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

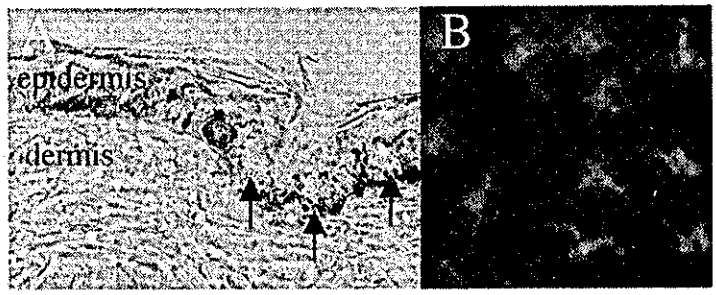


Figure 2. Langerhans cells in the epidermis. LCs in the suprabasal layer of the epidermis (A) (arrows, CD205-FITC staining, ×330). LCs distributed in a lattice-like formation (B) (mouse ear epidermal sheet, MHC class II-FITC staining, ×330).

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 $CD8\alpha^+$ 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*.

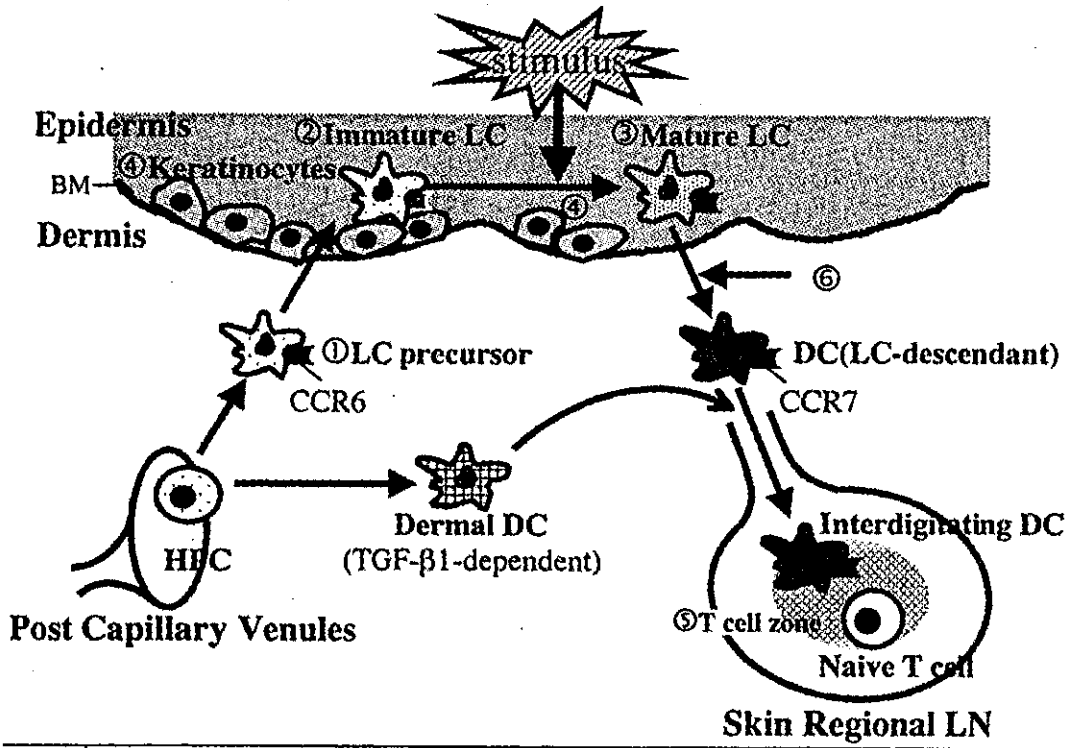
Migration of DCs/ LCs and relationship to immunity

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	CCR6↓, E-cadherin↓
			↓	IL-1β↑, α6-integrin↓
	②Immature LC	E-cadherin⁺	Interdigitating	MMP-2, 9↑ CCR7↑
		MHC classII⁺	DC	MHC classII⁺⁺
Skin/ LNs	④Keratinocytes	E-cadherin⁺	④Keratinocytes	TNF-α↑
		CCL20⁺	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,

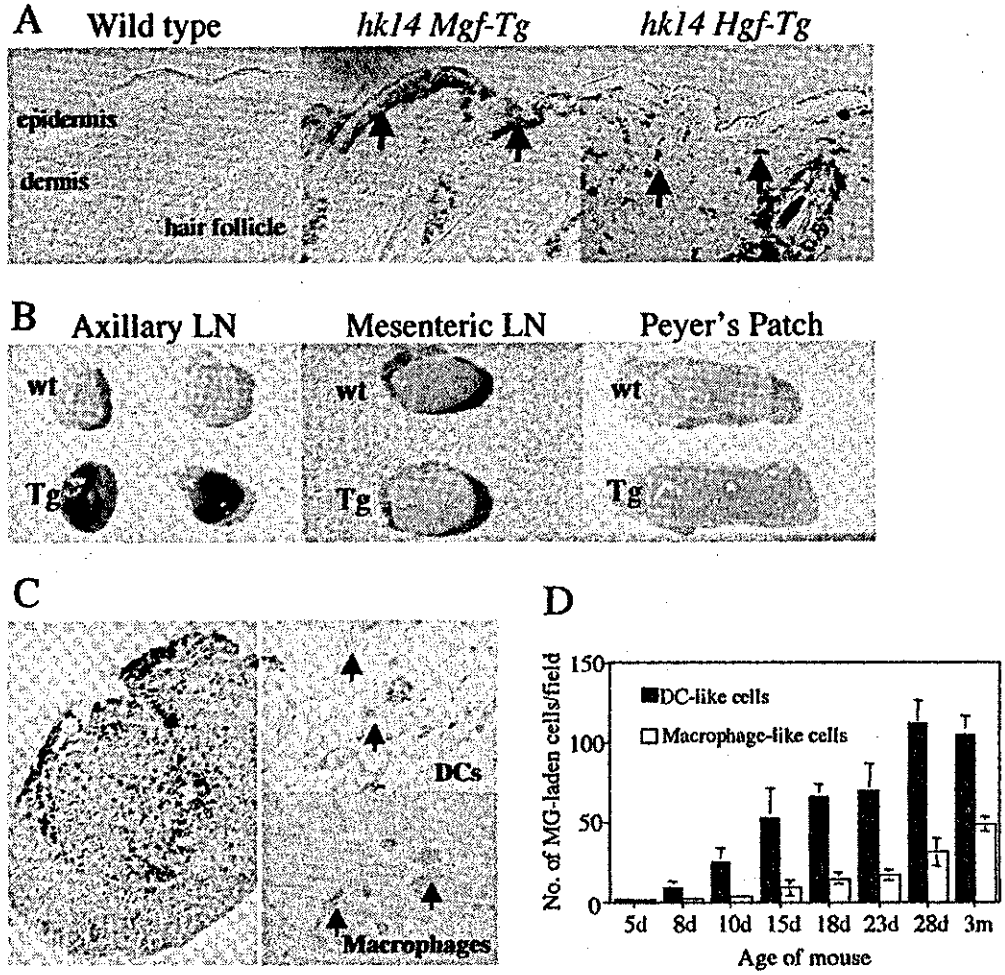


Figure 4. Skin and skin regional LNs of *hk14 Mgf-Tg* and *hk14 Hgf-Tg*. (A) Hyperproliferation of melanocytes (arrows) in the epidermis (*hk14 Mgf-Tg*) and the dermis (*hk14 Hgf-Tg*) (skin: E-cadherin staining, ×132). (B) Lymph nodes (LNs) and Peyer's patches of Tgs. Only skin regional LNs are pigmented (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).

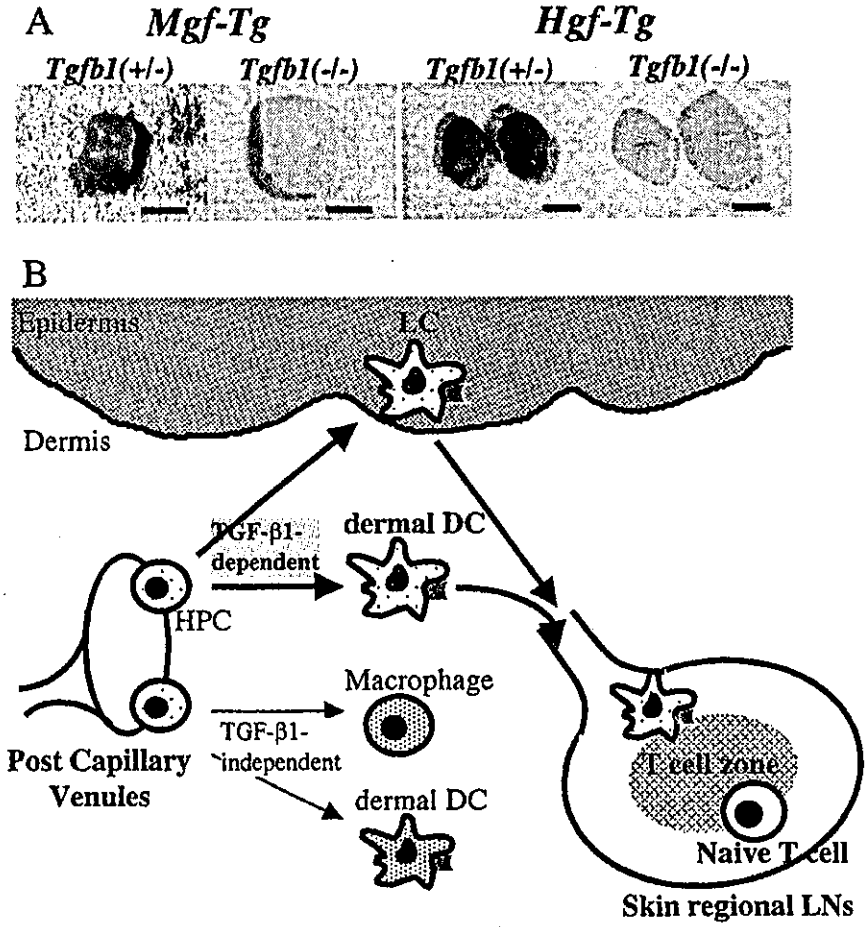


Figure 5. Complete abrogation of the migration of MG-laden DCs in *Tgfb1*-deficient Tgs. (A) Skin regional LNs of *Tgfb1*-deficient Tgs. Since the accumulation of MG-laden cells was abolished in both *Tgfb1(-/-) Mgf-Tg* and *Tgfb1(-/-) Hgf-Tg*, their LNs show no pigmentation. (B) A hypothetical schema 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

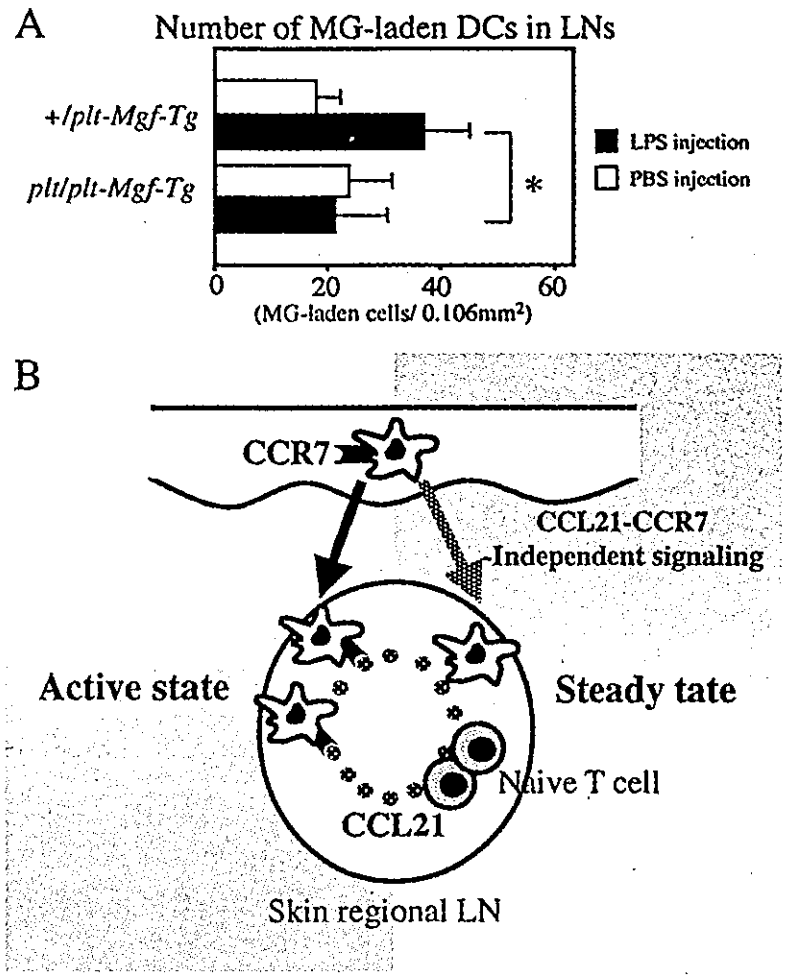


Figure 6. Distinct Ag-trafficking from skin in the steady and active state. (A) Number of MG-laden DCs in skin regional LNs of *+/plt-Mgf-Tg* and *plt/plt-Mgf-Tg* in the steady and active state. *+/plt-Mgf-Tg* and *plt/plt-Mgf-Tg* were injected with 20 μg of lipopolysaccharide (LPS) or PBS intravenously. 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.

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 $1,25(OH)_2D_3$. 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).

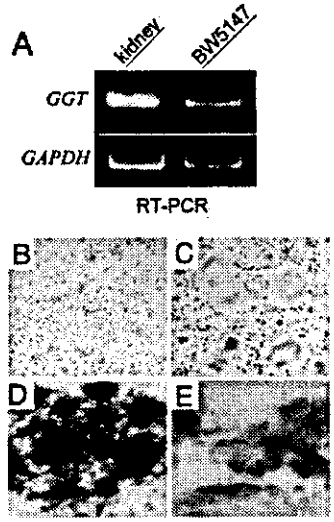

FIG. 1. **Expression of GGT in BW5147 cells and the osteoclast-forming activity of GGT.** RT-PCR analysis (*A*) indicates the expression of GGT mRNA in BW5147 cells. A sample of kidney was used as a control. Conditioned medium from *Xenopus* oocytes expressing GGT induced TRAP$^+$ MNC formation in bone 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

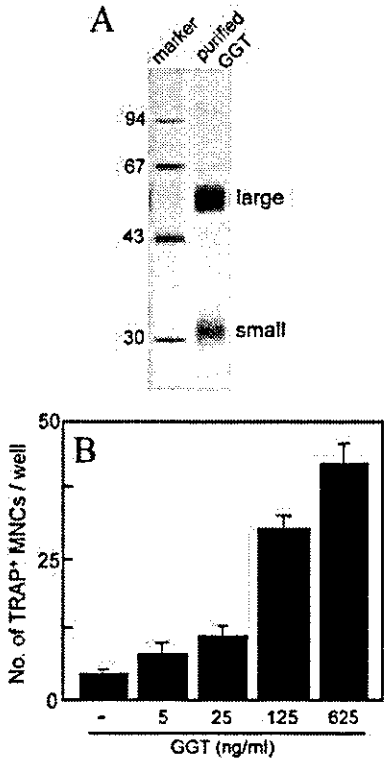

FIG. 2. **Osteoclast-forming activity of the purified GGT.** *A*, purified rat GGT was subjected to SDS-PAGE on 11% gels. The large and small GGT subunits were visualized by silver staining for protein. *B*, the mouse bone marrow cultures were maintained for 7 days with various concentrations of purified rat GGT. Data are expressed as the mean ± S.D. of the number of TRAP+ MNC/well.

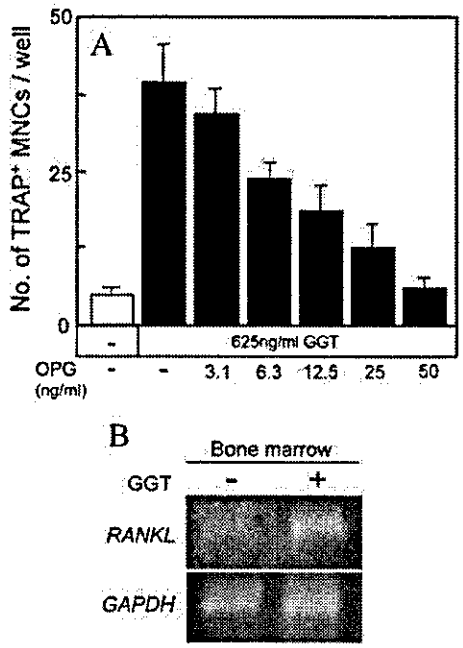

FIG. 3. **Inhibition study of osteoclast formation by OPG.** *A*, the effects of OPG (3.1–50 ng/ml) on TRAP+ MNC formation induced by GGT were examined. The cultures were maintained for 7 days in the presence of GGT (625 ng/ml), and the cells were counted. Data are expressed as the mean ± S.D. of the number of TRAP+ MNCs/well. *B*, RT-PCR showed up-regulation of RANKL mRNA expression in GGT-treated bone marrow culture. A blot loaded with 20 μg of total RNA from bone marrow cells cultured for 3 days in the presence of 625 ng/ml purified GGT was probed with RANKL and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

receptor autoradiographically (Fig. 1*D*). Furthermore, resorption pits were observed microscopically on the dentine slices or osteologic slides after 7 days of culture (Fig. 1*E*).

Osteoclast-forming Activity of GGT—To verify the osteoclast-forming activity of GGT, the enzyme purified from rat kidney was subjected to an *in vitro* osteoclast-forming assay using bone marrow cultures without other bone-resorbing factors. The GGT used in this study produced only two protein bands, which corresponded to the large and small subunits of GGT (Fig. 2*A*). Treatment with a range of 5–625 ng/ml of GGT (equivalent to 0.08–10 nM) for 7 days in culture induced TRAP+ MNC formation in a dose-dependent manner (Fig. 2*B*). Most of these cells expressed the CT receptor, and the resorption pit-forming activity was observed microscopically after 7 days of culture (data not shown). These results indicate that GGT serves as an inducer of TRAP+ MNCs, which satisfies the major criteria for osteoclasts. No endogenous GGT activity was detected in bone marrow cultures. The same results were obtained in additional examinations using recombinant human GGT, which was produced in *Spodoptera frugiperda* Sf21 cells with a baculovirus system as described previously (28) (data not shown).

Involvement of Expression RANKL in GGT-induced Osteoclast Formation—RANKL is the key regulator of osteoclastogenesis, and thus we examined whether RANKL is involved in the GGT-induced osteoclast formation. The osteoclast formation by GGT was inhibited dose dependently by OPG, a decoy receptor for RANKL (Fig. 3*A*). It was also found that GGT stimulated RANKL mRNA expression in bone marrow cells (Fig. 3*B*). These results suggest that the induction of osteoclastogenesis by GGT involves RANKL expression.

Inhibition Studies of GGT-induced Osteoclast Formation—The osteoclast-forming activity of GGT in the bone marrow cultures was further supported by an inhibition study using an anti-GGT antibody. In bone marrow cultures containing 625 ng/ml purified GGT, TRAP+ MNC formation was inhibited by the anti-GGT antibody in a dose-dependent manner (Fig. 4, *solid bars*), whereas substantial numbers of TRAP+ MNCs developed with non-immune antibodies (data not shown). The antibodies *per se* had no adverse effects on cell viability. Interestingly, this antibody did not have a significant effect on neutralizing the enzyme activity of GGT in these examinations (Fig. 4, *hatched bars*), suggesting that the osteoclast-forming activity of GGT is not associated with its enzymatic activity.

Osteoclast-forming Activity of Enzymatically Inactive GGT—To further confirm that the osteoclast-forming activity of GGT does not require the enzyme activity, we prepared an inactive form of GGT (I-GGT), in which the active site was blocked by covalent binding of acivicin, and tested whether I-GGT supported TRAP+ MNC formation in bone marrow cultures. TRAP+ MNC-forming activity was also tested using the murine hematopoietic cell/ST2 cell coculture system that generates osteoclasts *in vitro*. In both systems, I-GGT also induced TRAP+ MNC formation in a dose-dependent manner (Fig. 5). These results further support the suggestion that the osteoclastogenic activity of GGT does not require its own enzymatic activity.

Expression of RANKL in Stromal Cells—Most of the bone-resorbing factors act on the bone marrow stromal cells and stimulate RANKL production. To assess the induction of RANKL mRNA in response to GGT and I-GGT in stromal cells, we examined the expression of RANKL and OPG in bone marrow-adherent cells. Quantitative real time PCR analysis showed that both types of GGT induced a 4-fold increase in RANKL mRNA expression (Fig. 6*A*), whereas the level of OPG mRNA was slightly decreased (Fig. 6*B*). The same results were obtained in examinations using ST2 cells. In addition, the

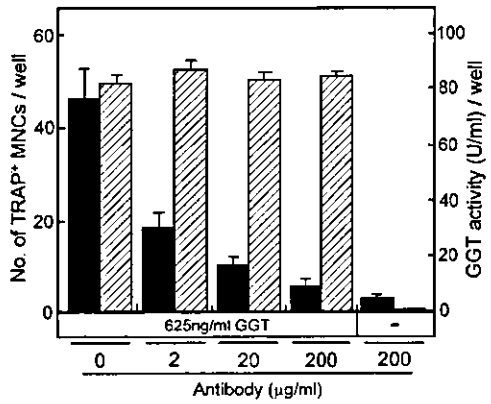

FIG. 4. **Inhibition of osteoclast formation by the anti-GGT antibody.** Cultures maintained for 7 days with various concentrations of goat anti-GGT IgG in the presence of 625 ng/ml purified GGT. The antibody inhibited TRAP⁺ MNC formation (*solid bars*) but not the enzyme activity (*hatched bars*).

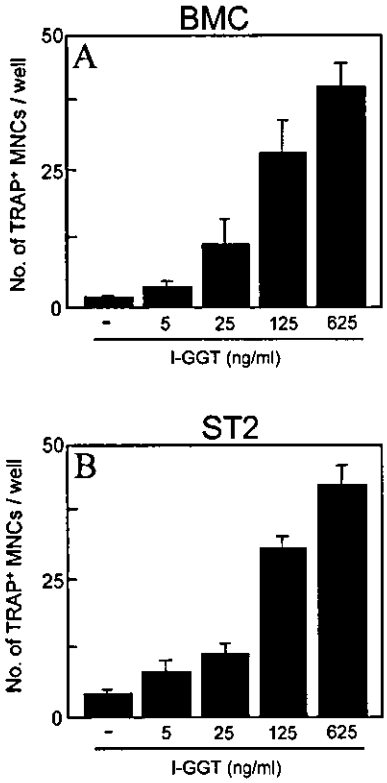

FIG. 5. **TRAP⁺ MNC-forming activity of inactive GGT.** The inactive form of GGT (*I-GGT*) also affected TRAP⁺ MNC formation in the bone marrow cultures (*BMC*) (*A*) and the cocultures of ST2 and bone marrow hematopoietic cells (*B*). The cultures were maintained for 7 days with various concentrations of I-GGT. The cells were then processed to count osteoclasts.

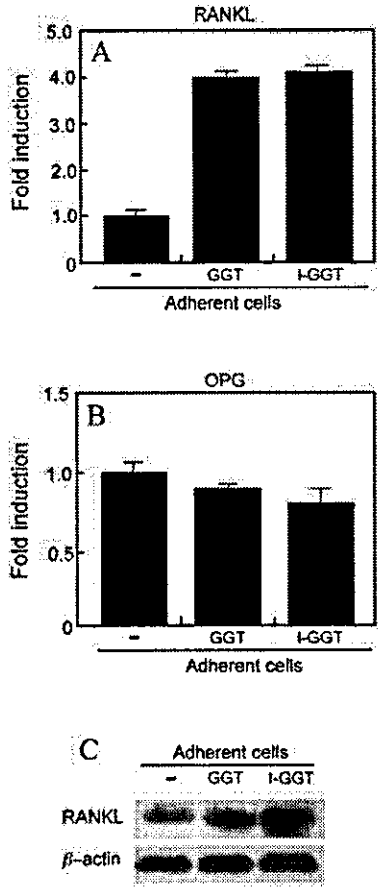

FIG. 6. **The induction of RANKL mRNA in stromal cells by GGT.** *A* and *B*, total RNA from the bone marrow-adherent cells cultured for 3 days in the presence of GGT/I-GGT (625 ng/ml) was probed with RANKL, OPG, and elongation factor-1α1. The ratio of RANKL: elongation factor-1α1 and OPG:elongation factor-1α1 amplimers was calculated from the PCR results using the Light Cycler™ system. *C*, immunoblot analysis indicated increased RANKL protein expression in bone marrow-adherent cells.

immunoblot analysis showed that there was increased RANKL protein expression (Fig. 6*C*). These results suggest that GGT stimulates RANKL expression in bone marrow stromal cells and thereby serves as a bone-resorbing factor in osteolysis. In contrast, GGT had no effect on RANKL production from bone marrow hematopoietic cells (data not shown).

DISCUSSION

This study used expression cloning, based on *in vitro* osteoclast-forming activity in mouse bone marrow cultures, which led to the successful cloning of a novel bone-resorbing factor from a cDNA library of mouse T-lymphoma, BW5147 cells. This factor was identified as GGT. Many previous studies have focused on the enzymatic functions of GGT. These studies have revealed that GGT catalyzes the first step in the degradation of glutathione and plays an important role in glutathione metabolism (29). In addition, the expression of GGT is elevated under certain conditions, such as carcinogenesis (30–33), and it is used as a marker enzyme for many diseases. However, other biological activities of or significance of GGT have not yet been demonstrated.

We showed that the purified GGT induced osteoclast formation in bone marrow culture. This induction was inhibited by OPG, and RANKL mRNA was elevated in the bone marrow cells stimulated by GGT. Therefore, it is most likely that GGT induces osteoclasts via expression of RANKL. Because BW5147 mouse T-lymphoma cells expressed GGT mRNA, GGT appeared to contribute to osteoclast-mediated bone destruction in bone metastasis involving BW5147 cells.

As shown by experiments using anti-GGT antibody and chemically modified inactive GGT, the osteoclast-forming activity of GGT is not associated with the its enzymatic activity, which catalyzes transpeptidation and hydrolysis of the γ-glutamyl moiety of glutathione and related compounds. In the induction of osteoclasts, therefore, it would be expected that GGT functions as a cytokine-like molecule, such as IL-1, via interaction with a presumable receptor. Furthermore, our results indicate that GGT acts on the bone marrow-adherent cells and the ST2 cells to express RANKL. Further investigation

would be required to elucidate osteoclastogenesis mediated by GGT-dependent RANKL expression.

The GGT plays an important role in normal development. GGT-deficient GGT^{tm1}/GGT^{tm1} mice have some metabolic defects and exhibit growth retardation (34). Nevertheless, it is unlikely that GGT is essential for physiological osteoclastogenesis because a recent study demonstrated that the number of osteoclasts was increased in GGT-deficient GGT^{tm1}/GGT^{tm1} mice (35). Although GGT^{tm1}/GGT^{tm1} mice have some bone abnormalities, including osteoclast, chondrocyte, and osteoblast, *N*-acetylcysteine treatment rescued most of these abnormalities. It was considered that the phenotypes observed in these mice are thought to result from impairment of cysteine metabolism (35). However, the osteoclast-forming activity of GGT is clearly independent of its enzymatic activity. GGT may act as an enhancer for RANKL expression under the pathological conditions, such as bone metastasis, and serve as a local bone-resorbing factor. Although the details remain to be clarified, this study has contributed to elucidating a distinct alternative mechanism or a mechanism that accelerates abnormal bone loss.

Expression of GGT is elevated in hepatic diseases and chronic alcoholism (33, 36, 37), which are frequently accompanied with osteopenia and osteoporosis (38, 39). Recently, it has been reported that OPG prevents an ethanol-mediated bone loss by inhibiting osteoclast formation (40). This is consistent with our inhibition study of GGT-induced osteoclast formation by OPG, and, thus, osteoclast formation by GGT may account for osteopenia induced by these hepatic diseases.

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