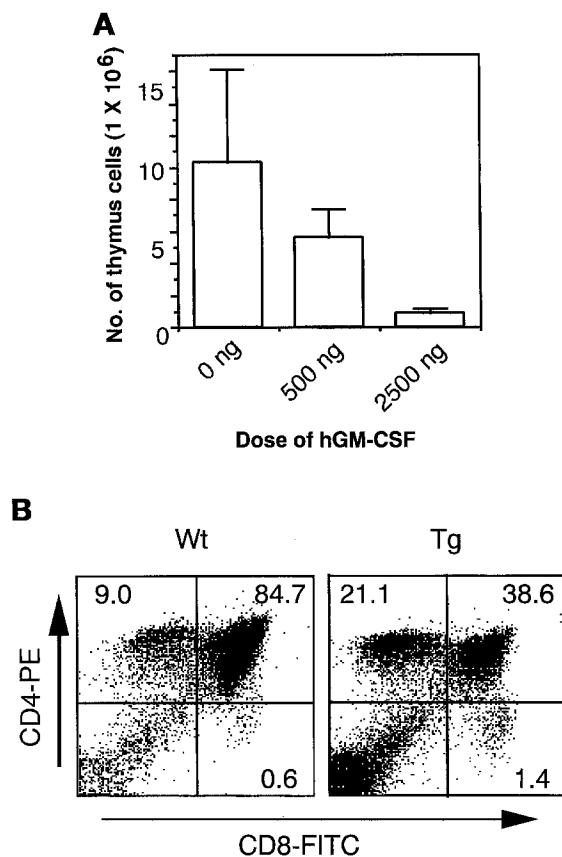
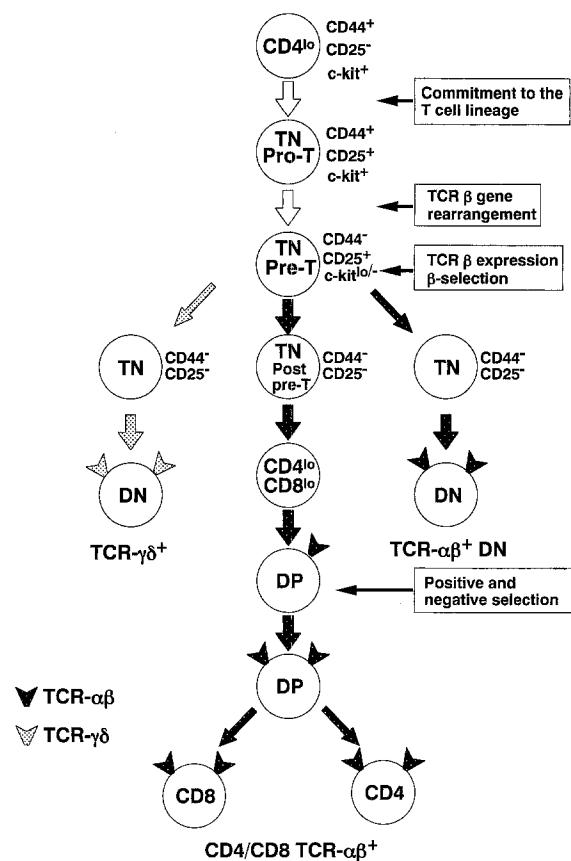


FIG. 4. Effect of hGM-CSF on thymocyte differentiation in vivo. Adult hGMR transgenic mice were injected with 500 ng or 2500 ng of hGM-CSF or with serum/PBS twice a day. Adult wild-type mice were also injected with 500 ng of hGM-CSF twice a day as a control. After 1 week of injection, thymuses were harvested from the mice and analyzed. **A**, Total number of cells recovered from thymuses in hGMR transgenic mice. **B**, Cells from hGMR transgenic mice and wild-type mice stained for expression of CD4 and CD8. CD4 and CD8 expression is indicated by a dot plot, and the quadrant percentages are shown. Tg, transgenic; Wt, wild-type. (From Nishijima I et al. Blood 1997;90:1031-8. Copyright 1997 Blood.)

the majority of day 14 fetal precursors have yet to reach this block point. Accordingly, inhibition by hGM-CSF occurs at a specific stage of thymocyte maturation.

In vivo effect of hGM-CSF

We also examined the effect of hGM-CSF on thymocytes in vivo by injecting exogenous hGM-CSF into hGMR transgenic mice.²¹ Adult transgenic mice were injected with 500 ng or 2500 ng of hGM-CSF or with serum/PBS, twice a day. As a control, adult wild-type mice were also injected with 500 ng of hGM-CSF twice a day. After 1 week of injection, thymuses were harvested from the mice and examined for differentiation by phenotyping for CD4 and CD8 expression. The number of thymocytes in hGMR transgenic mice was decreased by hGM-CSF in a dose-dependent manner (Fig. 4, A). The reduction of DP cell frequency with a corresponding



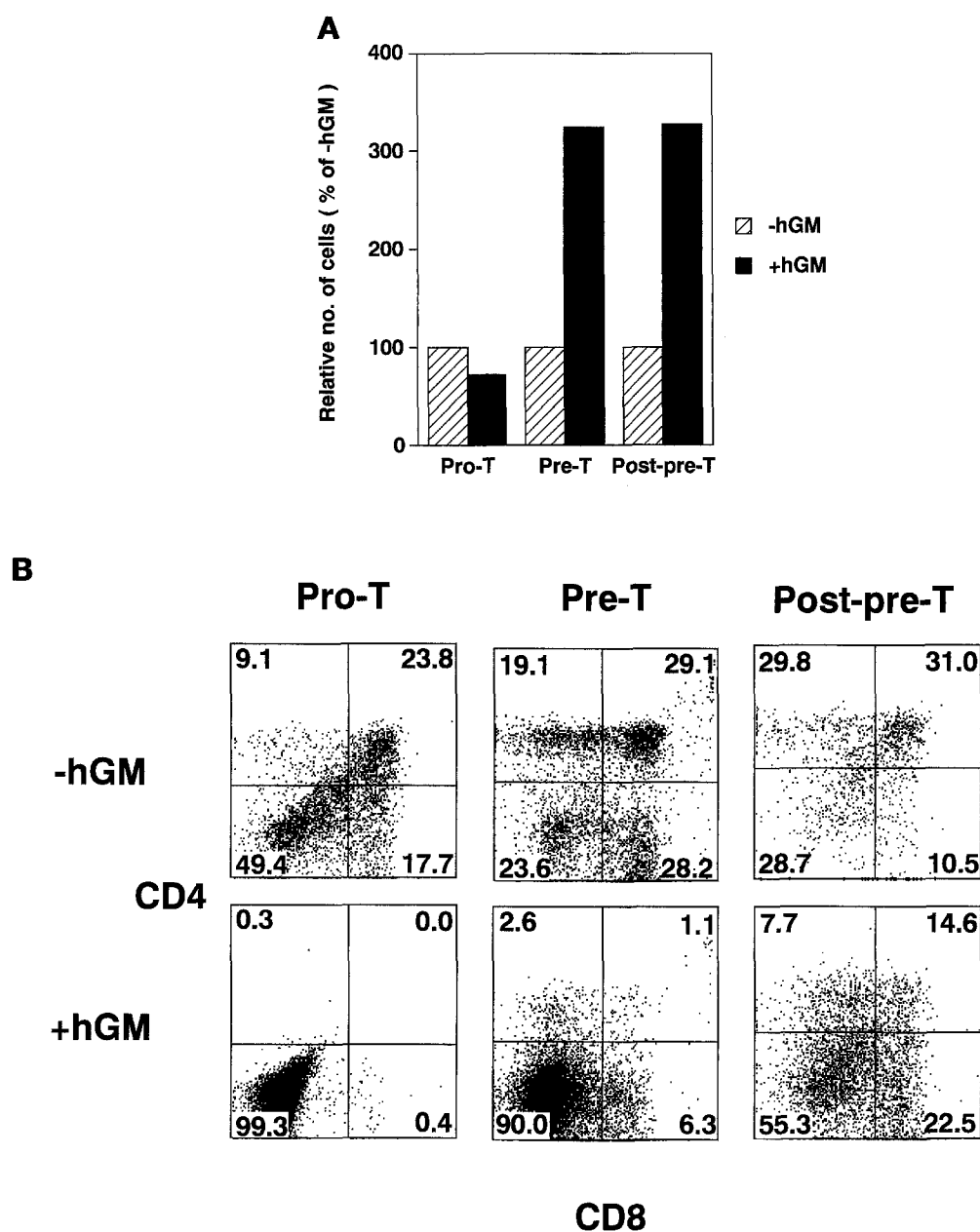


FIG. 6. Effect of hGM-CSF on differentiation of TN thymocyte precursor subsets from hGMR transgenic mice. Pro-T ($CD44^{+}25^{+}$), pre-T ($CD44^{-}25^{+}$), and post pre-T ($CD44^{-}25^{-}$) cells were sorted from transgenic mice, transferred into 2-deoxyguanosine-depleted wild-type fetal lobes, and then cultured with or without 50 ng/ml of hGM-CSF. Pro-T cell cultures were harvested on day 23, pre-T cell cultures on day 14, and post pre-T cell cultures on day 9. **A**, The number of cells within the lymphoid scatter gate per lobe is presented as a percentage of the value obtained from cultures without hGM-CSF. The ratio was calculated separately for each subset. **B**, Harvested cells stained for expression of CD4 and CD8. CD4 and CD8 expression is indicated by a dot plot, and the quadrant percentages shown. The results are representative of three different experiments. (Modified from Yasuda Y et al. *Blood* 1997;89:1349-56.)

pre-T, and post pre-T subsets were sorted from adult transgenic mice on the basis of CD44 and CD25 expression by flow cytometry and were cocultured with the T cell precursor-depleted lobes in hanging drops. The repopulated lobes were transferred to FTOC and cultured with or without hGM-CSF. Since transgene expression is controlled by a class I promoter, it is possible

that both thymic stromal cells and thymocytes express the hGMR. To exclude any potential effects mediated through activation of stromal cells by hGM-CSF, we repopulated wild-type fetal lobes with transgenic sorted thymocyte precursors. With this approach, any effect observed after the addition of hGM-CSF to the repopulated fetal lobes would be the result of a direct influ-

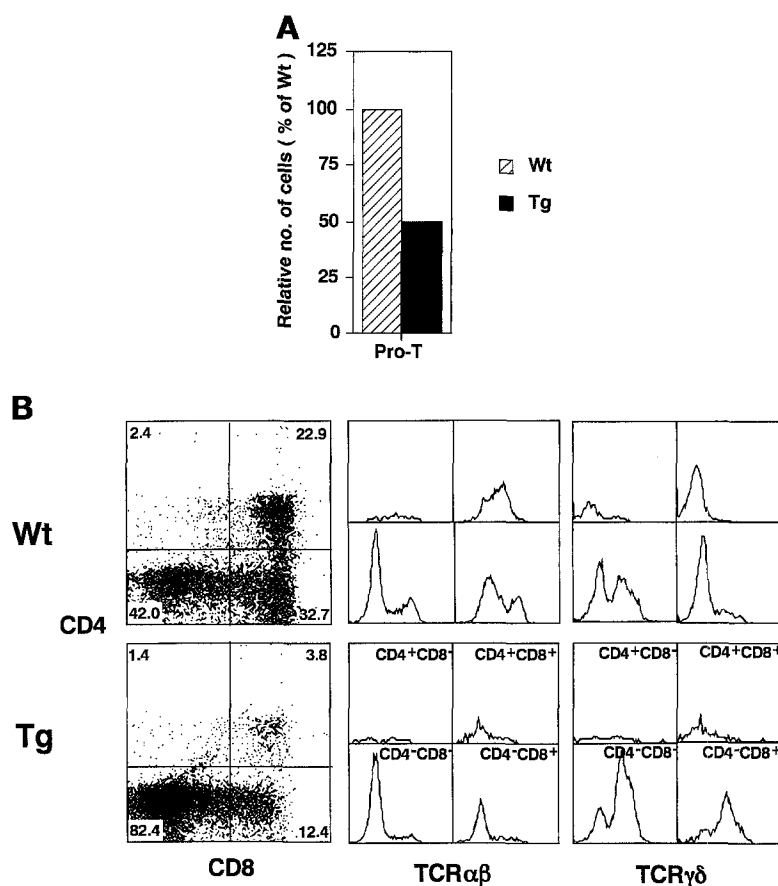


FIG. 7. Effect of hGM-CSF on differentiation of wild-type thymocyte precursor subsets mediated through hGMR transgenic stromal cells. Pro-T (CD44⁺25⁺) cells were sorted from wild-type mice, transferred into 2-deoxyguanosine-depleted hGMR transgenic or wild-type control fetal lobes, and then cultured with 50 ng/ml of hGM-CSF for 25 days. **A**, The number of cells within the lymphoid scatter gate per lobe is presented as a percentage of the value for wild-type FTOC. **B**, Harvested cells stained for expression of CD4, CD8, and $\alpha\beta$ - or $\gamma\delta$ -TCR. CD4 and CD8 expression is indicated by a dot plot, and the quadrant percentages shown (*left*). Cells within each quadrant were further analyzed for TCR $\alpha\beta$ (*center*) and TCR $\gamma\delta$ expression (*right*). The results are representative of two different experiments. Tg, transgenic; Wt, wild-type.

ence on thymocyte precursors and not one mediated via stromal cells. After 23 days of culture, the generation of DP and SP mature cells in hGMR transgenic pro-T cell-repopulated FTOC was completely blocked (Fig. 6, *B*, *left*). No mature DN $\alpha\beta$ -T cells were seen, but DN $\gamma\delta$ -T cells were detected, which suggested that these cells could arise without going through a DP intermediate stage.²⁰ Similar inhibition was also observed when hGMR transgenic pre-T cell-repopulated FTOCs were analysed after 14 days of culture in hGM-CSF (Fig. 6, *B*, *middle*). However, the inhibition of pre-T cells appeared to be less complete than that of pro-T cells, because a small percentage of DN and SP cells were observed. In contrast to pro-T cell and pre-T cell repopulated FTOC, post pre-T cell repopulated lobes contained DP and SP cells after culture with hGM-CSF for 9 days (Fig. 6, *B*, *right*). The higher frequency of DN and SP cells in post pre-T cell-repopulated FTOC may have been the result of the proliferation of mature DN and SP cells induced by hGM-CSF, because the majority of these cell popu-

lations expressed either the $\alpha\beta$ - or the $\gamma\delta$ -TCR.²⁰ Together, these data indicate that hGMR signaling blocks the normal differentiation of pro-T cells and most pre-T cells into DP and SP cells expressing the $\alpha\beta$ -TCR. The more mature post pre-T cells appeared to be downstream of this hGM-CSF-induced differentiation block because relatively normal differentiation occurred despite the presence of hGM-CSF. In contrast to our earlier data from conventional FTOC, cell yields within the lymphoid scatter gate were increased threefold for post pre-T cell and pre-T cell populations by the addition of hGM-CSF (see Fig. 6, *A*). The cell yield from pro-T cell-repopulated FTOC showed a slight decrease, although it was less marked than the decrease observed in conventional FTOC with both thymocytes and stromal cells expressing the hGMR. These divergent results are thought to be caused by secondary effects of hGM-CSF on transgenic stromal cells.

To confirm this possibility, we repopulated transgenic fetal lobes with wild-type sorted thymocyte

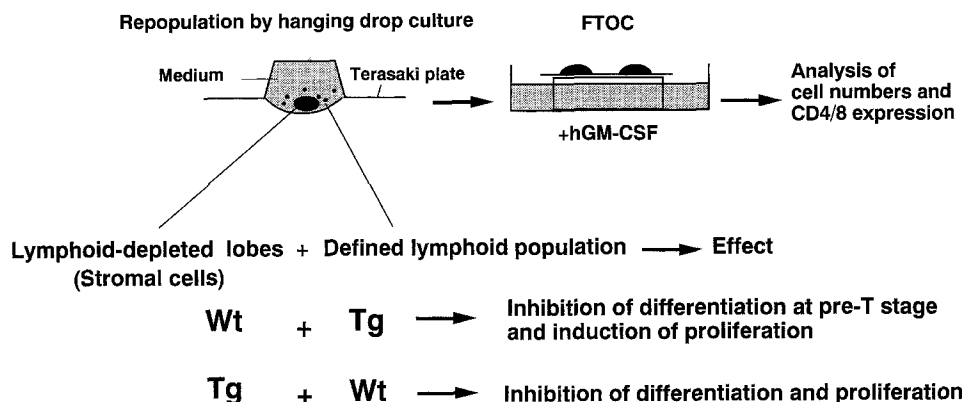


FIG. 8. Effect of hGM-CSF on repopulated FTOC. The combination of wild-type stromal cells and a transgenic lymphoid population showed different results from the combination of transgenic stromal cells and a wild-type lymphoid population.

precursors. This combination of stromal cells and thymocytes was the converse of that in the previous experiment, and thus any effect of hGM-CSF on the repopulated fetal lobes would be mediated through hGMR on stromal cells. We sorted pro-T cells from wild-type adult mice to repopulate fetal thymic lobes from hGMR transgenic mice on day 15 of gestation. We also repopulated day 15 fetal thymic lobes from wild-type mice as a control. FTOC was done with 50 ng/ml of hGM-CSF for 25 days in both instances. In agreement with our prediction, the cell yield within the lymphoid scatter gate was reduced to half of the wild-type level in transgenic FTOC, suggesting that the decrease of cells in conventional FTOC was due to the influence of stromal cells (Fig. 7, A). However, a reduction of DP cells was also observed in transgenic FTOC with hGM-CSF (Fig. 7, B). This effect of hGM-CSF was similar to the inhibition observed in transgenic pro-T cell-repopulated FTOC, although it did not completely block differentiation, because a few DP cells and mature DN and SP cells expressing the $\alpha\beta$ -TCR were observed. In addition, numbers of DN and CD8⁺SP cells expressing the $\gamma\delta$ -TCR were markedly increased. These data suggest that transgenic stromal cells responded to the hGM-CSF signal through the hGMR on their cell surface and then affected thymocyte proliferation and differentiation (Fig. 8).

DISCUSSION

We recently generated transgenic mice expressing both the α and the β subunit of the hGMR.⁵ In this study, we assessed the effects of hGMR signaling on the proliferation and differentiation of thymocytes. Proliferation assays showed that DN and SP populations proliferated in response to hGM-CSF *in vitro*, although they did not respond to murine GM-CSF. These results indicate that hGM-CSF can transduce proliferative signals in thymocytes as long as the cells express the hGMR, a finding in line with our previous report that

hGM-CSF supports the growth of erythroid precursor cells in the absence of erythropoietin when they express the hGMR.⁵ However, DP cells failed to proliferate in response to exogenous hGM-CSF, even though they expressed the hGMR. It is known that DP cells neither proliferate nor survive in response to a variety of cytokines, although the molecular basis of this phenomenon is not clear. The lack of expression of several cytokine receptors on DP cells has been reported,²⁻⁴ suggesting that this may be the cause of the unresponsiveness. However, our results suggest that the failure of DP cells to respond to cytokines is not simply due to the lack of receptor expression, but rather is due to differences in the signal transduction machinery between these cells and cells at other stages of differentiation.

Signaling through the hGMR not only induced thymocyte proliferation but also inhibited thymocyte differentiation in a stage-specific manner. The block point was analyzed by repopulation of wild-type fetal lobes with defined TN thymocyte precursor populations from hGMR transgenic mice. Pro-T cells and most pre-T cells were unable to differentiate into DP cells when placed back into FTOC in the presence of hGM-CSF. However, the more mature post pre-T cells were able to differentiate into DP and SP cells in FTOC. When pro-T cell-repopulated FTOC were phenotyped after culture with hGM-CSF, CD44⁺CD25⁺ pre-T cells were detected, which indicated that hGM-CSF did not prevent differentiation of pro-T cells to pre-T cells (data not shown). Pre-T cell-repopulated FTOC contained a small percentage of DP and SP cells, suggesting that the pre-T cells themselves are heterogeneous and that some of these cells have progressed past the hGM-CSF block point.

To account for the inhibition of thymocyte differentiation by hGM-CSF, it is tempting to speculate that hGM-CSF alters the lineage commitment of thymocyte progenitors. Interestingly, transgenic pro-T cell-repopulated FTOC contained a few large granulated cells, suggesting that a limited population of the progenitors

had differentiated to the myeloid lineage, although we did not phenotype this population. However, the inhibitory effect of hGM-CSF on the majority of thymic progenitors is reversible because removal of hGM-CSF from FTOC resulted in the normal differentiation.²⁰ Therefore, it appears that the block may not alter the differentiation potential of progenitors.

Previous studies examining the influence of cytokines such as transforming growth factor- β , IL-4, and IL-7 on T cell development in FTOC have detected effects similar to those of hGM-CSF.²³⁻²⁵ In particular, addition of IL-7 to FTOC resulted in a decrease of DP cells and a decrease in the number of $\alpha\beta$ -TCR-expressing T cells, with a corresponding increase of $\gamma\delta$ -T cells. While these findings are similar to our observations, these cytokines affected T cell differentiation at the CD44⁺ CD25⁻ cell or CD44⁺ CD25⁺ (pro-T) cell stage, that is, at a point earlier than that observed with hGMR signaling. Therefore, the mechanism of inhibition is likely to differ between hGM-CSF and these other cytokines. The block point of hGM-CSF seems to be close to the thymocyte maturation block point observed in mice with genetic deficiencies affecting pre-TCR expression or selection. The pre-TCR consists of a rearranged β -TCR chain in association with a pre-T α chain that forms a receptor transducing a signal required for continued maturation of $\alpha\beta$ -T cells in combination with CD3 ϵ and p56^{lck}. Mice lacking expression of recombinase-activating gene, TCR- β , pre-T α , CD3 ϵ , or p56^{lck} have a defect in their ability to generate $\alpha\beta$ -T cells, and thymic differentiation is arrested at the pre-T to post pre-T transition stage.^{14, 26-31} These findings are similar to those we obtained, suggesting that hGMR signaling inhibits either β -TCR gene rearrangement or β -selection via pre-TCR expression. Such a mechanism could explain why hGM-CSF inhibits differentiation of thymocytes but not that of other cells of the myeloid lineages.

Although both blocks may occur at a similar stage of differentiation, there are marked differences between hGMR transgenic mice and mice lacking pre-TCR function. The latter mice show a marked decrease of thymocyte numbers in addition to blocking of thymocyte differentiation.^{14, 26-31} It is possible that thymocyte proliferation is linked to differentiation and that blocking of differentiation results in the inhibition of proliferation. However, addition of hGM-CSF to transgenic pro-T cell-repopulated FTOC caused only a slight decrease of cell numbers, and pre-T cell-repopulated FTOC showed an increase of cell numbers despite the blocking of differentiation. These results suggest that these cells, although unable to differentiate beyond the pre-T cell stage, have the capacity to proliferate within the fetal thymic environment in response to hGM-CSF. Incubation of the transgenic pro-T, pre-T, or post pre-T cells with hGM-CSF in single cell suspension cultures also resulted in an increase of cell numbers for all three precursor populations (data not shown). It seems likely that cell proliferation induced by hGM-CSF is linked to the inhibition of differentiation. A recent

report showed that pro-T cells remain in the cell cycle but some pre-T and DP cells exit from the cell cycle, suggesting that a correlation exists between pre-TCR or TCR selection and cell cycle status.³² It is possible that unregulated proliferation of pre-T cells induced by hGM-CSF leads to the failure of these cells to differentiate.

In contrast, addition of hGM-CSF to conventional hGMR FTOC decreased cell yields. We showed that these divergent results were the result of secondary effects of hGM-CSF on transgenic stromal cells by repopulating hGMR transgenic lobes with wild-type pro-T cells lacking hGMR. Consistent with our hypothesis, the addition of hGM-CSF to repopulated FTOC decreased cell yields, although the frequency of DP cells also decreased unexpectedly. These results suggest that hGM-CSF influenced the proliferation and differentiation of wild-type pro-T cells via stromal cells expressing hGMR. The decrease of DP cells was similar to that induced by hGM-CSF via the hGMR on thymocytes. However, there were also several differences such as the existence of a few DP cells and mature DN and SP cells expressing the $\alpha\beta$ -TCR and a marked increase of DN and CD8⁺ SP cells expressing the $\gamma\delta$ -TCR. These results suggest that the inhibitory mechanisms mediated via hGMR differ between stromal cells and thymocytes. This idea is supported by the divergent effect on thymocyte proliferation, although we do not know whether or not the effects of hGM-CSF on proliferation and differentiation are linked. One possible mechanism to account for the effect of hGM-CSF mediated via stromal cells is that stromal cells exposed to hGM-CSF lose the ability to support thymocyte proliferation or differentiation. At present we do not know whether the inhibition is mediated by nonlymphoid hemopoietic cells in the thymus, such as macrophages, or by nonhemopoietic stromal cells, such as epithelial cells. Alternatively, stromal cells could produce molecules that inhibit thymocyte differentiation in response to hGM-CSF. For example, the inhibition by stromal cells was similar to that by IL-7. Addition of IL-7 to FTOC resulted in a decrease of DP cells and a decrease of $\alpha\beta$ -TCR-expressing T cells, with a corresponding increase of $\gamma\delta$ -T cells, especially CD8⁺ $\gamma\delta$ -T cells.²⁵ However, we have no evidence to support the existence of such inhibitory molecules, so further studies are needed to define the mechanism of inhibition by stromal cells.

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