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*J Immunol* 2012; 188:1809-1818; Prepublished online 16 January 2012; doi: 10.4049/jimmunol.1101746
http://www.jimmunol.org/content/188/4/1809

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**Supplementary Material**
http://www.jimmunol.org/content/suppl/2012/01/17/jimmunol.1101746.DC1

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Role of Mast Cells and Basophils in IgE Responses and in Allergic Airway Hyperresponsiveness

Minoru Sawaguchi,* Shinya Tanaka,* Yuiko Nakatani,† Yasuyo Harada,‡ Kaori Mukai,‡ Yuko Matsunaga,§ Kenji Ishiwata,§ Keisuke Oboki,‖ Taku Kambayashi,¶ Naohiro Watanabe,* Hajime Karasuyama,‡ Susumu Nakae,** Hiromasa Inoue,§ and Masato Kubo*†

We established a diphtheria toxin (DT)-based conditional deletion system using I If enhancer elements previously shown to be specific for IL-4 production in mast cells (MCs) or basophils (Mas-TRECK and Bas-TRECK mice). DT treatment of Bas-TRECK mice resulted in specific deletion of basophils, whereas both MCs and basophils were deleted in Mas-TRECK mice. DT-treated Mas-TRECK mice had impaired passive cutaneous anaphylaxis, IgE-mediated passive systemic anaphylaxis, and IgE-mediated chronic allergic inflammation, whereas DT-treated Bas-TRECK mice had impaired IgE-mediated chronic allergic inflammation. Using these mice, we also sought to tease out the role of MCs and basophils in airway hyperresponsiveness (AHR). Although MC deletion resulted in a slight increase in basal Ag-specific IgE levels and significant increases in basal IgE levels, we found that this deletion markedly impaired the AHR effector phase and was accompanied by decreased histamine levels. By contrast, basophil deletion had no effect on the AHR effector phase or on IgE production induced by systemic OVA immunization. Our results, using these newly established Mas-TRECK and Bas-TRECK models, demonstrated an indispensable role for MCs as effector cells in AHR. The Journal of Immunology, 2012, 188: 1809–1818.

Mast cells (MCs) are derived from bone marrow-derived hematopoietic progenitor cells that migrate into all vascularized tissues where they complete their maturation. Tissue and peritoneal MCs are typically long lived and normally reside proximally to epithelia, blood vessels, nerves, airways, gastrointestinal tract, and mucus-producing glands, but they do not recirculate into other vascularized tissues (1–3). When triggered, MCs release a spectrum of biologically active compounds stored within their cytosolic granules, including biogenic amines (histamine, serotonin), cytokines, serglycin proteoglycans, and various MC-specific proteases (chymase, tryptase, MC carboxypeptidase). MCs are central to many aspects of IgE-mediated allergic pathologic responses, such as the passive cutaneous anaphylaxis (PCA) reaction and systemic anaphylaxis.

In contrast to MCs, which are tissue resident, basophils circulate in the blood, where they constitute <1% of peripheral blood leukocytes and are thought to have a short half-life of only a few days (4, 5). Basophils contain fewer, but larger, granules compared with MCs and share many features with them, including expression of FcεRI, secretion of Th2 cytokines, and release of histamine after activation. Despite these similarities, basophils differ with respect to granule content and the types of eicosanoids that they produce. Recent studies highlighted a unique role for basophils in allergic responses and in immune regulation (6–8). Basophils appear to be a major source of IL-4 in allergen- and helminth parasite-activated human PBMCs, as well as in corresponding murine models. Basophils also can be activated directly by cytokine protease-containing allergens and parasites to produce Th2 cytokines. Furthermore, basophils play a critical role in cutaneous and pulmonary late-phase allergic responses (9).

Several MC-deficient mouse strains rely on mutations in c-kit (W allele) or c-kit ligand, which result in null or dysfunctional forms of these molecules (10, 11). More recently, KItW_sh/W_sh mice have emerged as an MC-deficient mouse model with fewer MC-unrelated abnormalities. However, even with these mice, it is still essential to demonstrate that any differences observed in MC-deficient mice compared with wild-type controls can be rectified by knock-in experiments (12). In contrast to MCs, studies of basophil function have relied on in vivo depletion using anti-FceRIα (MAR-1), which is specific for FcεRI. This regimen can be used to eliminate basophils at particular time points (6, 13, 14). However, MAR-1 cross-linking of FcεRI can lead to basophil and MC activation, which might lead to unanticipated secondary effects. The Mctp8Cre BAC Tg mouse was recently reported to be another tool...
(8), and this genetic system selectively eliminates basophils without considerable secondary effects. A knock-in system of the diphtheria toxin receptor (DTR) gene into the Mcpt8 locus is another promising tool that was recently established (15). However, there is still a need for tools to precisely distinguish and investigate MC and basophil functions in the same experimental system.

IL-4 is a cytokine produced by both MC and basophil lineages (16–18). In addition, the importance of the myeloid lineage as an innate source of IL-4 was suggested by a series of studies using bicistronic IL-4 reporter (4get) knock-in mice (19). We previously demonstrated that MCs and basophils use different enhancer elements to regulate Il4 gene expression (20). Our data indicated that the intronic enhancer (IE) is a regulatory element for MC, whereas a 3’-4-kb fragment that contains the 3’ untranscribed region (3’UTR) and HS4 elements is a specific enhancer for basophils.

Using a combination of these enhancers and a DTR-based transgenic (Tg) system, termed toxin receptor-mediated conditional cell knockout (TRECK), we set out to establish a model system that could be used to distinguish MC and basophil functions. We attempted to establish mice selectively lacking basophils (Bas-TRECK) or MCs (Mas-TRECK). The TRECK system relies on the fact that the mouse DTR binds diphtheria toxin (DT) very poorly compared with the human receptor (21). Thus, Tg expression of the human DTR confers sensitivity to DT and permits specific in vivo ablation of DTR+ cells when DT is injected. The analysis of these mice demonstrated that Mas-TRECK mice deplete both MC and basophils, whereas Bas-TRECK mice primarily deplete basophils. We used this model to analyze MC and basophil functions in PCA and chronic allergic inflammation. We also describe an independent role for MCs and basophils in airway hyperresponsiveness (AHR).

Materials and Methods

Mice

HS4 GFP reporter Tg mice were described previously (20). Mice expressing the human DTR under the control of IE element (for Mas-TRECK) and 3’UTR element (for Bas-TRECK) in the Il4 gene locus were generated by a Tg strategy. The basic pIL-4 construct was made by insertion of the 5’ enhancer (~863 to ~5448; the start codon is defined as 0) and the IL-4 promoter from position ~64 to ~827. The human DTR fragment was isolated from the DTR/pMs7 vector (provided by Dr. M. Tanaka, Research Center for Allergy and Immunology, the Institute of Physical and Chemical Research) and inserted into the basic pIL-4 and human DTR construct, respectively. Each Tg line was generated on a C57BL/6 (B6) background. All mice used in this study were maintained under specific pathogen-free conditions. Animal care was conducted in accordance with guidelines of Research Center for Allergy and Immunology, Yokohama Institute.

Reagents and Abs

Anti–c-Kit (2B8), FcεRI (MAR-1), CD49b (DX5), CD4 (L3T4), NK1.1 (PK136), CD11c (HC3), Siglec-F (E50-2440) and -TCR (H57-597) Abs (all from BD Biosciences, San Jose, CA) and anti-B220 (RA3-6B2), CD8a (53-6.7), IgM (eB121-15F10) and -Gr1 (RB6-8C5) (eBioscience, San Diego, CA), and anti-CD200R (BA91) Abs (23) were used for FACS analysis. Anti-DNP IgE mAb (Sigma Aldrich, St Louis, MO) was used for activation of MCs. Serum cytokines were measured using the 23-Plex panel Bioplex cytokine assay system (Bio-Rad Laboratories, Hercules, CA). A histamine ELISA kit (Sibaura Electronics, Japan) was used for analysis of serum histamine concentrations.

PCA reaction

The PCA reaction was induced by anti-OVA IgE and OVA. The left ears of mice were injected s.c. with anti-OVA IgE (TOS-2) (2 μg/10 μl PBS), a gift from Dr. Mamoru Kiniwa (Taiho Pharma), and their right ears were injected with an equal volume of PBS using a microsyringe under light anesthesia. After 12 h, the mice were injected i.v. with 250 μg OVA in 0.5% Evans blue dye. After 30 min, the ears were extracted by immersion in a formamide solution, and the dye concentrations were measured with an Ultrospec 2100 pro (GE Healthcare, Buckinghamshire, U.K.) at OD 620 nm.

IgE-mediated anaphylaxis

Mice were injected i.v. with 50 μg anti-IgE mAb (6D5). Rectal temperature was measured every 10 min with a digital thermometer (Sibaura Electronics, Japan).

IgE-mediated chronic allergic inflammation

Ascites of the trinitrophenyl (TNP)-specific IgE mAb was prepared from the IGEIb4 hybridoma (24). Mice were sensitized with TNP-specific IgE mAb i.v. 1 d before Ag challenge. The left ears of mice were injected s.c. with TNP11-OVA (Biosearch Tech, Novato, CA) in 10 μl PBS, and their right ears were injected with an equal amount of OVA using a microsyringe. Ear thickness was measured with a dial thickness gauge caliper (G-1A; Ozaki, Tokyo, Japan).

Airway hyperresponsiveness

Mice were immunized by i.p. injection of OVA (10 μg, grade V; Sigma Aldrich) eight times at 2-d intervals (priming) for 2 wk. Twenty-six days after the last injection, mice were treated with aerosolized OVA three times (challenge). AHR was measured, as described previously (25). Briefly, the OVA-immunized mice were challenged with OVA by inhalation, and acetylcholine-dependent AHR was measured. Cellular content in the bronchoalveolar lavage (BAL) fluid was assessed by Wright–Giems staining and cell counting.

Papain-induced IL-4 production and basophil migration

OVA-specific TCR Tg DO11.10 mice were crossed with Bas-TRECK mice (Bas-TRECK DO11.10), and mice were injected in the footpad with PBS containing OVA (50 μg) and 50 μg papain (Sigma). After 4 d, CD4+ T cells were isolated from draining lymph nodes (dLNs) using an IMag magnetic bead system (BD Biosciences). The CD4+ T cells (1 × 106 cells) were restimulated with OVA (10 μg/ml) for 48 h in the presence of irradiated syngeneic spleen cells (5 × 106 cells), and IL-4 production was assessed by ELISA. HS4 GFP reporter Tg mice (20) were injected with OVA (50 μg) and papain (50 μg) in the footpad, and dLNs were harvested at day 4. Frozen sections were prepared for immunohistochemical staining with anti-GFP Alexa Fluor 488 (BD Bioscience), anti-B220–PE (RA3-1B2), anti-TCR (BD Bioscience), and avidin-Alexa Fluor 647 (Invitrogen, Carlsbad, CA).

Statistical analysis

All data are presented as mean and SEM, and statistical analysis was performed with the two-tailed Student t test. Differences were recognized as significant at p < 0.01.

Results

Establishment of the Mas-TRECK system

We previously showed that the IE in intron 2 of the Il4 gene and a 4-kb region downstream of exon 4, termed proximal 3’, are specific enhancers for MCs and basophils, respectively (20). Based on these findings, a DTR Tg mouse system was developed under the control of IEs that specifically regulate IL-4 expression in MCs, leading to MC deletion. This established system was designated Mas-TRECK (Fig. 1A). DTR mRNA was highly expressed in MCs but not in neutrophils, eosinophils, T and B cells, or dendritic cells (DCs) (Fig. 1B). In contrast to the result from GFP reporter mice, a low, but detectable, level of mRNA was found in DX5+CD200R3+, FcεRIα+ basophils.

Treatment of Mas-TRECK mice with DT i.p. completely eliminated peritoneal MCs (c-Kit+ and FcεRIα+), and Alcian blue stained connective tissue MCs in the skin (Fig. 1C). To determine the optimal frequency of DT injections for MC depletion, mice were injected one, three, or five times with DT (250 ng), and MCs were found to be completely deleted after five injections in all MC-resident tissues, such as peritoneal cavity, skin, stomach, and
mesentery (Fig. 1D). We further examined whether this schedule of DT treatment had any effect on other cell types and found no significant deletion of neutrophils, eosinophils, T and B cells, or DCs. However, this treatment severely affected basophils because they express low levels of DTR (Fig. 1B, 1E). Even such a low level of DTR expression was adequate to accomplish complete deletion of basophils, because a single molecule of DT is sufficient to kill a mammalian cell (26). The proximal 3' enhancer inserted downstream of the Il4 promoter and a DTR cDNA, and this system was designated Bas-TRECK (Fig. 2A). We generated three independent lines (lines 1–3) of Bas-TRECK mice. Intraperitoneal injection with DT (500 ng/mouse) resulted in complete deletion of DXS* FcεRIα+ CD200R3* basophils both in bone marrow and in the peripheral blood of lines 1 and 3 but not line 2 (Fig. 2B, 2C). However, lines 1 and 2 showed unexpected deletion of peritoneal MCs (PMCs), indicating that line 3 was most useful for further experiments (Fig. 2D). We next examined expression of DTR mRNA among various cell types isolated from bone marrow of line 3. There was no effect on MCs, neutrophils, eosinophils, or T cell populations in DT-treated line 3 mice (Fig. 2F–H).

**FIGURE 1.** Deletion of MCs and basophils after DT treatment in Mas-TRECK mice. A. The mouse Il4 locus, cis-acting elements, and structure of the transgene used to generate Mas-TRECK mice. B. PMCs, basophils, neutrophils, and eosinophils were prepared from bone marrow cells, and CD4+ T cells, NK cells (NK1.1+TCR-β+), NKT cells (NK1.1+TCR-γδ+), CD8+ T cells (CD8+), B cells (B220+IgM+), and DCs (CD11c+IAb) were prepared from spleen of B6 and Mas-TRECK mice, and the expression of DTR mRNA was measured by RT-PCR. C. B6 and Mas-TRECK mice were injected i.p. with 250 ng of DT for 5 consecutive days. Three days after the last injection, MCs from the peritoneal cavity (PMCs; c-Kit+FceRIα+) were detected by flow cytometry (upper panel). MCs in ear skin were detected by Alcian blue and nuclear fast red dye staining of Carnoy’s solution-fixed sections (lower panel). Arrows indicate MCs. Original magnification ×100. D. Mas-TRECK mice were injected i.p. with 250 ng of DT for 3 or 5 consecutive days. Three days after the last injection, numbers of PMC and MC in mesentery, back skin, ear skin, and stomach (mucosal and submucosal) were counted using flow cytometry or Alcian blue dye staining, as described in C, respectively. MC numbers in these tissues are represented as counts/mm². E. Percentage deletion was calculated as the cell number observed in DT-treated Mas-TRECK mice and B6 mice treated five times with DT. PMCs, basophils, neutrophils, and eosinophils were prepared from bone marrow cells, and CD4+ T cells, NK cells, NKT cells, CD8+ T cells, B cells, and DCs were prepared from spleen. Data in C are representative of three independent experiments; data in B, D, and E are the mean and SEM of three independent experiments.
FIGURE 2. Deletion of basophils after DT treatment in Bas-TRECK mice. A, The mouse Il4 locus, cis-acting elements, and structure of the transgene used to generate Bas-TRECK mice. B, B6 and Bas-TRECK (line 3) mice were injected i.p. once with 500 ng of DT. The following day, basophils (DX5<sup>+</sup>CD200R3<sup>+</sup> FcεRIα<sup>+</sup>) were analyzed both in bone marrow (BM) and peripheral blood (PB). C, B6 mice and the three Bas-TRECK Tg lines (1–3) were treated with DT, as described in B, and basophils (DX5<sup>+</sup>CD200R3<sup>+</sup> FcεRIα<sup>+</sup>) in bone marrow (BM) were analyzed by flow cytometry. Percentage (left panel) and cell number (right panel) of DX5<sup>+</sup>CD200R3<sup>+</sup> FcεRIα<sup>+</sup> cells. D, Bone marrow-derived basophils (Baso; DX5<sup>+</sup>CD200R3<sup>+</sup> FcεRIα<sup>+</sup>) and PMCs (c-Kit<sup>-</sup>FcεRIα<sup>+</sup>) were obtained from the three Bas-TRECK Tg lines (1–3); percentage deletion was calculated against the number of basophils and PMCs in B6 mice. E, PMCs, basophils, eosinophils (Gr-1<sup>+</sup>Siglec-F<sup>+</sup>), and neutrophils (Gr-1<sup>+</sup>Siglec-F<sup>+</sup>) in bone marrow, as well as CD4<sup>+</sup> and CD8<sup>+</sup> T cells, B cells, and DCs in spleen were isolated from line 3 Tg mice. Expression of DTR mRNA was measured by RT-PCR. F and G, B6 and Bas-TRECK Tg mice (line 3) were treated with DT, as described in A. The following day, basophil deletion was monitored by histology (F) and flow cytometry (G). MCs in ear and back skin were detected by toluidine blue staining of Carnoy’s solution-fixed sections (upper panels in F). Original magnification ×100. Numbers of toluidine blue-stained cells in ear and back skin are represented as counts/mm<sup>2</sup> (lower panels in F). Data are representative of three independent experiments. H, Percentage deletion of the indicated cell types, which was calculated from the cell number in the DT-treated Bas-TRECK (line 3) versus B6 mice. Data in C–F and H are mean and SEM from three independent mice.

We also examined the effect of DT treatment on Th1/Th2 differentiation and natural helper cells in Mas-TRECK and Bas-TRECK mice. The lymphoid fraction was isolated from adipose tissue of DT-treated Mas-TRECK and Bas-TRECK mice; both lines showed a comparable proportion of natural helper cells (c-Kit<sup>+</sup> CD25<sup>+</sup> lymphoid cells) (Supplemental Fig. 1A). Moreover, DT treatment did not have any effect on development of Th1/Th2 cells derived from splenic CD4<sup>+</sup> T cells of Mas-TRECK and Bas-TRECK mice (Supplemental Fig. 1B). These results again excluded the possibility of nonspecific deletion in the Mas-TRECK and Bas-TRECK models.

To further confirm specific deletion of MCs and basophils, c-Kit<sup>+</sup> PMCs and DX5<sup>+</sup> bone marrow basophils from Mas-TRECK and Bas-TRECK mice were treated with DT in vitro. Cytokine production was then measured after stimulation by either IgE cross-linking or pharmacological activation (PMA plus ionomycin; data not shown). PMCs and basophils isolated from Mas-TRECK mice failed to produce IL-4. In Bas-TRECK mice, basophils, but not PMCs, showed a similar complete loss of IL-4 production, although PMCs from Bas-TRECK mice exhibit a slight decrease compared with control PMCs from B6 mice (Fig. 3A).

MCs maintain basal histamine levels in serum

In the TRECK system, DT exerts its cytotoxic effect by ADP ribosylating and inactivating eukaryotic elongation factor-2, leading to cell disruption. Thus, we asked whether DT treatment resulted in the release of inflammatory cytokines and chemical mediators stored in the disrupted MCs. To examine this possibility, we measured serum levels of cytokines (IL-1α, IL-1β, TNF-α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, and IFN-γ) and chemokines (oxatokin, RANTES, MIP-1α, MIP-1β, and MCP-1), either before or after DT treatment. DT treatment had no effect on B6 mice, and the profiles of both Mas-TRECK and Bas-TRECK mice were quite similar to the control mice (Fig. 3B), demonstrating that DT-induced cell disruption did not alter serum cytokine levels. In contrast, the deletion of MCs, but not basophils, resulted in a remarkable reduction in serum histamine levels in the steady state (Fig. 3C). A similar reduction was observed in Kit<sup>W_sh/W_sh</sup> mice, which are genetically deficient in MCs. These results demonstrated a requirement for MCs in the steady-state maintenance of histamine levels.

The role of MCs and basophils in the IgE-mediated allergic response

We next examined the IgE-mediated PCA reaction and IgE-mediated passive systemic anaphylaxis, which are MC-mediated allergic responses (27–29). After DT treatment, Mas-TRECK and Bas-TRECK mice were injected s.c. with anti-OVA IgE Ab. After 24 h, soluble OVA in Evans blue dye solution was injected i.v. The resulting increased vascular permeability was assessed by...
measurement of dye leakage at the challenge site. Although the control B6 and Bas-TRECK mice displayed a clear PCA reaction, Mas-TRECK mice showed no signs of dye leakage in the ear (Fig. 4A). To examine IgE-mediated passive systemic anaphylaxis, Mas-TRECK and Bas-TRECK mice were injected i.v. with anti-IgE mAb after the DT administration. In B6 and Bas-TRECK mice, this treatment induced a sudden decrease in body temperature due to anaphylactic shock, whereas the body temperature of Mas-TRECK mice remained unperturbed (Fig. 4B).

Next, we studied IgE-mediated chronic allergic inflammation (IgE-CAI), which is reported to be a basophil-mediated allergic response. DT-treated B6, Mas-TRECK, and Bas-TRECK mice were injected i.v. with TNP-specific IgE, challenged s.c. with TNP-OVA the next day, and monitored for ear thickness changes over 7 d. We detected a marked increase in ear thickness in B6 mice at day 2 after TNP-OVA challenge; however, Mas-TRECK and Bas-TRECK mice showed no increase (Fig. 4C). These results demonstrated that basophils are indispensable for IgE-CAI. Taken together, the Mas-TRECK and Bas-TRECK systems are useful tools to distinguish the in vivo roles of MCs and basophils.

**The essential role of MCs in AHR**

The role of MCs and basophils in the OVA-sensitized bronchial asthma model remains unclear, because few asthma model studies using conventional MC-deficient Kit<sup>W<sub>sh</sub>/W<sub>sh</sub></sup> mice reported the airway response (30). To examine this issue, we first studied the role of MCs and basophils in Mas-TRECK mice, because both cell types are deleted (Fig. 1). We found that the MC deletion was sustained for >18 d after five daily injections of DT (data not shown). The Mas-TRECK mice were treated with DT prior to either OVA immunization (priming) or aerosolized OVA treatment (challenge). After systemic immunization and aerosolized challenge with OVA, airway responsiveness was induced by the administration of acetylcholine aerosol. Control B6 mice and Mas-TRECK mice treated with DT before priming displayed AHR to acetylcholine, whereas DT treatment of Mas-TRECK mice before the aerosolized OVA challenge resulted in diminished AHR (Fig. 5A). DT treatment in the challenge phase had no significant effect on the number of eosinophils or the total number of infiltrating cells of all types in BAL fluid or on OVA-specific IgE levels in serum (Fig. 5B). However, these mice showed a remarkable reduction in serum histamine levels (Fig. 5C), and Ag-stimulated BAL-derived cells produced significantly less histamine (Fig. 5D). Interestingly, IL-4, but not other Th2 cytokines (IL-5 and IL-13), was also significantly reduced (Fig. 5D). A recent report indicated the importance of TNF secreted from MCs in AHR; however, TNF from the BAL-derived cells remained at low levels in both control and Mas-TRECK mice.

The Mas-TRECK system left open the possible involvement of both MCs and basophils in AHR. To distinguish the role of these two cell types, Bas-TRECK mice were treated with DT (500 ng/mouse) five times at 3-d intervals prior to and during the challenge phase. In contrast to Mas-TRECK mice, DT treatment of Bas-TRECK mice before the aerosolized OVA challenge had no effect on AHR-related outcomes, such as increased airway pressure, eosinophil attraction into BAL, and OVA-specific IgE production (Fig. 6A). These data suggested that histamine released from the MCs has an important role in the asthmatic response, especially in the challenge phase. MCs and basophils were both dispensable for the Ag-specific IgE production induced by systemic immunization with soluble Ag (Fig. 6A).

**Role of basophils in the IgE response and IL-4 production**

Basophils were recently suggested to be the primary IL-4 source for the induction of Th2 responses (14). Therefore, basophils would seem to have a role in IgE responses, because IL-4 is a critical cytokine to regulate IgE class switching in B cells. We further examined the role of basophils in the development of a systemic...
Basophils have been the focus of a papain-based immunization model that mimics parasite-derived protease activity and could induce IL-4 and MHC class II expression by basophils (6, 7). However, the role of basophils is still controversial. We generated DO11.10 TCR Tg mice on the Bas-TRECK background to explore the requirement for basophils in papain-mediated IL-4 production. After DT treatment, Bas-TRECK DO11.10 Tg mice were immunized in the footpad with soluble OVA together with papain; CD4+ T cells were isolated from the dLNs and restimulated with OVA peptide in the presence of APCs. In control DO11.10 mice, co-administration of papain and OVA induced significant levels of IL-4 production by lymph node T cells, whereas injection of soluble OVA alone failed to induce IL-4 (Fig. 6C). IL-4 production by DO11.10 cells in Bas-TRECK mice was significantly reduced (∼30%) compared with control DO11.10 mice. Thus, basophils appear to contribute partially to the Th2 response to protease-type allergens.

We next asked whether the papain treatment promoted migration of basophils into the T cell areas of the dLN. The localization of basophils was visualized using HS4 GFP Tg mice, in which basophils selectively express GFP (20). The GFP Tg mice were sensitized by injection of soluble OVA, with or without papain, into the footpad, and basophil migration was assessed by GFP localization. The basophils preferentially migrated into the T cell, but not the B cell, area of lymph nodes, and this was observed in papain-treated mice but not in untreated mice (Fig. 6D). These results indicated that basophil numbers increased in the dLNs when mice were treated with papain.

Deletion of MCs increases serum IgE levels

In Fig. 5B, MC deletion during immunization with soluble OVA appeared to cause enhanced IgE production. Therefore, we further examined the role of MCs and basophils in the immune response to OVA conjugated with alum adjuvant. Surprisingly, the initial DT treatment prior to OVA immunization resulted in elevation of basal IgE levels, such that they were already 10-fold higher than in the untreated Mas-TRECK mice (Fig. 7). In B6 mice, the basal IgE level was generally undetectable. The total IgE levels increased after immunization, and the mice also consistently showed a slight, but significant, increase in OVA-specific IgE. This increase was not due to a secondary effect of DT treatment, because the DT-treated B6 mice showed no change, and OVA-specific IgG1 and IgG2c levels in the DT-treated Mas-TRECK mice were comparable to those in the control B6 mice (Fig. 7). Therefore, our results suggested that IgE exists as a natural Ab that is continuously bound to FcεRI on tissue-type MCs residing in epithelia, blood vessels, gastrointestinal tract, and mucus-producing glands. Following MC depletion by DT treatment, this “sink” for soluble IgE no longer exists, and the level of serum IgE would, therefore, increase.

Discussion

We generated conditional-deletion systems, Mas-TRECK and Bas-TRECK, using MC- and basophil-specific enhancers of Il4 gene transcription. Based on previous results using adoptive transfer of bone marrow-derived MCs into Kitw-sh/Kitw-sh mice, the PCA reaction and the IgE-induced anaphylactic response were reported to be MC dependent (30). PCA is an acute cutaneous allergic response and s.c. allergen sensitization also elicits a delayed-type response manifested by ear swelling. The entire response consists of three phases: immediate, late, and chronic. The third-phase ear swelling response is defined as IgE-CAI and is mainly regulated by basophils (9). Furthermore, our studies using Mas-TRECK mice demonstrated that MCs regulate IgE-mediated anaphylactic
shock (31, 32). The combination of Mas-TRECK and Bas-TRECK systems allowed us to clearly distinguish the in vivo functions of MCs and basophils. Genetically MC-deficient mice, such as \( \text{Kit}^{W/W_v} \) and \( \text{Kit}^{W_{sh/W_{sh}}} \), are considered the best animal model in which to study MC function. Using these mice, a series of studies showed that MCs play an important role in the airway response (30, 33–36). Although some studies showed that MC deficiency resulted in attenuated eosinophil-mediated airway inflammation (34, 37), other studies indicated a lack of MC participation in AHR (38–40). These conflicting roles for MCs may be due to experimental variables, such as the inclusion or omission of artificial adjuvants, or the use of relatively low doses of Ag for sensitization (30, 33, 34, 36). In addition, MC-deficient mice have a variety of other abnormalities in several cell types. This is true, even in the \( \text{Kit}^{W_{sh/W_{sh}}} \) mouse, although the non-MC abnormalities are less severe than in \( \text{Kit}^{W/W_v} \) mice (41, 42). The TRECK systems that we established in this study were based on the Tg expression of DTR controlled by MC- and basophil-specific Il4 gene regulation. Some lines showed unexpected deletion because of difference in the copy number and the integration site of transgene. However, the Mas-TRECK and Bas-TRECK lines that we chose for this work had consistent specificity in DT-based deletion. Therefore, the TRECK system has the important advantage of minimizing potential confounding factors.

The Mas-TRECK system provides clear evidence that MCs are essential for the effector phase of AHR in a nonadjuvant immunization protocol but that these cells are not required for the regulation of eosinophil infiltration. These findings are consistent with the observation that nonadjuvant OVA-induced AHR was significantly reduced in MC-deficient \( \text{Kit}^{W_{sh/W_{sh}}} \) mice (43). The observed reduction in effector functions in AHR may be due to loss of cytokines and chemical mediators from MCs, a possibility supported by the striking decrease in serum histamine levels observed in Mas-TRECK mice. Reconstitution with bone marrow-derived MCs from \( \text{Tnf}^{2/2} \) mice failed to rescue the AHR in
FIGURE 6. AHR in Bas-TRECK mice treated with DT. A, Left and top panels, B6 (○) and Bas-TRECK mice (●) were immunized by i.p. injection of soluble OVA at 2-d intervals for 2 wk and challenged with OVA three times (black arrows). The unprimed B6 (▲) and OVA-immunized mice (○, ●) were challenged with OVA by inhalation, and acetylcholine-dependent AHR was measured. DT treatment was carried out before challenge, as indicated by red arrows. Middle panel, Cell number of total, macrophages (MΦ), neutrophils (Neu), lymphocytes (Lym), and eosinophils (Eos) in the BAL fluid was assessed by Wright–Giemsa staining and cell counting. Right panel, Serum was obtained at day 51 from immunized or unimmunized B6 and Bas-TRECK mice that were treated with DT in the challenge phase. OVA-specific IgE titers were measured by ELISA. B, Bas-TRECK mice were treated with DT (500 ng) at 3-d intervals at the time points indicated by the red arrows (left panel). Mice were immunized with OVA in alum on days 2 and 9 and boosted with OVA in PBS on day 16 (arrows indicate the times of immunization). Blood samples were collected on days 0, 1, 6, 13, and 20. Serum Ig levels were measured by ELISA (middle and right panels). C, DO11.10 Tg mice and DO11.10 Tg mice on a Bas-TRECK background (Bas-TRECK DO11.10) were treated with DT (500 ng) i.v. The following day, mice were immunized s.c. in the footpad with 50 μg of OVA in the presence or (Figure legend continues)
**Figure 7.** IgE production in Mas-TRECK mice treated with DT. B6 and Mas-TRECK mice were injected with DT (250 ng) five times and then treated with DT (100 ng) at 7-d intervals at the time points indicated (top panel). Mice were immunized with OVA in alum on days 7 and 14 and boosted with OVA in PBS on day 21 (†). Blood samples were collected on days 0, 5, 12, 19, and 26. Serum Ig levels were measured by ELISA (middle and bottom panels). Data represent mean and SEM of 10 individual mice. *p ≤ 0.05, **p ≤ 0.01.

Kit<sup>W-sh/W-sh</sup> mice (43). In Mas-TRECK mice, the deletion of MCs resulted in the loss of AHR, accompanied by a remarkable reduction in histamine levels. This correlation may be causative, because a defect in AHR was observed in histidine decarboxylase gene-targeted mice, which lack histamine (44), as well as in mice treated with a histamine receptor antagonist (45, 46). Therefore, it seems reasonable to conclude that the reduction in histamine levels may be a direct cause of the resistance to AHR observed in Mas-TRECK mice, although previous studies suggested that the histamine receptor antagonist also indirectly inhibited the Th2 response (45, 47).

Although basophils have been viewed as having functions similar to MCs, recent studies highlighted their unique functions in allergic responses. IL-4 production from a non-T cell source was shown to be necessary in the generation of functional Th2-mediated immunity (7, 14, 48). Basophils were shown to constitutively express MHC class II and costimulatory molecules, such as CD40, CD80, and CD86, and can be directly involved in Ag presentation to Th cells. After immunization with protease allergens, basophils are an initial source of IL-4 to dictate Th2 differentiation. Our data from this study are consistent, in that the complete deletion of basophils significantly reduced IL-4 production by T cells after immunization with a protease allergen, papain. However, T cells still express detectable levels of IL-4 in the absence of basophils, indicating the importance of other IL-4 sources. This finding is consistent with the recently described Mcpt8Cre BAC Tg system, in which basophils were shown to be dispensable for papain-induced Th2 differentiation (8). Moreover, the complete deletion of basophils failed to affect the IgE response induced by alum-based immunization. Our observations are inconsistent with a recent report using the MAR-1 Ab, which indicated that basophils are required for IgE production in the immune response to *T. muris* (14). This inconsistency might be due to the different experimental systems used in the two studies. Nevertheless, we propose that basophils probably contribute to very restricted and specialized Th2 responses, such as IL-4 production from T cells in response to protease-type allergens (e.g., papain). However, basophil functions are dispensable for the Ag-induced systemic IgE responses.

Interestingly, conditional deletion of MC caused an increase in basal IgE levels in serum, and this increase in IgE was also observed in an Ag-specific response. Serum IgE is undetectable in normal mice and Kit<sup>W-sh/W-sh</sup> mice, which constitutively lack MCs (data not shown). IgE alone, in the absence of Ag, on binding to FcεRI can enhance its surface expression though induction of signaling events that enhance stability of FcεRI (49) and also extend the survival of MCs in vitro and in vivo (50). Therefore, IgE Ab is apparently constantly generated as a natural Ab in the blood like other isotypes, but most of the IgE immediately binds to FcεRI expressed on the tissue-type MCs. This IgE binding to MCs may provide innate protection against pathogen invasion. The enhancement of IgE was also observed in an Ag-specific response; thus, deletion of MCs also influenced the systemic IgE response. This was also explained by an enhanced Th2 response including increased IL-4; however, MC deletion did not have any impact on total and specific IgG1 production (Fig. 7) or on Th2 development (Supplemental Fig. 1), indicating that this is not due to the effect of more IL-4 expression.

In conclusion, the combination of Mas-TRECK and Bas-TRECK mouse models provides a new approach to issues in al-
lergy that have previously been difficult to study experimentally; these systems eventually may allow us to understand the mechanisms underlying different types of allergic responses. We demonstrated in this study that MCs play essential roles in the effector phase of the asthmatic response. This newly established system should accelerate development of new therapeutic strategies for allergic asthma.

Acknowledgments

We thank Dr. Kiniwa (Taiho Pharma) for providing anti-OVA IgE (TO-25) and H. Fujimoto, Y. Hachiman, E. Hayashi, K. Ikari, and S. Law for technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References


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Supplemental Figure 1

B6 and Mas-TRECK mice were injected intraperitoneally with 250 ng of DT for 5 consecutive days, and Bas-TRECK mice were treated with 500 ng of DT. The lymphocytes fraction was obtained from adipose tissue in the peritoneal cavity of DT-treated B6, Mas-TRECK, and Bas-TRECK mice, and c-kit+CD25+ cells were analyzed as “Natural helper” cells. (B) CD4 T cells from DT-tREATED B6, Mas-TRECK, and Bas-TRECK mice were stimulated with anti-TCR mAb and anti-CD28 mAb under Th1 and Th2 skewing conditions, and the cells were then re-stimulated with anti-TCR mAb after 7 days. (A) and (B) are representative of two independent mice.