# Dramatic Increase in the Numbers of Functionally Mature Dendritic Cells in Flt3 Ligand-treated Mice: Multiple Dendritic Cell Subpopulations Identified

By Eugene Maraskovsky,* Ken Brasel,* Mark Teepe,* Eileen R. Roux,*
Stewart D. Lyman,‡ Ken Shortman,§ and Hilary J. McKenna*

From the *Department of Immunobiology and Department of ‡Molecular Genetics, Immunex
Corporation, Seattle, Washington 98101; and §The Walter and Eliza Hall Institute of Medical
Research, Melbourne, Victoria 3050 Australia

## Summary

Dendritic cells (DC) are the most efficient APC for T cells. The clinical use of DC as vectors for anti-tumor and infectious disease immunotherapy has been limited by their trace levels and accessibility in normal tissue and terminal state of differentiation. In the present study, daily injection of human Flt3 ligand (Flt3L) into mice results in a dramatic numerical increase in cells co-expressing the characteristic DC markers—class II MHC, CD11c, DEC205, and CD86. In contrast, in mice treated with either GM-CSF, GM-CSF plus IL-4, c-kit ligand (c-kitL), or G-CSF, class II$^+$ CD11c$^+$ cells were not significantly increased. Five distinct DC subpopulations were identified in the spleen of Flt3L-treated mice using CD8$\alpha$ and CD11b expression. These cells exhibited veiled and dendritic processes and were as efficient as rare, mature DC isolated from the spleens of untreated mice at presenting allo-Ag or soluble Ag to T cells, or in priming an Ag-specific T cell response in vivo. Dramatic numerical increases in DC were detected in the bone marrow, gastro-intestinal lymphoid tissue (GALT), liver, lymph nodes, lung, peripheral blood, peritoneal cavity, spleen, and thymus. These results suggest that Flt3L could be used to expand the numbers of functionally mature DC in vivo for use in clinical immunotherapy.

Dendritic cells (DC)[1] are rare bone marrow–derived cells that are predominantly found in the T cell–dependent areas of lymphoid tissue, as well as other tissues of the body (1). These cells express high levels of class I and class II MHC proteins, CD11c (2), the mannose receptor–like protein DEC205 (3, 4), and adhesion and costimulatory molecules (1). A substantial proportion of DC also express CD8$\alpha$ as a homodimer (5). DC specialize in processing and presenting foreign and self Ag to induce immunity (1) or tolerance (6, 7).

The lineage derivation of DC remains controversial, but there is growing evidence that DC can be subdivided into myeloid-derived (8–17) and lymphoid-derived populations (17–19). The myeloid relationship of DC is based mainly upon in vitro experimentation. In vitro, DC can be generated from bone marrow or cord blood progenitors as well as PBMC using GM-CSF but not M-CSF or G-CSF (9–11). Other growth factors that can enhance the GM-CSF–

dependent in vitro generation of DC are TNF-$\alpha$ (12–16), IL-4 (13, 14), and c-kitL (15, 16). These studies show that in the presence of GM-CSF, DC can be generated either directly from a myeloid-committed precursor (that also gives rise to monocytes and granulocytes) (11), or from a myelomonocytic intermediate that gives rise to monocytes (15–17). Alternatively, PBMC cultured with GM-CSF and IL-4 can transiently differentiate into DC-like cells (13, 14). DC can also arise from the most immature T cell precursors or bone marrow progenitors, which can also generate NK and B cells but not cells of the myeloid lineage (17–19). Interestingly, GM-CSF does not appear to be necessary for DC generation from lymphoid-committed precursors (19, 19a). In addition, although DC can be generated in vitro using GM-CSF, the increased level of GM-CSF in GM-CSF transgenic mice does not increase the number of DC in lymphoid tissue suggesting that other growth factors are important for DC generation in vivo (20).

By virtue of their highly developed Ag-presenting capacity, the use of DC as cellular vectors for anti-tumor and infectious disease vaccines or as inducers of transplantation tolerance is a promising immunotherapy strategy (21, 22, reviewed in reference 23). However, the clinical feasibility

---

[1]*Abbreviations used in this paper:* BM, bone marrow cells; c-kitL, c-kit ligand; DC, dendritic cells; Flt3L, Flt3 ligand; MSA, mouse serum albumin; PC, peripheral blood; PP, Peyer's patches

of using DC as immunotherapy vectors is hampered because only limited DC numbers can be generated in vitro from bone marrow progenitors or PBMC, and only one lineage of DC (myeloid-derived) is generated due to the use of GM-CSF (8, 9, 11–17, 21, 22). While studying the growth factor requirements for DC generation in vivo, we examined the effects of administering a recently identified hematopoietic growth factor, Flt3L (24). Flt3L has been shown to stimulate the proliferation of hematopoietic stem and progenitor cells (reviewed in reference 25, 26). Furthermore, in vivo administration of Flt3L has been shown to dramatically increase the numbers of hematopoietic progenitors in the bone marrow, peripheral blood and spleen, resulting in enhanced myelopoiesis and B lymphopoiesis (27, 28). We now report that the in vivo administration of Flt3L has a profound effect upon the generation of functionally mature DC in multiple organs in mice. Both lymphoid- and myeloid-derived DC populations have been identified in the spleens of these mice. Furthermore, the in vivo administration of either GM-CSF, or GM-CSF and IL-4, or c-kitL, or G-CSF did not significantly increase the numbers of spleen DC, suggesting a distinct effect of Flt3L on in vivo DC generation.

## Materials and Methods

*Mice and Cytokine Treatment Protocols.* Female C57Bl/6 mice (4–8 wk of age) were obtained from the Jackson Laboratory (Bar Harbor, ME) and kept in a specific pathogen-free facility. Mice (4–5 per group) were injected once daily (subcutaneously at the nape of the neck) with either mouse serum albumin (MSA) (1 μg) or with MSA plus human Chinese hamster ovary cell (CHO)-derived Flt3L (10 μg) for nine consecutive days. Additional treatment groups included daily subcutaneous injections for 11 d with either MSA alone (1 μg) or with MSA plus Flt3L, or human G-CSF (Neupogen; Amgen, Thousand Oaks, CA), or mouse GM-CSF (10 μg; Immunex, Seattle, WA), or c-kit ligand (10 μg; Immunex), or combinations of Flt3L and G-CSF or GM-CSF (10 μg each, respectively), or muGM-CSF and muIL-4 (10 μg each, respectively; Immunex).

*Cell Preparations and Flow Cytometric Isolation.* Flow cytometric analysis of various organs from cytokine-treated mice was performed on a FACStar® Plus (Becton Dickinson, San Jose, CA). Briefly, single cell suspensions from liver, inguinal and axillary LN, Peyer's patches (PP), spleen, and thymus were prepared by disrupting the organs between frosted glass slides and depleting RBC with $NH_2Cl$. PBMC were isolated after centrifugation of peripheral blood over a discontinuous density gradient using Lympholyte M (Cedalene, Ontario, Canada). Bone marrow cells (BM) were isolated by syringe extraction. Cells from the lungs were obtained by lung lavage using PBS. Cells from the peritoneal cavity were obtained by peritoneal washings with PBS. Cells from the various organs were then incubated with directly conjugated antibodies as indicated (PharMingen, San Diego, CA; NLDC145 [anti-DEC205] was a kind gift from Dr. G. Kraal, Free University, Amsterdam, The Netherlands) for 30 min at 4°C together with 50 μg/ml of anti-FcRIIγ mAb (2.4G2) to block Fc-receptors, and the cells were then analyzed with propidium iodide (1 μg/ml) to exclude dead and dying cells. Cytospins of sorted cells were prepared by spinning (500 rpm) 5 × 10⁴ cells

onto slides followed by staining with Wright-Giemsa according to the manufacturers instructions (Fisher Diagnostics, Pittsburgh, PA).

*Isolation of DC from Flt3L-treated and Control Mice.* Single cell suspensions of spleens were prepared from mice treated for either 9 or 11 d with Flt3L. Spleen cells were depleted of RBC and incubated with mAb to Thy-1, B220, NK1.1, and the erythroid Ag TER119 for 30 min at 4°C. The cells were incubated at 37°C for 30 min in serum-free RPMI-1640 medium containing 10% rabbit complement (Pel Freez Biologicals, Rogers, AK). The cells were then washed and residual mAb-coated cells were removed using anti-immunoglobulin (Ig)-coated magnetic beads (Dynabeads; Dynal, Oslo, Norway). The enriched cells were incubated with anti-CD11c, anti-CD11b, and anti-CD8α. The various cell populations were then sorted. Control DC were isolated as described (5). Briefly, C57Bl/6 spleen fragments were digested with collagenase and DNase for 25 min at room temperature, then treated with EDTA for 5 min. Light density cells were selected by centrifugation in Metrizamide medium (Nyegaard Diagnostics, Oslo, Norway; 1.068 g/cm³, pH 7.2, 4°C). These cells were incubated with mAb against CD3, CD4, Thy1, IL-2Rα, B220, Gr-1, CD11b, F4/80 Ag, FcRII, and TER119 followed by removal of non DC using anti-Ig-coated magnetic beads. The enriched cells (~70% DC) were stained with anti-class II and anti-CD11c and class II⁺ CD11c⁺ DC were sorted.

*Preparation and Purification of Alloreactive and Ag-specific CD4⁺ T Cells.* CD4⁺ T cells (90–95% pure) were isolated from either the LN of 4–8-wk-old DBA/2 (H-2ᵈ allogeneic) mice or from the inguinal draining LN of 8-wk-old C57BL/6 (H-2ᵇ syngeneic) mice 7 d after immunization with keyhole limpet hemocyanin (KLH) (50 μg; Pierce Chem. Co., Rockford, IL) in adjuvant (Ribi Immunochem Inc., Hamilton, MT; MPL™ + TDM + CWS emulsion). Lymph node cells were incubated with anti-CD8α, anti-class II (H-2ᵇ or H-2ᵈ) and anti-B220, and Ab-coated cells depleted with anti-Ig-coated magnetic beads. Depleted LN cells were composed of at least 95% CD4⁺ T cells as determined by FACS® analysis.

*Mixed Lymphocyte Reaction (MLR) and KLH-specific Presentation Assays.* The MLR and KLH presentation assays were performed in 96-well round-bottom culture plates. Allogeneic or KLH-specific CD4⁺ T cells (1 × 10⁵) were incubated with varying numbers of the indicated sorted spleen cell populations from Flt3L-treated mice or control DC in 0.2 ml modified DMEM medium containing 10% FCS and 10⁻⁴ M 2-ME (culture medium) in humidified 10% $CO_2$-in-air for 5 d. The cultures were pulsed with 0.5 μCi [³H]thymidine for 5 h and the cells were harvested onto glass fiber sheets for counting on a gas-phase β counter. The background counts for either T cells or DC cultured alone or KLH-specific T cells cultured with KLH were <100 cpm, <100 cpm, and <800 cpm, respectively.

*Priming of T Cells In Vivo with Ag Pulsed DC.* To detect in vivo Ag presenting capacity, class II⁺ CD11c⁺ spleen cells from Flt3L-treated mice (Flt3L DC) and control DC were cultured for 18 h in culture medium with or without 5 μg/ml KLH. The cells were then washed three times and 1 × 10⁵ cells were injected (in a volume of 0.02 ml PBS) into the foot pads of mice. Cells cultured in medium alone (-KLH) were injected into the contralateral foot pads as negative controls. Saline-only injected mice were also included as controls. After 7 d, the popliteal LN draining the foot pads were harvested, disrupted into a single cell suspension, and the LN cells were incubated with anti-CD8α, anti-class II (IAᵇ) and anti-B220, and the Ab-coated cells were then depleted with Ig-coated magnetic beads. CD4⁺ T cells were incubated (1 × 10⁵ T cells) with freshly isolated control DC (1 × 10⁴) in the

presence or absence of KLH (20 μg/ml) in 0.2 ml cultures for 5 d. The cultures were pulsed with 0.5 μCi [³H]thymidine for 5 h.

## Results

*Flt3L Treatment of Mice Resulted in a Significant Increase of Cells Co-expressing Class II MHC, CD11c, DEC205, and CD8a in Multiple Organs.* DC are very rare in dissociated spleen cell suspensions. The majority of DC require enzymatic digestion for release from the splenic stroma, but even then they are an infrequent component of the suspension (29). However, we found that unlike spleen cell suspensions from MSA-treated control mice, mice treated daily for 9 d with subcutaneous injections of human Flt3L (derived from CHO cells) resulted in 20% of spleen cells co-expressing class II MHC and the DC markers CD11c and DEC205, and 15% of spleen cells co-expressing class II MHC and CD8α (Fig. 1 *A*). Further phenotypic analysis revealed that MHC class II⁺ CD11c⁺ cells were CD3⁻, B220⁻, Gr-1⁻, NK1.1⁻, Ter119⁻, CD80⁻, but CD86⁺ (Fig. 1 *B*). These cells could be separated into three populations on the basis of CD11b expression (CD11b⁻, CD11b^dull and CD11b^bright) (Fig. 1 *B*). Flt3L-treated mice show a 2–3-fold increase in spleen and lymph node cellularity (26, 27). The absolute number of MHC class II⁺ CD11c⁺ cells was increased by 17-fold in the spleen, 4-fold in the LN and 6-fold in the peripheral blood (PB) by day 9 (Fig. 1 *C*). MHC class II⁺ CD11c⁺ cells were detected as early as day 5 in the spleen and by day 7 in LN and PB (Fig. 1 *C*).

*Five Distinct Populations of DC Can Be Identified in the Spleens of Flt3L-treated Mice.* To further define the cells expressing markers characteristic of DC, spleens from Flt3L-treated mice were depleted of T cells, B cells, NK cells and cells of the erythroid lineage by incubating the spleen cells with mAb directed against Thy1.2, B220, NK1.1, and Ter119, followed by depletion of mAb-coated cells with rabbit complement and two rounds of immunomagnetic bead depletion. Lymphocyte-depleted spleen cells from Flt3L-treated mice were then examined for the expression of CD11b and CD11c (Fig. 2 *A*). Five distinct populations were identified. As shown in Fig. 2 *B*, the majority of cells within population A (CD11b^bright CD11c⁻) were MHC class II⁻ and granulocyte marker Gr-1⁺. Population B (CD11b^bright CD11c^dull) was heterogeneous for both class II and Gr-1. The majority of cells within population C (CD11b^bright CD11c⁺) were MHC class II^bright and Gr-1⁻ (with 20% of cells expressing low levels of Gr-1). Unlike populations A or B, cells within populations D (CD11b^dull CD11c⁺) and E (CD11b⁻ CD11c⁺), as well as DC freshly isolated from the spleens of untreated mice (control DC) were MHC class II^bright and Gr-1⁻. Furthermore, populations A, B, and C were composed exclusively of CD8α⁻ cells, whereas populations D and E as well as control DC could be separated into CD8α⁻ and CD8α⁺ populations (Fig. 2 *B*).

Wright-Giemsa staining of cytospins indicated that cells within population A were highly enriched for immature
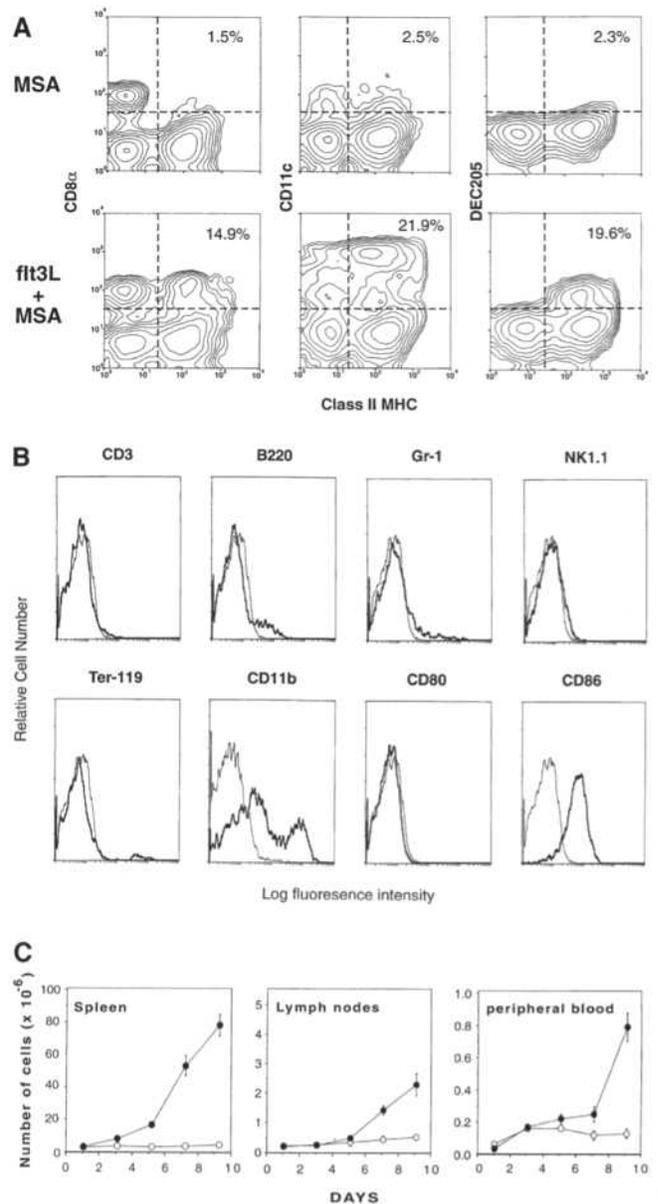


**Figure 1.** Flow cytometric analysis of spleen cells from mice treated with either MSA or MSA plus Flt3L. (*A*) The distribution of class II MHC and either CD8α, CD11c, DEC205, on spleen cells C57BL/6 mice (five per group) were injected once daily, subcutaneously, with either MSA (1 μg) or with MSA plus human Flt3L derived from CHO (10 μg) for nine consecutive days (*B*) The distribution of B220, CD3, Gr-1, NK1.1, Ter119, CD11b, CD80, and CD86 on class II⁺ CD11c⁺ spleen cells. (*C*) The kinetics of generation of class II⁺ CD11c⁺ cells in spleen, LN and peripheral blood from mice treated daily for 9 d with either MSA (*open circles*) or with MSA plus Flt3L (*closed circles*). Flow cytometric analysis of spleen cells, LN (inguinal, axillary), and PB was performed on a FACStar® Plus. Values represent the mean ± SD of five mice.

granulocytes or myeloid cells (Fig. 2 *C*). This is consistent with the expression of high levels of Gr-1 and CD11b and the lower levels of class II MHC. Population B was composed largely of immature myeloblasts. The blast-like morphology and heterogeneous levels of class II MHC and Gr-1 suggest that population B represents a population undergo-
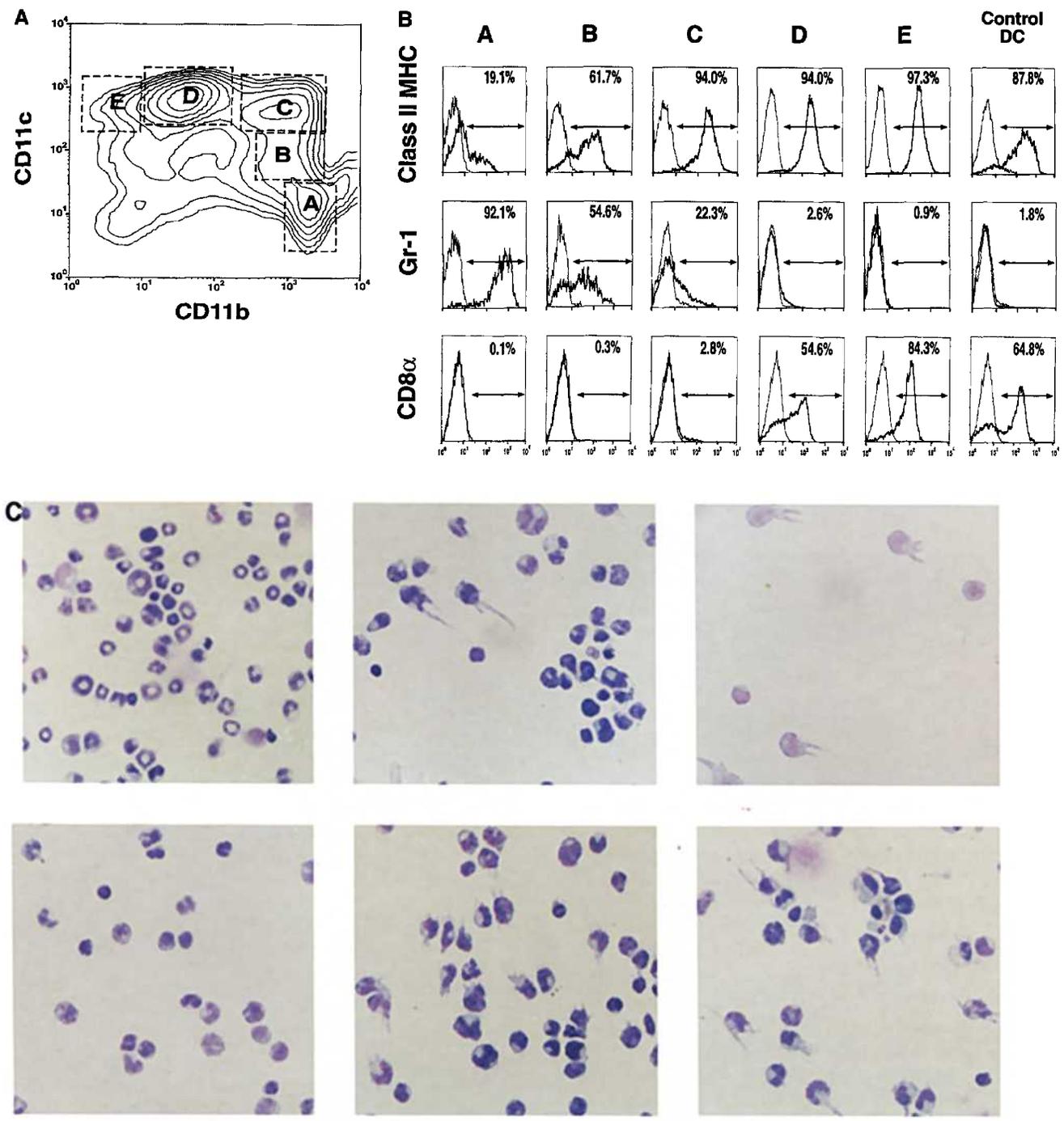
**Figure 2.** Detection and function of multiple spleen cell populations in mice after 11 d of treatment with Flt3L. (*A*) The distribution of CD11c and CD11b on depleted spleen cells from Flt3L-treated mice showing the gates used for analysis of class II MHC, Gr-1 and CD8α expression (shown in *B*) and for cell sorting. (*C*) Identification of spleen cell populations in Flt3L-treated mice with dendritic morphology. Cytospins were prepared for the various cell populations by spinning 5 × 10⁴ cells onto slides (500 rpm) followed by staining with Wright-Giemsa.

ing differentiation. Population C consisted of a mixture of immature myeloblasts (similar to those found within population B) and more mature myeloid cells with dendritic and veiled processes. Populations D and E that were either low or negative for CD11b and bright for CD11c were highly enriched for cells with veiled and dendritic processes that were indistinguishable from control DC (Fig. 2 C).

*Spleen DC from Flt3L-treated Mice Are Functional APC.* The various spleen populations from Flt3L-treated mice were examined for their in vitro capacity to stimulate the proliferation of allo-reactive T cells in an MLR. Spleen cells from Flt3L-treated mice were depleted of mature lymphocytes, and populations A, B, C, D, and E were isolated by flow cytometry on the basis of CD11c and CD11b ex-
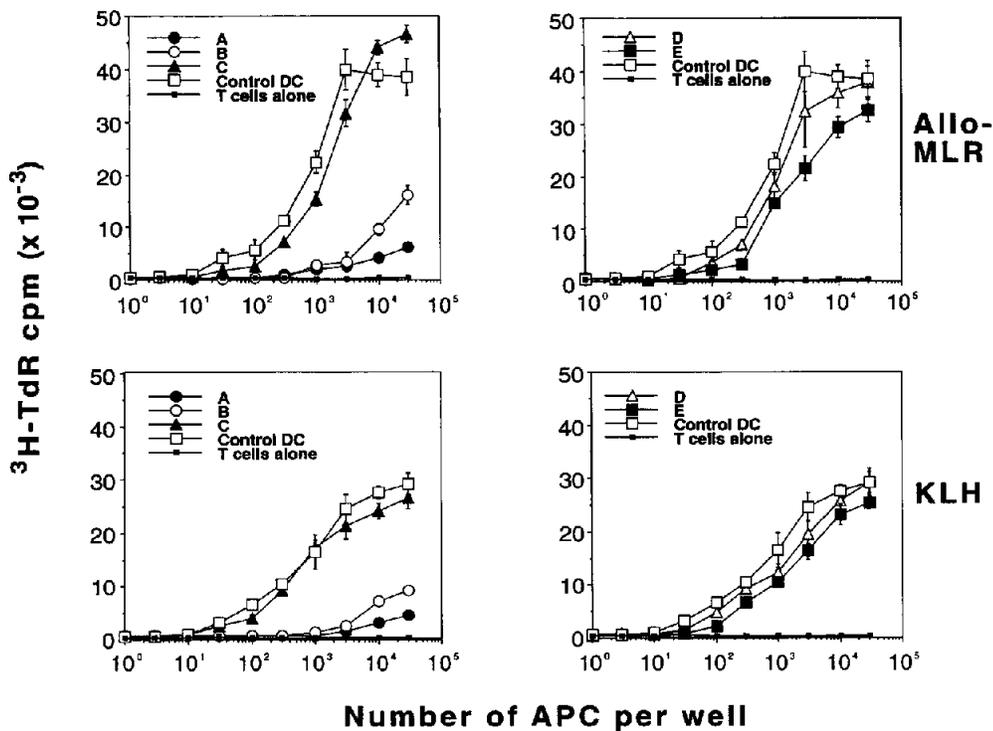
**Figure 3.** Comparison of the capacity of the various spleen cell populations in Flt3L-treated mice to stimulate the proliferation of allogeneic or KLH-specific $CD4^+$ T cells. Populations A (*closed circles*), B (*open circles*), C (*closed triangles*), D (*open triangles*), and E (*closed squares*) were compared with control DC (*open squares*) isolated from the spleens of untreated mice. Background control cultures (*closed star*) contained either DBA/2 allogeneic T cells alone or C57Bl/6 syngeneic KLH-specific T cells plus KLH alone. The background counts were <100 cpm or 800 cpm, respectively.
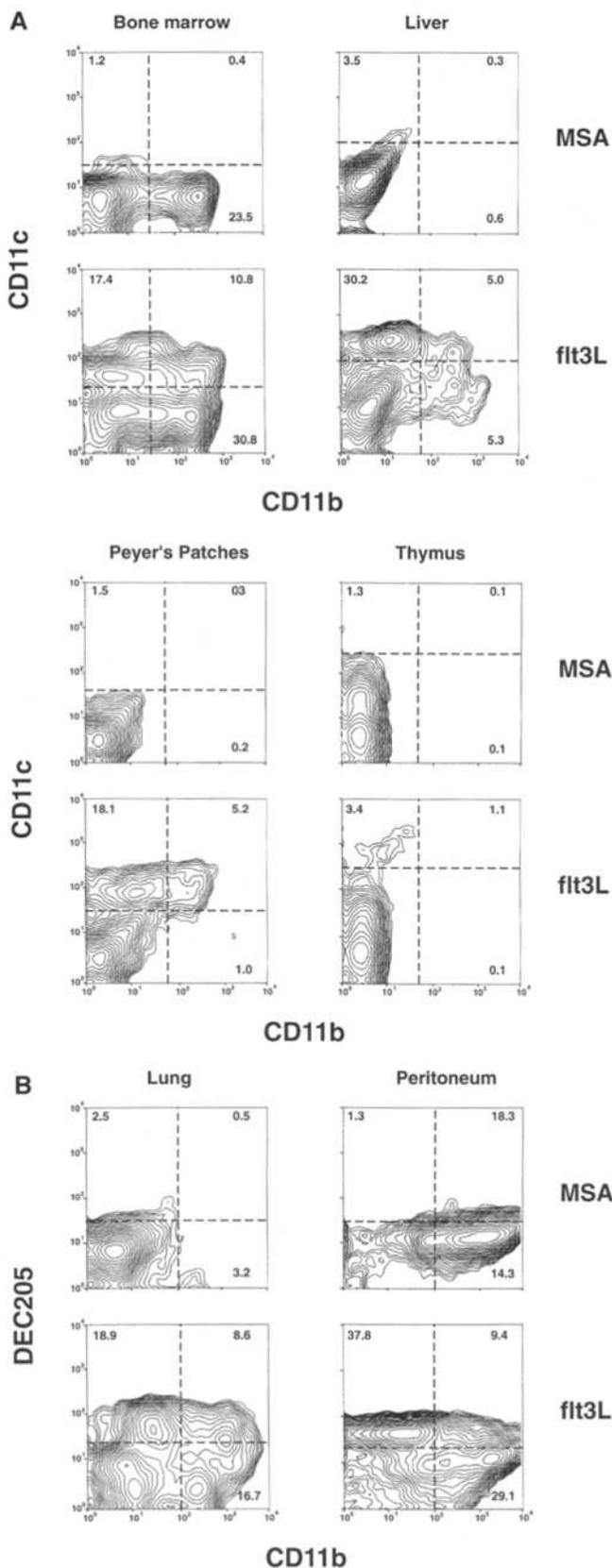
pression. When the various spleen populations from Flt3L-treated mice were compared with the rare freshly isolated control DC in an MLR, it was found that cells from populations C, D, and E were as efficient as control DC at stimulating the proliferation of allogeneic DBA/2-derived T cells (Fig. 3, *upper panels*). In contrast, cells from populations A and B were 30-fold less efficient as APC compared with control DC (Fig. 3, *upper panels*). Similarly, cells from populations C, D, and E, but not populations A and B, were as efficient as control DC at stimulating the proliferation of syngeneic KLH-specific T cells in a KLH presentation assay (Fig. 3, *lower panels*). This indicates that populations C, D, and E are not only composed of cells that appear morphologically like DC and express cell surface Ag characteristic of DC, but are also as efficient as control DC at stimulating alloreactive T cells and processing and presenting soluble Ag to Ag-specific T cells. Since populations D and E, which are either dull or negative for CD11b expression, can be further separated using CD8α expression, a total of five DC subpopulations can be detected in the spleens of Flt3L-treated mice.

*Ag-pulsed Spleen DC from Flt3L-treated Mice Are Efficient at Priming an Ag-specific Immune Response In Vivo.* DC have been previously shown to initiate an Ag-specific immune response when pulsed with soluble Ag ex vivo and then injected into mice (30). We compared the capacity of class $II^+$ $CD11c^+$ spleen DC generated from Flt3L-treated mice (Flt3L DC) with control DC to generate a T cell–specific immune response in vivo after exposure to soluble KLH ex vivo. Both Flt3L-DC and control DC were cultured in vitro with or without soluble KLH for 18 h. These cells were then washed and $1 \times 10^5$ KLH-pulsed DC were injected into the foot pads of naive mice. DC cultured in

medium alone were injected into the contralateral footpads as Ag-specificity controls. After 7 d, the popliteal LN draining the footpads were harvested and $CD4^+$ T cells were purified and restimulated in vitro with soluble KLH presented by freshly isolated control DC. KLH-pulsed Flt3L-DC were as efficient as KLH-pulsed control DC at generating KLH-specific T cells in the draining LN of mice
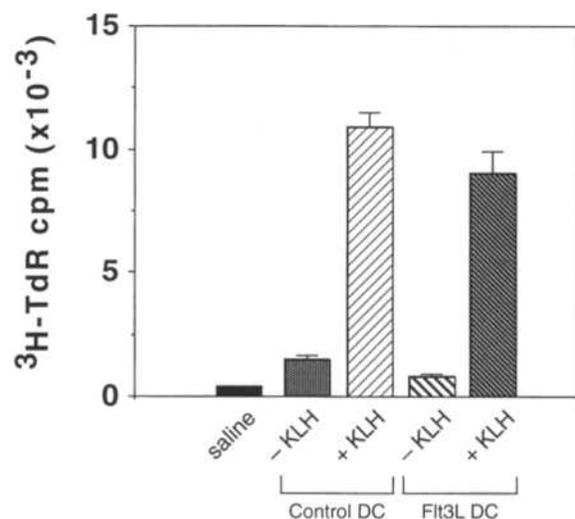


**Figure 4.** The induction of KLH-specific T cells in the draining LN of mice injected with KLH-pulsed DC. Class $II^+$ $CD11c^+$ spleen cells from Flt3L-treated mice and control DC were pulsed with (+KLH) or without (−KLH) KLH Ag and injected into the foot pads of mice (four per group). After 7 d, $CD4^+$ T cells were isolated from the popliteal LN draining the foot pads and cultured with freshly isolated control DC in the presence or absence of KLH for 5 d and [$^3$H]thymidine incorporation was measured.

**A**

Bone marrow    Liver

CD11c

| 1.2 | 0.4 |
| | 23.5 |

MSA

| 3.5 | 0.3 |
| | 0.6 |

| 17.4 | 10.8 |
| | 30.8 |

flt3L

| 30.2 | 5.0 |
| | 5.3 |

CD11b

Peyer's Patches    Thymus

CD11c

| 1.5 | 03 |
| | 0.2 |

MSA

| 1.3 | 0.1 |
| | 0.1 |

| 18.1 | 5.2 |
| | 1.0 |

flt3L

| 3.4 | 1.1 |
| | 0.1 |

CD11b

**B**

Lung    Peritoneum

DEC205

| 2.5 | 0.5 |
| | 3.2 |

MSA

| 1.3 | 18.3 |
| | 14.3 |

| 18.9 | 8.6 |
| | 16.7 |

flt3L

| 37.8 | 9.4 |
| | 29.1 |

CD11b

**Figure 5.** Detection of DC in multiple tissues and organs in Flt3L-treated mice but not MSA-treated controls. (*A*) The distribution of CD11c and CD11b on cells from bone marrow, liver, PP, and thymus in MSA- and Flt3L-treated mice. (*B*) The distribution of DEC205 and CD11b on cells from lung and peritoneal cavity in MSA- and Flt3L-treated mice.

(Fig. 4). KLH-specific T cells were not significantly detected in the contralateral LN draining the foot pads injected with either Flt3L-DC or with control DC cultured in medium alone (Fig. 4).

*Flt3L Treatment Generates Large Numbers of DC in Multiple Tissues and Organs in Mice.* We next examined whether the proportion of CD11c+ or DEC205+ cells was increased in other lymphoid and non-lymphoid tissues. The relative number of CD11c+ CD11b− and CD11c+ CD11b+ cells was significantly increased in the bone marrow (15- and 27-fold, respectively), liver (9- and 4-fold, respectively), PP (12- and 4-fold, respectively), and thymus (3-fold for CD11c+ CD11b− cells only) (Fig. 5 *A*). Because of the high level of CD11c expression by activated lung and peritoneal macrophages in normal and MSA-treated mice (data not shown), expression of DEC205 became a more definitive DC marker in these tissues. The relative number of DEC205+ CD11b− and DEC205+ CD11b+ cells was elevated by 8- and 15-fold, respectively, in the lung and 29- and 2-fold, respectively, in the peritoneal cavity (Fig. 5 *B*). Furthermore, a 2-fold increase in cellularity was observed in these tissues (data not shown). A 2–3-fold increase in nucleated cell number was observed in bone marrow, liver, PP, lung lavage, and peritoneal washings of Flt3L-treated mice (data not shown). Both MSA and Flt3L-treated mice displayed a 2–3-fold reduction in thymic cellularity which could be attributed to stress-induced hypoplasia as a result of daily manipulations of these mice.

*Flt3L but Not GM-CSF, GM-CSF Plus IL-4, c-kitL, or G-CSF Can Generate Large Numbers of DC When Administered In Vivo.* In vitro, DC can be generated from bone marrow progenitors or PBMC using GM-CSF (but not M-CSF or G-CSF) (1, 17). Furthermore, several studies have shown that IL-4, TNF-α and c-kitL can enhance the GM-CSF-dependent in vitro generation of DC (8–17). To determine the importance of GM-CSF upon DC generation in vivo, we compared the capacities of Flt3L or GM-CSF (as well as a non-DC generating cytokine, G-CSF) upon the generation of DC in the spleen. Class II+ CD11c+ cells were rare in the spleens of mice treated for 11 consecutive days with either MSA alone, or with GM-CSF or G-CSF (Fig. 6 *A*). However, treatment of mice with Flt3L alone or Flt3L plus GM-CSF or G-CSF significantly increased the relative numbers of class II+ CD11c+ cells in the spleen (Fig. 6 *A*). Although all the single growth factor treatments or Flt3L-containing growth factor combinations resulted in an increase in total spleen cellularity over 11 d, only mice treated with Flt3L or Flt3L-containing combinations showed a 20- to 30-fold increase in class II+ CD11c+ spleen cells (Fig. 6 *B*). Interestingly, the addition of either GM-CSF or G-CSF to Flt3L treatment increased the total number of class II+ CD11c+ cells only a further 1.2- and 1.3-fold, respectively, indicating that for in
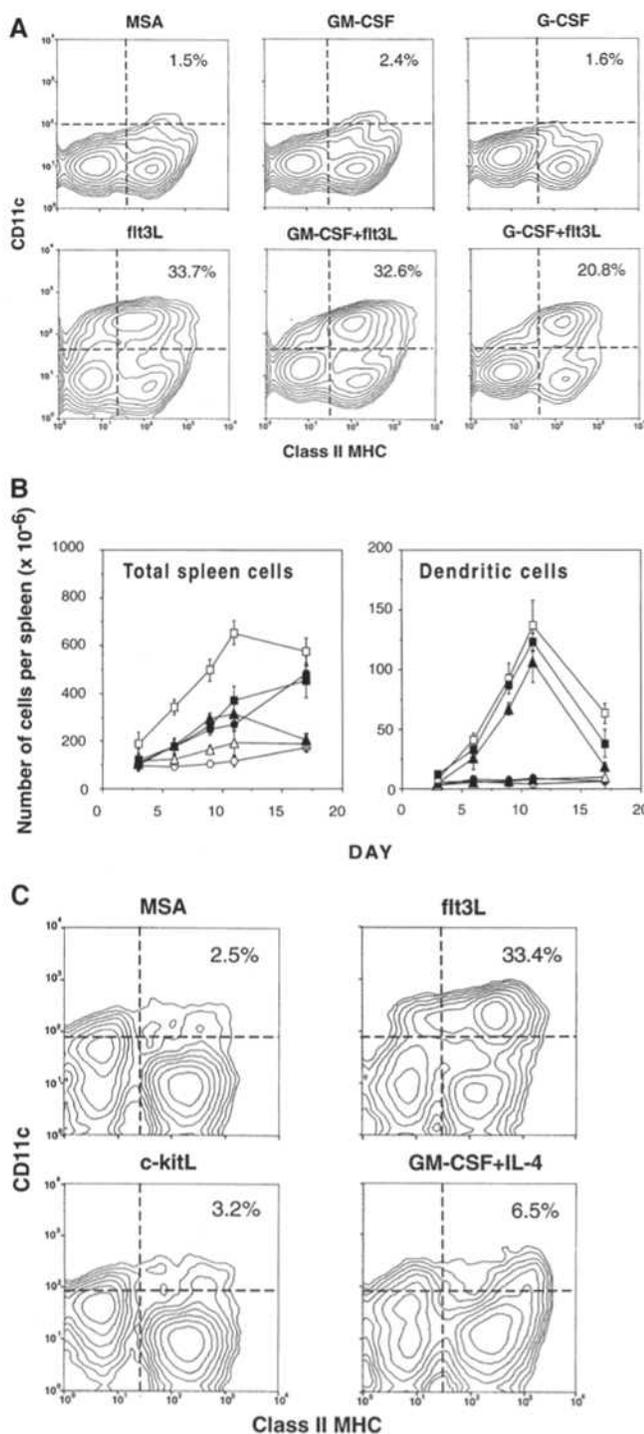
**Figure 6.** Detection of class II⁻ CD11c⁺ cells in growth factor–treated mice. (A) The distribution of class II MHC and CD11c in spleen cells from mice treated for 11 d with MSA alone, or with either Flt3L, G-CSF, GM-CSF, or Flt3L plus G-CSF or Flt3L plus GM-CSF. (B) Increases in total spleen cellularity and total numbers of class II⁺ CD11c⁺ DC over time in growth factor–treated mice Groups as described above: MSA alone (*open circles*), G-CSF (*closed circles*), GM-CSF (*open triangles*), Flt3L (*closed triangles*), Flt3L plus G-CSF (*open squares*), Flt3L plus GM-CSF (*closed squares*). Growth factor treatment ceased at day 11 and spleens from the various groups were also analyzed at day 17. Values represent the mean ± SD of 4 mice per group. (C) The distribution of class II MHC and CD11c in spleen cells from mice treated for 11 d with MSA alone, or with either Flt3L or c-kitL or GM-CSF plus IL-4.

vivo administration Flt3L (but not GM-CSF or G-CSF) is the principle growth factor in the generation of class II⁺ CD11c⁺ cells. In addition, cessation of growth factor treatment at day 11 resulted in a reduction in the total number of class II⁺ CD11c⁺ cells by day 17, indicating that the generation of these cells was transient (Fig. 6 B). Comparison of additional growth factors in vivo such as c-kit-L (10 μg/mouse/d) or GM-CSF plus IL-4 (10 μg of each/mouse/d) on the development of class II⁺ CD11c⁺ spleen cells is presented in Fig. 6 C. Although GM-CSF plus IL-4 are potent in vitro stimulators of DC development from bone marrow progenitors, this cytokine combination resulted in only a 3-fold increase in the relative numbers of class II⁺ CD11c⁺ spleen cells (Fig. 6 C). Interestingly, GM-CSF plus IL-4-treatment of mice did increase the total spleen cellularity by 2–3-fold (comparable to that seen in Flt3L-treated mice) suggesting that cells of other lineages were preferentially expanded in these mice (data not shown). Furthermore, although administration of c-kitL resulted in a 5-fold increase in CFU-GM in the spleen (data not shown) and a 1.5-fold increase in total spleen cellularity (data not shown), there was no significant increase in the relative numbers of class II⁺ CD11c⁺ cells in the spleen (Fig. 6 C). These results indicate that Flt3L is the most potent growth factor for the generation of large numbers of DC in vivo.

## Discussion

Our data indicate that in vivo administration of Flt3L results in a dramatic numerical increase of DC in multiple tissues in mice. These DC are as efficient at stimulating the proliferation of Ag-specific T cells in vitro or at inducing an Ag-specific T cell response in vivo as the rare DC isolated from the spleens of untreated mice. Elevated numbers of DC are detected in both lymphoid- and non-lymphoid tissue in Flt3L-treated mice, including the bone marrow, GALT, liver, lymph nodes, lung, peripheral blood, peritoneal cavity, spleen, and thymus. Of particular interest is the elevated numbers of DC in the GALT, lung, and peripheral blood which are sites amenable to vaccine delivery in clinical immunotherapy regimens.

At least three phenotypically distinct DC subpopulations have been identified on the basis of CD11b and CD11c expression (populations C, D, and E) and these appear to be functionally similar in the Ag-mediated T cell proliferation assays. Populations D and E, which are either dull or negative for CD11b expression, can be further separated using CD8α expression (a total of five DC subpopulations). This surface phenotype (CD11cᵇʳⁱᵍʰᵗ CD11bᵈᵘˡˡ CD8α⁺) is similar to that of the putative lymphoid-derived DC populations (5, 18). Although the generation of these DC from a lymphoid precursor has only been established for thymic DC, a similar CD8α⁺ population is found in spleen and LN (5, 31). A subclass of DC have recently been identified that co-express CD8α and high levels of FasL and kill activated Fas expressing CD4⁺ T cells in vitro (32). FasL-expressing DC may be involved in the regulation, rather

than stimulation, of primary peripheral T cell responses. It is notable that although cells within population E were highly efficient APC, they were consistently less efficient at stimulating the proliferation of Ag-specific T cells than cells within populations C, D, or control DC. Interestingly, the highest proportion of CD8$\alpha^+$ DC are found within population E. The less efficient APC function by population E may be due to deletion of activated CD4$^+$ T cells by FasL expression on the CD8$\alpha^+$ DC (32). We are currently examining the proportion of FasL-expressing cells within each DC subpopulation identified in Flt3L-treated mice.

Population C has not been previously described. The high CD11b expression by population C suggests a relationship to the myeloid lineage and it may represent an immature stage in development of the myeloid-derived DC population (11, 15, 16, reviewed in reference 17). It is also of note that mature splenic monocytes and macrophages are not clearly discernible in these mice. It is possible that the in vivo effects of Flt3L on myelopoiesis may result in the terminal differentiation of developing monocytes and macrophages into DC or DC-like cells analogous to human monocytes treated with GM-CSF and IL-4 (13, 14). Recently, phenotypic, histological, and functional studies suggest that the cells within population C are myeloid-derived DC (Pulendran B., J. Smith, M. Teepe, E. Roux, C. Maliszewski, and E Maraskovsky, manuscript in preparation).

Several studies have shown that GM-CSF is necessary for the in vitro generation of DC from bone marrow or cord blood progenitors, or from PBMC (reviewed in reference 17) and that this can be further enhanced by the addition of IL-4 or c-kitL (13–17). However, although GM-CSF appears to be necessary in vitro, overexpression of GM-CSF in GM-CSF transgenic mice does not increase the number of DC in lymphoid tissue, suggesting that other growth factors are important for DC generation in vivo (20). Interestingly, GM-CSF does not appear to be necessary for DC generation from lymphoid-committed precursors (19; Saunders, D., K. Lucas, J. Ismaili, L. Wu, E. Maraskovsky, D. Dunn, and K. Shortman, manuscript in preparation). We report here that neither GM-CSF and IL-4 nor c-kitL are as potent at stimulating the development of functionally mature DC in vivo as Flt3L. Furthermore, the multiple subpopulations of DC identified here and their apparent relationship to either the lymphoid or myeloid developmental pathways suggests that, unlike GM-CSF, Flt3L enhances the development of both lymphoid- and myeloid-derived DC populations.

It is unlikely that Flt3L treatment simply results in the mobilization of existing mature DC from other sites into the lymphoid tissue. First, Flt3 receptor is not detected on mature DC as assessed by flow cytometry and mature DC do not proliferate when cultured in Flt3L alone (data not shown). Second, elevated numbers of DC have been detected in multiple organs and tissues in Flt3L-treated mice, indicating that there is a generalized expansion of DC throughout these mice. Finally, Brasel et al. have recently shown that in vivo administration of Flt3L dramatically increases the numbers of hematopoietic progenitors in the

BM, peripheral blood and spleen, resulting in increased myelopoiesis and B lymphopoiesis (27, 28). These potent effects on hematopoiesis, as well as our observations on DC generation, suggest that in vivo administration of Flt3L facilitates the terminal development of a primitive, Flt3L-sensitive, rapidly expanding progenitor population into functionally mature DC. This hypothesis is further supported by studies showing that Flt3L can increase the absolute numbers of mature DC generated from cultured CD34$^+$ human bone marrow progenitors (33, 34). In addition, the early detection of DC in the secondary lymphoid organs of mice after Flt3L treatment may indicate that a more mature precursor population (such as a CFU-DC) (16) is also induced to differentiate into DC when exposed to Flt3L (or Flt3L-inducible signals) in vivo. We are currently investigating whether such committed precursor cells are responsive to Flt3L in vitro.

The use of DC as cellular vectors for tumor immunotherapy is a promising strategy (21, reviewed in reference 23). However, the fact that only limited numbers of DC can be generated in vitro from bone marrow progenitors or PBMC, and that only the myeloid-derived type of DC expand in GM-CSF containing cultures (11–17), may limit the utility of this clinical regimen. By expanding the numbers of functionally mature lymphoid and myeloid-derived DC in Flt3L-treated mice, we can not only assess whether these mice have a more robust or sensitized immune response to antigenic challenge, but also which DC lineage is most efficient at inducing effective tumor-specific immunity. In this regard, results from preliminary studies indicate that Flt3L-treated mice respond more efficiently to a tumor challenge in vivo. This appears to be associated with the induction of a T cell–mediated anti-tumor response which can mediate the rejection of even established tumors (Lynch, D.H., A. Andreasen, E. Maraskovsky, R.E. Miller, and J.C.L. Schuh, manuscript submitted for publication). Similar results have been obtained with Flt3L gene-transduced breast cancer cells that mediate the rejection of subsequently transplanted, untransduced breast cancer cells (35). These findings suggest that increasing the numbers of antigen presenting cells in vivo can enhance the generation of Ag-specific immunity.

It is not clear, however, that Flt3L-treatment will have the same effects with all antigens. The development of Ag-specific immunity is a multifaceted phenomenon. The nature of the Ag, the route of administration and the types and functional characteristics of the DC found at that site could all affect the magnitude and qualitative nature of the immune response generated. Therefore, a significant effort to identify the most efficient and appropriate methods to elicit Ag-specific immunity with various types of Ag will be required to assess the feasibility of using Flt3L-induced DC in various forms of cellular immunotherapy. With this in mind, we are currently examining the capacity of Flt3L-treated mice to more efficiently elicit immunity to soluble Ag in the absence of chemical adjuvants.

In conclusion, the results in the present study indicate that Flt3L is a potent inducer of DC when administered in

vivo and may be an important growth factor for both the in vitro and in vivo generation of large numbers of functionally mature DC. The feasibility of using DC as cellular adjuvants must now be tested in depth. Furthermore, the identification of different DC subpopulations may ultimately lead to the identification of differing functions (e.g., stimulatory versus regulatory and control of T cell cytokine production). The identification of functionally distinct DC subpopulations should in turn lead to their more focused and effective use in immunotherapy.

## References

1. Steinman, R.M. 1991. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* 9:271–296.
2. Metlay, J.P., M.D. Witmer-Pack, R. Agger, M.T. Crowley, D. Lawless, and R.M. Steinman. 1990. The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. *J. Exp. Med.* 171:1753–1771.
3. Kraal, G., M. Breel, M. Janse, and G. Bruin. 1986. Langerhans' cells, veiled cells, and interdigitating cells in the mouse recognized by a monoclonal antibody. *J. Exp. Med.* 163:981–997.
4. Jiang, W., W.J. Swiggard, C. Heufler, M. Peng, A. Mirza, R.M. Steinman, and M.C. Nussenzweig. 1995. The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. *Nature (Lond.).* 375:151–155.
5. Vremec, D., M. Zorbas, R. Scollay, D.J. Saunders, C.F. Ardavin, L. Wu, and K. Shortman. 1992. The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. *J. Exp. Med.* 176:47–58.
6. Matzinger, P., and S. Guerder. 1989. Does T-cell tolerance require a dedicated antigen-presenting cell? *Nature (Lond.).* 338:74–76.
7. Mazda, O., Y. Watanabe, J.-I. Gyotoku, and Y. Katsura. 1991. Requirement of dendritic cells and B cells in the clonal deletion of Mls-reactive T cells in the thymus. *J. Exp. Med.* 173:539–547.
8. Inaba, M., K. Inaba, M. Hosono, T. Kumamoto, T. Ishida, S. Muramatsu, T. Masuda, and S. Ikehara. 1991. Distinct mechanisms of neonatal tolerance induced by dendritic cells and thymic B cells. *J. Exp. Med.* 173:549–559.
9. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R.M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176:1693–1702.
10. Inaba, K., R.M. Steinman, M.W. Pack, H. Aya, M. Inaba, T. Sudo, S. Wolpe, and G. Schuler. 1992. Identification of proliferating dendritic cell precursors in mouse blood. *J. Exp.*

*Med.* 175:1157–1167.
11. Inaba, K., M. Inaba, M. Deguchi, K. Hagi, R. Yasumizu, S. Ikehara, S. Muramatsu, and R.M. Steinman. 1993. Granulocytes, macrophages, and dendritic cells arise from a common major histocompatibility complex class II-negative progenitor in mouse bone marrow. *Proc. Natl. Acad. Sci. USA.* 90:3038–3042.
12. Caux, C., C. Dezutter-Dambuyant, D. Schmitt, and J. Banchereau. 1992. GM-CSF and TNF-α cooperate in the generation of dendritic Langerhans cells. *Nature (Lond.).* 360:258–261.
13. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. *J. Exp. Med.* 179:1109–1118.
14. Romani, N., S. Gruner, D. Brang, E. Kämpgen, A. Lenz, B. Trockenbacher, G. Konwalinka, P.O. Fritsch, R.M. Steinman, and G. Schuler. 1994. Proliferating dendritic cell progenitors in human blood. *J. Exp. Med.* 180:83–93.
15. Young, J.W., P. Szabolcs, and M.A.S. Moore. 1995. Identification of dendritic cell colony-forming units among normal human CD34+ bone marrow progenitors that are expanded by c-kit-ligand and yield pure dendritic cell colonies in the presence of granulocyte/macrophage colony-stimulating factor and tumor necrosis factor α. *J. Exp. Med.* 182:1111–1120.
16. Szabolcs, P., M.A.S. Moore, and J.W. Young. 1995. Expansion of immunostimulatory dendritic cells among the myeloid progeny of human CD34+ bone marrow precursors cultured with c-kit ligand, granulocyte-macrophage colony-stimulating factor, and TNF-α. *J. Immunol.* 154:5851–5861.
17. Caux, C., and J. Banchereau. 1996. In vitro regulation of dendritic cell development and function *In* Blood Cell Biochemistry, Vol.7: Hemopoietic growth factors and their receptors. A. Whetton and J. Gordon, editors. Plenum Press, London. In press.
18. Ardavin, C., L. Wu, C.-L. Li, and K. Shortman. 1993. Thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population. *Nature (Lond.).* 362:761–763.

19. Galy, A., M. Travis, D. Cen, and B. Chen. 1995. Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity*. 3:459–473.

19a.Saunders, D., K. Lucas, J. Ismaili, L. Wu, E. Maraskovsky, D. Dunn, and K. Shortman. 1996. Dendritic cell development in culture from thymic precursor cells in the absence of granulocyte-macrophage colony stimulating factor. *J. Exp. Med.* In press.

20. Metcalf, D., K. Shortman, D. Vremec, S. Mifsud, and L. Di Rago. 1996. Effects of excess GM-CSF levels on hematopoiesis and leukemia development in GM-CSF/max 41 double transgenic mice. *Leukemia*. 10:713–719.

21. Mayordomo, J.I., T. Zorina, W.J. Storkus, L. Zitvogel, C. Celluzzi, L.D. Falo, C.J. Melief, S.T. Ildstad, W.M. Kast, A.B. Deleo, and M.T. Lotze. 1995. Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity. *Nat. Med.* 1:1297–1302.

22. Thomson, A.W., L. Lu, N. Murase, A.J. Demetris, A.S. Rao, and T.E. Starzl. 1995. Microchimerism, dendritic cell progenitors and transplantation tolerance. *Stem Cells*. 13:622–639.

23. Young, J.W., and K. Inaba. 1996. Dendritic cells as adjuvants for class I major histocompatibility complex-restricted antitumor immunity. *J. Exp. Med.* 183:7–11.

24. Lyman, S.D., L. James, T. VandenBos, P. de Vries, K. Brasel, B. Gliniak, L.T. Hollingsworth, K.S. Picha, H.J. McKenna, R.R. Splett et al. 1993. Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells. *Cell*. 75:1157–1167.

25. Lyman, S.D. 1996. Biology of Flt3 ligand and receptor. *Int. J. Hematol.* 62:63–73.

26. McKenna, H.J., and S.D. Lyman. 1996. Biology of Flt3 ligand, a novel regulator of hematopoietic stem and progenitor cells. In Bone Marrow Transplantation: Basic and Clinical Studies. S. Ikehara, F. Takaku, and R.A. Good, editors. Springer Publishing Co., New York. 85–93.

27. Brasel, K., H.J. McKenna, K. Charrier, P. Morrissey, D.E. Williams, and S.D. Lyman. 1995. Mobilization of peripheral blood progenitor cells with Flt3 ligand. *Blood*. 86:463a.

28. Brasel, K., H.J. McKenna, P.J. Morrissey, K. Charrier, A.E. Morris, C.C. Lee, D.E. Williams, and S.D. Lyman. 1996. Hematological effects of Flt3 ligand in vivo in mice. *Blood*. 88: 2004–2012.

29. Crowley, M., K. Inaba, M. Witmer-Pack, and R.M. Steinman. 1989. The cell surface of mouse dendritic cells: FACS analyses of dendritic cells from different tissues including thymus. *Cell. Immunol.* 118:108–125.

30. Inaba, K., J.P. Metlay, M.T. Crowley, and R.M. Steinman. 1990. Dendritic cells pulsed with protein antigens in vitro can prime antigen-specific, MHC-restricted T cells in situ. *J. Exp. Med.* 172:631–640.

31. Wu, L., D. Vremec, C. Ardavin, K. Winkel, G. Süss, H. Georgiou, E. Maraskovsky, W. Cook, and K. Shortman. 1995. Mouse thymus dendritic cells: kinetics of development and changes in surface markers during maturation. *Eur. J. Immunol.* 25:418–425.

32. Süss, G., and K. Shortman. 1996. A subclass of dendritic cells kills CD4 T cells via Fas/Fas-ligand—induced apoptosis. *J. Exp. Med.* 183:1789–1796.

33. Siena, S., M. Di Nicola, M. Bregni, R. Mortarini, A. Anichini, L. Lombardi, F. Ravagnani, G. Parmiani, and A.M. Gianni. 1995. Massive ex vivo generation of functional dendritic cells from mobilized CD34$^+$ blood progenitors for anticancer therapy. *Exp. Hematol.* 23:1463–1471.

34. Maraskovsky, E., E. Roux, M. Teepe, H.J. McKenna, K. Brasel, S.D. Lyman, and D.E. Williams. 1995. The effect of Flt3 ligand and/or c-kit ligand on the generation of dendritic cells from human CD34$^+$ bone marrow. *Blood*. 86:420a.

35. Chen, K., S.E. Braun, E. Wiebke, S.D. Lyman, Y. Chiang, H.E. Broxmeyer, and K. Cornetta. 1995. Retroviral-mediated gene transfer of the Flt3 ligand into murine breast cancer cells prevents tumor growth in vivo. *Blood*. 86(Suppl. 1):244a.