developed ideas about the relation between the steady-state migration of DCs and the regulation of immune tolerance.

In the first half of this review we describe the general features of DCs, and in the second half we discuss the migration of DCs from the skin to regional LNs and the possible functions of DCs that migrate under active- and steady-state conditions.

Features of DCs

DCs are heterogeneous cell populations with respect to their function, origin and distribution. In this section we introduce the features of DCs.

Functions of DCs as immune stimulators

DCs are professional APCs expressing major histocompatibility complex (MHC) class II molecules, like macrophages and B cells (11-13). DCs are the major APCs, which prime immune responses (1). They are widely distributed in many organs (14), especially in the mucosae and epithelia of the intestine, airway and skin (15-17). Because mucosae and epithelia are the first barrier against foreign antigens (Ags), DCs are considered to be "sentinels" of the immune surveillance system.

DCs regulate at least three distinct functions of immune responses. First, they prime and activate Ag-specific adaptive immunity, which is mediated by T lymphocytes (1). DCs capture foreign Ags that have invaded the epithelia or mucosae, migrate to regional LNs via the lymphatic system, process and present the Ags, and then activate Ag-specific naive T cells. Blood-borne Ags in the circulation are captured by DCs residing in the white pulp of the spleen, where DCs present processed Ags to naive T cells, as in regional LNs. Second, DCs bridge innate and adaptive immunity. Innate immunity is a non-T cell-mediated, non-Ag-specific immune response against pathogens. It is activated by recognition of microbial and viral patterns by APCs (e.g., cell wall components of bacteria, viral DNA and RNA) (18-20). DCs express pattern-recognition receptors of the Toll-like receptors (TLRs) family (21), and when DCs recognize pathogens via TLRs they activate and further skew suitable adaptive immune responses such as Th1 or Th2 responses (22). Third, DCs are considered to regulate immune tolerance. Although this mechanism has still not been completely elucidated, a variety of DC subsets, distinct distributions of DC subsets and differences of migration status might be related to directing whether DC function is immunogenic or tolerogenic (described later).

Naive T cell activation requires two types of signaling from DCs: a signaling via T cell receptor (TCR)-provided Ag-bound MHC, and signaling(s) via "co-stimulatory molecules" such as CD80 (B7-1) or CD86 (B7-2) (23-24). Without stimulation, DCs are considered to be "immature" and still have

Ag-capturing capacity. When DCs have captured Ags that act as stimuli they are induced to "mature" and upregulate co-stimulatory molecules. These mature DCs begin to migrate to regional LNs, and have lost their Ag-capturing capacity (25). In the absence of signal(s) via co-stimulatory molecules, T cells lose responsiveness against Ags, a condition called "anergy" (26). Thus, DC migration, maturation and activation of immune responses are deeply linked.

The origin and differentiation factors of murine DCs

There are two major subpopulations of DCs: myeloid DCs and lymphoid DCs (Figure 1). Myeloid DCs are differentiated from myeloid-lineage precursors from which granulocytes and monocyte-macrophage-lineage cells also arise (27-28), whereas lymphoid DCs arise from lymphoid-lineage precursors in the thymus (29). The most critical factor for myeloid DC differentiation from bone marrow-derived hematopoietic precursor cells (HPCs) is granulocyte-macrophage colony stimulating factor (GM-CSF) (27). Supplementation with interleukin-4 (IL-4) plus GM-CSF is often used to

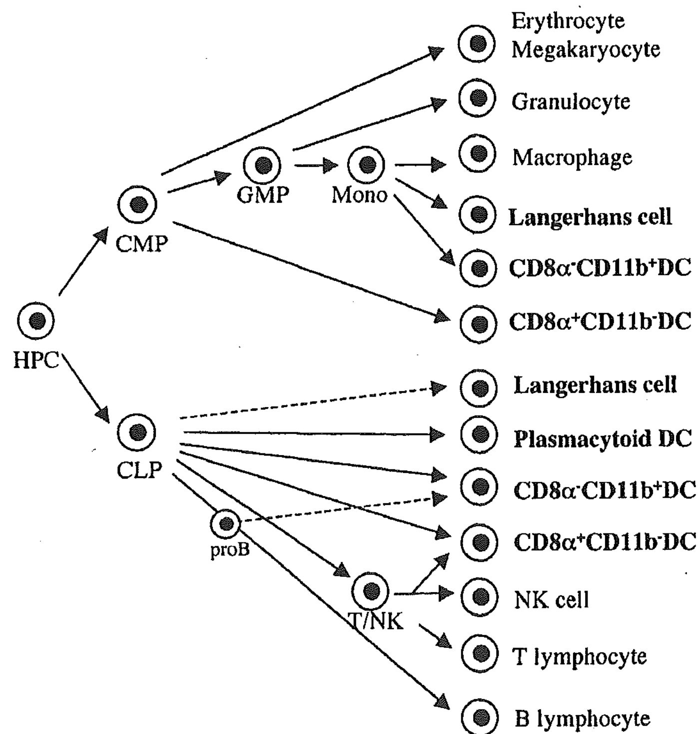


Figure 1. Proposed murine DC subpopulations. Both common myeloid precursor (CMP) and common lymphoid precursor (CLP) cells can give rise to CD8α⁻ and CD8α⁺ DCs with similar efficiency; however, peripheral DCs are mainly derived from CMPs *in vivo* (Ref. 33). HPC: Hematopoietic precursor cell, GMP: granulocyte-macrophage precursor, Mono: monocyte, T/NK: T cell/ NK cell precursor, proB: pro B cell. Dashed arrows indicate possibly rare pathways (Ref. 65 (LCs), 118 (CD8α⁻DC)).

efficiently induce the production of myeloid DCs (30). Lymphoid DCs can be induced without GM-CSF or IL-4 (29). Although Flt3 ligand (Flt3L) affects the expansion of both lineages of DCs *in vitro* and *in vivo* (31-32), Flt3L seems to be more critical for the development of lymphoid DCs (33). Other cytokines such as Kit ligand (KitL), IL-3 or IL-7 also affect the induction of DCs (32,34-36).

Recently, another subtype of murine DCs referred to as plasmacytoid DCs (pDCs) was described (37-38). Their generation is accelerated by the addition of Flt3L in culture (32) and injection of Flt3L plus GM-CSF in mice (38). It has been speculated that pDCs are of lymphoid origin (39,40), and that their function is tolerogenic (41,42).

Markers and distribution of murine DCs

It is difficult to detect DCs using a single specific surface marker, because DCs consist of several populations and change their expression of surface markers during Ag-processing.

Although MHC class II molecules are useful markers for detecting DCs, they are also expressed on macrophages and B cells. Nowadays, the most widely accepted surface marker of murine DCs is CD11c, an αX integrin that binds fibrinogen. All myeloid, lymphoid and pDCs ($Gr1^+B220^+CD11b^-$) express CD11c in mice (29,37,38).

To distinguish between myeloid and lymphoid DCs, CD8α, which is broadly accepted as a cytotoxic T cell marker, has been used as a lymphoid DC marker in mice. However, recent studies have revealed that $CD8\alpha^+$ and $CD8\alpha^-$ DCs arise from both myeloid and lymphoid precursors (33,43,44). Recently, $CD8\alpha^+$DCs ($CD8\alpha^+CD11b^-$) and $CD8\alpha^-$DCs ($CD8\alpha^-CD11b^+$) have often been used to specify murine DC subsets.

DCs also express markers according to their functions: TLRs for recognizing bacteria and viruses (20,21) and C-type lectin receptors CD205 (DEC205) and CD209 (DC-SIGN) as receptors for carbohydrate Ags (45) and so on. To prime/ activate immune responses, co-stimulatory molecules are upregulated on DCs upon their activation (described above).

The distributions of different subsets of DCs also differ. $CD8\alpha^+$ DCs are mainly localized in the thymus, T-cell-rich zone of the spleen, Peyer's patches and LNs, whereas $CD8\alpha^-$ DCs are mainly localized in the marginal zone of the spleen, the subepithelial dome of Peyer's patches, LNs and the skin (e.g., Langerhans cells and dermal DCs; described later) (46,47). Each subset is considered to play distinct roles in immune regulation. For example, murine $CD8\alpha^+$DCs and $CD8\alpha^-$DCs skew Th1 and Th2 responses respectively (48), while $CD8\alpha^+$ but not $CD8\alpha^-$ splenic DCs may induce immune tolerance via the uptake of apoptotic cells (49) (Table 1).

Table 1. Main distribution of murine DC subsets

Subsets	Distributions
$CD8^+$DCs ($CD11b^-CD11c^+CD8^+$)	Thymus, T cell zone of spleen, Peyer's patches and LNs
$CD8^-$DCs ($CD11b^+CD11c^+CD8^-$)	Marginal zone of spleen, Subepithelial dome of Peyer's patches, LNs, Epidermis (LCs), Dermis (dermal DCs)
Plasmacytoid DCs ($Gr1^+B220^+CD11b^-$)	Thymus, T cell zone of spleen, Peyer's patches and LNs

Human DCs

Although human and mouse DCs function similarly as an immune stimulators, human DCs differ from murine DCs in some respects. Induction of human myeloid DCs (DC1s) requires interleukin-4 (IL-4) (28) or tumor necrosis factor-α (TNF-α) (50) in addition to GM-CSF. DC1s express CD11c, whereas human lymphoid DCs (DC2s) lack CD11c expression, unlike murine lymphoid DCs (51). Human DC1 and DC2 skew Th1 and Th2 responses, respectively, so their functions seem opposite those of murine DCs (52,53).

Langerhans cells as DCs in the skin

Langerhans cells (LCs) in the epidermis are a skin-resident DC population (54) that can activate immune responses (55-57). LCs are dendritic-shaped cells characterized by the presence of racket-shaped Birbeck granules in their cytoplasm (58). They are distributed in the basal and suprabasal layers of the epidermis in a lattice-like formation (Figure 2). When stimuli (e.g., microbial or viral infection, injury, etc.) are given to the epidermis, LCs capture the foreign Ags, and then migrate from the epidermis through the basement membrane (the epidermal-dermal junction) into regional LNs. Migrated LCs (called "interdigitating DCs") then present Ags to naive T cells in LNs (56,59,60).

Many studies of the surface markers and differentiation pathway of LCs support the idea of their myeloid origin (61-65), although Anjuére and colleagues suggest that LCs may arise from $CD4^{low}$ lymphoid-committed precursor cells in mice (66). Transcriptional factor *Ikaros* dominant-negative mice, which lack all lymphoid lineage cells (T and B cells, NK cells, lymphoid-related DCs) have myeloid-lineage cells and normal LCs (67,68). LCs in the epidermis are $CD8\alpha^-$, but mature LCs express CD8α on their surface (63), suggesting that CD8α might be a maturation marker for LCs, or else this finding may indicate the heterogeneity of LCs themselves.

Murine LCs also express various markers: CD11c and MHC class II; co-stimulatory molecules CD80 and CD86 with stimulation; lipid and glycolipid receptor CD1 (69,70); adhesion molecules E-cadherin (71,72) and gp40 (Ep-CAM) (73); Birbeck granule-specific antibody Lag (74); C-type lectin receptors

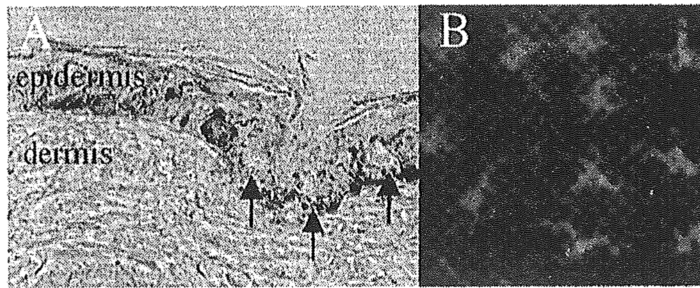


Figure 2. Langerhans cells in the epidermis. LCs in the suprabasal layer of the epidermis (A) (arrows, CD205-FITC staining, ×330). LCs distributed in a lattice-like formation (B) (mouse ear epidermal sheet, MHC class II-FITC staining, ×330).

CD205 (DEC205) (75) and CD207 (Langerin) (76,77). CD207 is regarded as a potent specific LC marker in the skin (77); however, because the expression of surface molecules on LCs changes depending on their phases of migration and maturation, tracing LCs by their surface markers is not easy.

An indispensable factor for LC differentiation is transforming growth factor-β1 (TGF-β1) (35,61,64). TGF-β1-deficient mice lack LCs (78). Recently the helix-loop-helix transcriptional factor inhibitor of DNA binding or differentiation 2 (Id2) has been found to regulate LC differentiation acting downstream of TGF-β1. Id2-deficient mice lack LCs in addition to having significant decreases of NK cells and CD8α^+ DCs (79,80).

There is another skin DC subset referred to as dermal DCs. They are considered to have several subpopulations, which may include LC precursors (81). However, the sparse evidence about specific surface markers (82,83) and the presence of other cells in the dermis (e.g., macrophages, fibroblasts, etc.) make it difficult to analyze them precisely. Dermal DCs have the capacity to perform Ag-presentation *in vitro* (82), but it remains unclear whether they can migrate to LNs like LCs, or whether other cells such as macrophages transport Ags in the dermis *in vivo*.

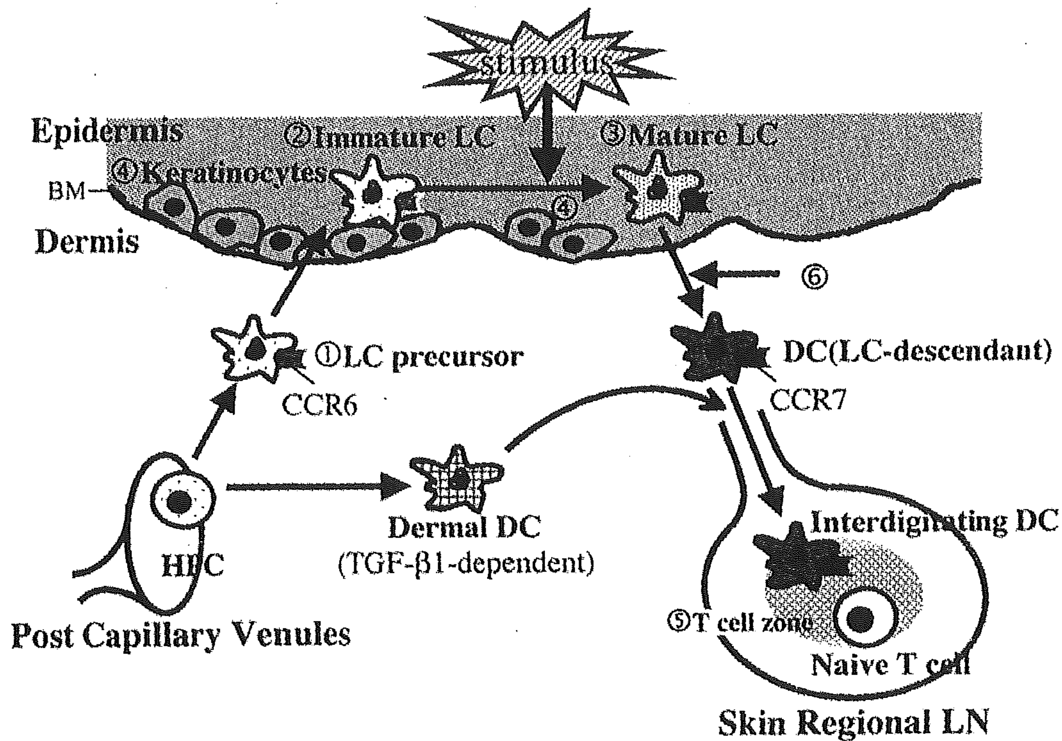
Migration of DCs/ LCs and relationship to immunity

Hereafter we discuss the relationship between DC migration and immune responses.

Molecules associated with LC migration

LC migration to regional LNs is considered to be link with stimulation. (25). LC precursors migrate out of post-capillary venules in the dermis, and enter into the epidermis. In humans, this process is regulated by the interaction of a chemokine receptor, CCR6, on LC precursors and its ligand, CCL20 (MIP-3α), which is secreted by keratinocytes (84,85). In mice, CCR2 might regulate this process (86). In the epidermis, LCs that are still immature adhere to

keratinocytes via E-cadherin (87). Upon stimulation, Ag-capturing LCs downregulate E-cadherin and CCR6, and upregulate CCR7 instead. The subsequent migration is regulated by CCR7 on LCs and its ligand, CCL21 (secondary lymphoid chemokine; SLC), which is expressed in the T cell zone in regional LNs (88). DCs that have begun to migrate have already lost the Ag-capturing capacity, and they are considered to die thereafter by apoptosis in LNs (89). After the activated LCs migrate out of the epidermis, new LC precursors are recruited into the epidermis (Figure 3).



	Stable		Stimulated	
LC/ DC	①LC precursors	CCR6⁺(human)	③Mature LC ~ Interdigitating DC	CCR6↓, E-cadherin↓ IL-1β↑, α6-integrin↓ MMP-2, 9↑ CCR7↑ MHC classII⁺⁺
	②Immature LC	E-cadherin⁺ MHC classII⁺		
Skin/ LNs	④Keratinocytes	E-cadherin⁺ CCL20⁺	④Keratinocytes	TNF-α↑
			⑤T cell zone stromal cells	CCL21⁺
	⑥Other migration-associated molecules CD40-CD40L, CD44-osteopontin, MRP1, PGE2-EP4			

Figure 3. Migration pathway of LCs. Each process of LC migration is regulated by molecules expressed on LCs molecules expressed in the skin and in the LNs (see attached table). Upon stimulation, LCs dramatically change their expression of surface molecules and migrate into LNs. HPC: hematopoietic precursor cells, BM: basement membrane (the junction of the epidermis and the dermis).

Proinflammatory cytokines such as TNF-α secreted by keratinocytes, interleukin-1β (IL-1β) secreted by LCs, and bacterial lipopolysaccharide (LPS) also induce LC migration (90-93). Novel molecules that regulate LC migration from the epidermis have also been discovered: α6-integrin (94), the signaling of CD40-CD40 ligand (95), matrix metalloproteinases 2 and 9 (MMP-2, 9) (96,97), Leukotriene C_4 transporter multidrug resistance-associated protein 1 (MRP1) (98), hyaluronate receptor CD44 (99), the ligand for CD44 and αvβ3-integrin osteopontin (100), and prostaglandin E2-EP4 signaling (101). Most of these molecules are related to inflammatory responses, further suggesting the deep relationship between LC migration and the stimulated condition.

DC/LC migration in "the steady state"

As described above, LC migration had been thought to occur in inflammatory situations, in other words, in the active state. However, recent studies have revealed an overlooked phenomenon: steady-state migration of DCs and LCs.

In the conventional notion of DCs as APCs, DCs have been thought to be immunogenic as a result of their capturing foreign Ags. On the other hand, several findings have suggested that DCs present "self Ags" in LNs (8,102). These self Ags are considered to be the products of apoptotic self tissues/ cells. Huang and colleagues suggested that DCs constitutively transport apoptotic intestinal epithelial cell remnants to mesenteric LNs in the germ- free rat intestine (5). Because apoptotic cell death is thought not to induce inflammatory responses (103), these findings have suggested the migration of DCs under non-inflammatory, "steady state" conditions.

Recently, we demonstrated the constitutive migration of skin DCs to regional LNs even in the steady state using skin-hyperpigmented transgenic mice (Tgs) (6). These Tgs express KitL (*Mgf*) or hepatocyte growth factor (*Hgf*) in keratinocytes using the human keratin 14 promotor (*hk14*) and they show hyperproliferation of melanocytes in the epidermis (*hk14-Mgf-Tg: Mgf-Tg*) or the dermis (*hk14-Hgf-Tg: Hgf-Tg*). Their skin regional LNs were always pigmented by melanin granules (MGs) transported by skin DCs even under steady-state conditions (9) (Figure 4). The findings strongly suggest that the steady-state migration of DCs also occurs in the skin. Although subcutaneous injection of stimulants, painting of contact sensitizers (2,4,6,-trinitrochloro-benzene (TNCB), 2,4-dinitrofluorobenzene (DNFB), FITC, etc.), UV-irradiation and skin allografts have been traditional methods to analyze LC migration from the skin (57,59,60,104,105), these methods irritate the skin, which suggests that any LC migration or function observed with these methods may be occurring only in the active state. We think that our Tg system has some advantages over traditional methods for analyzing skin DC migration. MGs are endogenous,

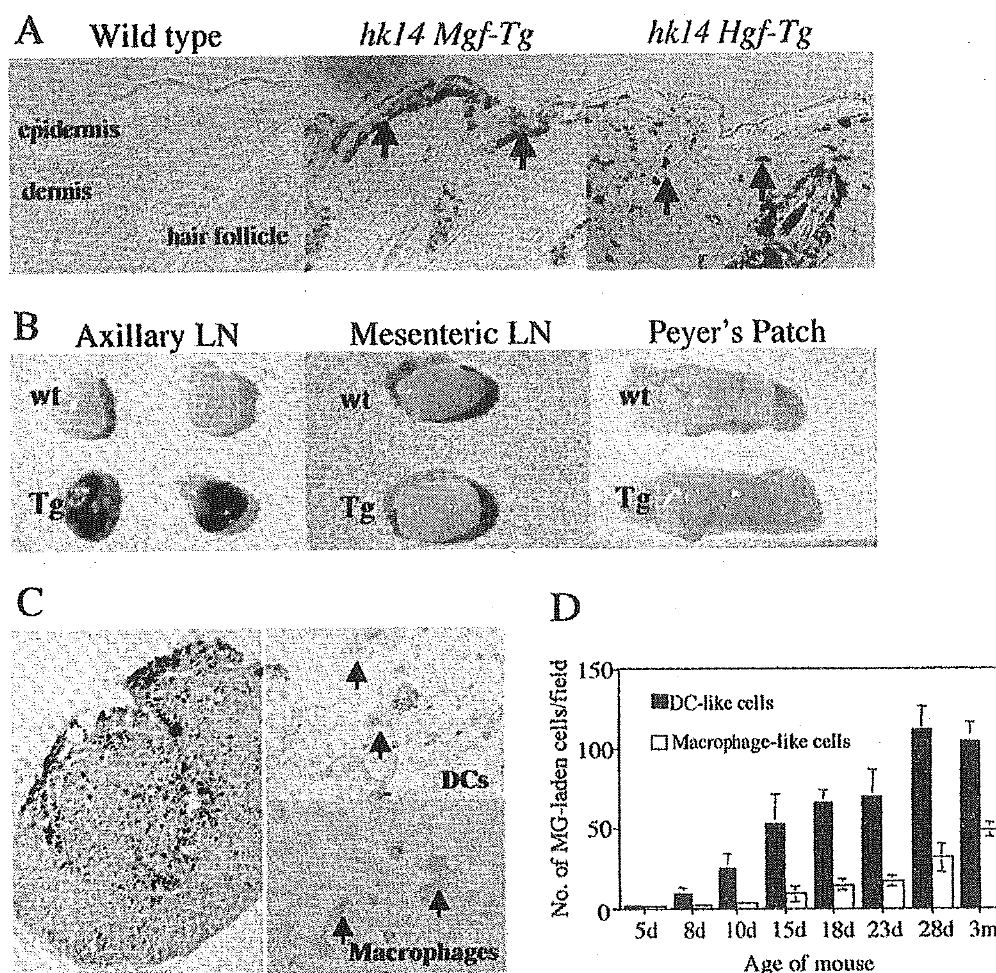


Figure 4. Skin and skin regional LNs of *hk14 Mgf-Tg* and *hk14 Hgf-Tg*. (A) Hyperproliferation of melanocytes (arrows) in the epidermis (*hk14 Mgf-Tg*) and the dermis (*hk14 Hgf-Tg*) (skin: E-cadherin staining, ×132). (B) Lymph nodes (LNs) and Peyer's patches of Tgs. Only skin regional LNs are pigmented (see Axillary LN), but non-skin regional LNs and other organs are not pigmented (see Mesenteric LN and Peyer's patch). (C) LNs of Tgs. Melanin granule (MG)-laden cells are distributed mainly in the T cell zone (left). DCs (MG-transporting cells) and macrophages bear MGs in LNs. MG-laden macrophages are considered to phagocytose apoptotic DCs. (left panel: ×8, H-E staining, right panels:×330, CD205 (DCs)- and CD68 (macrophages)-FITC staining). (D) Accumulation of MG-laden cells in regional LNs. MG-laden cells increased with age, suggesting continual trafficking of skin Ags even in the steady state. The numbers of MG-laden DC-like cells and macrophage-like cells in regional LNs were counted. A field was 0.0589 mm^2 and 10 fields were assessed.

non-resolved Ags, so by employing the MGs as a visible tracer we will be able to trace the migration pathway of self Ags to their terminus. In addition, the MGs accumulate in regional LNs with age, so we would be able to estimate the total amount of transported self Ags by measuring the amount of MGs even in the steady state.

Detection of skin Ag-transporting cells in the dermis

First, we crossed mice with LCs lacking TGF-β1 (encoded by the *Tgfb1* gene) with each of the Tgs described above and bred *Tgfb1(-/-)-Mgf-Tg* and *Tgfb1(-/-)-Hgf-Tg*. Skin regional LNs of both types of mice showed no pigmentation, suggesting a lack of migration of Ag-transporting cells both from the epidermis and the dermis to LNs, even in the steady state. This finding seems reasonable in *Tgfb1(-/-)-Mgf-Tg*, because they lack LCs, the only Ag-transporting cells in the epidermis. On the other hand, we were surprised by the result with *Tgfb1(-/-)-Hgf-Tg*. This result suggested that there are TGF-β1-dependent cells like LCs in the dermis, and they are the only transporters of dermal Ags, with neither TGF-β1-independent other dermal DC subpopulations nor macrophages in the dermis able to perform this function (6) (Figure 5).

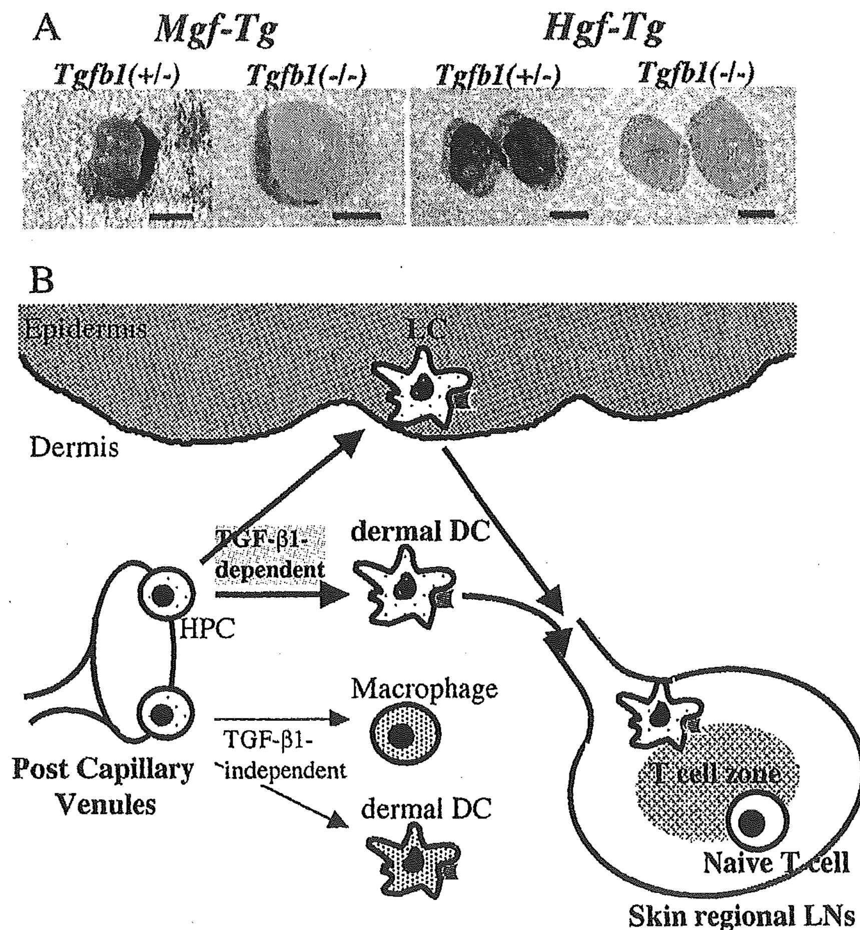


Figure 5. Complete abrogation of the migration of MG-laden DCs in *Tgfb1*-deficient Tgs. (A) Skin regional LNs of *Tgfb1*-deficient Tgs. Since the accumulation of MG-laden cells was abolished in both *Tgfb1(-/-) Mgf-Tg* and *Tgfb1(-/-) Hgf-Tg*, their LNs show no pigmentation. (B) A hypothetical schema of skin Ag-transport in the steady state. Only TGF-β1-dependent cells transport skin Ags both from the epidermis and the dermis in the steady state.

There is a possibility that LC precursors before entering the epidermis or LCs that have migrated out from the epidermis might capture dermal Ags. We do not have direct evidence against the possibility; however, we did not find any MG-laden cells in the epidermis of *Hgf-Tg*, suggesting that there is little possibility that LC precursors captured dermal Ags before entering the epidermis. Similarly, emigrated LCs are considered to lose the capacity of capturing Ags, as described above. Thus, we have precisely identified the population of Ag-transporting cells in both the epidermis and the dermis.

Distinct Ag trafficking in the steady and active state

Next we found distinct migration of LCs from the skin in the steady versus active state using *Mgf-Tg* and *p*aucity of *l*ymph node *T* cell mice (*plt/plt*). *plt/plt* lack expression of chemokines CCL21 and CCL19 in T cell zones of LNs and the spleen, which causes homing defects of T cells and DCs, resulting in decreased T cell and DC numbers in these organs (106,107). We expected that skin regional LNs of *plt/plt-Mgf-Tg* might be less pigmented because interaction of CCR7 and CCL21, which regulates LC migration to the T cell zone of regional LNs (88) is disrupted. Unexpectedly, we found normal steady-state migration of LCs into LNs in *plt/plt-Mgf-Tg*. In contrast, the active-state migration of LCs in *plt/plt-Mgf-Tg* was impaired, as reported by Gunn and colleagues (107). We concluded that distinct mechanisms controlled LC migration into LNs in the steady and active state. CCR7-CCL21 interaction regulates only the active state migration, while the steady state migration is regulated by other unknown molecules (10) (Figure 6). The Tgs allowed us to definitely detect the steady-state migration of LCs/DCs from the skin.

The relationship of steady-state migration of DCs and immune tolerance

DCs that migrate in the steady state seem to play distinct roles in the immune system compared with conventional immunogenic DCs. DCs in regional LNs of stable *Mgf-Tg* showed no upregulation of co-stimulatory molecules such as CD80, CD86, or CD40 (6). Sauter and colleagues showed that only immature DCs exposed to necrotic cells became immunogenic, whereas DCs exposed to apoptotic cells did not (108). Considering that abundant self Ags are produced from apoptotic self tissues, it is no wonder that the self Ag-presenting DCs represent the immature phenotype. These immature DCs are considered to induce immune tolerance, which is necessary to suppress aberrant, autoreactive immune responses.

Naive T cell activation requires both signalings of TCR- peptide-bound MHC complexes and co-stimulatory molecules (23,24), and if the signaling of co-stimulatory molecules is not provided from DCs, T cells undergo anergy

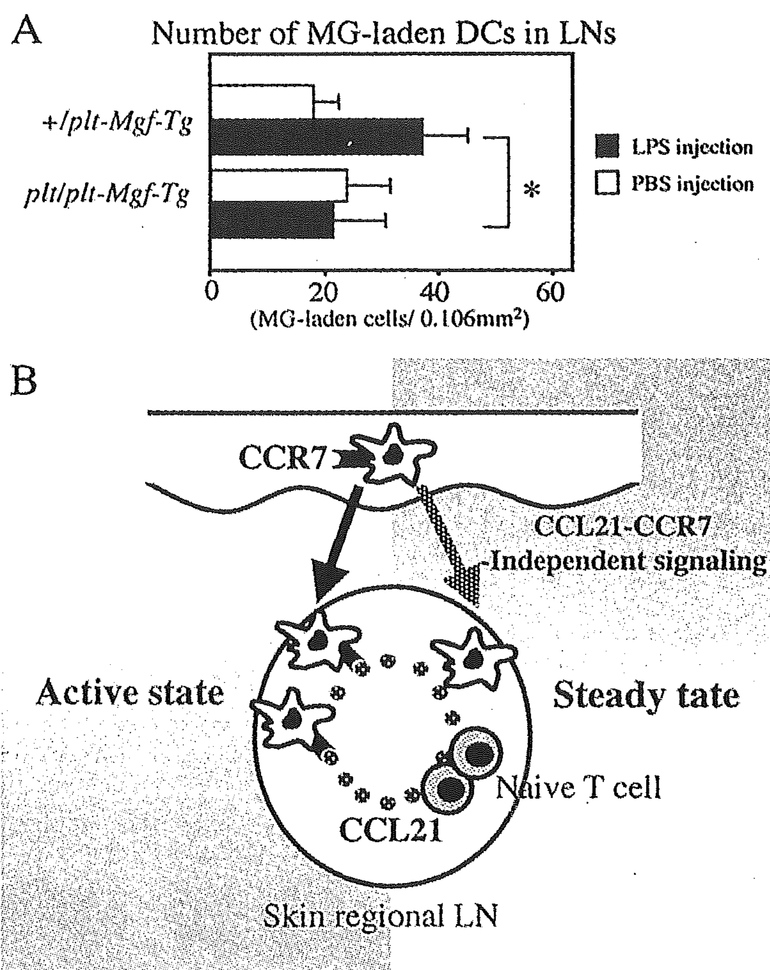


Figure 6. Distinct Ag-trafficking from skin in the steady and active state. (A) Number of MG-laden DCs in skin regional LNs of *+/plt-Mgf-Tg* and *plt/plt-Mgf-Tg* in the steady and active state. *+/plt-Mgf-Tg* and *plt/plt-Mgf-Tg* were injected with 20 μg of lipopolysaccharide (LPS) or PBS intravenously. Four days after the injection, skin regional LNs (brachial and inguinal) were subjected to immunostaining with CD205-FITC antibody. A field was 0.106 mm^2 and 10 fields were assessed. The number of MG-laden DCs in LPS-injected *+/plt-Mgf-Tg* was increased compared with the number in the PBS- injected control, whereas the number of MG-laden DCs was not increased by LPS injection in *plt/plt-Mgf-Tg* (closed bars, $*p<0.001$). Note that the number of MG-laden DCs is comparable in both *+/plt-Mgf-Tg* and *plt/plt-Mgf-Tg* with PBS injection (open bars). This indicates that the steady-state migration of LCs is not impaired in *plt/plt-Mgf-Tg*. (B) A hypothetical scheme of LC migration. Active-state migration of LCs is regulated by CCL21-CCR7 interaction, whereas steady-state migration is independent of CCL21-CCR7 interaction.

(26). This system is very important for establishing peripheral immune tolerance to self Ags, because almost all self-reactive T cell clones are removed in the thymus; however, a few self-reactive clones escape from the clonal deletion and move into the peripheral circulation. Our results using Tgs and *Tgfb1(-/-)* mice

showed complete abrogation of skin Ag-trafficking to LNs, and, interestingly, *Tgfb1(-/-)* mice suffer from fulminating autoimmune disease (6,109). In contrast, *plt/plt* mice, in which only active-state Ag-trafficking was abolished, did not suffer from autoimmune disease. These results might emphasize the role of the steady-state migration of DCs as one of the systems regulating immune tolerance.

Perspectives

It is important to be able to evaluate immune responses based on the localization of migrated DCs or the total amount of transported Ags, without taking note of the characteristics of DCs. Observations of DC functions using surface markers are sometimes unstable because of the heterogeneity of DCs. Our Tg system is useful in this regard because it enables us to detect migrated DCs themselves, employing MGs as a tracer.

Using alymphoplastic mutant mice (*aly/aly*), which lack LNs and Peyer's patches (110), and *Mgf-Tg*, we observed previously that MG-laden DCs migrated aberrantly to the liver, spleen, kidney and lung (6), and interestingly, *aly/aly* mutant mice suffer from autoimmune disease (110,111). The findings suggest that normal migration of DCs into regional LNs themselves might have a key role in regulating the normal immune responses. A few other researchers have also suggested the importance of the migration of DCs into the proper site to achieve the proper immune responses (112,113). We also hope to elucidate the relationship between abnormal localization of migrated DCs in the LNs or spleen and immune disregulation using our system.

Furthermore, we plan to examine whether the amount of trafficked Ags is related to regulation of immune responses. It will be interesting to know if the quantitative regulation of Ags in the steady state maintains the immune tolerance.

Conclusion

We discussed DCs as a dual-fated immune regulator controlling both immune responses against foreign Ags and immune tolerance to self Ags. Self Ags are transported by DCs constitutively in the steady state and this transport is regulated by distinct mechanism(s) from that in the active state. The molecules or mechanisms regulating steady-state DC migration might play a key role in establishing immune tolerance.

Although we focused on the relationship between DC migration and immunity, the varieties and distributions of DC subsets would also have a great impact on regulating distinct immune responses. In addition, recently some reports have shown that DCs induce or cause proliferation of $CD4^+CD25^+$

regulatory T cells (Tregs) (114-116). Tregs are considered to maintain immune tolerance and suppress autoimmunity (117). These findings further emphasize the role of DCs as a tolerogenic immune regulator.

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γ-Glutamyltranspeptidase Stimulates Receptor Activator of Nuclear Factor-κB Ligand Expression Independent of Its Enzymatic Activity and Serves as a Pathological Bone-resorbing Factor*

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A novel bone-resorbing factor was cloned using an expression cloning technique, which involved a *Xenopus* oocyte expression system and an assay for osteoclast formation. A candidate clone was isolated from a BW5147 mouse T-lymphoma cell cDNA library. Sequencing analysis identified the factor as γ-glutamyltranspeptidase (GGT), which is an enzyme involved in glutathione metabolism. The addition of purified GGT protein to mouse bone marrow culture effectively induced formation of osteoclasts. An antibody against GGT inhibited osteoclast formation but not the enzymatic activity. We also demonstrated that an inactive form of GGT, the enzymatic activity of which had been blocked by chemical modification with a specific inhibitor, acivicin, supported osteoclast formation. These results indicate that GGT acts on osteoclast formation independent of its own enzymatic activity. Furthermore, both native GGT and inactive GGT stimulated the expression of the receptor activator of nuclear factor-κB ligand (RANKL) mRNA and protein from bone marrow stromal cells. This report is the first demonstration of a novel biological activity of GGT protein in a manner independent of its enzymatic activity.

Osteoclasts are potent bone resorbing cells that are derived from hematopoietic cells of the monocyte/macrophage lineage (1–3). Osteoclast differentiation is regulated by the simultaneous stimulation of colony stimulating factor-1 (CSF-1/M-CSF) and the receptor activator of nuclear factor-κB ligand (RANKL),[1] which are produced by osteoblasts/stromal cells (3–8). The expression of these essential factors is stimulated by systemic bone-resorbing factors such as 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), parathyroid hormone, and interleukin (IL)-11 (9, 10). Increased osteoclast activity is responsible for progressive bone loss in postmenopausal osteoporosis and Paget disease (11, 12). Local bone destruction has also been observed in bone metastasis and rheumatoid arthritis (13, 14). Tumor cells that have metastasized to bone induce osteoclastogenesis via the secretion of bone-resorbing factors such as parathyroid hormone-related protein, IL-11, and prostaglandin E_2 (13).

Our previous study (15) demonstrated that the injection of BW5147 mouse T-lymphoma cells into AKR mice led to bone metastasis, accompanied by severe bone destruction. Conditioned medium from BW5147 cell cultures stimulated tartrate-resistant acid phosphatase-positive ($TRAP^+$) multinucleated osteoclast (MNC) formation in the mouse bone marrow culture. Because TRAP activity is expressed specifically in osteoclasts lineage (16–18), it has been suggested that the BW5147 cells produce an osteoclast-forming factor that may play a role in pathological bone resorption such as metastatic lesions.

To identify and characterize the factor that promotes pathological bone resorption, we cloned a cDNA for the osteoclast-forming factor(s) from a BW5147 cell cDNA library by expression cloning using a *Xenopus* oocyte translation system. Consequently, we identified γ-glutamyltranspeptidase (GGT) as the bone-resorbing factor. GGT is an ectoenzyme that plays an important role in regulating glutathione metabolism (19) and is well known as a clinical marker for a number of diseases. In this study, we showed a possible mechanism by which GGT induces osteoclast formation using a mouse bone marrow culture system.

EXPERIMENTAL PROCEDURES

Cell Culture—A mouse T-lymphoma cell line, BW5147 (CRL-1588), (20) was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Expression Cloning—Poly$(A)^+$ RNA was prepared from BW5147 cells and size-fractionated by means of sucrose density gradient centrifuga-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) E15738 and U30509.

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[1] The abbreviations used are: RANKL, receptor activator of nuclear factor-κB ligand; IL, interleukin; TRAP, tartrate-resistant acid phosphatase; MNC, multinucleated osteoclast; GGT, γ-glutamyltranspeptidase; 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; I-GGT, inactive GGT; OPG, osteoprotegerin.

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tion. The 2–4-kb fraction that had the greatest osteoclast-forming activity was used to construct a directional cDNA library using a ZAP-cDNA synthesis kit (Stratagene) and SuperScript II (Invitrogen). Then 50 ng of poly(A)$^+$ RNAs or cRNAs, which were synthesized *in vitro* from the pools of clones, were injected into *Xenopus* oocytes. The conditioned media were assayed for osteoclast-forming activity, which was determined using the assay described below. Each positive pool was further subdivided and analyzed until a single clone was obtained.

Mouse Bone Marrow Culture—Bone marrow cells (2×10^6 cells/ml) obtained from the tibias and femurs of 5–12-week-old C3H/HeJ mice (Nippon Clea) were cultured in 96-well plates in 180 μl of α-minimal essential medium containing 10% fetal bovine serum and 10^{-8} M 1,25(OH)$_2$D$_3$. A 20-μl aliquot was added on day 1 of the culture. The culture medium was replaced with fresh medium containing a 20-μl aliquot at 3-day intervals. After 7 days of culture, the cells were fixed with 4% paraformaldehyde and stained for TRAP activity. The TRAP staining solution containing 50 mM sodium tartrate detects osteoclasts specifically (16, 17). The number of TRAP$^+$ MNCs (>3 nuclei) was scored under a microscope. Bone marrow stromal ST2 cells, which support hematopoiesis, were also co-cultured with bone marrow hematopoietic cells (2.5×10^5 cells/well) that were passed through a Sephadex G-10 column (Amersham Biosciences) under the same conditions.

Calcitonin Binding Assay—For autoradiography using ^{125}I-salmon calcitonin, the cells were cultured on a chamber slide and incubated with 0.2 nM ^{125}I-salmon calcitonin in α-minimal essential medium for 1 h at 37 °C after TRAP staining as described previously (18).

Pit Formation Assay—To determine the resorption activity of TRAP$^+$ MNCs, the bone marrow cells were seeded on the dentine slices placed in 96-well plates or on calcium phosphate-coated slides (osteologic multitest slide, Millennium Biologix). The dentin slices and osteologic slides were stained for TRAP activity. After staining, the TRAP$^+$ cells on the dentin or slides were removed with a scraper, and the number of pits was counted under a phase-contrast microscope.

GGT and Antibody—GGT was purified from rat kidney as described previously (21). When enzymatically inactive GGT (designated as I-GGT) was prepared, the purified enzyme was allowed to react with acivicin (L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid), which potently inhibits the enzyme by covalently binding to the active site (22, 23). After the residual activity was decreased to less than 0.1%, unreacted reagents were removed by gel filtration using Toyopearl HW40-F (Tosoh) pre-equilibrated with phosphate-buffered saline. A goat IgG against rat GGT was prepared as described in the previous report (24). Non-immune goat IgG (Sigma) was used as a control antibody. Endotoxin was undetectable in these antibodies, as verified using an endospec kit (Seikagaku Co.).

GGT Assay—The enzymatic activity of GGT was measured using a GGT 419 assay kit (Sigma).

Electrophoresis and Immunoblotting—The purified GGT was subjected to SDS-PAGE on 11% gels according to the Laemmli procedure (25) and was visualized using silver staining (26). The bone marrow-adherent cells were solubilized in radioimmune precipitation assay buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1% w/v SDS, 1% w/v Nonidet P-40, 0.5% w/v sodium deoxycholate, and a protease inhibitor mixture (Complete, Roche Diagnostics). The lysates were electrophoresed on SDS-PAGE gels, and Western blotting was performed using anti-mouse RANKL (Imgenex) antibody and horseradish peroxidase-conjugated anti-IgG secondary antibodies. RANKL was detected using ECL Western blotting detection reagents (Amersham Biosciences). Total protein extracted from mouse and rat kidneys was used as control for GGT.

Reverse Transcriptase-PCR—Total RNA was extracted from BW5147 cells and bone marrow cultures using Isogen (Nippongene). All of the RNA samples were then transcribed into first strand cDNA using a Gene Amp RNA PCR kit (PerkinElmer Life Sciences). The cDNAs were amplified by hot start PCR with the primers from the sequences of GGT or RANKL. The primers used were as follows: mouse GGT, 5′-ATCATCGGCCTCTGTATCTG-3′ (sense) and 5′-GCTGTTGTAGATGGTGAAGA-3′ (antisense); mouse RANKL, 5′-ACACCTGGAATGAAGAAGATAAATG-3′ (sense) and 5′-AGCCACTACTACCACAGAGATGAAG-3′ (antisense); glyceraldehyde-3-phosphate dehydrogenase, 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ (sense) and 5′-CATGTAGGCCATGAGGTCCACCAC-3′ (antisense). The PCR products were separated by electrophoresis on a 1% agarose gel.

For the quantitative real time PCR analysis of RANKL and osteoprotegerin (OPG) mRNA levels, total RNA was extracted from bone marrow-adherent cells. The Light Cycler™ system (Roche Diagnostics) was used with the LightCycler-FastStart DNA Master SYBRGreen I mix, according to the manufacturer's protocol. The reactions were carried out up to 43 cycles with denaturing at 95 °C exposure, annealing at 55 °C, and extension at 72 °C. The primers used for this system were as follows: mouse RANKL, 5′-TCTGCAGCATCGCTCTGTT-3′ (sense) and 5′-GCAGTGAGTGCTTTTGTCTTCTGA-3′ (antisense); mouse OPG, 5′-CTTGCCTTGATGGAGAGCCT-3′ (sense) and 5′-TCGCTCGATTTGCAGGTCT-3′ (antisense); elongation factor-1α1 (EF-1α1) as an internal standard, 5′-GGTGATTATCCTGAACCATC-3′ (sense) and 5′-ATATCAACAATGGCAGCATC-3′ (antisense).

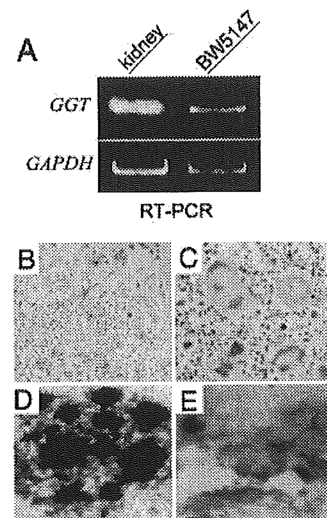

FIG. 1. **Expression of GGT in BW5147 cells and the osteoclast-forming activity of GGT.** RT-PCR analysis (*A*) indicates the expression of GGT mRNA in BW5147 cells. A sample of kidney was used as a control. Conditioned medium from *Xenopus* oocytes expressing GGT induced TRAP$^+$ MNC formation in bone marrow cultures. The cultures were maintained for 7 days in the presence of a conditioned medium of oocytes injected with distilled water (*B*) and GGT cRNA (*C*). Most of the TRAP$^+$ MNCs expressed calcitonin receptors (*D*). The *black grains* in the autoradiograph indicate the binding of ^{125}I-salmon calcitonin. Resorption pit formation (stained *red*) was observed on a dentin slice (*E*).

RESULTS

Expression Cloning of Osteoclast-forming Factor—The culture medium of *Xenopus* oocytes injected with poly(A)$^+$ RNA from BW5147 cells induced the formation of a significant number of osteoclast-like cells in mouse bone marrow cultures, as indicated by an increased number of TRAP- and CT-receptor-positive MNCs (data not shown). After fractionation of the poly(A)$^+$ RNA by sucrose density gradient centrifugation, to enrich the mRNA that encodes for this factor, the fraction from 2–4 kb, which contained the greatest amount of this factor, was used as a template to prepare a cDNA library. As a result, ~630,000 individual clones were obtained, which were then divided into 63 pools, each of which contained ~10,000 clones. These pools were grown separately, and cRNA was prepared from each pool by *in vitro* transcription. Following expression in the *Xenopus* oocytes as a result of cRNA injection, the resulting culture media were screened for osteoclast-inducing activity. One positive pool was found to contain high levels of activity, and this pool was further subdivided and screened. After several rounds of screening, several positive clones including colony stimulating factor-1, RANKL, IL-1, and IL-6 were obtained. One unknown positive clone remained, and this cDNA clone was sequenced (GenBank™ accession number E15738). A data base homology search indicated that the factor cloned was identical to mouse GGT (Ref. 27 and GenBank™ accession number U30509). We confirmed that the culture medium had 40 units/ml of *ggt* activity and that the BW5147 cells expressed GGT mRNA (Fig. 1*A*). The conditioned medium of oocytes injected with GGT mRNA induced many TRAP$^+$ MNCs in the bone marrow culture (Fig. 1*C*). More than 50% of the GGT-induced TRAP$^+$ MNCs induced by GGT expressed the CT