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The IL-1 Receptor-Related T1 Antigen Is Expressed on Immature and Mature Mast Cells and on Fetal Blood Mast Cell Progenitors[1]

Dirk R. Moritz,[2]* Hans-Reimer Rodewald,† Jacqueline Gheyselinck,* and Roman Klemenz[3]*

Expression of the *T1* gene, also known as ST2, DER4, and Fit-1, has been shown to be associated with cell proliferation. It gives rise to two different mRNAs that encode a receptor-like protein and a soluble molecule representing the ectodomain of the receptor form. Although T1 is a member of the IL-1R family, its biologic function is currently unknown. In this study, we have analyzed the expression of the T1 surface Ag in murine hemopoietic organs. Mast cells (MCs) were shown to be the only identifiable cell lineage that expressed T1 at high levels. T1 expression was found on cultured bone marrow-derived immature MCs. Similarly, freshly isolated connective tissue-type MCs from the i.p. cavity were also shown to express high levels of T1. Interestingly, the earliest detectable committed MC precursor isolated from fetal blood (FB) at day 15.5 of gestation, but not circulating hemopoietic stem cells in FB, also expresses high level of T1. Since FB promastocytes lack expression of the high affinity IgE receptor (FcεRI), T1 expression precedes expression of FcεRI in MC ontogeny. The finding that the T1 Ag is selectively expressed at several stages during development of the MC lineage suggests that this cell surface molecule, in combination with the well-established markers c-Kit and FcεRI, should be valuable for studying the MC lineage. ***The Journal of Immunology,* 1998, 161: 4866–4874.**

The *T1* gene, also designated *ST2* or *DER4*, has been originally identified as a gene that was inducible by Ha-*ras* and v-*mos* oncogenes (1, 2) or by serum stimulation (3, 4) in mouse fibroblasts. Homologous cDNAs have been cloned from humans (5) and rats (Fit-1) (6). In fibroblasts, transcription of *T1* results in the production of two mRNA species of 2.7 and 5 kb as a consequence of alternative 3′ processing (7). The 2.7-kb *T1* transcript is expressed in fibroblast cell lines (1–4); nonhemopoietic tissues such as embryonic bone, skin, and retina (8); in the developing mammary gland; and in Ha-*ras*-induced mammary adenocarcinomas (9). The murine 5-kb *T1* mRNA transcript has been detected in cell populations of the major hemopoietic organs such as in fetal liver, in spleen and bone marrow (BM),[4] as well as in the lung (6, 8). Several cell lines of macrophage, erythroid progenitor, and T cell origin (6, 8), established MC lines, and primary MCs (8, 10) also express *T1* mRNA. The longer 5-kb T1 transcript encodes a 567-amino-acid integral membrane protein (7) that, based on its high structural similarity, has been classified as a member of the IL-1R family (7, 11, 12). Two different groups have recently reported the identification of putative T1 ligands (13, 14), but it is as yet unclear whether the T1-binding proteins described indeed represent the physiologic T1 ligands.

In this study, we have analyzed the expression of the T1 Ag in the hemopoietic system. For this, we utilized the T1-specific mAb DJ8, which we have recently generated (47). We found that MCs were the only identifiable cell lineage that expressed T1 at high levels. MCs are critical effectors in the pathogenesis of IgE-dependent allergic disorders (15, 16), but are also implicated in host defense against bacterial infections (17–19) and parasites (20, 21). MCs can be classified into two subtypes: the mucosal MC (MMC), which is widely distributed in mucosal tissues of the respiratory tract and the intestinal lamina propria, and the connective tissue-type MC, which is found throughout the skin, musculature, in perivascular tissues, and in the peritoneal cavity (16). MCs contain characteristic secretory granules loaded with mediators such as histamine, heparin, proteases, and others, which can be released upon stimulation. MCs also produce and secrete a number of proinflammatory cytokines such as IL-1α and TNF-α (22). Ag-mediated engagement of FcεRI triggers MC degranulation and mediator release (23, 24). A second surface receptor, c-Kit, is also characteristic for MCs. The c-*kit* proto-oncogene encodes a protein tyrosine kinase receptor that is activated by binding to its ligand, stem cell factor (SCF). Activation of c-Kit results in essential signals for MC differentiation and function (25).

MCs derived from multipotent hemopoietic stem cells (HSC) (26, 27). In contrast to other hemopoietic cells that leave the BM after having undergone partial or complete differentiation, it is thought that putative, undifferentiated, and as yet unidentified MC precursors leave the adult BM, migrate via the peripheral blood, and invade mucosal and connective tissues, where they differentiate into morphologically distinct mature MCs. In this study, we report that the T1 receptor is expressed during different developmental stages of the MC lineage. T1 was detected on both immature BM-derived cultured MCs (BMCMCs), as well as on mature i.p. MCs (IPMCs). Importantly, the earliest committed progenitor of the MC lineage, which has recently been purified from fetal

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[4] Abbreviations used in this paper: MC, mast cell; AP, alkaline phosphatase; BM, bone marrow; BMCMB, bone marrow-derived cultured mast cell; BMMϕ, bone marrow-derived macrophage; FB, fetal blood; HSC, hemopoietic stem cell; IPMϕ, intraperitoneal macrophage; IPMC, intraperitoneal mast cell; MMC, mucosal mast cell; PE, R-phycoerythrin; Sav-APC, streptavidin-allophycocyanin; SCF, stem cell factor.

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blood (FB) (28), but not circulating progenitors containing HSC, expresses T1 on the cell surface. Our results indicate that the T1 Ag, along with the FcεRI and c-Kit surface receptors, is within the hemopoietic system selectively expressed on MCs, and that it might be a useful novel surface Ag marker for studying the MC lineage.

Materials and Methods

Cell culture

Nonhemopoietic cell lines (NIH3T3, F-2, s.End.1, TME-3H3, L cells, HC11, B16-F10, and 3LL) were maintained in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated FCS (Life Technologies), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). All hemopoietic cell lines (P815, BW5147, P388, X63.Ag8, SciET27/F) were grown in lymphoid cell growth medium: Iscove's modified Dulbecco's medium (IMDM; Life Technologies), supplemented with 10% heat-inactivated FCS (Life Technologies), 50 μM 2-ME, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml).

T1 expression in established cell lines

A total of 1×10^6 viable cultured cells was stained with a 1/1000 pretitrated dilution of an anti-T1 polyclonal rat antiserum (47) or with a 1/1000 dilution of the preimmune serum of the same animal for 30 min at 4°C. After washing the cells in FACS buffer (PBS, 10% FCS, 0.1% sodium azide), 1 μg of a FITC-conjugated goat anti-rat Ig (Southern Biotechnology, Birmingham, AL) was added to the cells and incubated for 30 min at 4°C. Cells were washed and resuspended in FACS buffer, and 10,000 forward scatter/side scatter-gated viable cells were acquired and analyzed on a Becton Dickinson (San Jose, CA) FACSCalibur flow cytometer.

mAbs for flow cytometry

The following primary mAbs were utilized in this study: biotinylated or FITC-labeled DJ8 (anti-T1 mAb) (47); biotinylated ACK-4 (anti-c-Kit) (29); R-phycoerythrin (PE)-conjugated 3C1 (anti-c-Kit); PE-conjugated CT-TH1 (anti-Thy-1); PE-conjugated RA3-6B2 (anti-B220); PE-conjugated M1/70.15 (anti-Mac-1); PE-conjugated F4/80 (anti-mouse macrophage; all from Caltag Laboratories, San Francisco, CA); PE-conjugated H129.19 (anti-CD4); biotinylated 53-6.7 (anti-CD8α); PE-conjugated TER-119 (antierythroid BM cells); IgE-3 (antitrinitrophenol mouse IgE, κ); and unlabeled rat IgG1, κ isotype control (all from PharMingen, San Diego, CA). PE-conjugated isotype control Abs IgG2a-PE and IgG2b-PE were from Caltag. Second-step reagents were: streptavidin-allophycocyanin (Sav-APC; PharMingen) and FITC-labeled goat anti-mouse Ab (Southern Biotechnology, Birmingham, AL). The anti-T1 DJ8 mAb was purified from hybridoma supernatants on protein G-Sepharose (Pharmacia, Uppsala, Sweden). DJ8 was biotinylated with NHS-LC-biotin (Pierce, Rockford, IL), following the instructions of the manufacturer. DJ8 and rat IgG1 isotype control mAbs were identically treated for labeling with FITC (isomer I on celite; Sigma, St. Louis, MO) following standard procedures (30). After the labeling procedure, all labeled Abs were carefully titrated by FACS analysis, and the determined optimal concentration was used in subsequent experiments.

BM cultures

BM cells were obtained from 8- to 10-wk-old C57BL/6 mice by flushing femora and tibiae with HBSS buffer (Life Technologies). BM cells were cultured in lymphoid culture medium (see above) supplemented with 3% conditioned culture supernatant from murine IL-3-secreting X63/IL-3 cells (31). BM cultures were enriched for MCs by repetitively transferring the suspension cell fraction into fresh culture flasks every 5 days in the presence of IL-3. MCs were identified by double staining for IgE receptors and c-Kit (32). To this end, BM suspension cells were first incubated with IgE mAb at 10 μg/ml and anti-c-Kit-PE (10 μg/ml), followed by a FITC-labeled goat anti-mouse Ab (1:100). After about 4 wk in culture, >98% of the cells displayed typical MC-like $IgE^+c\text{-}Kit^+$ phenotype as well as metachromatic staining with Giemsa. BMCMCs were analyzed for T1 expression by incubating cells with IgE, c-Kit-PE, and DJ8-biotin (0.5 μg/ml), followed by the secondary step reagents goat anti-mouse Ig-FITC and Sav-APC (1:200), respectively.

To follow the appearance of BMCMCs in BM cultures, BM cells were prepared and cultured as outlined above. At different time points, suspension cells were removed from the culture flasks and stained with c-Kit-PE and DJ8-FITC.

For analysis of T1 expression in the $c\text{-}Kit^+$ cells present in freshly isolated BM suspensions, cells were incubated with DJ8-FITC. To visualize $c\text{-}Kit^+$ BM cells, they were additionally stained with biotinylated anti-c-Kit, followed by Sav-APC and a mixture of PE-conjugated lineage-specific Abs (Thy-1-PE, B220-PE, TER-119-PE, and Mac-1-PE).

In the presence of IL-3, BM-derived monocytes also differentiate into macrophages that are present in the adherent fraction of the BM culture (30, 33). To assay the expression of T1 on BM-derived macrophages, adherent cell fractions of 6-day-old BM cultures were trypsinized and simultaneously stained with DJ8-FITC (0.5 μg/ml) and the macrophage-specific Abs Mac-1-PE or F4/80-PE (both at 10 μg/ml).

Flow cytometry of primary lymphoid cells

The spleen, axillary lymph nodes, and the thymus were removed surgically from 8-wk-old C57BL/6 mice, and the organs were mechanically homogenized to obtain a single-cell suspension. From the same animals, BM cells were prepared as described above. PBMCs were obtained from whole heparinized peripheral blood after removal of RBC through osmotic lysis. Fetal livers were derived from day 13 and day 15 timed-pregnant mice. For the analysis of T1 expression, single-cell suspensions were directly stained with DJ8-FITC in the presence of 10% FCS. Background staining was determined with IgG1-FITC, an isotype-matched control Ab.

T1 expression in spleen-derived T cells was assessed by double staining with either CD4-PE, Thy-1-PE, and CD8-biotin, followed by avidin-PE vs T1-FITC or IgG1-FITC. For the analysis of B cells, splenocytes were double stained with B220-PE and T1-FITC. All stainings were performed in PBS, 10% FCS, and 0.05% sodium azide at 4°C for 30 min.

Immunoprecipitation of T1 M in BMCMCs

BMCMCs were grown as described above. For each immunoprecipitation, 1×10^7 viable BMCMCs were solubilized in lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris/HCl, pH 8, 5 mM EDTA, 10 mM iodoacetamide), supplemented with a mixture of protease inhibitors (Complete; Boehringer Mannheim, Mannheim, Germany). Postnuclear cell lysates were incubated with 30 μl protein G-Sepharose (Pharmacia) that had been coated with 3 μg anti-T1 mAbs DJ4 and DJ8 (47) or IgG1 isotype control mAb for 4 h at 4°C. After washing the resin three times with PBS, the beads were boiled in reducing Laemmli sample buffer, and the eluted proteins were electrophoresed through 10% SDS-PAGE gels. Proteins were transferred onto a polyvinylidene difluoride membrane (Millipore), and unspecific binding sites were blocked with PBS-T (PBS, 0.4% Tween-20) containing 5% skim milk powder (Fluka, Buchs, Switzerland). The immunoblot was probed with a rabbit polyclonal antiserum directed against bacterially produced murine T1 protein (34), followed by an AP-conjugated goat anti-rabbit Ab (Promega, Madison, WI). After extensively washing with PBS-T, the blot was developed using the Western Blue AP substrate according to the manufacturer's recommendations (Promega).

Intraperitoneal MCs

Unfractionated cell suspensions were obtained from the peritoneal cavity by peritoneal lavage of 6- to 8-wk-old C57BL/6 mice. The fraction of i.p. MCs in this preparation was assessed by cytologic staining with Giemsa or Berberine sulfate and found to be 2 to 3%. For flow cytometry, FcR were blocked with FACS buffer (containing 10% FCS) and stained for IgE receptor, c-Kit, and T1, as described above for BMCMCs. In a second experiment, IPMCs were prepared identically and stained with DJ8-FITC and c-Kit-PE. Double-positive $T1^+c\text{-}Kit^+$ cells were identified and sorted on a FACStar flow cytometer. Cytocentrifuge preparations of the total, unsorted cells, as well as the sorted cell population that was found to be about 94% pure upon reanalysis, were examined with May-Grünwald-Giemsa stain.

FB mast cell progenitors

The preparation of FB progenitor as well as the FACS staining and sorting of pro-MCs was performed as described (28, 35). In short, fetuses of timed-pregnant C57BL/6 mice were obtained at day 15.5 of gestation. The uterus was excised and washed in PBS, and the embryos were removed without injuring the umbilical cord. Fetuses were placed on their backs, and the jugular veins and cervical arteries were cut. About 20 μl of FB was collected from each fetus. The heparinized FB was washed in PBS and 5% FCS, and the fetal leukocytes were purified by discontinuous Percoll (60% v/v) (Pharmacia, Uppsala, Sweden) gradient centrifugation.

The obtained FB leukocytes were blocked with 0.5 mg/ml normal mouse Ig (Jackson ImmunoResearch, West Grove, PA) and stained with Thy-1-PE (1/50 diluted) and biotinylated c-Kit (5 μg/ml) mAb, followed by Sav-APC (1:200). The fetal MC progenitors were identified as the $Thy\text{-}1^{low}c\text{-}Kit^{high}$ population and sorted on a FACStar flow cytometer. As a negative control, the $Thy\text{-}1^-c\text{-}Kit^+$ multipotent progenitor population (35) was also sorted. The two sorted cell populations that were found to be

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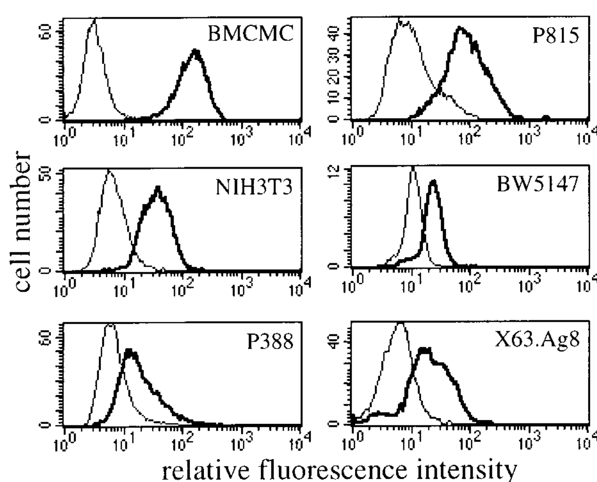

Expression of T1M not detectable on:

• endothelial cell lines	F-2 s.End.1 TME-3H3
• fibroblastoid cell line	L-cells
• epithelial cell line	HC11
• pro-T cell line	SciET27/F
• tumor cell lines	B16-F10 3LL

FIGURE 1. T1 expression in lymphoid and nonlymphoid cell lines and in BMCMCs. A total of 1×10^6 cultured cells was incubated with a rat anti-T1 polyclonal antiserum, followed by a FITC-conjugated goat anti-rat Ig as a second-step reagent (bold line). Negative control stainings were performed with the rat preimmune serum as the first step reagent (fine line). BMCMCs are BM-derived cultured MCs, P815 is a MC line, NIH3T3 cells are fibroblasts, BW5147 is a T cell hybridoma, X63. Ag8 is a B cell myeloma line, and P388 cells are macrophages. A total of 10,000 life-gated cells was acquired and analyzed on a Becton Dickinson FACSCalibur flow cytometer.

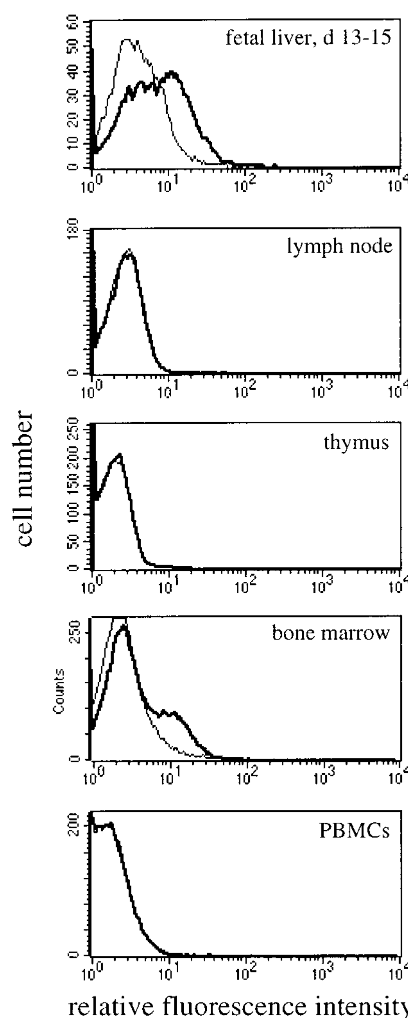

FIGURE 2. T1 expression in primary and secondary hemopoietic organs. Lymphoid organs were prepared as described in *Materials and Methods*. Single-cell suspensions were prepared and incubated with DJ8-FITC (bold line). Negative control stainings were performed using an isotype-matched control IgG1 Ab that has been FITC conjugated in exactly the same way as DJ8 (fine line). A total of 10,000 life-gated cells was acquired and analyzed on a Becton Dickinson FACSCalibur flow cytometer.

>99% pure upon reanalysis were subsequently stained with the anti-T1 mAb DJ8-FITC (0.6 μg/ml).

A second independent preparation was performed with day 15.5 fetuses of timed-pregnant BALB/c mice. The obtained FB leukocytes from this preparation were directly analyzed without cell sorting after triple staining with Thy-1-PE, c-Kit-biotin, and DJ8-FITC or IgG1-FITC, followed by Sav-APC.

Results

Expression of T1 in established cell lines and primary MC

The expression levels of the T1 Ag were analyzed in a number of hemopoietic and nonhemopoietic established cell lines by flow cytometry. Lymphoid cell lines such as the BW5147 T cell hybridoma, the X63.Ag8 B cell myeloma, and the P388 macrophage cell line were all shown to express low but significant levels of the T1 receptor (Fig. 1). In agreement with previous results (7, 10, 47), substantial amounts of T1 were also detectable on NIH3T3 fibroblasts. However, the highest levels of T1 expression were observed in primary bone BMCMCs and the P815 mastocytoma cell line. T1 expression was undetectable in three endothelial cell lines (F-2, s.End.1, amd TME-3H3), another fibroblast-like cell line (L cells), in HC11 breast epithelial cells, in a pro-T cell line, and in the two tumor cell lines B16-F10 (melanoma) and 3LL (Lewis lung carcinoma).

T1 is not expressed abundantly in primary lymphoid organs

On the basis of the results obtained with established cell lines, we next analyzed T1 expression in freshly isolated cells that were prepared from a number of murine lymphoid organs (Fig. 2). Weak staining with the anti-T1 DJ8-FITC mAb was observed consistently in a subpopulation of BM cells comprising ~5 to 7% of all cells (Fig. 2; see also Fig. 5, *first panel*, and Fig. 8*B*). Similar weak staining intensities were detected on fetal liver cells of about days 13 and 15 of gestation. In contrast, all other lymphoid organs analyzed, such as lymph nodes, spleen, thymus, and PBMCs, were negative for T1 expression.

Since T1 has been detected on B cell, T cell, and macrophage cell lines (Fig. 1), and T1 mRNA expression has been found in the

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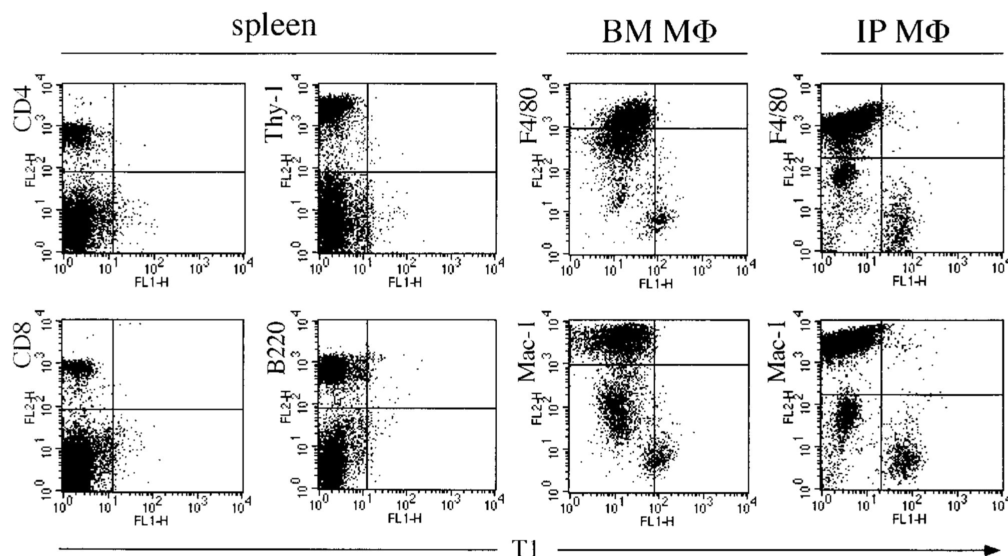

FIGURE 3. T cells, B cells, and macrophages do not express T1. Lymphocyte suspensions were prepared from spleen and double stained with T cell markers (CD4-PE, CD8-PE, and Thy-1-PE) or a B cell marker (B220-PE) vs DJ8-FITC. Macrophages were obtained from BM suspensions that were cultured in the presence of IL-3 (BMMϕ) or from i.p. lavage (IPMϕ). BMMϕ and IPMϕ cells were double stained with DJ8-FITC and the macrophage-specific markers F4/80-PE and Mac-1-PE. Quadrant markers were adjusted after negative control stainings with FITC- or PE-labeled isotype-matched control mAbs.

same cell types (6, 8), we analyzed primary lymphocytes and macrophages in greater detail (Fig. 3). For this, splenocytes were double stained for T or B cell markers vs T1. Thy-1^+, $CD4^+$, and $CD8^+$ T cells were clearly negative for T1 expression, as were $B220^+$ B cells. A very small fraction of cells that did not express any of the tested lymphoid lineage markers did express low levels of T1. Next, T1 expression was measured in BM-derived macrophages (BMMϕ) that grew out of primary BM cultures in the presence of IL-3 (33) and on freshly isolated i.p. macrophages (IPMϕ). All macrophages were identified by staining with F4/80 or anti-Mac-1 macrophage-specific Abs. Both BM-derived and i.p. macrophages did not express significant levels of T1 (Fig. 3). The small population of cells that express T1, but not the macrophage-specific markers, represents BMCMCs and IPMCs, as will be shown below.

T1 is expressed at high levels in BMCMCs

In the comparative expression analysis shown in Figure 1, highest levels of T1 were detected on the surface of BMCMCs. To further substantiate this finding, we analyzed BMCMCs by triple-color analysis. The hallmark of mature MCs is the expression of the high affinity IgE receptor, FcεRI, and the c-Kit receptor (16). The FcεRI on BMCMCs was detected by binding of a purified IgE mAb, and the c-Kit receptor by staining with an anti-c-Kit mAb. Most cells of a 6-wk-old BMCMC culture that was grown in the presence of IL-3 homogenously expressed both FcεRI and c-Kit (Fig. 4*A*). Gi-

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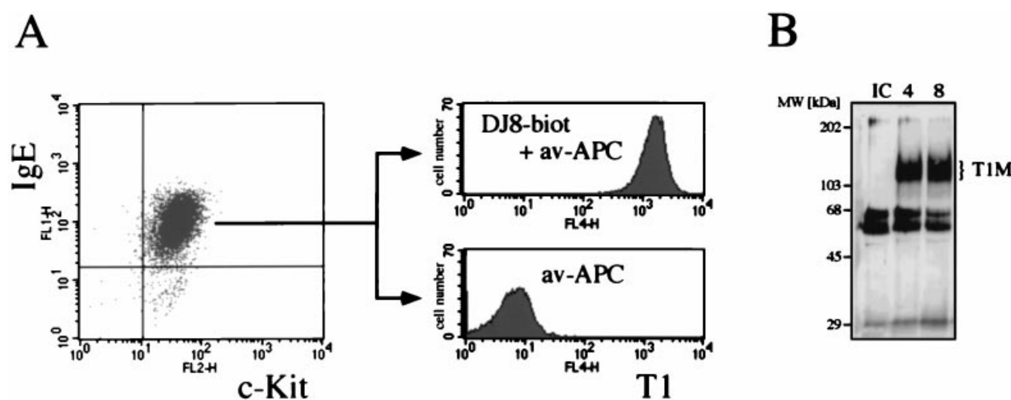

FIGURE 4. BMCMCs express high levels of T1. *A*, Flow cytometry. BMCMCs were triple stained with IgE/anti-IgE-FITC, c-Kit-PE, and DJ8-biotin/Sav-APC. A total of 10,000 forward scatter/side scatter-gated cells was analyzed by two-color dot plot revealing that >99% of the cells were IgE^+c-Kit^+ double positive. T1 expression was analyzed in histogram mode by gating on the IgE^+c-Kit^+ double-positive population. Negative control staining of IgE^+c-Kit^+ cells after staining with Sav-APC second-step reagent alone is shown in the *lower histograms*. *B*, Immunoprecipitation of the membrane form of T1 (T1 M) from BMCMCs. A total of 1×10^7 viable BMCMCs was solubilized in Nonidet P-40 lysis buffer. Postnuclear lysates were incubated with protein G-Sepharose beads coated with anti-T1 mAbs DJ4 (4), DJ8 (8), or IgG1 isotype control (IC). Bound proteins were eluted by boiling the beads in Laemmli reducing sample buffer. Proteins were electrophoresed through 10% SDS-polyacrylamide gels and electroblotted onto a polyvinylidene difluoride membrane. After blocking unspecific binding with 5% skim milk in PBS, the membrane was probed with a rabbit polyclonal Ab directed against a bacterially produced T1 protein. Binding of the Ab was revealed by incubation with an AP-conjugated goat anti-rabbit Ab (Promega). The lower band of about 50 kDa present in all three lanes is due to a cross-reactivity of the goat anti-rabbit antiserum with the rat Ig heavy chain.

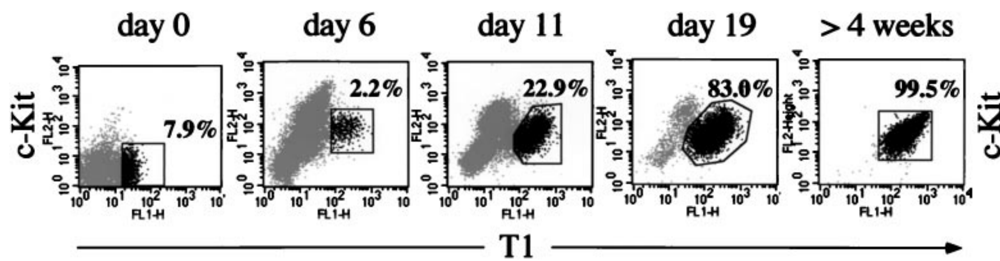

FIGURE 5. Kinetics of MC induction in primary BM cultures. BM suspensions were prepared from C57BL/6 mice, as described in *Materials and Methods*. BM cells were cultivated in IL-3-containing medium, and suspension cells were transferred into fresh cultureflasks every 5 days. At the indicated time points, nonadherent cells were removed from the culture wells and double stained with c-Kit-PE and DJ8-FITC. A total of 10,000 forward scatter/side scatter-gated cells was acquired and analyzed by two-color dot-plot analysis on a Becton Dickinson FACSCalibur flow cytometer. Gates were defined after comparison with negative control stainings using PE- and FITC-conjugated isotype-matched control mAbs.

emsa staining of metachromatic granules further confirmed the MC nature of those cells (not shown). All of the IgE$^+$c-Kit$^+$ MCs were shown to express uniformly high levels of T1 when stained with biotinylated DJ8 mAb followed by Sav-APC. In contrast, incubation with the secondary staining reagent alone resulted in background staining.

This finding was biochemically confirmed by immunoprecipitation analysis. Whole IgE$^+$c-Kit$^+$ BMCMCs were solubilized in Nonidet P-40-containing lysis buffer and T1 protein immunoprecipitated with the two anti-T1 mAbs DJ4 and DJ8 (47). The precipitated T1 was subsequently detected by immunoblotting using a polyclonal anti-T1 rabbit antiserum. Both DJ4 and DJ8, but not the IgG1 isotype control mAb, precipitated abundant amounts of T1, which was apparent as a 110- to 120-kDa band (Fig. 4*B*).

We next analyzed the emergence of T1$^+$c-Kit$^+$ immature MCs in IL-3-induced primary BM cultures (Fig. 5). As shown in a previous experiment (Fig. 2), about 8% of freshly isolated BM cells expressed low amounts of T1 (T1^{low}). Importantly, no T1$^+$c-Kit$^+$ double-positive cells were detectable in freshly isolated BM cells. However, when the BM cells were cultivated in the presence of IL-3, T1$^+$c-Kit$^+$ double-positive immature MCs were readily detectable at day 6, comprising about 2% of all cells. The expression level of T1 observed in the T1$^+$c-Kit$^+$ BMCMCs was at least 10 times higher than the level measured on the T1^{low} cells at the onset of the culture. Over time, the T1$^+$c-Kit$^+$ BMCMC population constantly expanded and comprised about 99% of all cells after 4 wk of culture.

Mature IPMCs express high levels of T1 in vivo

After having demonstrated that immature BMCMCs express high levels of T1, we were interested to investigate whether mature in vivo MCs do also express T1. Hence, we isolated cells from the peritoneal cavity of C57BL/6 mice and analyzed them by flow cytometry. For this, the peritoneal cells were triple stained with IgE, anti-c-Kit, and anti-T1 mAbs. Mature IPMCs were clearly identifiable as a IgE$^+$c-Kit$^+$ double-positive population (Fig. 6*A*). The IgE$^+$c-Kit$^+$ IPMC population comprised about 2% of the total cell number. When only analyzing the IgE$^+$c-Kit$^+$ MC population, it was revealed that all of them homogeneously expressed high levels of T1. In contrast, IgE$^-$c-Kit$^-$ non-MCs in the peritoneal lavage did not express significant levels of T1 (not shown). Our result was confirmed by quantification of the T1$^+$ cells present in the total peritoneal cell preparation. As shown in the histogram of DJ8-stained ungated cells (Fig. 6*A*), about 2% of the total cell population expressed high levels of T1. Since this percentage of T1$^+$ cells is identical to the percentage of IgE$^+$c-Kit$^+$ MCs present in the total cell population, we conclude that all and only MCs express T1. Interestingly, the T1-staining intensities detected on IPMCs and on BMCMCs were almost identical (compare histograms in Figs. 4 and 6*A*).

To further substantiate the finding that T1 is expressed on IPMCs, we again isolated i.p. cells but, this time, double stained them with anti-c-Kit and anti-T1 mAbs. The analysis revealed that all of the c-Kit$^+$ cells were also positive for T1. The T1$^+$c-Kit$^+$ population was sorted to high purity by flow cytometry. The characteristic May-Grünwald-Giemsa metachromatic staining of the purified population unambiguously confirmed the MC nature of T1$^+$c-Kit$^+$ cells (Fig. 6*B*). It is important to note that a single staining of peritoneal cells with the DJ8 mAb alone (see Fig. 6*A*, *upper left panel*, "ungated cells") will suffice to identify and sort-purify the IPMC population.

T1 is expressed on the earliest detectable Thy-1^{low}c-Kit^{high} MC precursor

Stimulated by the fact that both mature IPMCs as well as immature BMCMCs express high levels of T1, we investigated T1 expression in MC precursors. The earliest committed precursor of the MC lineage has been identified recently in FB of day 15.5 of gestation (28). It is defined as a population that expresses low levels of the Thy-1 surface glycoprotein and high levels of c-Kit (Thy-1^{low}c-Kit^{high}). To isolate the MC progenitors, we collected FB from fetuses at day 15.5 of gestation. FB leukocytes were enriched by discontinuous Percoll gradient centrifugation. MC-committed precursors were identified by staining the FB leukocytes with anti-Thy-1 and anti-c-Kit mAbs. This staining resolved three typical cell populations that differ in their respective expression of Thy-1 and c-Kit. The Thy-1$^+$c-Kit^{low} population of FB cells has been found to contain prothymocytes, whereas Thy-1$^-$c-Kit$^+$ cells were shown to be HSC (35, 28). The rarest population, defined by the Thy-1^{low}c-Kit^{high} phenotype, represents MC lineage-committed progenitors. In the first experiment (Fig. 7*A*), the Thy-1^{low}c-Kit^{high} pro-MCs and the Thy-1$^-$c-Kit$^+$ HSC population were purified by flow-cytometric cell sorting. The two sorted populations were shown to be >99% pure upon reanalysis. Staining of the highly purified cells with the T1-specific Ab DJ8 revealed that all of the Thy-1^{low}c-Kit^{high} pro-MCs expressed high levels of T1. In contrast, the Thy-1$^-$c-Kit$^+$ HSC and the Thy-1$^+$c-Kit^{low} pro-T cell (not shown) populations did not stain with the anti-T1 Ab. These results were confirmed in a second, slightly modified experiment. Percoll gradient-separated FB leukocytes of 15.5-day-old BALB/c fetuses were directly triple stained with Thy-1, c-Kit, and T1 Abs without cell sorting. Analysis of the Thy-1^{low}c-Kit^{high} pro-MC population revealed again that all cells stained brightly for T1 (Fig. 7*B*). In contrast, the Thy-1$^-$c-Kit$^+$ HSC population did not exhibit significant levels of T1. Negative control stainings using an Ab

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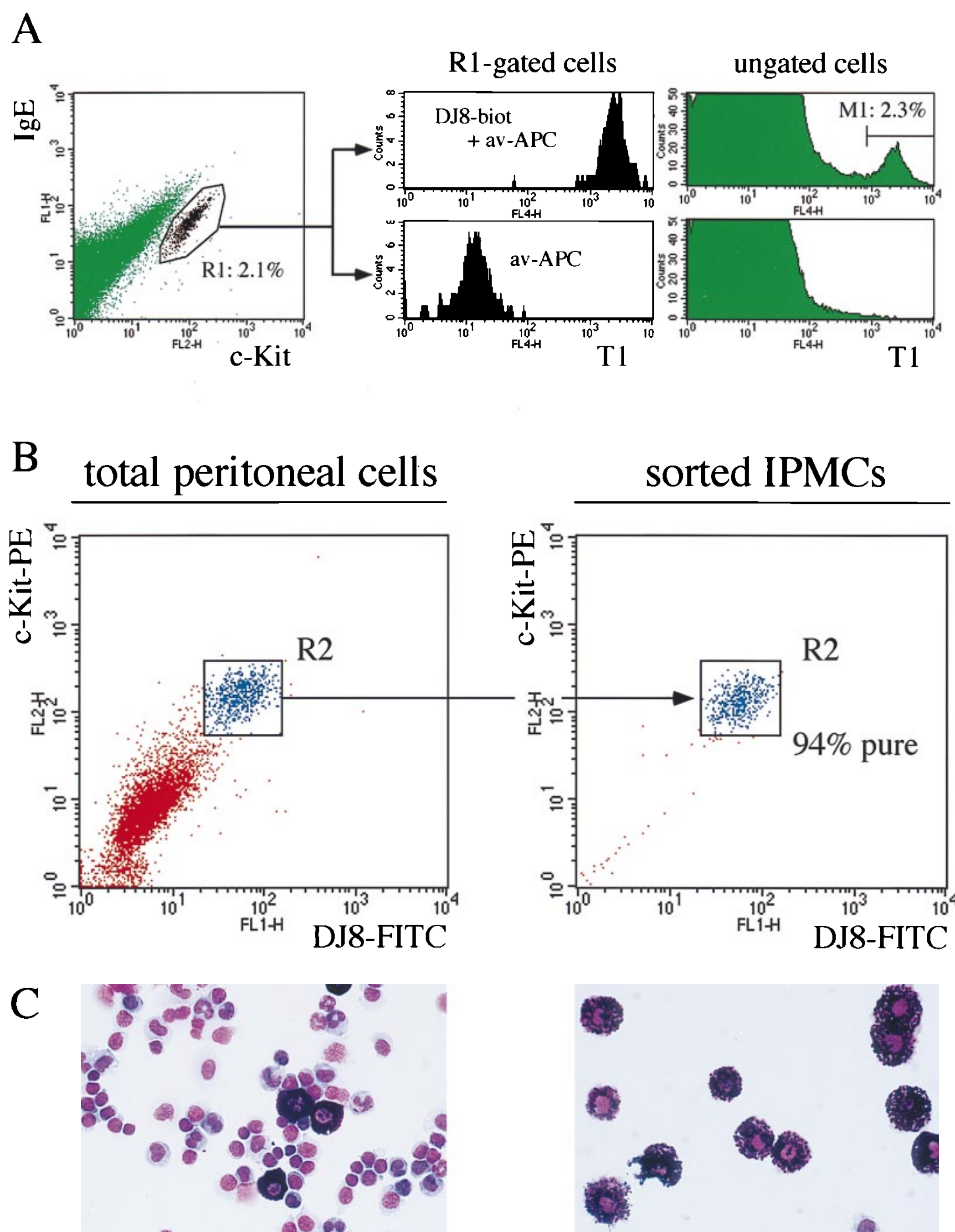

FIGURE 6. Freshly isolated IPMCs express high levels of T1. Intraperitoneal cells were obtained, as described in *Materials and Methods*, by lavage of the i.p. cavity of C57BL/6 mice. *A*, Flow cytometry. Cells were triple stained with IgE/anti-IgE-FITC, c-Kit-PE, and DJ8-biotin/Sav-APC. A total of 100,000 forward scatter/side scatter-gated cells was acquired and analyzed by two-color dot plot revealing that about 2% of all cells were IgE$^+$c-Kit$^+$ double positive. T1 expression was analyzed in histogram mode by gating on the IgE$^+$c-Kit$^+$ MCs (gate R1). To ascertain that all of the IgE$^+$c-Kit$^+$ MCs did uniformly express T1, the percentage of T1$^+$ cells in the ungated total cell population was also determined. Negative control staining of IgE$^+$c-Kit$^+$ cells is shown by staining with the second-step reagent Sav-APC alone (*lower histograms*). *B*, IPMC cell sorting. Intraperitoneal cells were double stained with DJ8-FITC and c-Kit-PE. Forward scatter/side scatter-gated T1$^+$c-*kit*$^+$ double-positive cells were identified (gate R2) and FACS sorted. *C*, Unsorted and sorted cells were cytospun and May-Grünwald-Giemsa stained.

that matches the isotype of the anti-T1 DJ8 mAb resulted in background staining.

Thy-1^{low}c-Kit^{high} pro-MCs are not present in adult BM

The in vitro formation of MC colonies in the presence of SCF (the c-Kit ligand) and IL-3 suggests the existence of MC precursors in the BM (36). Inspired by this, we attempted to identify a phenotypic equivalent of the FB Thy-1^{low}c-Kit$^+$ promastocyte in the BM. To this end, unfractionated preparations of freshly prepared BM cells from 4-wk-old C57BL/6 mice were double stained with Thy-1 and c-Kit mAbs. This staining resolved two distinct populations, a Thy-1$^+$c-Kit$^-$ and a Thy-1$^-$c-Kit$^+$ population (Fig. 8*A*). The first population probably consists of Thy-1$^+$ T cell lineage

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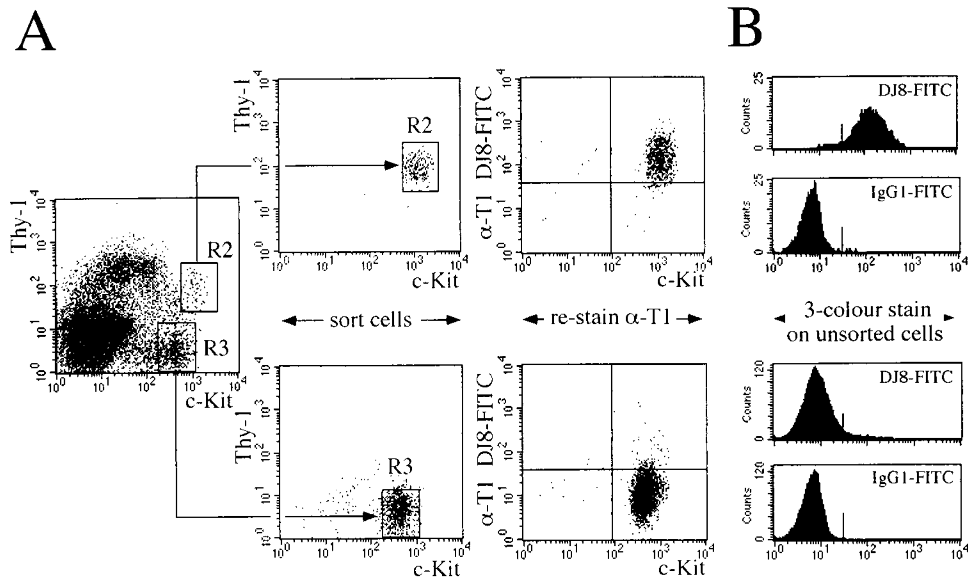

FIGURE 7. T1 is expressed on Thy-1^{low}c-Kit^{high} promastocytes. Day 15.5 FB leukocytes were prepared as described in *Materials and Methods*. *A*, To visualize pro-MCs, FB cells were double stained with Thy-1-PE and c-Kit-biotin/Sav-APC. Forward scatter/side scatter size-gated cells were analyzed by two-color dot plot. Thy-1^{low}c-Kit^{high} pro-MCs (gate R2) and the control Thy-1^{-}c-Kit^{+} multipotent population (gate R3) were identified and FACS sorted on a Becton Dickinson FACStar flow cytometer. Sorted cells in R2 and R3 were >99% pure upon reanalysis. The two sorted cell populations were restained with DJ8-FITC, and again analyzed by two-color dot plot. *B*, In a second independent experiment, FB leukocytes were directly triple stained with Thy-1-PE, c-Kit-biotin/Sav-APC, and DJ8-FITC or IgG1-FITC isotype control. T1 expression was analyzed in histogram mode on gated Thy-1^{low}c-Kit^{high} promastocytes (cells in gate R2, *upper histogram pair*) and on the control Thy-1^{-}c-Kit^{+} HSC population (cells in gate R3, *lower histogram pair*). The marker for background staining was adjusted by negative control staining with IgG1-FITC.

cells, whereas the latter probably represents c-Kit^{+} early progenitors including HSC. However, a population corresponding to the FB Thy-1^{low}c-Kit^{high} pro-MCs was clearly not detectable. We reasoned that the Thy-1 expression level of the putative BM MC precursors might be lower than of the FB progenitors, and consequently considered the existence of Thy-1^{-}c-Kit^{high} MC precursors. Based on this assumption and on our previous results, we investigated the possibility to employ T1 as a marker for pro-MCs in the BM. To better distinguish the c-Kit^{+} population from cells of the mature hemopoietic lineage (lin) such as B cells, T cells,

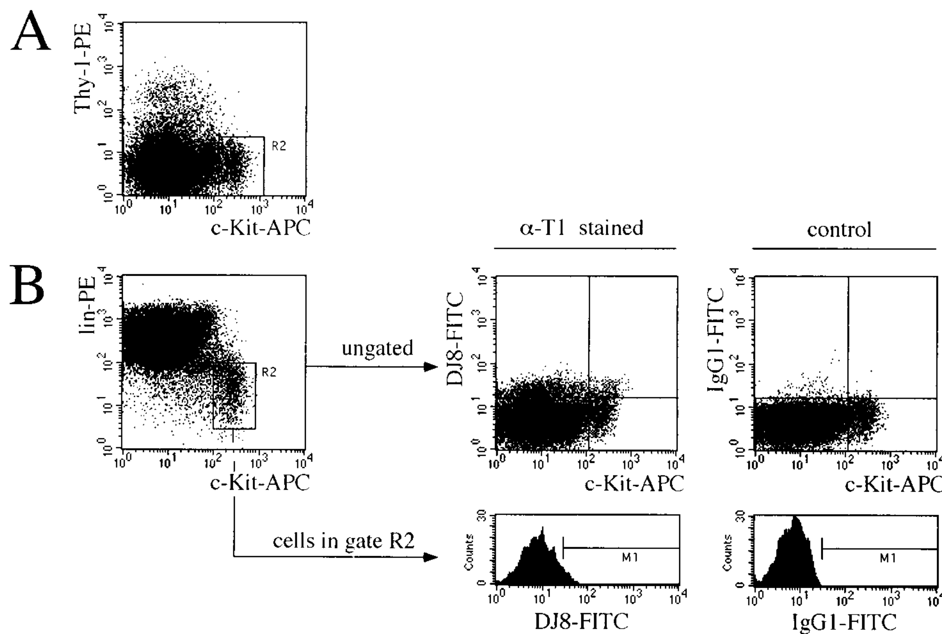

FIGURE 8. A population analogous to FB Thy-1^{low}c-Kit^{high} pro-MC population is not detectable in adult BM. *A*, BM cells of C57BL/6 mice were double stained with Thy-1-PE and c-Kit-biotin/Sac-APC and analyzed by two-color dot plot. *B*, BM suspensions were tricolor stained with a mixture of PE-conjugated mAbs specific for hemopoietic lineages (lin-PE: B220-PE, Thy-1-PE, TER-119-PE, Mac-1-PE), c-Kit-biotin/Sav-APC, and DJ8-FITC or IgG1-FITC. T1 expression was either analyzed by two-color dot plot on ungated cells (*top panels*) or by histogram analysis after gating on the c-Kit^{+}lin^{-} population (*bottom panels*).

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macrophages, and erythroid cells, we stained BM cells with a combination of different lineage-specific markers. This resulted in two clearly separable populations, the lin^+c-Kit^- and the lin^-c-Kit^+ cells. Triple-color analysis and gating on the lin^-c-Kit^+ cells clearly revealed that T1 is not expressed in this population at levels comparable with FB promastocytes. From this result, we conclude that the adult BM might not contain a pro-MC precursor that can be defined on the basis of the simultaneous expression of c-Kit and T1.

Discussion

The objective of this study was to gain insights into the biologic importance of the T1 receptor within the hemopoietic system. To this end, we determined which hemopoietic cells express T1 and found that the MC lineage were the only identifiable cells that consistently expressed the T1 Ag at high levels at different stages of their ontogeny. Immature c-$Kit^+$$IgE^+$ BMCMCs that were derived in vitro by cultivating murine BM cells in the presence of IL-3 (37) expressed highest levels of T1 when compared with T1-expressing cell lines or T1 expression in primary lymphoid organs (Figs. 1 and 2). Our results are consistent with the presence of *T1* mRNA in BMCMCs (10) and in several established MC lines (8, 10). In primary BM cultures, T1 expression, in combination with c-Kit, was shown to be instrumental for studying the kinetics of BMCMC appearance (Fig. 5). Interestingly, we detected no $T1^+$c-Kit^+ cells, i.e., a phenotype consistent with that of immature BMCMCs, in freshly isolated BM cells (see below). However, 6 days after cultivation in IL-3-containing medium, we observed a small population of $T1^+$c-Kit^+ cells that were identifiable as BMCMCs by morphology. This population continuously expanded over time until, at 4 wk, the culture homogeneously consisted of $T1^+$c-Kit^+ BMCMCs. To address the in vivo relevance of this finding, we tested T1 expression in freshly isolated IPMCs and found that all mature c-$Kit^+$$IgE^+$ IPMCs also uniformly express high levels of T1 in vivo.

BMCMCs resemble immature MCs that exhibit some characteristics of MMCs. However, it is important to note that cultured BMCMCs have the potential to differentiate into both MC types, depending on the local microenvironment (38). Whereas T cell-derived factors such as IL-3 and IL-4 promote the development of MMCs, the presence of fibroblast-derived SCF induces the maturation of connective tissue-type MCs (38). IPMCs represent an example of the latter cell type (39). Our finding that T1 is expressed in both mature IPMCs as well as immature BMCMCs precursors suggests that T1 expression is a general characteristic of MCs.

An interesting aspect of our study is the expression of T1 in the earliest committed MC progenitors, which have recently been identified in FB as cells that express low levels of Thy-1 and high levels of c-Kit (Thy-1^{low}c-Kit^{high}). FB pro-MCs already contain metachromatic granules, but do not yet express the FcεRI (28). From this, we conclude that T1 precedes FcεRI expression in MC ontogeny. This fact makes T1 an interesting novel surface Ag for the identification of MC progenitors. It is thought that MC precursors exist in the BM of adult mice, peripheral blood, and fetal liver (26, 40, 41). However, a morphologically defined MC precursor has not yet been purified from these sites (28, 36, 42). Our attempts to utilize T1 expression as a surrogate marker for the identification of a putative $T1^+$ MC precursor population in the adult BM were unsuccessful. Neither freshly isolated, unfractionated BM cells nor the c-Kit-expressing population that was devoid of cells belonging to mature hemopoietic lineages (c-$Kit^+$$lin^-$) did contain a cell population expressing substantial amounts of T1. In agreement with our findings, other authors were also unsuccessful in isolating committed MC progenitors in the BM. Lantz and Huff (43) reported the immunomagnetic purification of c-$Kit^+$$lin^-$ BM cells and showed that this population did not contain cells that either expressed FcεRI or showed metachromatic granules. This c-$Kit^+$$lin^-$$Fc\epsilon RI^-$ metachromatic $granule^-$ population was shown to be highly enriched for CFU-mast, i.e., cells that proliferated in response to SCF/IL-3 and gave rise to immature granulated MCs. However, since c-$Kit^+$$lin^-$ BM cells include HSC, these experiments could not resolve the question as to whether the MC colony-forming progenitor in the BM is a multipotent cell type, or a MC lineage-committed cell, or a mixture of both. Within the CFU mast-enriched, c-$Kit^+$$lin^-$ population, a subpopulation of granulated cells, a phenotype consistent with that of the MC precursors found in FB (28), has not yet been identified.

These results, in combination with our own data, suggest that a granulated FB-like MC precursor with a c-$Kit^+$$T1^+$ phenotype may not be found in the BM. To explain this finding, we consider the following possibilities: 1) T1 might only be expressed on fetal, but not adult MC progenitors; 2) $T1^+$c-Kit^+ promastocytes might exist in the BM, but at frequencies too low to allow detection by flow cytometry; 3) the CFU-mast activity found in the BM (26) does not result from a FB-like committed pro-MC, but rather from a more primitive pluripotent HSC; and 4) a FB-like promastocyte does not reside in the BM, but circulates in the blood or is localized in other tissues. Taken together, we have established T1 as a novel marker for the MC lineage that is expressed during all stages of its ontogeny. This raises the question of the biologic function of T1 in MCs. Since T1 is expressed throughout MC development, we consider a role of T1 in the induction of MC differentiation unlikely. T1, as c-Kit (44), might function as a survival factor for MCs. We also consider a role of T1 as a modulator of MC effector functions. Interestingly, the cytoplasmic domain of T1 bears an amino acid sequence (IIYPRV) (7) that fits the consensus sequence I/VxYxxL/V (D.R.M., unpublished observation) of an immunoreceptor tyrosine-based inhibition motif that is found in the cytoplasmic portions of NK cell receptors (killer cell inhibitory receptors, KIRs) and members of the gp49 receptor family (45, 46). However, the possible roles of T1 discussed in this work remain speculative. Future studies, such as the targeted inactivation of the T1 gene in mice, will have to clarify the physiologic role of T1 in MC biology.

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