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Generation of Macrophages from Early T Progenitors In Vitro

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Early T progenitors in the thymus have been reported to have the capacity to develop into B cells, thymic dendritic cells, and NK cells. Here we describe conditions that induce early T progenitors to develop into macrophages. Initially, we observed that early T progenitors could be induced to develop into macrophages by cytokines produced from a thymic stromal cell line, TFGD, and later we found that the cytokine mixture of M-CSF plus IL-6 plus IL-7 also induced macrophage differentiation from pro-T cells. M-CSF by itself was unable to induce macrophage differentiation from early T progenitors. To correlate this observation with the developmental potential of early T progenitors, mouse embryonic thymocytes were sorted into four populations, pro-T1 to pro-T4, based on the expression of CD44 and CD25, and then cultured with TFGD culture supernatant. We found that pro-T1 and pro-T2 cells, but not pro-T3 and pro-T4 cells, generate macrophages. Limiting dilution analysis of the differentiation capability of sorted pro-T2 cells also confirmed that pro-T2 cells could generate macrophages. These results suggest that T cells and thymic macrophages could originate from a common intrathymic progenitor.


T cells develop in the thymus from precursor cells seeded continuously from the bloodstream (reviewed in Ref. 1). T cell precursors proliferate, rearrange TCR genes, and then are subjected to a series of positive and negative selection events (reviewed in Ref. 2). A major issue in early T cell development was the developmental potential of the early T progenitors in the thymus (reviewed in Ref. 3). T cell precursors derive, ultimately, from multipotent hemopoietic stem cells (HSCs) in the fetal liver or in the adult bone marrow, and although some HSCs enter the thymus (reviewed in Refs. 4–6), most hemopoiesis has been attributed to more specialized progenitors whose "lymphoid" commitment is imposed before entering the thymus (7). The earliest intrathymic T progenitors have been defined as CD4lowCD8lowCD3+CD44+CD25− (pro-T1) cells. Pro-T1 cells, which are distinguished from HSCs by the expression of CD4 and CD44, were shown, in addition to T cells, to generate B cells, NK cells, and dendritic cells (DCs), but not myeloid or erythroid cells, when transferred into irradiated mice (8–13). Pro-T2 cells, the next stage in the T cell pathway, are CD4−CD8−CD3−CD44+CD25− and have lost the precursor activity for B cells and NK cells, but were shown to retain the capacity to generate DCs following transfer to irradiated recipients in vivo, or in vitro with a mixture of cytokines (14–15). Later stages in the T cell pathway, CD4+CD8−CD3−CD44+CD25+ (pro-T3) cells and CD4+CD8−CD3−CD44+CD25− (pro-T4) cells, were shown to have rearranged TCR-β and -γ genes and to be committed to the T cell lineage. 

Thymic DCs play a key role in presenting self Ags to developing thymocytes. The preceding observations have suggested that thymic DCs, rather than deriving from myeloid precursors, actually develop from the same thymic progenitors, pro-T1 and -T2 cells, as do T lymphocytes (16). 

Thymic macrophages play an important role in eliminating apoptotic thymocytes (17), which die at a high rate because of failure of their Ag receptors to recognize self Ags with appropriate avidity. Part of the recognition of apoptotic thymocytes by thymic macrophages is via scavenger receptors (18). Thymic macrophages are heterogeneous in surface markers (19). The origin of thymic macrophages has not been established. On one hand, they could derive from blood monocytes; however, it is also possible that they develop locally based on evidence of myelopoiesis in the thymus (20). If thymic macrophages were generated locally, it would be expected that they derive from multipotential HSCs rather than from pro-T cells, because the latter failed to generate macrophages under the conditions tested previously (reviewed in Ref. 16). Here, we report that early T progenitors have the potential to generate macrophages in vitro culture incorporating M-CSF plus IL-6 plus IL-7. Pro-T1 and -T2 cells were shown, through cell sorting and single-cell cloning, to generate macrophages under these conditions, whereas pro-T3 and pro-T4 cells had lost this capacity. Our results extend the lineage potential of early T progenitors and suggest that thymic macrophages, like thymic DCs, could be generated within the thymus from a common progenitor.

Materials and Methods 

Cell line and cell culture 

The mouse thymic stromal cell line, TFGD, was obtained from a thymoma mass that spontaneously developed in a p53−/− mouse. Briefly, a single-cell suspension of the tumor was prepared by treatment with trypsin and...
cultured in RPMI 1640 supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FBS (HyClone, Logan, UT) in a tissue culture plate. Vigorously growing adherent cells were subcultured three times a week thereafter. At the 28th subculture, this cell line was identified to produce cytokines that induce differentiation of thymic progenitor cells into macrophages.

**Cytokines and mAbs**

Recombinant human M-CSF, mouse GM-CSF, mouse IFN-γ, mouse IL-6, and mouse IL-7 were purchased from PeproTech (Rocky Hill, NJ). The mAbs recognizing murine cell surface markers, anti-CD11b (clone M1/70), anti-CD11c (clone HL3), anti-CD25 (clone 7D4), anti-CD40 (clone 3/23), anti-CD44 (clone IM7), anti-CD45 (clone RA3-6B2), anti-HSA (clone M1/69), anti-ICAM-1 (clone 3E2), anti-I-Ak (clone AF6-120.1), anti-B7-1 (clone 16-10A1), anti-B7-2 (clone GL1), and anti-Gr-1 (clone RB6-8C5) were purchased from BD PharMingen (San Diego, CA). Anti-F4/80 (clone A3-1) was purchased from Serotech (Oxford, U.K.). Anti-Dec-205 and anti-CSFIR were provided by Drs. K. Komschlies and J. Keller (National Cancer Institutes, Frederick, MD), respectively. As isotype-matched control Abs, anti-TCR-Vβ4 (clone KT4), anti-TCR-Vβ2 (clone B20.6), anti-TCR-Vβ4 (clone K25), anti-TCR-Vβ1.8 (clone MR5-2) and anti-TCRβ (clone G235-1) were purchased from BD Pharmingen and were used respectively.

**Preparation and sorting of fetal thymocytes**

C57BL/6 mice were mated overnight, and plugs were checked the following day, which was designated as day 1 of gestation. At day 14 or 15 of gestation, mothers were sacrificed by CO2 asphyxiation and embryos by chilling on ice. Fetal thymuses were removed under dissection microscope. Fetal thymocytes were prepared from fetal thymus lobes by gentle dissection after treatment with 0.2% collagenase (Sigma, St. Louis, MO) on ice. Thymocytes were then cultured in a 24-well tissue culture plate (1 x 10^6/well) in a total volume of 200 μl. After overnight stimulation, 50 μl of cell-free supernatant was incubated with 50 μl of Griess reagent (1% sulfanilamide/0.1% naphthylenediamine dihydrochloride/2.5% HPO_4_2^-) at room temperature for 5 min, and the absorbance at 550 nm was determined in a Dynatech MR900 microplate reader (Dynatech Laboratories, Chantilly, VA). The concentration of NO^-3 was determined from a least squares linear-regression analysis of a sodium nitrite standard curve.

**Generation of macrophages from fetal thymocytes**

Adherent cells were first removed from total day-14 fetal thymocytes by plastic adherence after incubating fetal thymocytes overnight in a 6-well or 24-well plate in a culture medium supplemented with the culture supernatant of TFGD (50%, final concentration) or with defined amounts of cytokines. After the adherent cell-depleted thymocytes were counted, we adjusted the cell number (1 or 2 x 10^4/ml) with the same medium and then distributed them to wells of a 24-well tissue culture plate (1 ml/well). Cells were fed with the same medium every 3 to 4 days by replacing half of the culture medium with fresh medium. For sorted populations of fetal thymocytes, cells were cultured directly in a 24-well tissue culture plate (1 x 10^5/well) in a culture medium supplemented with the culture supernatant of TFGD.

**Phenotypic analysis**

Macrophages (2 x 10^5) were washed in a staining solution of PBS containing 5% FBS and 0.1% NaN_3, and then resuspended in 50 μl of staining solution containing 10 μg of anti-CD16/CD32 mAbs (clone 2.4G2) and 10% normal mouse serum to block nonspecific binding of Abs to Fc receptors. Cells were incubated for 10 min on ice, mixed with 50 μl of prelabeled Ab solution and then incubated for another 20 min on ice. Unbound Abs were removed by washing the cells twice with staining solution. For biotinylated Abs, the cell pellet was resuspended in 50 μl of PE- or FITC-conjugated avidin solution, incubated for 10 min on ice, and then washed twice with staining solution. For unconjugated Abs, Dec-205 and CSFIR, the cell pellet was resuspended in 50 μl of FITC-goat anti-rat IgG (BD Pharmingen) incubated for 10 min on ice, and then washed twice with staining buffer. After a final washing, cells were fixed in 1% paraformaldehyde in PBS, and flow cytometric analysis was performed on a FACS-Calibur (BD Biosciences, Mountain View, CA) as previously described (12). The purity of sorted populations was generally >98%.

**APC function**

APC function of macrophages derived from pro-T cells was assessed by examining their ability to stimulate proliferation of T cells with anti-CD3. T cells were isolated from the spleen of C57BL/6 mice by depleting adherent cells with nylon wool adherence and then sorting the adherent cell-depleted cells with anti-IA mAb (clone 25-9-3a; American Type Culture Collection, Manassas, VA) plus rabbit serum (Low-Tox-M; Cedarlane Laboratories, Ontario, Canada). Purified T cells (1 x 10^5/ml) were mixed with anti-CD3 mAb (50 ng/ml; BD Pharmingen), and then 100 μl of the cell suspension was added to each well of U-bottom plates containing pro-T derived macrophages. Adherent cells that were isolated from the spleen cells of C57BL/6 mice by plastic adherence (37°C, 2 h) were used as a control APC. DNA synthesis was measured by [3H]thymidine (DuPont Pharmaceuticals, Wilmington, DE) incorporation (1 μCi/well) for the final 6 h of the 3-day culture period.

**Results**

**Generation of macrophages from pro-T cells**

A thymic stromal cell line, TFGD, was derived from a thymic tumor mass that developed spontaneously in a p53^-/-^ mouse. At the 28th passage, supernatant from the TFGD line was observed to induce pro-T cells to differentiate into a cell type that did not resemble a lymphoid cell, and which was later identified as a macrophage. As a source of early T progenitors, we used day-14 embryonic fetal thymocytes, which are composed of the first two stages of pro-T cells, pro-T1 and pro-T2, in approximately equal proportions. To insure that the macrophages generated in this culture system did not derive from macrophages in the starting population, adherent cells in the fetal thymocyte preparations were first removed by plastic adherence after being cultured overnight in a culture medium containing the culture supernatant from TFGD cells (50%, final concentration). The adherent cell-depleted fetal thymocytes were then cultured in the same type of medium for additional 7–8 days. Most of the fetal thymocytes formed a layer of adherent cells when observed at day 4 from the initiation of the culture, and the adherent cells, appearing to undergo cell division, differentiated into macrophages. Approximately 6 x 10^5 macrophages could be recovered consistently at day 8 from 2 x 10^5 adherent cell-depleted day-14 fetal thymocytes.

Shown in Fig. 1 are the phenotypic and functional characteristics of the macrophages generated after 8 days of culture of pro-T cells in supernatant from TFGD cells. Pro-T-derived macrophages exhibited variable degrees of cytoplasmic vacuolation but lacked cytoplasmic granules, as shown in forward and side scatter profiles. As shown in Fig. 1A, the cells expressed surface markers typical of mouse macrophages including CD11b (Mac-1), receptors for CSF-1 (CSF-1R), CD24 (heat-stable Ag), CD44 (pp1), IgG-opsonized sheep RBC (SRBC, 5%). The slide chambers were incubated for 1 h at 37°C, rinsed with PBS, and then treated with ACK lysis buffer for 3 min to lyse unengulfed SRBC. Opopsonized SRBC were prepared by incubating SRBC with a 1:256-diluted mouse anti-SRBC IgG Ab (Cordis, Miami, FL) for 30 min at 37°C in a shaking water bath.
CD45 (leukocyte common Ag), and CD54 (ICAM-1). Pro-T-derived macrophages did not express lymphoid lineage markers such as CD25 and CD45R (B220). The granulocyte lineage marker, Gr-1, was not expressed. DC-restricted markers, Dec-205 and CD11c, were also negative in the macrophages, although a low level of expression was observed for F4/80, which is also expressed on some macrophages. We examined surface molecules important in interacting with T cells and observed high levels of expression of both the costimulatory molecule B7-1 (CD80) as well as the activating receptor CD40. B7-2 (CD86), another costimulatory molecule, was not expressed, and the constitutive level of MHC class II (I-Ab) was low. However, the level of MHC class II was markedly up-regulated upon stimulation with IFN-γ, as shown in Fig. 1B.

Functional assays of pro-T-derived macrophages showed that they produced NO in response to IFN-γ and LPS stimulation (Fig. 1C). The macrophages produced higher amounts of NO than the control mouse macrophage cell line ANA-1, which was established by infecting fresh bone marrow-derived cells from C57BL/6 with the J2 (v-raf/v-myc) recombinant retrovirus (21) and has been well characterized to produce NO. Pro-T-derived macrophages were also highly phagocytic for IgG-opsonized SRBC, as shown in Fig. 1D. APC function of pro-T-derived macrophages was weak, as shown in Fig. 2, adding to the criteria distinguishing these macrophages from DCs, which are potent APCs.

Both Pro-T1 and T2 cells can generate macrophages

Mouse embryonic thymocytes were sorted into four populations, pro-T1 to pro-T4, the first four stages of development, and then examined for their developmental potential to macrophages.
Pro-T1 cells, cells in the most primitive stage, effectively generated macrophages when cultured in a medium with TFGD culture supernatant (Fig. 3, upper panel). Even pro-T2 cells could generate macrophages (Fig. 3, lower panel) under these conditions, although fewer, when compared with pro-T1 cells, initiated with the same cell number as can be seen comparing the results found in Fig. 3, upper vs lower panels. Generation of macrophages from pro-T1 and T2 cells was further confirmed by a single-cell cloning assay we recently used to determine the clonal origins of NK, αβ, and γδ T cells (12). Pro-T1 and T2 cells were sorted, plated at a frequency of one cell per well in a round-bottom microtiter plate, and then cultured for an additional 7 days in a medium supplemented with TFGD culture supernatant. Microscopic observation of the wells showed a range of 48–74% (two experiments) of pro-T1 cells vs 6–15% (three experiments) of pro-T2 cells differentiated into adherent cells with morphology of macrophages (data not shown). The later stages (pro-T3 and pro-T4) were unable to generate macrophages under these conditions (data not shown). Single cells underwent very few cell divisions during this culture period, just one to three divisions for pro-T1 and none or one division for pro-T2 cells. To verify that single cells generated macrophages, we observed that the cells readily phagocyted, opsonized latex beads.

Cytokine combination of M-CSF, IL-6, and IL-7 supports the development of macrophages from pro-T cells

To identify the cytokines responsible for the generation of macrophages from early T progenitors, we first assayed for a number of cytokines in the TFGD culture supernatant by ELISA. High levels of M-CSF and IL-6 were detected, whereas no GM-CSF, IL-4, IL-5, IL-9, or IL-13 was detectable (data not shown). Because M-CSF was present, and it is an effective inducer of macrophage generation from bone marrow cultures and IL-6 is an effective cofactor in macrophage generation, we examined the ability of M-CSF and IL-6 alone and in combination with other cytokines to induce the development of macrophages from pro-T cells. As shown in Fig. 4, M-CSF alone or IL-6 alone failed to induce differentiation of day-14 thymocytes into macrophages. Combining IL-6 with M-CSF could induce macrophage differentiation from early T progenitors, although the differentiation-inducing activity was low compared with that of TFGD culture supernatant. Combining IL-7 with M-CSF and IL-6 resembled TFGD culture supernatant in strongly increasing the differentiation to macrophages. Comparison of TFGD culture supernatant with the mixture of IL-7, M-CSF, and IL-6 showed that the defined cytokine mixture induced about half the frequency of macrophage potential from individual pro-T cells, suggesting that TFGD culture supernatant may contain additional activities.

Discussion

We demonstrate that early T progenitors can generate macrophages in vitro following stimulation by a mixture of cytokines. The macrophages generated from early T progenitors expressed...
surface markers characteristic of mature macrophages in the periphery, were strongly phagocytic for IgG-opsonized SRBC, and produced NO in response to LPS and IFN-γ stimulation. The macrophages expressed a low level of class II MHC molecules that increased significantly following IFN-γ stimulation.

We show that macrophages could be generated from sorted populations of pro-T1 or T2 cells. Limiting dilution analysis indicated that sorted pro-T1 and T2 cells showed a range of cloning efficiency of 48–74% and 6–15%, respectively, making it unlikely that the macrophages were generated from contaminating multipotential stem cells. An earlier study showed that mouse fetal thymocytes could generate macrophages in vitro when cultured with a stromal cell line (5) and was interpreted to indicate the presence of multipotential stem cells that have been shown to be present in the fetal thymus (4), whereas our study defines conditions under which true pro-T cells can generate macrophages.

Our results extend the lineage potential of early T progenitors in the thymus. As reviewed by Shortman et al. in detail (16), the c-kit“CD44 “CD25” (pro-T1) cells have previously been shown to have the capacity to form B cells, NK cells, and thymic DCs, as well as T cells, but not erythroid or myeloid cells, under the conditions tested at that time (15). The next downstream precursor, pro-T2 cells, appeared to have lost B cell and NK cell potential but still retained the capacity to form DCs. Thus, thymic DCs are now thought to derive from the common lymphoid precursor rather than from a myeloid precursor. Our results show that, like DCs, which were previously thought to be of myeloid origin, macrophages, too, can derive from a “lymphoid” progenitor.

Some parallels between B and T cell development can now be seen with regard to the potential to generate other cell types. A bipotential B macrophage progenitor has been demonstrated previously (22). Those bipotential B macrophage progenitors, like the T lineage progenitors in this study, respond to IL-7 (23). DCs have also been shown to be generated from pro-B cells (24) under similar conditions required to generate DCs from pro-T cells. In both T and B lineages, VDJ recombination marks a stage of loss of macrophage and DC potential. If the onset of VDJ recombination was coupled to lymphoid commitment, it could explain why, in our study, a lower proportion of pro-T2 cells had macrophage potential than did pro-T1 cells, because VDJ recombination accelerates at the pro-T2 stage (25).

M-CSF alone was unable to induce macrophage differentiation from early T progenitors, whereas it alone is sufficient to induce bone marrow precursors to generate macrophages. The combination of M-CSF and IL-6 induced significant macrophage differentiation from pro-T cells, a phenomenon similar to the observation of Jansen et al. that IL-6 is required for optimal colony formation by bone marrow cells (26). Addition of IL-7 to M-CSF plus IL-6 dramatically increased the frequency of macrophages generated from pro-T cells and presumably reflects the dependency of pro-T cells on survival signals from IL-7 (27, 28). The IL-7 effect also distinguishes pro-T cells from bone marrow cells in the signals required for macrophage generation and may explain the previous failures to detect macrophage generation from this cell type. We noted that the TFGD culture supernatant was about a mixture. Our study shows that macrophage diversion can be induced through receptors naturally expressed on pro-T cells.

It remains to be shown whether in vivo thymic macrophages, like thymic DCs, derive from pro-T cells. However, this possibility is supported by findings that the three cytokines that we show generate macrophages in vitro are produced in the thymus: M-CSF (30), IL-6 (31), and IL-7 (32). Local production of macrophages would be a means of coordinating the numbers of thymocytes with the numbers of macrophages required to dispose of dead thymocytes, which some estimate as high as a third of the thymocytes dying each day.

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References
18. Platt, N., H. Suzuki, Y. Kurihara, T. Kodama, and S. Gordon. 1996. Role for the IL-2 receptor into lymphoid progenitors from bone marrow en-...