IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Transgenic Expression of GM-CSF in T Cells Causes Disseminated Histiocytosis

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Recent studies highlight surprising roles for granulocyte-macrophage colony-stimulating factor (GM-CSF) production by T cells. T-cell–derived GM-CSF is required for the differentiation of monocyte-derived inflammatory dendritic cells during inflammation and for the pathogenicity of IL-17 producing T helper cells in autoimmunity. To gain further insight into these findings, we engineered *in vivo* overexpression of GM-CSF specifically in T cells, under the control of the *Lck* promoter. *Lck*-GM-CSF transgenic mice displayed a dramatic phenotype, characterized by splenomegaly, lymphadenopathy, thymic atrophy, and multiple abnormalities in blood cell populations. Thymocyte differentiation was severely affected, and there was a dramatic increase in regulatory T cells in the thymus and peripheral lymphoid organs. *Lck*-GM-CSF transgenic mice developed a disseminated histiocytosis and had increased circulating IL-17 producing T helper cells–related cytokines. The pathological characteristics in *Lck*-GM-CSF transgenic mice resemble those of histiocytic human diseases, such as Langerhans cell histiocytosis. The etiology of many histiocytic disorders is unknown, but our findings suggest that over-production of GM-CSF by T cells could be a pathogenic factor and raise the possibility that GM-CSF may represent a novel therapeutic target. (*Am J Pathol* 2014, 184: 184–199; <http://dx.doi.org/10.1016/j.ajpath.2013.09.014>)

Although granulocyte-macrophage colony-stimulating factor (GM-CSF) was first described as a hematopoietic regulator that stimulates the expansion and differentiation of myeloid cells from bone marrow progenitors, it is recognized to have multiple functions.¹ Interestingly, GM-CSF is produced by T cells and is transiently up-regulated in CD4 and CD8 T cells on stimulation through the T-cell receptor and costimulatory molecules, such as CD28.² Recently, IL-23 and the transcription factor, retinoic acid receptor–related orphan receptor γ t (ROR- γ t), were also found to drive GM-CSF production by type 17 helper T cells (Th17).³ However, GM-CSF is unlikely to act directly on T cells, because these cells lack expression of the GM-CSF receptor.^{4,5}

We recently reported that T-cell–derived GM-CSF plays a pivotal role in acute inflammatory peritonitis and during acute inflammatory arthritis.⁶ Production of GM-CSF by murine T cells was NF- κ B1 dependent, showing that transcription factors other than ROR- γ t can drive its production. T-cell–derived GM-CSF stimulated the differentiation of monocyte-derived

inflammatory dendritic cells (MoDCs), most likely through the recruitment of monocyte precursors via chemokine ligand (CCL) 2 (monocyte chemoattractant protein-1). Other recent studies showed that GM-CSF promotes the induction and survival of Th17 cells via IL-6 and IL-23, and that GM-CSF is required for the pathogenicity of Th17 cells in experimental allergic encephalomyelitis.^{3,7} These discoveries, and the knowledge that GM-CSF–deficient mice are protected from several T-cell–dependent experimental disease models,^{8,9} suggest that GM-CSF might be of much greater importance for T-cell function than previously thought.

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Several models of transgenic overexpression of GM-CSF, either systemically¹⁰ or within nonhematopoietic tissues, have been previously described.^{11–14} A common feature of these models was local macrophage and dendritic cell (DC) recruitment/differentiation, with phenotypes including blindness due to accumulation of macrophages in the eye¹⁰ and the induction of autoimmune gastritis.¹⁴ To further dissect the role that T-cell-derived GM-CSF might play during inflammatory disease, we generated transgenic (TG) mice that overexpress GM-CSF specifically in T cells, using the murine *Lck* (p56lck) promoter. We found a dramatic phenotype in *Lck*-GM-CSF TG mice, characterized by splenomegaly, lymphadenopathy, and a remarkable infiltration of both lymphoid and nonlymphoid organs by histiocytes. Histiocytes possess hybrid characteristics of DCs, macrophages, and monocytes and are a classic feature of a range of poorly understood human diseases. Our study suggests that increased production of GM-CSF by T cells can drive histiocytosis and raise the possibility that GM-CSF inhibition may be beneficial in some of the histiocytic disorders.

Materials and Methods

Production of *Lck*-GM-CSF TG Mice

A 446-bp cDNA containing the coding sequence for murine GM-CSF (mGM-CSF) was inserted into the BamHI cloning site of the p1017S1 plasmid, which is flanked by the T-cell-specific murine *Lck* proximal promoter and the human growth hormone poly-A tail (hGHpA).¹⁵ A linearized 5715-bp mGM-CSF transgene construct was injected into the pronucleus of fertilized C57BL/6 embryos. Two founder lines of *Lck*-GM-CSF TG mice were generated, with a different transgene copy number (as determined by Southern blot analysis). These founders were designated *Lck*-GM^{hi} and *Lck*-GM^{lo}, respectively. TG mice were maintained in the heterozygous state on a C57BL/6 background.

Southern Blot Analysis for *Lck*-GM-CSF Transgene Detection

Genomic DNA from TG mice and wild-type (WT) littermate controls was purified from spleen by standard protocols and 5 µg (*Lck*-GM^{hi}) or 10 µg (*Lck*-GM^{lo}) of DNA was digested overnight with PstI at 37°C. DNA was run on a 0.8% Tris-acetate-EDTA agarose gel, transferred to a Hybond N+ nylon membrane, probed with a murine GM-CSF-specific ³²P-deoxy-ATP-labeled 446-bp cDNA, and visualized by a Phospho Imager (BioRad, Gladesville, Australia).

Quantitative RT-PCR for GM-CSF and ROR-γt Expression

Total RNA was purified using RNeasy columns (Qiagen, Chadstone, Australia). cDNA was prepared by reverse

transcription. Quantitative PCR was performed using an ABI Prism 7900 instrument (Applied Biosystems, Mulgrave, Australia) and SYBR Green reagent, according to the manufacturer's instructions (Qiagen). Results were normalized (ΔC_T) for hydroxymethylbilane synthase (hmbs) RNA content.

$\Delta\Delta C_T$ was calculated as ΔC_T (TG) – ΔC_T (WT) for each sample.

Primer sequences were as follows: hmbs, 5'-CC-TGGTTGTTCACTCCCTGA-3' (forward) and 5'-CA-ACAGCATCACAAGGGTTT-3' (reverse); GM-CSF, 5'-CCAGCTCTGAATCCAGCTTCTC-3' (forward) and 5'-TCTCTCGTTTGTCTTCCGCTGT-3' (reverse); and ROR-γt, 5'-CCGCTGAGAGGGCTTCAC-3' (forward) and 5'-TGCAGGAGTAGGCCACATTACA-3' (reverse).

Mice

Rag1-deficient (*Rag1*^{−/−}) mice¹⁶ and GM-CSF-deficient (*Csf2*^{−/−}) mice¹⁷ (both backcrossed to the C57BL/6 background for 15 generations) were obtained from the Walter and Eliza Hall Institute (WEHI) animal services (Kew, Australia).

Cell Culture

Single-cell suspensions of pooled superficial lymph nodes (LNs), LN cells enriched for CD4⁺/CD8⁺ cells by magnetic bead purification (Miltenyi Biotec, Macquarie Park, Australia), or thymocytes were cultured for 3 days at 0.5 × 10⁶ cells per well in 96-well culture plates at 37°C, 5% CO₂ in RPMI 1640 medium containing 10% fetal calf serum, 20 µmol/L 2-mercaptoethanol, 2 mmol/L glutamine, and 1 mmol/L sodium pyruvate [complete medium (CM)]. Cells were stimulated on plates coated with 10 µg/mL anti-CD3 (clone 145-2C11; WEHI, Bundoora, Australia) and 2 µg/mL anti-CD28 (clone 37.51; WEHI) monoclonal antibodies. For analysis of cytokine production by histiocytes, purified histiocytes (sorted by flow cytometry) were cultured for 10 hours in CM at 37°C, 5% CO₂.

Immune Activation *in Vivo*

Mice were injected with 100 µL complete Freund's adjuvant (CFA; Sigma, Castle Hill, Australia) s.c. at the base of the tail. Control mice received PBS only. Seven days after CFA injection, superficial LNs were pooled, single-cell suspensions were prepared, and cells were restimulated *in vitro* with 50 ng/mL phorbolmyristate ester and 1 µg/mL ionomycin, or medium alone (un-stimulated), for 4 to 6 hours. Production of cytokines was determined by intracellular flow cytometry.

Measurement of GM-CSF in Supernatants by Enzyme-Linked Immunosorbant Assay

GM-CSF was measured in culture supernatants with the antibody pair, MP1-22E9 and biotinylated MP1-31G6 (both

from BD Pharmingen, North Ryde, Australia), used according to the manufacturer's instructions.

GM-CSF Bioassay

The bioactivity of GM-CSF in fresh culture supernatant was tested using the GM-CSF-dependent FDC-P1 cell line (WEHI). Different concentrations of culture supernatant were added to 1000 FDC-P1 cells per well in the presence of 10 µg/mL neutralizing anti-IL-3 monoclonal antibody (clone MP2-8F8; WEHI). Cells were cultured in 96-well black optical culture plates (Thermo Scientific, Scoresby, Australia) for 2 days at 37°C, 10% CO₂, in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Proliferation was determined by adding 20 µL per well resazurin (Sigma), and relative fluorescence units were measured at 535 nm (excitation) and 590 nm (emission) after 1, 2, 3, and 4 hours, respectively. Optimum fluorescence was obtained at 4 hours.

Measurement of Multiple Analytes in Serum and Supernatants

Serum or cell supernatant cytokines and chemokines were measured using the Bio-Plex mouse cytokine 23-plex panel (BioRad), used according to the manufacturer's instructions. The assay was read on a Bio-Plex 200 instrument and analyzed using Bio-Plex Manager software version 5.0 (BioRad, Gladesville, Australia).

Antibodies for Flow Cytometry

The following anti-mouse antibodies were used: Fcγ receptor II/III (FcR Block) 2.4G2, CD3-biotin, CD4-fluorescein isothiocyanate (FITC), CD4-biotin, CD5-phycoerythrin (PE), CD8-Alexa700, CD8-biotin, CD11b-biotin, CD11c-FITC, CD24-PE.Cy5, CD25-PE, CD28-allophycocyanin (APC), CD44-FITC, CD103-Alexa700, CD115-FITC, CD205-PE, CD205-Alexa700, F4/80-FITC, F4/80-Alexa700, F4/80-Alexa647, and H-2A^b-biotin (all from WEHI); CD3-PE, CD207-Alexa647, Foxp3-APC, and Ly-6C-peridinin-chlorophyll-protein-cyanin5.5 (PerCp-Cy5.5) (all from eBioscience, Kensington, Australia); CD4-PerCp-Cy5.5, CD8-APC, CD8-APC-cyanin7 (APC-Cy7), CD11b-APC, CD11b-PE-Cy7, CD11c-PE, CD11c-APC, CD40-PE, CD43-biotin, CD62L-APC, CD62L-APC.Cy7, CD64-PE, CD207-Alexa647, GM-CSF-PE, IL-17A-APC, c-kit-APC, major histocompatibility complex (MHC) II-PE, NK1.1-biotin, Ly6C/G-APC, Ly6G/C-PE, SiglecF-PE, and streptavidin-PE.Cy7 (all from BD Pharmingen); CD62L-PE-Texas Red (Invitrogen, Mulgrave, Australia); CD25-Alexa700, Ly6C-Pacific Blue (PacBlue), F4/80 PacBlue, Qa2-biotin, and T-cell receptor (TCR) β-PE.Cy7 (all from BioLegend, Karyaryup, Australia); and CX3CR1-PE, CCR2-APC, GM-CSF receptor (GM-CSFR) α-APC, and GM-CSFRβ-APC (all from R&D Systems, East Brisbane, Australia).

Flow Cytometry

Single-cell suspensions were prepared by passing organs through a 70-µm cell strainer or by density gradient, as described later. Erythrocytes were lysed and cells were then washed with PBS containing 2 mmol/L EDTA and 1% fetal calf serum, treated with 10 µg/mL FcR block and stained for flow cytometric analysis. Dead cells were excluded by propidium iodide, Fluoro-Gold, or Aqua live/dead (Invitrogen) staining. For intracellular staining of CD207, cells were treated with BD Cytofix/cytoperm (BD Biosciences, North Ryde, Australia), according to the manufacturer's instructions. For intracellular staining of IL-17A and GM-CSF, cells were cultured for 4 to 6 hours in CM containing monensin and brefeldin (GolgiStop, Golgiplug; BD Pharmingen), according to the manufacturer's instructions, followed by fixation in Cytofix/cytoperm, as previously described. An LSRII or LSR Fortessa instrument was used for flow cytometry (BD BioSciences). Histiocytes were sorted on an MoFlo automated cell sorter (Beckman-Coulter, Gladesville, Australia).

Enrichment of DCs in Superficial LNs

To enrich for DCs, light-density cells (LDCs) were purified as previously described.¹⁸ Cells were then stained for flow cytometric analysis of DC populations and subdivided into subpopulations based on surface and intracellular markers, as previously described.^{19,20}

Histological Analysis of Organs and Cytospins

TG mice and WT littermates (two males and two females of each genotype at 6 weeks of age) were analyzed independently [through Australian Phenomics Network, by pathologist Dr. John Finnie, Adelaide, Australia, who is expert in the diagnosis of lymphoma; and at WEHI D.M.]. Tissue sections (5 µm thick) were stained with H&E and scanned using a Zeiss Mirax digital slide scanner (Zeiss, North Ryde, Australia). Cells were cytopun onto polylysine-coated slides, stained with May-Grunwald Giemsa or Diff Quick stain (Lab Aids, Narrabeen, Australia), and examined using a Zeiss Axioplan 2 microscope, fitted with an AxioCam HR camera (Zeiss). AxioVision software version 3.1 (Zeiss) was used for all analyses of microscopic images.

Differential Cell Counts of Blood and Peripheral Lymphoid Organs

Blood was collected by retro-orbital bleed in sodium-EDTA tubes. Spleen, LN, and thymus were depleted of erythrocytes using ammonium chloride. Automated differential cell counts were performed using an ADVIA 120 instrument calibrated for murine hematopoietic cells (Siemens, Bayswater, Australia).

Figure 1 Generation of *Lck*-GM-CSF transgenic (TG) mice. **A**: DNA construct used to inject fertilized C57BL/6 embryos. **B**: Agarose gel and Southern blot of genomic DNA from wild-type (WT) or *Lck*-GM-CSF TG mice probed with a ³²P-labeled transgene-specific probe. **C**: Survival curves of male and female TG and WT mice ($n = 10$ to 20 mice per group). **D**: Production of GM-CSF by thymocytes or LN cells, unstimulated, or after 3 days of *in vitro* stimulation with anti-CD3 and anti-CD28 (CD3/CD28). ND, not detected. Results are representative of two independent experiments ($n = 2$ mice per group). **E**: Bioactivity assay for GM-CSF. Proliferation was measured (by fluorescence) for FDC-P1 cells. Cells were incubated with 1:1000 diluted culture supernatant of anti-CD3/anti-CD28-stimulated thymocytes or CD4⁺/CD8⁺ LN cells, in the presence of anti-IL-3. Results are representative of two independent experiments ($n = 2$ mice per group). **F**: Quantitative RT-PCR for GM-CSF on purified mRNA from thymocytes, total LN cells, sorted LN CD4⁺ or CD8⁺ T cells, and sorted LN CD3⁺ non-T cells. ΔCt was determined by normalization for expression of the *hmbs* gene. $\Delta\Delta Ct$ was defined as ΔCt TG $- \Delta Ct$ of the corresponding WT cell population. Results are representative of two independent experiments ($n = 1$ to 3 mice per group). Data are given as means $\pm$ SEM. **G**: GM-CSF levels measured by BioPlex in serum of *Lck*-GM TG mice ($n = 16$ mice per group). $*P < 0.05$ (unpaired *t*-test).

In Vitro Clonogenic Analysis of Bone Marrow and Spleen Cells

Colony formation by bone marrow and spleen cells was performed as previously described.²¹

In Vivo Phagocytosis of Fluorescent Latex Beads

Phagocytic cells were labeled *in vivo*, as previously described.²² Briefly, mice were injected i.v. with 250 μ L of 1:25 diluted 0.5 to 1.0 μ mol/L YG fluorescent latex beads (PolySciences, Gynea, Australia). Organs were analyzed for the presence of latex beads by flow cytometry, as previously described.

Mixed Leukocyte Reaction

Splenic antigen-presenting cell populations from *Lck*-GM^{hi} mice were sorted by flow cytometry, based on expression of F4/80, CD11b, and CD11c. Where indicated, cells were fixed with 2% paraformaldehyde (PFA) for 30 minutes on ice. CD3⁺ responder T cells from BALB/c spleen were purified by magnetic bead sorting (AutoMacs; Miltenyi Biotec, North Ryde, Australia), using anti-CD3-PE-labeled antibody (BD Pharmingen) and anti-PE magnetic beads (Miltenyi Biotec), according to the manufacturer's instructions. Sorted TG splenocytes were co-cultured with 1×10^5 CD3⁺ responder cells in a final volume of 200 μ L for 4 days at 37°C and 5%

Figure 2 Splenomegaly, lymphadenopathy, thymic atrophy, perturbed CD8/CD4 T cells in the thymus, and increased ROR- γ t expression in thymocytes and T cells of *Lck*-GM-CSF TG mice. **A:** Photographs of spleen, LN, and thymus of *Lck*-GM^{hi} TG and WT controls at 6 weeks of age. Scale bar = 1 cm. **B:** Flow cytometric analysis of T-cell populations in spleen and LN. Single-cell suspensions were stained for TCR β , CD4, CD8, Foxp3, CD25, CD44, and CD62L. Cells were gated on TCR β ⁺, and Tregs were defined as CD4⁺Foxp3⁺. Naïve T cells were defined as CD62L^{lo}/CD44^{hi}. Effector/memory (eff/mem) T cells (eff/mem) were defined as CD62L^{lo}/CD44^{hi}. **C:** Single-cell suspensions from thymus were analyzed by flow cytometry for mature/immature CD4⁺ and CD8⁺ T-cell populations and triple-negative (CD3/CD4/CD8⁻) differentiation. Mature CD4⁺ T cells were defined as CD24⁻Qa2⁺. Mature CD8⁺ T cells were defined as TCR β ⁺CD24⁻Qa2⁺. Lineage⁻ TN1, TN2, TN3, and TN4 were defined by the expression of CD44 and CD25, as shown. Data are given as means $\pm$ SEM. **D:** Intracellular flow cytometry for the detection of IL-17 and IFN- γ in CD4⁺ and CD8⁺ T cells after priming with CFA and restimulation of LN cells (phorbolmyristate ester/ionomycin) *in vitro*. **E:** Quantitative PCR for ROR- γ t expression on RNA from purified thymocytes and LN cell populations. Expression in TG cells is shown as $-\Delta\Delta C_T$, normalized for expression of the *hmbs* gene and normalized for ROR- γ t expression of the corresponding WT cells. **P* < 0.05, Student's *t*-test. Stim, stimulated; Uns, unstimulated.

Table 1 Weight and Absolute Numbers of Main Cell Subsets in Peripheral Lymphoid Organs

Variable	Spleen			Lymph nodes			Thymus		
	<i>Lck</i> -GM ^{hi}	<i>Lck</i> -GM ^{lo}	WT	<i>Lck</i> -GM ^{hi}	<i>Lck</i> -GM ^{lo}	WT	<i>Lck</i> -GM ^{hi}	<i>Lck</i> -GM ^{lo}	WT
Weight (grams)	0.37 ± 0.04	0.11 ± 0.01	0.09 ± 0.01	0.10 ± 0.02	0.05 ± 0.02	0.03 ± 0.004	0.03 ± 4.0	0.06 ± 0.01	0.07 ± 0.01
WBC	2920.54 ± 396.78	615.99 ± 121.9	479.64 ± 53.88	88.22 ± 19.94	16.50 ± 4.8	27.71 ± 12.15	21.59 ± 6.69	460.11 ± 152.2	401.18 ± 93.9
Neut	103.97 ± 20.13	6.61 ± 2.6	3.28 ± 0.77	1.53 ± 0.18	0.64 ± 0.18	1.05 ± 0.38	0.70 ± 0.22	2.03 ± 1.15	1.54 ± 0.66
Lymph	2023.61 ± 191.75	581.67 ± 113.5	463.15 ± 51.01	78.66 ± 17.91	15.08 ± 4.93	25.90 ± 11.72	18.59 ± 5.77	452.18 ± 147.3	395.51 ± 91.26
Mono	69.53 ± 16.24	2.48 ± 1.45	1.32 ± 0.41	0.46 ± 0.08	0.27 ± 0.0	0.28 ± 0.02	0.16 ± 0.12	2.30 ± 0.0	0.64 ± 0.56
Eos	530.78 ± 185.04	9.79 ± 7.17	3.01 ± 0.43	1.53 ± 0.26	1.59 ± 1.04	0.43 ± 0.06	0.77 ± 0.12	2.22 ± 1.03	1.74 ± 0.6
Baso	105.42 ± 13.66	8.03 ± 1.94	6.40 ± 1.16	1.07 ± 0.33	ND	0.30 ± 0.0	0.87 ± 0.25	32.97 ± 8.4	28.95 ± 5.33

Data are given as means ± SEM × 10⁻⁶/μL. *n* = 4 to 7 mice per group.

Baso, basophils; Eos, eosinophils; Lymph, lymphocytes; Mono, monocytes; ND, not detected (lower than the detection limit); Neut, neutrophils; WBC, white blood cell.

CO₂. Tritiated thymidine, 1 μCi per well (Amersham Bio-Sciences, Rydalmere, Australia), was added for the final 24 hours of culture; then, the supernatant was removed and cells were harvested onto glass-fiber filters for determination of tritiated thymidine incorporation using a Topcount scintillation counter (Perkin Elmer, Glen Waverley, Australia).

Statistical Analysis

P values were determined by Mann-Whitney or Student's *t*-test, as indicated in the table and figure legends.

Results

Generation and Validation of TG Mice

TG mice were generated by injecting a construct comprising the murine proximal *Lck* promoter, mGM-CSF cDNA, and the hGHpA site into fertilized mouse embryos (Figure 1A). TG mice were identified by PCR using primers spanning the hGHpA/mGM-CSF junction. Two founders were selected, and transgenic offspring were confirmed by Southern blot analysis of genomic DNA (Figure 1B). One founder had a higher copy number of integrated transgene DNA, as judged by a more intense band for the transgene, despite lower loading of DNA (Figure 1B). Founders were, therefore, designated *Lck*-GM^{hi} and *Lck*-GM^{lo}. The probe for GM-CSF could also detect endogenous GM-CSF, which served as a loading control (Supplemental Figure S1). Survival analysis showed that *Lck*-GM^{hi} mice appeared healthy for up to approximately 23 weeks, then gradually succumbed to splenomegaly and anemia (Figure 1C). *Lck*-GM^{lo} mice appeared healthy for >12 months and did not show increased mortality (Figure 1C).

To assay expression of the *Lck*-GM-CSF transgene, we cultured thymocytes and LN cells, with or without anti-CD3 and anti-CD28 antibodies. Three days later, GM-CSF levels in the supernatant were measured by enzyme-linked immunosorbent assay. Unstimulated thymocytes from both TG lines and unstimulated LN cells from the *Lck*-GM^{hi} line showed increased production of GM-CSF, compared with WT cells. Stimulated thymocytes and LN cells from both transgenic lines showed markedly increased production of GM-CSF compared with WT cells (Figure 1D). To verify the bioactivity of GM-CSF produced by *Lck*-GM TG lymphocytes, these supernatants were diluted and added to GM-CSF-dependent FDC-P1 cells. Possible interference from IL-3, which is also a growth factor for FDC-P1 cells, was blocked by the addition of neutralizing anti-IL-3 antibodies. FDC-P1 cells displayed a dose-dependent proliferation after incubation with T-cell supernatants, and this was greater with TG supernatants, demonstrating that the *Lck*-GM-CSF transgene produced functional protein (Figure 1E). Increased production of GM-CSF was also confirmed using quantitative RT-PCR on unstimulated *Lck*-GM^{hi} thymocytes and purified LN cell populations (Figure 1F). We found a clear increase in GM-CSF transcript (shown by an increase in $-\Delta\Delta C_T$) in thymocytes, total LN cells, and purified CD8⁺ LN cells, a slight increase in purified CD4⁺ LN cells, but no increase in CD3⁻ non-T cells. Together, these data show that *Lck*-GM mice express increased levels of functional GM-CSF specifically in the T-cell lineage, with the strongest expression in thymocytes and peripheral CD8⁺ T cells.

Lymphoid Abnormalities in *Lck*-GM-CSF TG Mice

To determine the effects of increased T-cell-derived GM-CSF on the lymphoid system, spleen, LN, and thymus were

Table 2 BioPlex Analysis of Serum Cytokines

Mouse	Analyte (pg/mL)									
	IL-1β	IL-2	IL-5	IL-6	IL-10	IL-17	GM-CSF	CCL2	CCL4	TNF
<i>Lck</i> -GM ^{hi}	60.7 ± 13.3	24.1 ± 2.9	11.4 ± 1.6	26.8 ± 12	14.2 ± 2.7	7.3 ± 1.4	32.7 ± 6.6	254 ± 39.4	54.4 ± 14	372 ± 68.4
WT	22.6 ± 5	15.7 ± 2.9	6.7 ± 0.8	1.1 ± 0.6	10 ± 3.3	3.4 ± 1.5	10 ± 3.3	156 ± 34	19.3 ± 8	204 ± 76.6
<i>P</i> value*	0.008	0.04	0.037	0.2	0.23	0.019	0.002	0.083	0.074	0.091

Data are given as means ± SEM. *n* = 12 to 14 mice per group.

*Two-tailed Mann-Whitney test.

harvested from 6-week-old WT and TG mice. Although TG mice and WT littermates had comparable body weights (data not shown), the weight, cellularity, and size of the spleen and LNs of *Lck-GM^{hi}* mice were increased, compared with WT littermates (Figure 2 and Table 1). In contrast, these parameters were reduced in the thymus (Figure 2A and Table 1). Some of these differences were also present (spleen and LN weight and cell numbers), but were less pronounced, in *Lck-GM^{lo}* mice (Table 1), consistent with a lower transgene copy number (Figure 1B). Analysis of the main cell subsets (neutrophils, lymphocytes, monocytes, eosinophils, and basophils) in spleen and LNs revealed that the increase in total white cells of spleen and LNs included all subsets (Table 1). Eosinophil numbers were increased dramatically in the spleen, in accordance with other reports of GM-CSF overexpression.¹⁰ Likewise, the reduction in cellularity of the thymus was caused by decreased numbers of each subset, with the exception of monocytes (Table 1).

Analysis of T-cell subsets in these organs revealed relatively normal proportions of CD4⁺ and CD8⁺ T cells in the spleen and lymph nodes of *Lck-GM^{hi}* mice (Figure 2B). However, a substantial increase in the number of CD4⁺Foxp3⁺ regulatory T cells was observed in the TG mice, as well as a switch from naïve to effector/memory phenotype (Figure 2B), consistent with a systemic inflammatory process.²³ In the TG thymus, thymocyte differentiation was severely impaired, with the proportion of immature double positive cells (DP, CD4⁺CD8⁺) decreased, whereas double negative cells (DN, CD4[−]CD8[−]) and mature single positive subsets were increased (Figure 2C). To examine whether an early block in thymocyte differentiation might explain the lower numbers of cells, we analyzed progression through the CD3/CD4/CD8 triple-negative (TN) stages. There was an increase in TN1 cells, suggestive of an early differentiation block, but some progression through the TN stages was observed (Figure 2C), including β -selection [as judged by CD28 up-regulation in the TN3 subset (data not shown)]. The distribution of the semimature and mature single-positive thymocytes was severely altered, with large proportional increases of the most mature SP cells (defined as CD24^{lo}/Qa2^{hi}), at the expense of the semimature SP cells. We also observed a substantial increase in the proportion of CD4⁺Foxp3⁺ regulatory T cells in the thymus. In the context of markedly reduced thymocyte cellularity, these changes are consistent with a severe (but incomplete) block in thymic lymphopoiesis, with some recirculation of mature T cells.

Increased Th17 Pathway Cytokines and Expression of ROR- γ t in Thymocytes and LN Cells

In light of the recently reported role for GM-CSF in the differentiation of effector Th17 cells,³ we analyzed serum from mice aged between 6 and 12 weeks by Bio-Plex bead array (BioRad). Of the 23 cytokines and chemokines measured, IL-1 β , IL-2, IL-5, IL-12p40, GM-CSF, and IL-17A were markedly increased in TG mice, when

Figure 3 Lymphopenia, monocytopenia, thrombocytopenia, neutrophilia, eosinophilia, and an increase in large myeloid cells in peripheral blood of *Lck-GM-CSF* TG mice. Peripheral blood from TG mice and littermate controls between 6 and 12 weeks of age, analyzed by automated differential cell count. * $P < 0.05$, *** $P < 0.001$ (unpaired t -test).

compared with WT littermate controls (Table 2). All of these cytokines, except for IL-5, are implicated in the Th17 pathway.⁷ These findings suggest there might be enhanced Th17 (CD4⁺) or Tc17 (CD8⁺) differentiation in the *Lck-GM-CSF* TG mice. In contrast, serum concentrations of other cytokines and chemokines [IL-1 α , IL-3, IL-4, IL-9, IL-10, IL-12(p70), IL-13, eotaxin, G-CSF, interferon (IFN) γ , keratinocytes, monocyte chemoattractant protein-1 (CCL2), macrophage inflammatory protein-1 α (CCL3), macrophage inflammatory protein-1 β (CCL4), regulated on activation normal T cell expressed and secreted (CCL5), and tumor necrosis factor (TNF) α] were no different from WT littermates (data not shown).

To formally assess Th17 and Tc17 differentiation in *Lck-GM* TG mice during global immune activation, we injected mice with CFA. LN cells were restimulated *in vitro*, and we compared the percentage of IL-17- and IFN- γ -producing CD4⁺ and CD8⁺ cells (Figure 2D). The percentage of CD4⁺ IL-17- or IFN- γ -producing T cells in the TG mice was increased on *in vitro* restimulation, regardless of CFA injection. CFA injection boosted the number of CD4⁺ IL-17-producing T cells by approximately twofold. Surprisingly, when stimulated in this manner, the percentage of CD8⁺ IL-17-producing T cells increased by approximately threefold in the *Lck-GM^{lo}* mice when primed with CFA. This increase was not seen in the *Lck-GM^{hi}* mice. In contrast, the percentage of

Table 3 Colony Formation by Bone Marrow Cells

Mouse	Stimulus	No. of colonies/25,000 bone marrow cells					
		Blast	G	GM	M	Eo	Meg
TG	GM-CSF	0	11 ± 4	2 ± 1	24 ± 5	1 ± 1	0
	G-CSF	0	8 ± 2	0	0	0	0
	M-CSF	0	3 ± 2	1 ± 1	32 ± 17	0	0
	IL-3	3 ± 4	13 ± 6	8 ± 4	12 ± 3	2 ± 2	3 ± 1
	SCF	4 ± 1	6 ± 2	3 ± 1	2 ± 1	0	0
	SCF + IL-3 + EPO	10 ± 3	20 ± 4	12 ± 5	20 ± 9	2 ± 0	12 ± 5
	IL-6	0	7 ± 3	0	0	0	0
	Saline	0	0	0	0	0	0
WT	GM-CSF	0	27 ± 4	11 ± 11	45 ± 6	4 ± 3	0
	G-CSF	0	16 ± 5	0	0	0	0
	M-CSF	0	4 ± 3	11 ± 1	63 ± 13	0	0
	IL-3	2 ± 1	42 ± 2	22 ± 1	24 ± 11	5 ± 0	11 ± 1
	SCF	9 ± 4	24 ± 7	2 ± 3	2 ± 0	0	0
	SCF + IL-3 + EPO	8 ± 1	47 ± 1	23 ± 3	37 ± 1	5 ± 4	36 ± 11
	IL-6	0	6 ± 7	1 ± 0	1 ± 1	0	0
	Saline	0	0	0	0	0	0

Data are given as means ± SD. *n* = 2 mice per group.

Blast, blast cell, precursor cell; Eo, eosinophil; EPO, erythropoietin; G, granulocyte; GM, granulocyte/macrophage; M, macrophage; Meg, megakaryocyte; SCF, stem cell factor.

CD8⁺ IFN-γ-producing T cells was independent of CFA injection, and was increased by approximately twofold and 10-fold in the *Lck*-GM^{lo} and *Lck*-GM^{hi} mice, respectively. We also assessed the production of GM-CSF in this experiment (Supplemental Figure S2). As expected, GM-CSF production was stronger in the *Lck*-GM^{hi} mice, in particular in stimulated CD8⁺ T cells. This result suggests a link between IFN-γ and GM-CSF production after stimulation.

Quantitative PCR for ROR-γt, which is a transcription factor required for Th17 differentiation,²⁴ was performed. We found markedly increased ROR-γt mRNA in TG thymocytes, total LN cells, and purified CD8⁺ LN cells (Figure 2E), suggesting an increased population of Tc17 cells in these organs (Figure 2E). Consistently, LN cells from both lines produced more IL-17, as well as GM-CSF, when stimulated with anti-CD3 and anti-CD28 (data not shown). Interestingly, the mRNA expression of ROR-γt in purified TG CD4⁺ LN cells was unchanged. Although these cells displayed a clear increase in GM-CSF mRNA, this increase was modest when compared with purified CD8⁺ LN cells (Figure 1E), suggesting a quantitative relationship between GM-CSF expression and ROR-γt expression.

Peripheral Blood Cell Populations Are Altered in *Lck*-GM-CSF TG Mice

To analyze whether other hematopoietic cell types were affected by increased GM-CSF production by T cells, TG mice and WT littermates from both lines were bled at 6 weeks of age. Absolute numbers of total white blood cells were no different between WT and TG mice (data not shown); however, TG mice had a markedly increased percentages of neutrophils, eosinophils, and large myeloid cells (identified by the

absence of the typical physical features of common blood cell populations) (Figure 3). Accordingly, there were reduced percentages of lymphocytes, monocytes, and platelets in TG mice. These changes in blood cell populations were observed in both lines, but were more pronounced in *Lck*-GM^{hi} mice. The *Lck*-GM^{hi} mice were also anemic (hematocrit, 60% ± 0.8% for WT versus 46.3% ± 6.4% for TG). We, therefore, chose *Lck*-GM^{hi} mice for more detailed analysis.

Decreased Colony-Forming Potential in Bone Marrow but Increased Colony-Forming Potential in Spleen in *Lck*-GM^{hi} Mice

In light of the perturbations to TG myeloid populations, we performed colony-forming assays to assess myeloid precursors in the bone marrow and spleen (Table 3 and Table 4). TG bone marrow cells had decreased granulocyte, granulocyte/macrophage, macrophage, eosinophil, and megakaryocyte colony-forming potential (Table 3). Cell counts of bone marrow revealed normal cellularity, but increased percentages of mature neutrophils (data not shown). In contrast, TG splenocytes displayed increased colony-forming potential (Table 4), suggesting extramedullary myelopoiesis. We confirmed this result by manual differential cell counts of splenocytes (data not shown). Assays with saline alone did not yield any colonies from cultures of bone marrow or spleen.

Altered Lymphoid Organ Architecture Due to Histiocytic Infiltration in *Lck*-GM^{hi} Mice

Histopathological analysis of the spleen and LNs of TG mice revealed marked effacement of the normal architecture

Table 4 Colony Formation by Spleen Cells

Mouse	Stimulus	No. of colonies/50,000 spleen cells					
		Blast	G	GM	M	Eo	Meg
TG	GM-CSF	0	2 ± 1	2 ± 1	5 ± 4	0	0
	M-CSF	0	0	0	5 ± 4	0	0
	SCF + IL-3	20 ± 0	4 ± 5	2 ± 2	6 ± 2	1 ± 1	11 ± 6
	+ EPO						
WT	Saline	0	0	0	0	0	0
	GM-CSF	0	0	0	0	0	0
	M-CSF	0	0	0	1 ± 1	0	0
	SCF + IL-3	1 ± 1	0	1 ± 0	3 ± 2	0	5 ± 1
	+ EPO						
	Saline	0	0	0	0	0	0

Data are given as means ± SD. *n* = 2 mice per group.
Blast, blast cell, precursor cell; Eo, eosinophil; EPO, erythropoietin; G, granulocyte; GM, granulocyte/macrophage; M, macrophage; Meg, megakaryocyte; SCF, stem cell factor.

by histiocyte-like cells, although a follicular pattern was still discernible (Figure 4). The spleen displayed an increase in nucleated erythroid cells, consistent with extramedullary erythropoiesis. Occasional multinucleated giant cells were observed, suggesting fusion of histiocytes and/or tissue macrophages. The TG thymus lacked normal lobes and had an atrophic appearance. There was effacement of the thymic medulla by histiocytes, as observed in spleen and LN, and the cortex was markedly reduced (Figure 4), consistent with the severe defects we observed in thymocyte differentiation. TG lungs showed multiple nodules, consisting of polymorphonuclear cells and histiocytes (Figure 4). There was an absence of mitotic figures in these nodules, arguing against neoplastic transformation. TG bone marrow (BM) showed a marked reduction in lymphocytes and an increase in granulocytes (Figure 4), in agreement with the differential cell counts. Nonlymphoid organs, including lung, kidney, and liver, showed varying degrees of infiltration by histiocytes and polymorphonuclear cells (Figure 4 and data not shown). In summary, the histological findings in *Lck*-GM-CSF TG mice were consistent with a disseminated histiocytosis.

Lck-GM^{hi} Histiocytes Have Hybrid Features of Monocytes, Macrophages, and DCs

Analysis of myeloid markers revealed dramatic expansion of CD11b⁺ F4/80^{int/+} cells in the lymphoid organs of TG mice (Figure 5). In TG blood and BM, these cells were F4/80^{lo} (data not shown). This population could be further divided, based on CD11c staining and on morphological characteristics. The CD11c⁺ population (histiocytes) was preferentially expanded in *Lck*-GM^{hi} mice (Figure 5, B and C). Histiocytes were extensively immunophenotyped by flow cytometry. These cells also expressed CD115, MHCII, and low levels of CCR2 and CD62L, but not CD43, CD205, Ly6C, or CX3CR1; thus, they showed hybrid features of monocytes, macrophages, and DCs (Table 5). Histiocytes also expressed GM-CSFR α and GM-CSFR β (Figure 5D)

and intracellular CD207, which is a hallmark of Langerhans cells (Figure 5E). CD11b⁺ F4/80^{int/+}CD11c[−] cells expressed the eosinophil marker, SiglecF, and were morphologically identical to eosinophils (Figure 5C). We next evaluated the production of cytokines and chemokines by *Lck*-GM^{hi} histiocytes, *in vitro*. CD11b⁺ F4/80^{int/+}CD11c⁺ histiocytes produced high levels of CCL4 and CCL5, suggesting that these cells might engender an

Figure 4 Histiocytic infiltrates in organs of *Lck*-GM-CSF TG mice. Organs and bone from *Lck*-GM^{hi} mice were collected at 6 weeks of age, and sections were stained with H&E. Original magnifications: ×50 (LN and thymus); ×100 (spleen, lung, and BM). C, cortex; F, follicle; M, medulla.

Figure 5 Characterization of histiocytes from TG mice. **A:** Flow cytometric analysis of CD11b and F4/80 expression on leukocytes from spleen, LN, and thymus of *Lck*-GM^{hi} mice. **B:** Histiocytes can be distinguished from eosinophils by CD11c expression. Numbers represent percentage of live cells. **C:** Cytospins of CD11b⁺ F4/80^{int/+} CD11c⁻ eosinophils and CD11b⁺ F4/80^{int/+} CD11c⁺ histiocyte cells, sorted from TG spleen. Cells were stained with May-Grunwald Giemsa stain. Original magnification, $\times 1000$. **D:** Detection of GM-CSFR α and β on CD11b⁺ F4/80⁺ cells from TG spleen (black line) by flow cytometry. **E:** Detection of CD207 (Langerin) on histiocytes (CD11c⁺, clear histogram) and eosinophils (CD11c⁻, shaded histogram) by flow cytometry. Control (gray line): unstained cells (**D** and **E**). **F:** Analysis of cytokines and chemokines in culture supernatant from purified histiocytes from TG spleen by BioPlex bead array ($n = 2$ mice). **G:** Mixed leukocyte reaction of purified, unfixed histiocytes (CD11c⁺, white circles) or eosinophils (CD11c⁻, black squares), or PFA-fixed histiocytes (gray circles), with purified splenic BALB/c CD3⁺ T cells. Proliferation after 4 days was measured by ³H thymidine incorporation. Pooled data from two experiments shown ($n = 2$ mice per experiment). * $P < 0.05$ (unpaired *t*-test). **H:** Uptake of fluorescent-labeled latex beads *in vivo* by CD11b⁺ F4/80^{int/+} CD11c⁺ histiocytes. Beads were injected i.v., and spleen cells were analyzed by flow cytometry. CPM, counts per minute.

autocrine loop for recruitment of monocyte/macrophage and DC precursors (Figure 5F). In contrast, under these conditions, histiocytes did not produce nitric oxide (data not shown).

Histiocytes from *Lck*-GM^{hi} Mice Can Mature to Become Functional Antigen-Presenting Cells

To determine whether histiocytes can stimulate naïve T cells, purified histiocytes and eosinophils were co-cultured with purified allogeneic CD3⁺ BALB/c T cells for 4 days. Histiocytes, but not eosinophils, could stimulate alloreactive T cells (Figure 5G). Histiocytes that were fixed with 2% PFA before culture could not stimulate T cells, showing that cell maturation *in vitro* is required, as is the case for immature DCs. Immature DCs can also efficiently absorb antigens for processing and presentation.²⁶ We, therefore, tested the ability of histiocytes to phagocytose latex beads *in vivo* (Figure 5H).

Approximately 10% of histiocytes phagocytosed latex beads (Figure 5H), whereas, as expected, eosinophils did not.

Increased Langerhans DCs in LNs from *Lck*-GM^{hi} Mice

The human disease Langerhans Cell Histiocytosis (LCH) is characterized by tissue infiltration with Langerhans-type cells, commonly referred to as LCH cells, to distinguish them from Langerhans cells (LCs).²⁷ LCH cells express LC antigens, CD207 and CD1a, and the monocyte markers, CD68 and CD14. Expression of CD205, which is present on mature LCs, has been detected on tissue sections of human LCH lesions, but to our knowledge, the co-expression of CD207 and CD205 has not specifically been addressed in human LCH cells.²⁷ In the mouse, functionally mature LCs in the LNs share a phenotype similar to human LCs and express CD207, CD11b, and CD205.²⁰ TG histiocytes expressed low levels of CD207 and were negative for

Table 5 Surface Phenotype of CD11b⁺ F4/80^{int/+}CD11c⁺ Histiocytes

Cell type	Membrane marker														
	CD11b	CD11c	CD43	CD62L	CD115	CD205	CD207	Ly6C	F4/80	CX3CR1	CCR2	MHCII	SiglecF	GM-CSFR α	GM-CSFR β
Histiocytes	+	+	—	+/-	+	—	Lo	—	Int	—	Lo	+	—	+	+
Eosinophils	+	—	+/-	+/-	+/-	—	—	+/-	Int	+	Lo	—	+	+	+
Res mono	+	+	+	—	+	—	—	—	Lo	+	—	—	—	+	+
Infl mono	+	—	+	+	+	—	—	+	Lo	+	+	—	—	+	+
cDC	+/-	++	—	—	—	—	—	—	—	Int	—	+	—	+	+
Langerhans	+	+	—	—	+	++	++	—	—	—	+	++	—	+	+
Infl mac	+	—	—	—	—	—	—	+	+	+	+	+/-	+	+	+

Surface phenotypes of monocytes, DCs, and macrophages. Data obtained from previous studies.^{4,20,25}

cDC, conventional DC; Infl mac, inflammatory macrophage; Infl mono, inflammatory monocyte; Int, intermediate levels; Lo, low levels; Res mono, resident monocyte; +, positive cells; ++, high expressing positive cells; +/-, both positive and negative cells; —, negative cells.

CD205 (Figure 5D). To determine whether DC populations were skewed in TG mice, we enriched for DCs via a density gradient. The absolute number of light-density cells per mg of tissue was marginally increased in the TG mice, but this did not reach statistical significance (data not shown). However, DC populations of TG mice did differ from those in WT littermates (Figure 6). In the spleen, there was an increase in the percentage of CD11c⁺CD11b[—] (resident DCs or migratory CD11b[—] DCs) and CD11b⁺CD11c⁺ DCs in the light-density fraction (Figure 6A). With these markers, we could also distinguish CD11c^{int}CD11b⁺ histiocytes/histiocyte precursors (and possibly DC precursors), which were absent in the WT spleen (Figure 6A). The DCs were further analyzed for CD205 and CD207 expression (Figure 6A). This revealed the presence of Langerhans cells in the TG spleen, defined by the expression of CD205 and CD207. LCs are normally only present in LNs.²⁰ In addition, TG mice displayed reduced CD8⁺ DCs in the spleen (Figure 6B). The proportion of CD8[—] DCs was comparable to WT spleen. In the LNs, there was a slight reduction in CD8⁺ DCs and an increase in CD8[—] DCs (Figure 6C). The percentage of Langerhans cells was variable, but there was a trend toward an increase of these cells. In contrast, CD11c⁺CD11b[—] DCs were reduced in *Csf2*^{−/−} mice (data not shown), consistent with a direct effect of GM-CSF on at least some DC populations in peripheral lymphoid organs.

Histiocytosis Can Be Transferred to Lymphopenic Mice by Mature *Lck*-GM^{hi} T Cells and Thymocytes

To examine whether the histiocytosis observed in TG mice was directly due to over-production of GM-CSF by T cells, we transferred purified TG or WT CD3⁺CD4⁺ (CD4⁺ T cells), CD3⁺CD8⁺ (CD8⁺ T cells), and CD3[—] (non-T) LN cells, or unfractionated thymocytes, into *Rag1*^{−/−} mice. A week after transfer, mice that received TG CD4⁺, CD8⁺ T cells, or TG thymocytes had altered peripheral blood populations, which were similar to the changes found in TG mice. Recipient mice had a markedly increased percentage of large myeloid cells in the peripheral blood, which increased over time (Figure 7A). Histiocytes could be

identified on histological analysis of spleens from mice injected with TG CD4⁺ or CD8⁺ T cells (data not shown) and thymocytes, but not in spleens from mice that received non-T cells (Figure 7B). In addition, spleens from mice injected with TG thymocytes or CD8⁺ T cells were larger than spleens from mice injected with WT cells or TG CD3[—] non-T cells (Figure 7C). This effect was not seen when mice were injected with CD4⁺ T cells, most likely reflecting the difference in GM-CSF production between these cell types (Figure 1).

Differential cell counts of the spleen showed that the population of large myeloid cells was specifically expanded (Figure 7D), and these cells were CD11b⁺F4/80⁺ (shown for mice transferred with TG thymocytes in Figure 7E). In contrast, mice that received WT cells or TG non-T cells, and non-injected *Rag1*^{−/−} mice (data not shown), did not accumulate histiocytes and had normal splenic architecture. These data demonstrate that histiocytosis is a cell-intrinsic consequence of the TG T cells.

Discussion

Recent studies highlight the importance of GM-CSF production by T cells in mediating immunity and inflammation.^{3,6,7} Following our earlier work,⁶ we hypothesized that increased production of GM-CSF by T cells might expand MoDCs during inflammation and cause increased differentiation of Th17 cells. We tested this hypothesis by over-expressing GM-CSF specifically in T cells, under the control of the *Lck* promoter. We show strong expression of the *Lck*-driven GM-CSF transgene in the thymus and expression in peripheral lymphoid organs, in accordance with earlier reports using this promoter.^{28–30} Transgenic mice displayed a dramatic phenotype, characterized by splenomegaly, lymphadenopathy, thymic atrophy, and multiple abnormalities in thymic and blood cell populations. *Lck*-GM-CSF^{hi} mice had a decreased life span, which was due to splenomegaly (caused by histiocyte infiltration and extramedullary hematopoiesis) and anemia. *Lck*-GM-CSF TG mice developed disseminated histiocytosis, with an increased Foxp3⁺ T-cell population, increased levels of

Figure 6 Flow cytometric analysis of DC populations. Light-density spleen cells were stained for CD11b and CD11c, CD8, CD205, and CD207. CD11c^{hi}/CD11b^{hi} cells were gated (**A**) and further analyzed for the expression of CD8, CD205, and CD207 (**B**). **C**: Percentage of CD8⁺, CD8⁻ conventional DCs, and Langerhans DCs in WT and TG LN. Representative plots shown from two separate experiments. Mean and range from one experiment given in **C**. $n = 2$ to 3 mice per genotype per experiment. His, histiocyte/histiocyte precursor population.

ROR- γ t mRNA, and increased circulating Th17-related cytokines. The increase in CD4⁺ IL17⁺ cells was even more obvious when *Lck*-GM-CSF mice were injected with CFA, linking GM-CSF production in the context of global immune activation with Th17 differentiation. Interestingly, basal expression of GM-CSF was strongest in thymocytes

and CD8⁺ T cells, suggesting the increase in circulating Th17 cytokines in naïve *Lck*-GM-CSF mice is most likely the result of more Tc17 cells, rather than Th17 cells. *In vitro*—stimulated *Lck*-GM TG CD4⁺ and CD8⁺ T cells produced high levels of IFN- γ , which was increased to up to approximately 10-fold in the *Lck*-GM-CSF^{hi} mice compared with WT mice. These findings suggested that a link exists between the T-cell-derived production of GM-CSF and the production of IL-17 and IFN- γ by T cells. In view of our findings in CD8⁺ T cells, it is possible that GM-CSF plays a role in the development of Tc17/IFN- γ cells; these cells have been shown to have potent anti-tumor properties, which might explain the absence of tumors in the *Lck*-GM TG mice.³¹ In addition, infiltrating IFN- γ -secreting T cells induce macrophage activation and inflammation, and play a significant role in target organ damage in autoimmunity.³² Increased IFN- γ has also been implicated in the pathophysiological characteristics of some histiocytic disorders.³³

Histiocytic disorders encompass a range of malignant and nonmalignant conditions that are incompletely understood, partly through lack of suitable animal models. LCH is a clonal proliferative disease, characterized by tissue infiltration with Langerhans-like cells (as recently reviewed³⁴) and mutations in *BRAF*.^{35,36} Not all cases of LCH are aggressive, and some have a better prognosis.^{34,37} A previous model of histiocytosis was generated by infecting mice with malignant histiocytosis sarcoma virus, leading to transitional macrophages/DC sarcomas.³⁸ Another experimental model was induced by transgenic expression of the SV40 T oncogene in DCs.³⁹ These mice developed infiltration of spleen, liver, bone marrow, thymus, and mesenteric lymph node with CD11c⁺CD8⁺CD207⁺ DCs, resembling the aggressive form of LCH. Mice in both these models invariably died early from the disease.

In TG mice, we found many infiltrating cells that expressed CD115, F4/80, and CD11b in the lymphoid organs, peripheral blood, BM, and nonlymphoid organs, such as the kidney, lung, and liver. These cells were phenotypically similar to immature monocytic Langerhans cell precursors.⁴⁰ A subpopulation of these cells expressed CD11c and low levels of CD207, but not CD205. Although these markers are not exclusively expressed by LCs,¹⁹ the presence of CD11b suggests TG histiocytes are more similar to LCs than to other DC types. In WT mice, LCs are only found in the LNs, but we found mature CD205⁺CD207⁺ LCs in the spleen of TG mice. This finding may suggest that LCH in the TG mice might involve immature monocytic Langerhans cell precursors. Spleen and LN showed a dramatically increased size and cellularity, but with preserved follicular structure. High levels of chemokines, such as CCL2, CCL4, and CCL5, were produced by MoDCs⁶ and TG histiocytes, which could act as chemoattractants for histiocytic precursors to enter tissues and differentiate under the influence of local GM-CSF. The histiocytes of *Lck*-GM-CSF Tg mice express GM-CSFR α and β , CD207, and low levels of MHCII and can phagocytose latex beads

Figure 7 Histiocytosis can be transferred to *Rag1*^{-/-} mice by TG thymocytes and LN T cells. The 1×10^6 thymocytes or purified LN CD4⁺ T cells, CD8⁺ T cells, or non-T (CD3⁻) cells per mouse were transferred to *Rag1*^{-/-} mice by i.v. injection. **A**: Percentages of large myeloid cells were determined in peripheral blood by automated differential cell counts weekly after transfer ($n = 2$ to 3 mice per group per time point). **B**: H&E-stained spleen sections from *Rag1*^{-/-} recipients receiving thymocytes or non-T cells. Original magnification, $\times 200$. $n = 3$ mice per group. Data from one experiment representative of two experiments is shown. E, eosinophils; H, histiocytes; R, red pulp; W, white pulp. **C**: Spleen weight and blood cell populations of *Rag1*^{-/-} mice on day 28 after transfer of donor cells and differential counts of splenocytes ($n = 2$ to 6 mice per group). **D**: Percentage of large myeloid cells in spleen determined by differential cell count on day 28 after transfer. Data are given as means $\pm$ SEM ($n = 3$ mice per group). **E**: Flow cytometric analysis of CD11b and F4/80 expression on splenocytes from *Rag1*^{-/-} mice on day 28 after transfer of WT or TG thymocytes. * $P < 0.05$ (*t*-test, Holm-Sidak method).

in vivo, and freshly isolated cells do not stimulate naïve T cells in mixed leukocyte reaction, suggesting an immature antigen-presenting cell phenotype. Human LCH histiocytes also resemble DCs held in an immature state *in vivo* by extrinsic factors, such as IL-10.⁴¹ We show that, *in vitro*, *Lck*-GM-CSF-derived histiocytes can mature to become functional antigen-presenting cells, and we also observed marginally higher serum IL-10 in *Lck*-GM-CSF TG mice. Collectively, these observations in *Lck*-GM-CSF TG mice are also reminiscent of human LCH. The clinical and histological features and the absence of mitotic figures and of spontaneous cellular proliferation in our model thus suggest a nonneoplastic process in *Lck*-GM^{hi} mice, which has many similarities to LCH.

Transgenic expression of GM-CSF in nonhematopoietic tissue has been shown to cause local accumulation of macrophages and neutrophils.^{10–14} GM-CSF is a potent

growth factor for eosinophils,⁴² and more of these cells were observed in another GM-CSF transgenic mouse.¹⁰ We also found increased eosinophils and show that expression of GM-CSF in T cells causes accumulation of histiocytes, which has not been observed in previous GM-CSF transgenic mice. Interestingly, eosinophils and histiocytes can be found together in eosinophilic granuloma of bone.⁴³ We, therefore, hypothesize that histiocytes differentiate in tissues that provide proximity to GM-CSF-producing T cells. This hypothesis is supported by earlier studies that showed increased serum levels of GM-CSF in patients with LCH, expression of the GM-CSF receptor on LCH cells, and increased production of GM-CSF by T cells, macrophages, and LCH cells in LCH lesions.^{44–46}

In contrast to increased cellularity in peripheral LNs, the thymi of *Lck*-GM^{hi} mice were atrophic and had a severely reduced cortex. However, thymocyte differentiation appeared

to still proceed, but was severely impaired. Increased local GM-CSF might favor retention of mature thymocytes in the medulla, or perhaps more likely, promote the recirculation of peripheral T cells. A direct effect of GM-CSF on thymocytes is unlikely due to the absence of GM-CSF receptors on T cells,⁴⁷ and the observation that GM-CSF receptor-deficient mice did not display an overt T-cell phenotype.⁴⁸ In the thymus and LN, we also observed a major increase in the proportion of CD4⁺Foxp3⁺ regulatory T cells (Treg). Treg differentiation in the thymus requires both high-avidity TCR interactions and an additional signal from IL-2 or other γ -chain cytokines.⁴⁹ Treg expansion in the *Lck*-GM TG mice is most likely due to increased recirculation from the periphery, but could be secondary to locally increased IL-2, TNF, or transforming growth factor β expression, caused by GM-CSF. Of potential relevance, we detected elevated IL-2 and slightly increased TNF in the serum. Interestingly, increased Treg numbers have been detected in lesions and peripheral blood from patients with LCH.⁵⁰ Tregs were detected in proximity of LCH cells, and it was suggested that LCH cells could be involved in Treg differentiation.⁵⁰ Our findings in the *Lck*-GM TG thymus support this conclusion. We hypothesize that overproduction of GM-CSF by T cells induces the local differentiation of LCH cells, which produce cytokines or chemokines that promote Treg, Tc17, and Th17 differentiation. Expansion of Tregs might explain why *Lck*-GM^{hi} mice are not overtly sick despite the infiltrates in many organs. Elevated serum levels of Th17-related cytokines (IL-1 β , IL-2, and IL-12p40) are also consistent with a contribution of T-cell-derived GM-CSF to the Th17 pathway,⁷ and are most likely the result of increased GM-CSF production by CD8⁺ T cells, driving cytokine production by macrophages and DCs. However, whether the IL-17 family plays a direct role in the pathogenesis of LCH remains controversial.^{51,52}

In the bone marrow, histiocytes might compete with myeloid progenitor cells for a common bone marrow niche, which might explain the decreased colony-forming potential we found from that site and the increased extramedullary hemopoiesis seen in the spleen. Administration of exogenous GM-CSF in itself does not cause histiocytosis,⁵³ suggesting a requirement for direct cellular interaction. Our observation that transfer of *Lck*-GM TG T cells into lymphopenic recipients causes histiocytosis illustrates how this T-cell cytokine might affect myeloid differentiation. Our previous study showed that NF- κ B1-deficient mice, which had reduced GM-CSF production in CD4⁺ T cells, also had a decrease in inflammatory monocyte-derived DCs.⁶ Our results, therefore, shed new light on the possible *in vivo* consequences of GM-CSF production by T cells and are important for two reasons. First, the *Lck*-GM-CSF TG mice provide a novel murine model for histiocytosis. This heterogeneous group of human diseases has been difficult to understand and to treat. We provide evidence that overproduction of GM-CSF by T cells could be a pathogenic factor in histiocytic disorders, and LCH in particular. Second, and most important, our results raise the possibility that

specific inhibitors of GM-CSF should be considered in the management of histiocytic disorders.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.09.014>.

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