Understanding the Dendritic Cell Lineage through a Study of Cytokine Receptors

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Summary

Dendritic cells form a system of antigen presenting cells that are specialized to stimulate T lymphocytes, including quiescent T cells. The lineage of dendritic cells is not fully characterized, although prior studies have shown that growth and differentiation are controlled by cytokines, particularly granulocyte/macrophage colony-stimulating factor (GM-CSF). To further elucidate the nature and control of the dendritic cell lineage, we have studied the expression of specific cytokine receptors. Sufficient numbers of dendritic cells were purified from spleen and skin to do quantitative binding studies with radiolabeled M-CSF, GM-CSF, and interleukin 1 (IL-1). To verify the nonlymphoid nature of dendritic cells, we made an initial search for rearrangements in T cell receptor and immunoglobulin genes and none were found. M-CSF binding sites, a property of mononuclear phagocytes, also were absent. In contrast, GM-CSF receptors were abundant on mature dendritic cells, with ~3,000 binding sites/cell with a single K_d of 500–1,000 pM. Substantial numbers of high affinity (<100 pM) IL-1 binding sites were identified as well; cultured epidermal dendritic cells (i.e., epidermal Langerhans cells) had 500/cell and spleen dendritic cells ~70/cell. Cross-linking approaches showed the 80-kD species that is expected of high-affinity type 1 IL-1 receptor. Anti-type 1 IL-1 receptor (R) mAbs also visualized these receptors by flow cytometry on freshly isolated epidermal dendritic cells. These results provide new evidence that dendritic cells represent a differentiation pathway distinct from lymphocytes and monocytes. Together with recent findings on the effects of IL-1 and GM-CSF on epidermal dendritic cells in situ (see Results and Discussion), the data lead to a proposal whereby IL-1 signals IL-1R to upregulate GM-CSF receptors and thereby, the observed responsiveness of dendritic cells to GM-CSF for growth, viability, and function.

Gradually, mechanisms are being identified to explain how dendritic cells (DC)[1] operate in situ to initiate T cell–dependent immune responses (for review see 1, 2). Epidermal dendritic cells (Langerhans cells, [LC]) have provided a good deal of information (for review see 3, 4). These cells are equipped to acquire antigen in the skin, to transport the antigen to lymphoid organs, and there to sensitize T cells most likely in the T cell areas. IL-1 can initiate this stepwise series of DC functions in situ (5, 6). GM-CSF likely plays a subsequent role in the development and maturation of immunostimulatory functions (7, 8). Other populations of DC, such as those in rat lung (9, 10) and human blood (11), likewise develop their typical features and functions in response to cytokines.

It follows that the responsiveness of DC to cytokines, like IL-1 (7, 12) and GM-CSF (7, 13), could underlie early events in the development of T cell immunity. We now have looked for the frequency and types of cytokine receptors on DC in a nonlymphoid tissue, skin, and in the lymphoid organ, spleen. This topic has not previously been approached directly, in part because the isolation of the required numbers of DC is not straightforward. We report that mature DC lack M-CSF binding sites, but express relatively high levels of a single class each of GM-CSF and IL-1 binding sites. These and other findings are used to propose that DC are a nonlymphoid, nonmacrophage lineage that is mobilized by the sequential action of IL-1 and GM-CSF on their respective receptors.

[1] *Abbreviations used in this paper:* d1, cultured for 1 day; d3, cultured for 3 days; DC, dendritic cells; EC, epidermal cells; hIL-1ra, human IL-1 receptor antagonist; LC, Langerhans cells; R, receptor.

Materials and Methods

Mice. Specific pathogen-free BALB/c (H-2^d) and C3H/He (H-2^k) mice (6–12 wk old of both sexes) were obtained from Charles River Wiga GmbH (Sulzfeld, Germany). We used mainly BALB/c mice, but pilot experiments were also performed with C3H/He mice, and yielded comparable results.

Culture Medium. RPMI 1640 supplemented with 10% FCS (56°C, 0.5 h; Seromed, Biochrom KG, Berlin, Germany), 1 mM L-glutamine, 5×10^{-5} 2-ME, and 50 μg/ml gentamicin sulfate.

Spleen DC. DC were prepared from the low density fractions of collagenase-treated spleens. After overnight culture the nonadherent cells were harvested and depleted of residual macrophages and B cells by rosetting with EA to provide an EA^- DC fraction of >90% purity as described (14). In most experiments the low density fractions were pretreated with anti-B220 (TIB-146; American Type Culture Collection [ATCC], Rockville, MD) and anti-Thy 1 (TIB 99, ATCC) mAbs plus C′ to reduce the number of B and T lymphocytes, respectively. The purity of the final DC preparation (yield: 0.3×10^6/spleen) was then consistently at least 95% as determined by cytology and flow cytometry analysis of relevant surface markers (14).

Epidermal LC. Epidermal cell (EC) suspensions (containing 1–3% LC) were prepared from ear skin by exposure to 1% trypsin (Cat. No. 16-893-49; Flow Laboratories, Irvine, Scotland), and treated with anti-Thy 1-mAb and rabbit C′ followed by a brief trypsin exposure (0.125%, 10 min, 37°C) to remove dead cells exactly as described (15). This treatment removes most keratinocytes as well as the dendritic, γ/δ + Thy-1+ EC, and results in a viable (>90%) EC suspension containing about 15% LC (15). To obtain d3 LC the anti-Thy1/C′-treated EC were cultured (20×10^6/100 mm petri dish) for 72 h exactly as described (16), and the nonadherent fractions floated on dense albumin columns as described (15). These nonadherent low density cells contained most of the LC in the culture and were 60–90% LC. For those experiments requiring more highly enriched LC, we enriched LC to >95% by a "mismatched" panning technique (15–17) from either fresh or nonadherent fractions of 16–72 h cultured anti-Thy 1/C′-treated EC (yield: $3–6 \times 10^4$ fresh or cultured LC/2 mouse ears).

Thymocytes. Thymic lobes were thoroughly teased, passed through a metal sieve, and the resulting thymic cell suspension filtered through a nylon mesh (Nitex 3-325-44; Tetko, Elmsford, NY).

Peritoneal Macrophages. Peritoneal macrophages were exudate cells harvested by lavage using 10 ml of PBS 5–7 d after a single injection of 2 ml thioglycollate medium (yield: $8–15 \times 10^6$ cells/lavage).

Cell Lines. PAM 212, J774, and 70Z/3 lines were obtained from ATCC, the EL4-NOB1 cell line from the European Collection of Animal Cell Cultures (ECACC; Porton Down, Salisbury, UK). Adherent PAM 212 and J774 cells were harvested by exposure to 0.5 mM EDTA in Ca^{2+}, Mg^{2+} free PBS followed by vigorous pipetting.

TCR Gene Rearrangement. DNA was prepared according to standard techniques (18) by lysis of cells in 50 mM Tris-HCl (pH 8.0), 100 mM EDTA, 0.1% SDS buffer, followed by proteinase K digestion (0.5 mg/ml, 55°C, overnight), phenol and chloroform extractions and isopropanol precipitation, digested with EcoRI, electrophoresed on a 0.7% agarose gel (5 μg DNA/lane), transferred to ζ probe (Bio-Rad Laboratories GesmbH., Vienna, Austria) by alkaline blotting (19), and then hybridized as described (20) using the following probes which were ^{32}P-labeled by random priming (21): (*a*) Jδ1, a 500-bp PvuII/PstI fragment including the Jδ1 coding region (22) and Cα, a 700-bp NcoI fragment from a Cα cDNA clone (23) probes; or (*b*) a Cγ1 probe (20). Liver and AKR thymoma 110 (24) DNA served as positive and negative controls, respectively.

Ig Gene Rearrangement. DNA was isolated from DC, LC, the Avison B cell line (serving as a positive control) (provided by Dr. C. Peschel, Gutenberg University, Mainz, Germany) and BALB/c kidney (germ line control), digested with EcoRI, electrophoresed, and then transferred onto nylon membranes and finally hybridized employing a ^{32}P-labeled J11 probe (25) (kindly provided by Dr. C. Peschel). The J11 probe spans the J_{H3} and J_{H4} region of the Cμ flanking sequences in the mouse Ig μ genes.

^{125}I-GM-CSF Binding Assays. Radiolabeling of carrier-free recombinant muGM-CSF and equilibrium binding assays were essentially done as previously described (26, 27). In brief, purified rmu GM-CSF (specific activity $>4 \times 10^7$ U/mg protein; Immunex Co., Seattle, WA) was radiolabeled using the Enzymobead radioiodination reagent (Bio-Rad Laboratories) to a specific activity of 60,000 cpm/ng (= 1.1×10^{15} cpm/mmol). ^{125}I-GM-CSF lacked high molecular weight aggregates, and retained >90% of its biological activity as assessed by survival of purified LC after 3 d of culture (7, 8). For binding assays 10^6 DC, 10^6 LC, and 2×10^6 J774 cells each were washed three times in cold binding buffer (PBS, 2% BSA, 20 mM Hepes buffer, pH 7.2, 0.2% sodium azide), and resuspended in 100 μl binding buffer containing the specified amounts of ^{125}I-GM-CSF with or without a 50-fold molar excess of unlabeled GM-CSF to determine nonspecific binding. Incubations were carried out in 96-well flexible assay plates (Falcon Labware, Oxnard, CA) at 37°C for 45 min (this incubation time was found in pilot experiments to achieve binding equilibrium). Cell suspensions were then layered atop 200 μl of a phthalate oil mixture [1.5 parts of dibutylphthalate, 1 part of bis(2-ethylhexyl)-phthalate; Sigma GmbH, Deisenhofen, Germany] precooled to 4°C in 400 μl polyethylene centrifuge tubes. Samples were centrifuged for 2 min at 11,000 *g*. Cell-bound (cell pellet) and free (supernatant) ^{125}I-GM-CSF was quantitated using a gamma counter (LKB-Wallac, Turku, Finland). Equilibrium binding data were analyzed according to the method of Scatchard (28) and by computerized linear regression analysis.

^{125}I-M-CSF Binding Assays. Purified carrier-free human recombinant M-CSF produced by transfected CHO cells (1.9×10^6 U/mg, courtesy of Dr. Steven Clark, Genetics Institute, Cambridge, MA) was radiolabeled using the Enzymobead radioiodination reagent (Bio-Rad Laboratories GesmbH.) to a specific activity of 80,000 cpm/ng (= 1.1×10^{15} cpm/mmol) (29). ^{125}I-M-CSF retained >90% of its biological activity as determined by a mouse bone marrow proliferation assay. Binding assays were performed as described above for GM-CSF except that incubation was for 120 min, and all steps were strictly performed on ice to avoid ligand-induced receptor internalization (29). In pilot experiments 1 μg/ml polymyxin B was included to neutralize any contaminating LPS (30) with identical results.

^{125}I-IL-1 Binding Assays and Affinity Cross-linking. To study IL-1 receptors (R) on DC, d1 and d3 LC, and control cells (i.e., the EL4-NOB1 murine T cell lymphoma cell line expressing the type 1 IL-1R and the 70Z/3 murine pre-B lymphoma cell line that expresses the type 2 IL-1R) (31, 32) we performed binding assays essentially as described (33). In brief, after two washes in cold binding buffer (see above) samples of cells ($1–5 \times 10^6$ each resuspended in 100 μl) were incubated (2 h, 4°C, permanent shaking) in increasing concentrations of ^{125}I-IL-1α or β (2,000 Ci/mmol

specific activity, fully active in the standard murine D10.G4.1 proliferation assay, purchased from Amersham International, Little Chalfont, UK) with or without a 50- or 100-fold molar excess of recombinant human IL-1β (British Biotechnology, Abingdon, UK). In pilot experiments we also prewashed cells in pH 3.0 glycine buffer to remove any prebound IL-1 (31), but this procedure did not markedly change results, and was, therefore, omitted in further binding assays. In selected experiments blocking mAbs to the type 1 IL-1R either mAb 1593-01 (obtained from Genzyme Corp., Cambridge, MA) or mAb M15 (34) or human recombinant IL-1R antagonist (kindly provided by Dr. A. Steinkasserer, MRC Immunochemistry Unit, University of Oxford, Oxford, UK, [35]) were added 30 min before the addition of IL-1. Duplicate aliquots of cells with bound IL-1 were separated from free IL-1 by centrifugation through a phthalate oil mixture, and equilibrium data analyzed according to Scatchard (28) as described above for ^{125}I-GM-CSF binding. For affinity cross-linking of ^{125}I-IL-1β we followed the protocol described by Dower et al. (33). In short, 2–3 × 10^6 highly enriched d1 or d3 LC, 5 × 10^6 DC, EL 4-NOB1, and 70Z/3 cells were incubated in binding medium for 2 h at 4°C with 10^{-9} M ^{125}I-IL-1β in the presence or absence of a 50-fold molar excess of unlabeled IL-1β. After washes, cell samples were resuspended in 100 μl PBS and 2 μl disuccinimidyl suberate (DDS; Pierce Chemical, Rockford, IL) solution (50 mg/ml DMSO) was added. Samples were incubated for 30 min at room temperature, then washed, and finally resuspended in 20–50 μl PBS containing 1% Triton X-100 and 2 mmol/liter PMSF to prevent proteolytic degradation. After a 15-min detergent extraction and centrifugation to remove nuclei and debris, 15–40 μl supernatant was taken off and 2–4 μl aliquots were analyzed by SDS/PAGE under reducing conditions employing a 10–15% gradient or 7.5% homogenous gels and a Phast System (Pharmacia LKB, Uppsala, Sweden).

Cytofluorography Analysis of Cytokine Receptors. We used cytofluorography (FACScan®, Becton Dickinson & Co., Mountain View, CA) of viable, propidium iodide excluding cells to detect surface expression of cytokine receptors. For the detection of M-CSF receptors spleen DC, d3 LC, and J774 cells (this macrophage cell line served as a positive control) were incubated (40 min each on ice) in goat anti-CSF-1 R antiserum (diluted 1:200 in PBS, 1% BSA, 0.2% sodium-azide) (kindly provided by Dr. E. R. Stanley, Albert Einstein College of Medicine, Bronx, New York) or control normal goat serum, followed by biotinylated swine anti-goat IgG (Tago, Inc., Burlingame, CA), and finally FITC-streptavidin diluted 1:500 (Amersham International). For staining of the IL-1R type 1, the cells were incubated with three different rat IgG2a anti-mouse IL-1R type 1 mAbs (M5 [34], M15 [34], or 1593-01 [obtained from Genzyme Corp.] at 10 μg/ml followed by FITC-conjugated mouse anti-rat Ig mAb [Cat. no. 605540; Boehringer Mannheim Corp., Indianapolis, IN]). To identify BALB/c LC this incubation sequence was extended by rat Ig to block free anti-rat Ig binding sites, biotinylated anti-I-$A^{b,d}$ mAb (clone B21-2, rat IgG2b, TIB 229 from ATCC), and, finally, streptavidin-phycoerythrin (Dianova, Hamburg, Germany). To stain LC of C3H/He mice we used murine anti-I-$E^{k,d}$ mAb (clone 14-4-4S, mouse IgG2a, HB32 from ATCC) followed by phycoethrin-conjugated donkey anti-mouse Ig (Cat. no. 715-116-151; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Results and Discussion

TCR and Ig Genes Are in Germline State in Spleen DC As Well As LC. Despite the accumulating evidence (including single cell assays) that DC are myeloid cells (1, 2, 36, 37), we considered the formal possibility that DC might be related to lymphocytes. For example several T cell–associated molecules (CD1, CD4, CD5, and CD8) are expressed by certain human and murine DC subsets (1, 2, 11, 38–40) and Shortman et al. (41) identified a population of early murine precursors that gave rise to both DC and T cells (but not B cells or phagocytes) when transferred into irradiated thymi. When we studied lymphoid DC (highly enriched from spleen by a method [40] ensuring recovery of loosely as well as more strongly tissue-bound DC) as well as fresh or cultured LC (a homogenous and well-characterized subset of nonlymphoid tissue DC) (3, 4) we found no evidence for a rearrangement of either TCR or Ig genes (see Fig. 1, *A* and *B* and Fig. 2) supplementing the observation by Shortman et al. (40) that thymic DC have TCR in germline. These data show, therefore, that DC would have to deviate before lymphocyte gene rearrangement from a putative common DC/T cell precursor (41).

Spleen DC and LC Express a Single Class of Receptors for GM-CSF. We performed three complete equilibrium binding experiments with BALB/c DC and d3 LC, and, as a positive control, the J774 macrophage line. Pilot experiments using DC and LC prepared from C3H/He mice yielded comparable results. Scatchard analysis of the binding data revealed a single class of GM-CSF-R on DC as well as LC (Fig. 3) with a K_d in the range of 500–1,000 pM, i.e., an affinity similar to that seen on other cell types including myeloid ones (26, 27, 42). About 3,000 specific binding sites per DC or LC were found (see Table 1). This is a relatively large number and, by using J774 as a reference for comparison with published data, would translate into two to three times the receptor

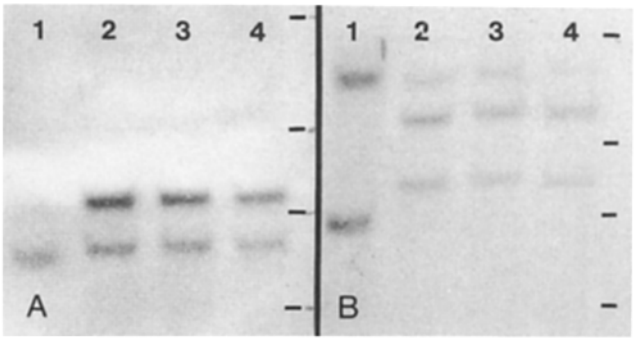


Figure 1. Southern blot analysis of TCR gene status. 5 μg EcoRI digested DNA samples/lane in AKR thymoma cells (lane *1*), d3 LC (lane *2*), spleen DC (lane *3*), and BALB/c liver (lane *4*). (*A*) Jδ1 and Cα probes: both probes were hybridized at the same time to give an internal control for the amount of DNA. In the TCR α/β expressing AKR thymoma cells the Jδ1 band is absent as the δ locus is obligatorily deleted during VJα rearrangements (63). The ratio of Jδ1 to Cα bands stays, however, constant in LC, DC, and BALB/c liver (= germline control) samples indicating that no TCR rearrangement has occurred. (*B*) Cγ probe: LC and DC show the same three bands as the BALB/c liver germline control, whereas a Cγ rearrangement is obvious in the AKR thymoma cells. Horizontal bars indicate DNA size markers (21, 9.4, 6.6, and 4.3 kb from top to bottom).

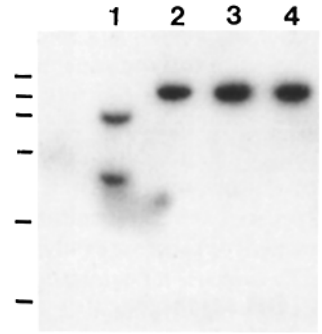


Figure 2. Southern blot analysis of Ig gene status. 5 μg EcoRI digested DNA in Avison B cell line (lane *1*), spleen DC (lane *2*), d3 LC (lane *3*), and BALB/c kidney (lane *4*) using the J_{11} probe which spans over the J_{H3} and J_{H4} region of the Cμ flanking sequences in the BALB/c germ line J_H region. Note that an Ig gene rearrangement is detectable in the Avison B cell line, but not in DC, LC, or the kidney (= germline control) DNA. Horizontal bars indicate DNA size markers (7, 6, 5, 4, 3, 2, 1.6, 1 kb from top to bottom).

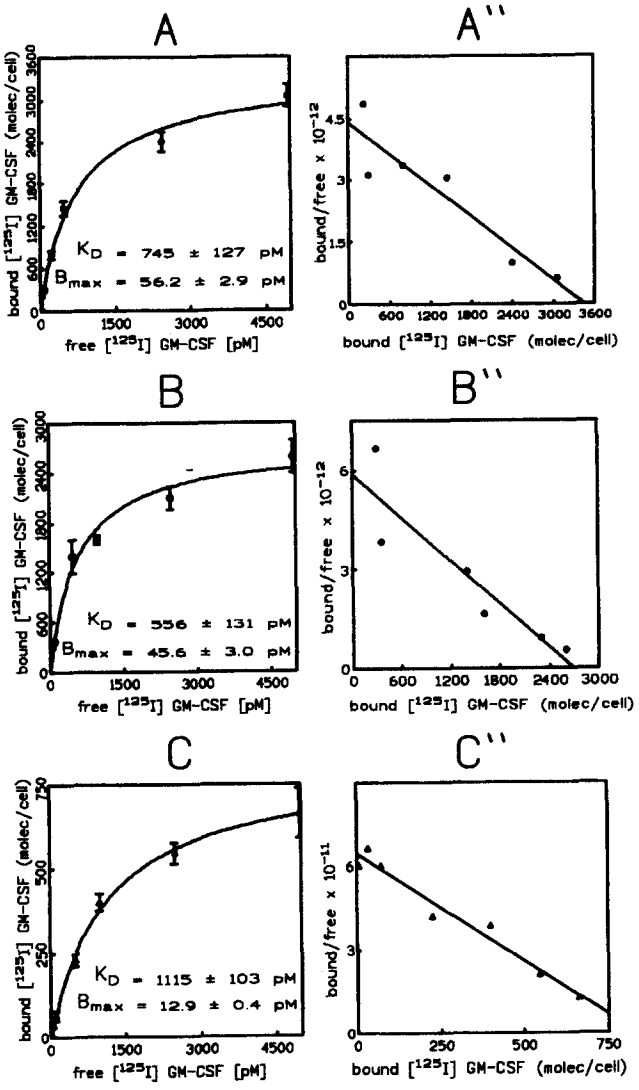


Figure 3. Equilibrium binding of ^{125}I-GM-CSF. Highly enriched BALB/c d3 LC (*A* and *A''*), spleen DC (*B* and *B''*), or J774 cells (*C* and *C''*) were incubated for 45 min at 37°C with various concentrations of rmu ^{125}I-GM-CSF (see Materials and Methods for experimental details). *A*, *B*, and *C* show specific binding data; *A''*, *B''*, and *C''* show a Scatchard representation of the specific binding data replotted from *A*, *B*, and *C*, respectively. K_d, dissociation constant, B max, maximum binding.

Table 1. *Binding Characteristics of rmu ^{125}I-GM-CSF to Murine Cultured LC, Spleen DC, and J774 Cells*

Cell type	GM-CSF-R	K_d
	number/cell	*pM*
d3 LC	3,248 (± 168)	745 (± 198)
DC	2,644 (± 174)	544 (± 65)
J774 macrophage line	858 (± 41)	1,170 (± 244)

Data represent the mean (± SD) of three independent equilibrium binding assays performed as outlined under Materials and Methods. DC and LC were prepared from BALB/mice, but pilot experiments using C3H/He LC and DC yielded comparable results.

number reported for peritoneal exudate or normal bone marrow cells. 3,000 binding sites would be comparable to receptor numbers found on purified granulocyte/macrophage progenitor cells (26, 27, 42). With regard to the detection of solely a single type of receptors on the trace population of DC and LC we could not use >2 × 10^6 cells/assay point, and might, therefore, have missed a small subset of additional receptors with lower or higher affinity. The observation that ~60 pM GM-CSF is needed to maintain the viability of LC and DC is in congruence with the expression of a single class of GM-CSF-R on LC and DC. It is known that a subset of occupied receptors, and, thus, a concentration several orders of magnitude below the K_d is sufficient for maximal biological activity (27). We were unable to detect GM-CSF receptors on freshly prepared LC. This finding is, however, inconclusive as trypsin exposure is needed for preparation of EC and trypsin removes GM-CSF–binding sites on cultured LC (data not shown).

Recent single cell assays have shown that DC, macrophages, and granulocytes share a common progenitor (36, 37), and, therefore, represent three distinct pathways of myeloid differentiation. The expression of a substantial number of GM-CSF-R on mature DC (i.e., spleen DC as well as d3 LC) also indicates a myeloid origin of DC, as previous studies (43) have shown that essentially all cells within the myeloid lineage (monocytes, polymorphs, eosinophils) display GM-CSF-R whereas erythroid cells and lymphoid cells (except certain T cell lines [27, 42]) are negative.

GM-CSF induces the proliferation of DC precursors (37, 44–47), maintains DC viability (7, 8, 13, 48), and mediates the development from less mature but nonproliferating precursors into fully mature DC (7, 8). To more fully understand these events it will clearly be important to monitor the expression of GM-CSF-R including any changes in binding affinity and to analyze respective regulatory mechanisms (see below).

Spleen DC and LC Lack Receptors for M-CSF. We next studied a classical marker for macrophages, i.e., the expression of M-CSF-R. Neither equilibrium binding (data not shown) nor cytofluorography analysis (using an anti-M-CSF-R

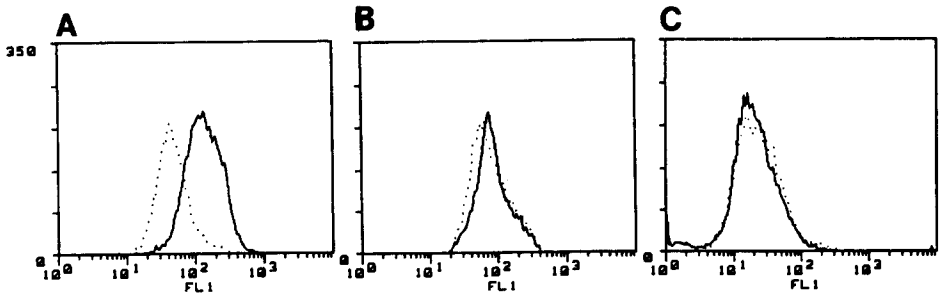
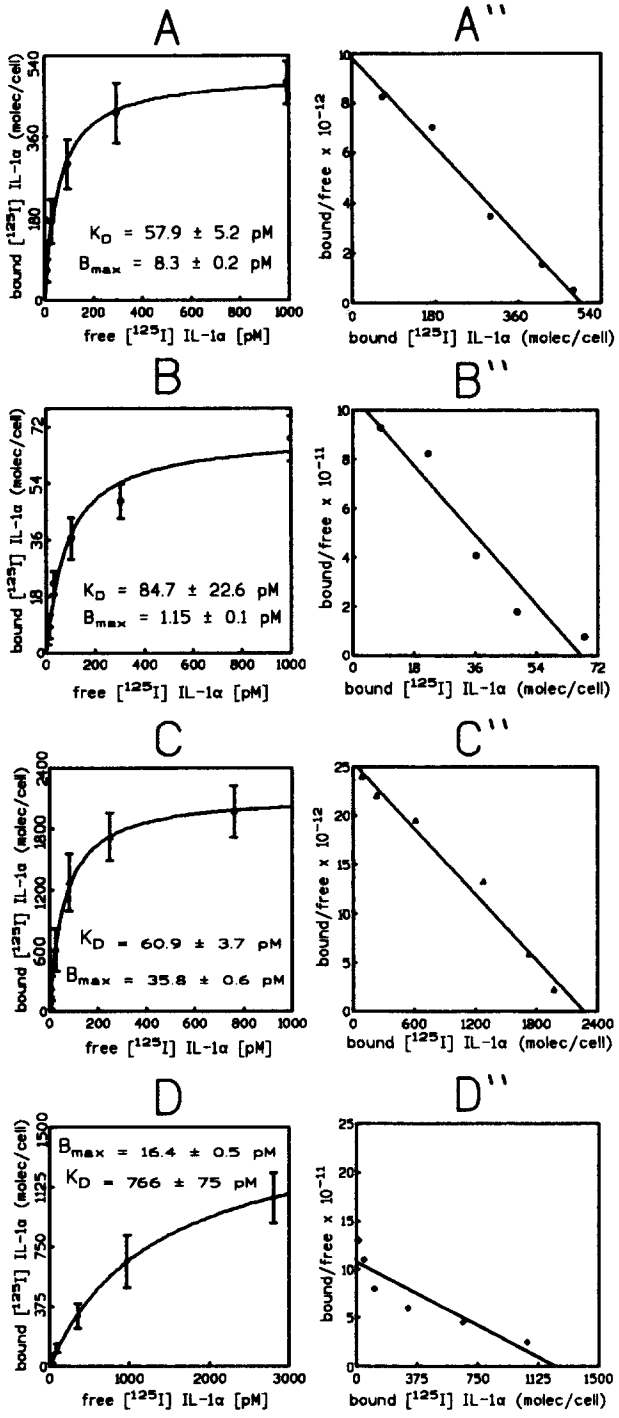


Figure 4. Cytofluorography analysis of M-CSF-R expression. J774 macrophage cell line (*A*), d3 LC (*B*) and spleen DC (*C*) were stained with goat-anti-CSF-1 R antiserum (*solid line*) or with control goat serum (*dashed line*) as outlined in Materials and Methods. Binding of the antibody is only detectable to J774 cells but not to LC and DC.



antibody kindly provided by Dr. R. E. Stanley) (Fig. 4) revealed any M-CSF-R on spleen DC or d3 LC. Receptors were, as expected, readily detectable on peritoneal macrophages (~30,000 receptors/cell). Thymocytes lacked receptors for M-CSF (negative control).

The absence of M-CSF-R on mature DC is further proof that DC are not a specialized subset of macrophages, as the number of M-CSF-R increases on monocytes/macrophages with cell maturation. For example, adherent macrophages display 50,000 or more of these receptors (43). The lack of M-CSF binding sites also explains why LC (8, 15) and DC (Koch, F., unpublished observations) do not respond to M-CSF, and why the numbers of DC/LC are virtually normal in osteopetrotic mice (49, 50) that carry a defective M-CSF gene.

Spleen DC and LC Express the High Affinity Type I IL-1R. IL-1 is known to enhance the function of skin (7), spleen (12), and thymic (51) DC, and to regulate the expression of GM-CSF receptors (52, 53). We, therefore, studied IL-1Rs on DC. Three complete equilibrium binding experiments with spleen DC and d3 LC prepared from BALB/c mice as well as control EL4-NOB1, and 70Z/3 cells were performed using human recombinant ^{125}I-IL-1α, which binds to both type 1 and type 2 murine IL-1R (32). Pilot experiments using DC and d3 LC prepared from C3H/He mice gave comparable results. Scatchard analysis of the binding data revealed a single class of high affinity (K_d <100 pM) IL-1R on DC (mean 69/cell) as well as d3 LC (mean 490/cell). The results were comparable to EL4-NOB1 cells (known to express the high-affinity type 1 IL-1R), but different from 70Z/3 cells (known to exhibit the 10-fold lower affinity type 2 IL-1R) (Fig. 5, Table 2). Pilot experiments employing human recombinant ^{125}I-IL-1β also revealed a single class of IL-1 binding sites in similar numbers, but the K_d was somewhat lower (~500 pM). Preincubation of DC and LC in pH 3.0 glycine buffer to remove prebound IL-1 (31) did not significantly change

Figure 5. Equilibrium binding of rhu ^{125}I-IL-1α. Highly enriched BALB/c d3 LC (*A* and *A''*), spleen DC (*B* and *B''*), and, as a control, EL4-NOB1 cells (known to express the high-affinity type 1 IL-1 R) (*C* and *C''*) as well as 70Z/3 cells (known to express the low-affinity type 2 IL-1R) (*D* and *D''*) were incubated for 2 h at 4°C with various concentrations of rhu-IL-1α (see Materials and Methods for experimental details). Panels *A–D* show specific binding data, panels *A''–D''* show a Scatchard representation of these data. K_d, dissociation constant, *B max*, maximum binding.

Table 2. *Binding Characteristics of rhu ^{125}I-IL-1α to Murine Cultured LC, Spleen DC, and Control Cells*

Cell type	IL-1	K_d
	number/cell	*pM*
d3 LC	490 (± 20)	58 (± 5)
DC	69 (± 11)	85 (± 22)
EL4-NOB1	2,150 (± 40)	61 (± 4)
70Z/3	1,230 (± 240)	480 (± 132)

Data represent the mean (± SD) of three independent equilibrium binding assays performed as outlined under Materials and Methods. DC and LC were prepared from BALB/mice, but pilot experiments using C3H/He LC and DC yielded comparable results.

the number of IL-1Rs detected. Human IL-1β as well as the human IL-1 receptor antagonist (hIL-1ra) effectively competed for binding of ^{125}I-IL-1α to spleen DC and LC, demonstrating that the IL-1R on DC/LC can bind all three forms of IL-1 (Table 3). It is known that the IL-1ra readily binds to the murine type 1 IL-1R yet only poorly, if at all, to the type 2 murine IL-1R (32, 54). Blocking of ^{125}I-IL-1α binding by hIL-1ra is, therefore, further evidence that DC/LC express the type 1 high-affinity IL-1R. Additional support was the finding that the antimurine type 1 IL-1R mAbs M15 (34) and 1593-01 (Genzyme Corp.) were able to completely block the binding of ^{125}I-IL-1α to spleen DC and LC (Table 3). Affinity cross-linking of ^{125}I-hIL-1β to IL-1R (33) revealed complexes of ~100 kD which, upon subtraction of ~18 kD for human IL-1β, correspond to the ~80 kD molecular mass described for the type 1 IL-1R (but the complexes are distinctly larger than those produced by the 60-kD type 2 IL-1R of 70Z/3 cells [Fig. 6 *A*]). These data clearly show that d3 LC and spleen DC express high-affinity type 1 IL-1R.

Table 3. *Effect of rhu IL-1R Antagonist (IL-1ra) and M15 Antimurine Type 1 IL-1R mAb on rhu ^{125}I-IL-1α Binding*

	Percent inhibition	
Cell type	IL-1-ra	M15 mAb
DC	95–100	95–100
d3 LC	95–100	90–100
EL4-NOB1	96–100	96–100
70Z/3	0–10	0–10

The data (three experiments) are expressed as percent inhibition of rhu ^{125}I-IL-1α binding (at 3 × 10^{-10} and 10^{-9} M concentration with DC and LC, and 10^{-11} to 10^{-9} M concentration with cell lines) to 1.5 or 2 × 10^6 cells in the presence of rhu IL-1ra (300-fold molar excess) or M15 mAb (50 μg/ml) when compared with the specific binding in the absence of inhibitor (= total binding of rhu ^{125}I-IL-1α minus binding in the presence of 100-fold molar excess of rhu IL-1β). For experimental details see Materials and Methods.

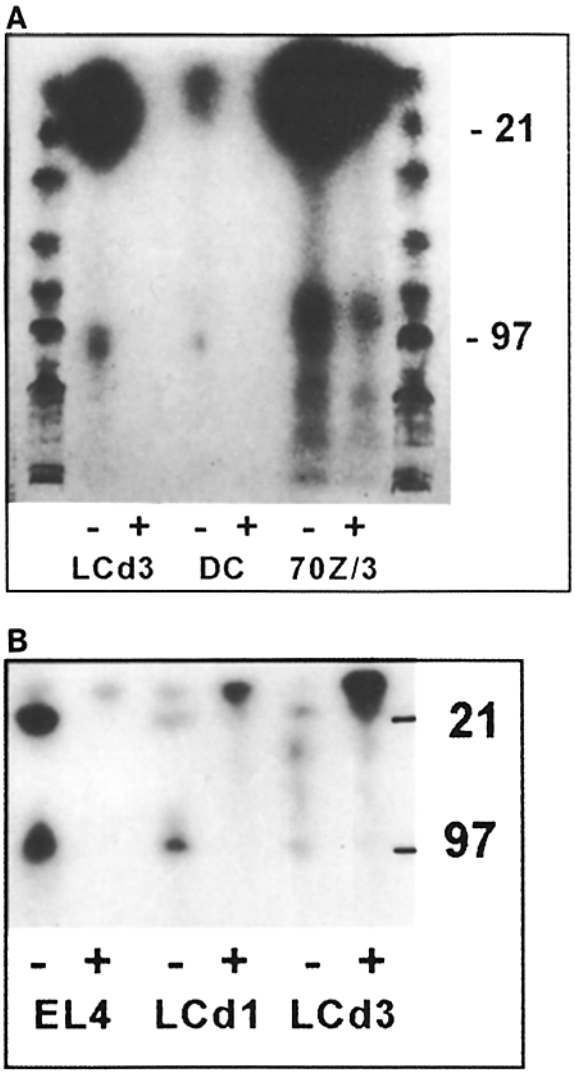


Figure 6. Characterization of IL-1R by affinity cross-linking. Highly enriched BALB/c spleen DC (5 × 10^6), epidermal LC cultured for 1 d (= LCd1) (2 × 10^6 cells) and 3 d (= LCd3) (3 × 10^6 in *A*, 2 × 10^6 in *B*) as well as EL4-NOB1 cells (5 × 10^6) (expressing the murine type 1 IL-1R) and 70Z/3 cells (5 × 10^6) (expressing the murine type 2 IL-1R) were cross-linked to rhu ^{125}I-IL-1β in the absence (−) or presence (+) of 50-fold molar excess of unlabeled IL-1, extracted, electrophoresed (7.5% homogenous gel in *A*, 10–15% gradient gel in *B*), and autoradiographed as described in Materials and Methods. Note that LC and DC, like EL4-NOB1 cells, reveal complexes of ~100 kd that are distinctly larger than the complexes produced by the 70Z/3 cells, and that d1 LC produce a more intense band than d3 LC. Lanes on the *right* and *left* side represent ^{14}C-labeled molecular mass markers (code CF.756, molecular mass from top to bottom 14.3, 21, 30, 46, 69, 97.4, and 200 kD; Amersham, Braunschweig, Germany).

In pilot experiments we had been unable to detect specific binding of ^{125}I-IL-1α to freshly prepared LC. This was not surprising given the protease sensitivity of the IL-1R molecules (1% trypsin treatment for ≥20 min removes IL-1 binding sites on cultured LC and EL4-NOB1 cells, data not shown) and two sequential trypsin exposures during the LC enrichment procedure (see Materials and Methods). We therefore turned to cytofluorography analysis with antitype 1 IL-1R

mAbs (55), since this approach does not require the enrichment of LC. We prepared EC solely from cartilage-free thin dorsal ear halves, which allows preparation of EC by mild trypsin exposure (0.4% for 15 min). Using this protocol we found unequivocal staining of LC (identified by their expression of MHC class II molecules) with all three antitype 1 IL-1R mAbs used (Fig. 7). Interestingly, the keratinocytes, which in situ express mainly type 2 IL-1R mRNA (56), were not stained. These data suggest that freshly prepared LC (as in vitro equivalents of resident LC) express type 1 IL-1R's. We could not detect binding of antitype 1 IL-1R mAbs to either d1 LC, d3 LC (expressing 500 IL-1R/cell, see above), or spleen DC (70 IL-1R/cell). We suspect, therefore, that freshly prepared, immature LC (as in vitro equivalents of LC in situ) express large numbers of type 1 IL-1Rs that are downregulated upon culture and development into fully mature DC. This notion is also supported by our preliminary finding (only one experiment as it is particularly demanding to purify sufficient numbers of d1 LC) that d1 LC as shown by

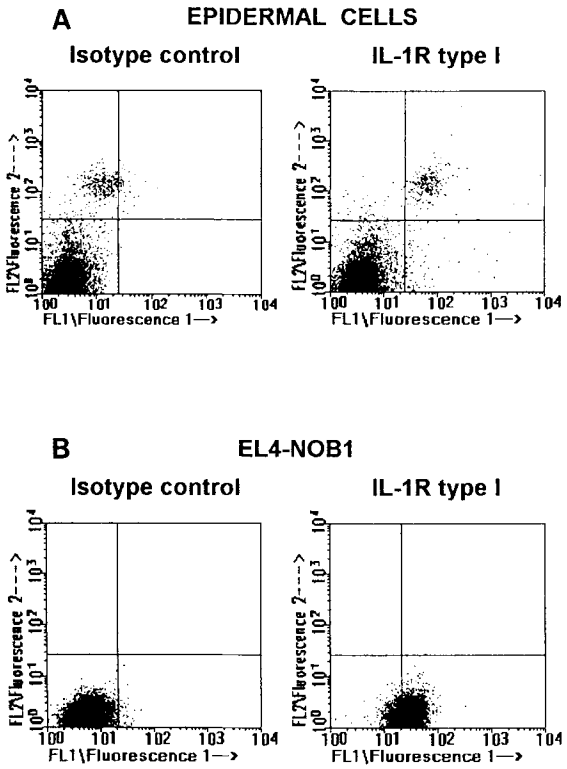


Figure 7. Cytofluorography analysis of type 1 IL-1R expression. Dot plot representation of IL-1R type 1 staining (= fluorescence 1 on horizontal axis) of EL4-NOB1 cells and freshly prepared BALB/c epidermal cells using the anti-IL-1R type 1 mAb M15 (34). Staining with two other anti-IL-1R type 1 mAbs (mAb 1593-01 [obtained from Genzyme Corp.] as well as mAb M5 [34]) yielded comparable results with BALB/c as well as C3H/He epidermal cells. EL4-NOB1 cells (*B*), which express the type 1 IL-1R (~2140 per cell) have been shown to be stained by the anti-IL-1R type 1 mAb M15 (55). Fresh LC (*A*), identified by staining their MHC class II antigens (= fluorescence 2 on vertical axis), are clearly stained above background (isotype control). MHC class II negative epidermal cells (primarily keratinocytes), do not stain above background. 10,000 viable, propidium iodide negative cells were evaluated.

equilibrium binding analysis express at least 1,200 IL-1R, i.e., 2.5 times the number found on d3 LC, and upon affinity–cross-linking produce a more intense band relative to d3 LC (Fig. 6 *B*).

The expression of high-affinity type 1 IL-1R by DC might be important for the regulation of their development and maturation (see discussion below), and also further supports the notion that dendritic cells are distinct from monocytes/macrophages which typically express the lower affinity type 2 IL-1R (31).

Possible Relevance of Differential Cytokine Receptor Expression on DC. As outlined in the introduction it has been known for some time that GM-CSF (7, 8, 13) and IL-1 (7, 12) have profound effects on LC and spleen DC, whereas M-CSF (8, 15) does not seem to affect these cells. It is also well established that cytokine receptor expression controls target cell responsiveness and thus affects the biological net effect of cytokines. However, there has been no information on the expression of cytokine receptors on DC, likely because it is difficult and costly to purify sufficient numbers of these trace cell populations. Our experiments provide data on cytokine receptors and (*a*) support the notion that DC represent a distinct myeloid subset plus (*b*) provide clues to the mechanism and regulation of DC maturation and development. Freshly isolated LC (as equivalents of resident LC) develop into fully mature (i.e., potent immunostimulatory) DC upon culture in the presence of GM-CSF, whereas IL-1 has an enhancing effect but is not essential (7, 8, 57). In vivo, the intradermal injection of even large doses of GM-CSF has, however, quite surprisingly no discernible effect on resident LC (Koch, F., unpublished results), whereas IL-1 upon intradermal injection induces LC maturation as first observed by Nylander Lundqvist et al. (5) and more recently studied in more detail by Enk et al. (6). These discrepant findings can be reconciled, however, in a working model that takes into account the ample expression of type 1 IL-1Rs on immature LC reported here as well as the observation that IL-1 can upregulate the β subunit of the GM-CSF-R (52, 53). We suggest that IL-1 (released in situ from keratinocytes [IL-1α] and/or LC [IL-1β] upon deposition of antigen [58] or in vitro during preparation of epidermal cells and isolation of LC [16, 59, 60]) mediates the upregulation of GM-CSF receptors on epidermal LC (i.e., immature DC) by interacting with their type 1 IL-1R, and hereby induces the GM-CSF–dependent maturation of LC (7, 8) (and possibly of other immature DC as well). The proposal that IL-1 regulates DC function at the level of the GM-CSF-R may also explain prior experiments that IL-1 boosts DC function (12).

Our finding that mature LC/DC express a single class of intermediate-affinity GM-CSF-R is in concordance with such a model. Recent progress in elucidating the molecular basis of GM-CSF-R has revealed, that binding affinities reflect the different relative numbers of GM-CSF-R α and β chains. Park et al. (61) showed that COS cells that express solely the α subunit of the murine GM-CSF-R exhibit low-affinity binding (K_d >10,000 pM), whereas coexpression of the β subunit produces a subpopulation of high-affinity GM-CSF binding

sites. Interestingly, Budel et al. (62) recently demonstrated in the human system that upon myeloid maturation and upregulation of the β subunit the high affinity (K_d ~50 pM) converted into intermediate affinity GM-CSF binding (K_d ~300–700 pM). Using COS cell transfection it was proven that overexpression of the β chains relative to the GM-CSF-Rα subunits indeed causes a change from high to intermediate affinity binding. In view of these findings it is, of course, tempting to speculate that our observation of a single class of intermediate affinity GM-CSF-R on mature DC (i.e., spleen DC and cultured LC) is due to a high β to α subunit ratio possibly as a result of a marked upregulation of the β subunit during the development of less mature, nonproliferating precursors into fully mature DC.

GM-CSF has more recently also been shown to support in vitro the development of murine DC from MHC class II negative to more committed, rapidly proliferating, MHC class II positive precursors that finally give rise to fully mature, nondividing DC (36, 44, 46). It will, therefore, also be of interest to study whether IL-1 and/or other cytokines regulate GM-CSF-R expression during growth, as well as maturation of DC progenitors.

In summary, our data (*a*) provide further evidence that DC represent a distinct subset of the myeloid lineage; and (*b*) suggest that IL-1 regulates DC function by upregulating GM-CSF receptors, and thereby, the established responsiveness of DC to GM-CSF for growth, viability, and function. Further studies of cytokine receptors are likely to be critical to fully understand the control of DC growth and maturation, and might also allow the design of protocols for modulating these processes in clinical situations.

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