A

B

Figure 6. Immunoblotting and immunohistochemical analyses of PG synthase proteins in basophils and mast cells. **A:** Cell lysates were prepared from BMBAs (CD49b^{+} fraction of bone marrow cells cultured with rIL-3 for 10 days) and BMMCs at various stages of their development (c-kit^{+} fractions of bone marrow cells cultured with rIL-3 for 10, 20, or 30 days) and were subjected to immunoblotting with the indicated antibodies. Actin was used as a loading control. **B:** Cytospin slides prepared from bone marrow CD49b^{+} cells (primary basophils) were stained with Alexa 488-conjugated anti-mMCP-8 mAb and Alexa 647-conjugated anti-H-PGDS mAb or Alexa 647-conjugated anti-mPGES1 mAb. Intracellular H-PGDS and mPGES1 proteins were detected in mMCP-8 (+) cells. Phase contrast images are at left.

was consistent with the findings that levels of H-PGDS and L-PGDS expression were higher in BMMCs than in BMBAs (Figure 5, A and B).

The class of PGES expressed also varies according to cell type.[35–37] Expression of mPGES1 is markedly induced by pro-inflammatory stimulation in various tissues.[35] PGES1 is involved in the COX-2–mediated PGE2-biosynthetic pathway[35]; mPGES2 is abundant in brain, heart, skeletal muscle, kidney, and liver[37]; cPGES is distributed ubiquitously in the cytosol of various cells[36]; and COX-1 contributes to PGE2 generation by PGES2 and cPGES.[36,39,40] We immunohistochemically confirmed the presence of mPGES1 in primary basophils, but we were unable to assess the protein expression of the other PGES enzymes, because antibodies against mPGES2 and cPGES were not available in our laboratory. Nevertheless, transcripts encoding mPGES1, mPGES2, and cPGES were all detected in BMBAs. The subtype of PGES that is actually involved in PGE2 generation in basophils is uncertain. However, a COX-1–coupled PGES, such as mPGES2 or cPGES, may mediate immediate PGE2 production. In mast cells, COX-1, but not COX-2, is responsible for immediate PGD2 production after cell stimulation with IgE + Ag.[41]

In contrast to the high production of PGD2 from BMMCs, PGE2 generation from BMMCs was low, despite that transcripts of all of the genes that encode PGES were detected. IgE-mediated signals may not provide an effi-

cient stimulus for induction of mast cell-derived PGE2 production.

Basophils promote the initiation of IgE-CAI associated with marked eosinophil infiltration.[18] Treatment with a basophil-depleting Abs prevents the development of IgE-CAI,[42] indicating the essential role of basophils in this reaction. Our prior study showed that the number of infiltrative basophils in skin lesions of IgE-CAI was higher than that of dermal mast cells (19.4 cells/mm^2 versus 8.2 cells/mm^2).[31] Thus, basophils may be equally or even more important than mast cells as sources of PGD2 production in IgE-CAI. PGD2 is one of the important mediators responsible for the development of IgE-CAI as evidenced by unpublished observation (Y. Matsushima) that the administration of an antagonist against the PGD2 receptor CRTH2 ameliorated skin inflammation.

Although stimulation of the IgG receptors induces PAF release from basophils,[19] in the current study these stimulations did not result in PGD2/E2 synthesis by basophils. Although we have not been able to assess actual differences in the signals mediated by IgE and IgG in basophils, FcγR signals, unlike FcεRI signals, may preferentially activate the PAF synthesis and the 5-liooxygenase pathway rather than the COX-PG synthesis pathway.

In this study, we confirmed that human basophils are also capable of producing PGD2. This result was in striking contrast to a prior study which reported that basophils do not produce PGs.[27] This discrepancy could be due to differences in preparation of the basophil samples or in the assay systems or cell priming methods. The previous study isolated basophils with the use of the method by MacGlanshan et al,[43] and cell purity ranged from 64% to 92%. Some of these cells might have been non-releasing basophils, because basophils from 10% to 20% of human peripheral blood donors show defects in histamine release.[44] There have also been previous attempts to detect PG expression with the use of radioimmunoassay and gas chromatography mass spectrometry which gave negative results.[27] In the present study, we only prepared highly enriched basophils from volunteers who had histamine-releasing basophils on the basis of the results of a
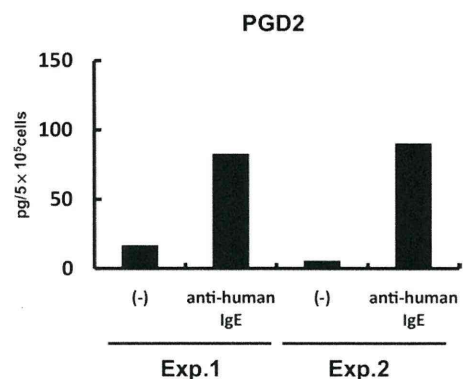

Figure 7. Human blood basophils also secrete PGD2 in response to IgE-mediated stimulation. Human blood basophils obtained from the venous blood of healthy donors were primed with 10 ng/mL human IL-3 and then stimulated with 1 μg/mL anti-human IgE Ab for 30 minutes. The concentration of PGD2 and PGE2 in the supernatant fluids was determined with EIA. Human blood basophils produced PGD2 after IgE-mediated stimulation. The results of two separate experiments (exp. 1 and exp. 2) are shown.

basophil activation test (see *Materials and Methods*). Furthermore, these basophils were primed with IL-3, because IL-3 has been shown to induce phosphorylation of phospholipase A_2.[28] In addition, in our study, PGs were detected with EIA, which is a more sensitive detection system than radioimmunoassay and gas chromatography mass spectrometry. These combined approaches may have been the reason why our study, in contrast to earlier studies, could successfully detect PG expression in basophils.

Unlike mouse basophils, human basophils did not generate a large amount of PGE2. More intense stimulation than anti-human IgE might be required for the induction of PGE2 release. Alternatively, arachidonic acid metabolite cascades in human basophils may be different from those of mice. Thus, human basophils may favor the H-PGDS pathway over the PGES pathway.

IgE-CAI in mice appears to share morphologic similarities with some of the skin lesions of atopic dermatitis, long-lasting urticaria, and/or prurigo reaction in humans. Our prior study suggested that there was a possible involvement of the PGD2-CRTH2 interaction in these diseases.[45] Most notably, the skin lesions found in these diseases are histologically characterized by a marked basophil infiltration.[46] Thus, basophil-derived PGD2 may at least partly contribute to the pathogenesis of these inflammatory skin reactions.

In summary, the present study showed an overlooked function of basophils: the production of PGD2 and PGE2 through FcεRI-mediated signaling. Basophils may be involved in IgE-mediated allergic inflammation and immune responses via the release of PGD2 and/or PGE2.

Acknowledgment

References

1. Narumiya S, Sugimoto Y, Ushikubi F: Prostanoid receptors: structures, properties, and functions. Physiol Rev 1999, 79:1193–1226
2. Lewis RA, Soter NA, Diamond PT, Austen KF, Oates JA, Roberts LJ 2nd: Prostaglandin D2 generation after activation of rat and human mast cells with anti-IgE. J Immunol 1982, 129:1627–1631
3. Tanaka K, Ogawa K, Sugamura K, Nakamura M, Takano S, Nagata K: Cutting edge: differential production of prostaglandin D2 by human helper T cell subsets. J Immunol 2000, 164:2277–2280
4. Shimura C, Satoh T, Igawa K, Aritake K, Urade Y, Nakamura M, Yokozeki H: Dendritic cells express hematopoietic prostaglandin D synthase and function as a source of prostaglandin D2 in the skin. Am J Pathol 176:227–237
5. Kabashima K, Narumiya S: The DP receptor, allergic inflammation and asthma. Prostaglandins Leukot Essent Fatty Acids 2003, 69:187–194
6. Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, Takamori Y, Ichimasa M, Sugamura K, Nakamura M, Takano S, Nagata K: Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. J Exp Med 2001, 193:255–261
7. Theiner G, Gessner A, Lutz MB: The mast cell mediator PGD2 suppresses IL-12 release by dendritic cells leading to Th2 polarized immune responses in vivo. Immunobiology 2006, 211:463–472
8. Miadonna A, Tedeschi A, Brasca C, Folco G, Sala A, Murphy RC: Mediator release after endobronchial antigen challenge in patients with respiratory allergy. J Allergy Clin Immunol 1990, 85:906–913
9. Fujitani Y, Kanaoka Y, Aritake K, Uodome N, Okazaki-Hatake K, Urade Y: Pronounced eosinophilic lung inflammation and Th2 cytokine release in human lipocalin-type prostaglandin D synthase transgenic mice. J Immunol 2002, 168:443–449
10. Satoh T, Moroi R, Aritake K, Urade Y, Kanai Y, Sumi K, Yokozeki H, Hirai H, Nagata K, Hara T, Utsuyama M, Hirokawa K, Sugamura K, Nishioka K, Nakamura M: Prostaglandin D2 plays an essential role in chronic allergic inflammation of the skin via CRTH2 receptor. J Immunol 2006, 177:2621–2629
11. Oiwa M, Satoh T, Watanabe M, Niwa H, Hirai H, Nakamura M, Yokozeki H: CRTH2-dependent. STAT6-independent induction of cedar pollen dermatitis. Clin Exp Allergy 2008, 38:1357–1366
12. Harris SG, Padilla J, Koumas L, Ray D, Phipps RP: Prostaglandins as modulators of immunity. Trends Immunol 2002, 23:144–150
13. Hilkens CM, Vermeulen H, van Neerven RJ, Snijdewint FG, Wierenga EA, Kapsenberg ML: Differential modulation of T helper type 1 (Th1) and T helper type 2 (Th2) cytokine secretion by prostaglandin E2 critically depends on interleukin-2. Eur J Immunol 1995, 25:59–63
14. Yao C, Sakata D, Esaki Y, Li Y, Matsuoka T, Kuroiwa K, Sugimoto Y, Narumiya S: Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion. Nat Med 2009, 15:633–640
15. Sheibanie AF, Tadmori I, Jing H, Vassiliou E, Ganea D: Prostaglandin E2 induces IL-23 production in bone marrow-derived dendritic cells. FASEB J 2004, 18:1318–1320
16. Macfarlane AJ, Kon OM, Smith SJ, Zeibecoglou K, Khan LN, Barata LT, McEuen AR, Buckley MG, Walls AF, Meng Q, Humbert M, Barnes NC, Robinson DS, Ying S, Kay AB: Basophils, eosinophils, and mast cells in atopic and nonatopic asthma and in late-phase allergic reactions in the lung and skin. J Allergy Clin Immunol 2000, 105:99–107
17. Plager DA, Weiss EA, Kephart GM, Mocharla RM, Matsumoto R, Checkel JL, Schwartz LB, Gleich GJ, Leiferman KM: Identification of basophils by a mAb directed against pro-major basic protein 1. J Allergy Clin Immunol 2006, 117:626–634
18. Mukai K, Matsuoka K, Taya C, Suzuki H, Yokozeki H, Nishioka K, Hirokawa K, Etori M, Yamashita M, Kubota T, Minegishi Y, Yonekawa H, Karasuyama H: Basophils play a critical role in the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells. Immunity 2005, 23:191–202
19. Tsujimura Y, Obata K, Mukai K, Shindou H, Yoshida M, Nishikado H, Kawano Y, Minegishi Y, Shimizu T, Karasuyama H: Basophils play a pivotal role in immunoglobulin-G-mediated but not immunoglobulin-E-mediated systemic anaphylaxis. Immunity 2008, 28:581–589
20. Yoshimoto T, Yasuda K, Tanaka H, Nakahira M, Imai Y, Fujimori Y, Nakanishi K: Basophils contribute to T(H)2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to CD4+ T cells. Nat Immunol 2009, 10:706–712
21. Denzel A, Maus UA, Rodriguez Gomez M, Moll C, Niedermeier M, Winter C, Maus R, Hollingshead S, Briles DE, Kunz-Schughart LA, Talke Y, Mack M: Basophils enhance immunological memory responses. Nat Immunol 2008, 9:733–742
22. Voehringer D: The role of basophils in helminth infection. Trends Parasitol 2009, 25:551–556
23. Wada T, Ishiwata K, Koseki H, Ishikura T, Ugajin T, Ohnuma N, Obata K, Ishikawa R, Yoshikawa S, Mukai K, Kawano Y, Minegishi Y, Yokozeki H, Watanabe N, Karasuyama H: Selective ablation of basophils in mice reveals their nonredundant role in acquired immunity against ticks. J Clin Invest 120:2867–2875
24. Schulman ES, MacGlashan DW Jr, Schleimer RP, Peters SP, Kagey-Sobotka A, Newball HH, Lichtenstein LM: Purified human basophils and mast cells: current concepts of mediator release. Eur J Respir Dis Suppl 1983, 128(Pt 1):53–61
25. Peters SP, Naclerio RM, Schleimer RP, MacGlashan DW Jr, Pipkorn U, Lichtenstein LM: The pharmacologic control of mediator release from human basophils and mast cells. Respiration 1986, 50(Suppl 2):116–122
26. Bull HA, Courtney PF, Bunker CB, Rustin MH, Pearce FL, Dowd PM: Basophil mediator release in atopic dermatitis. J Invest Dermatol 1993, 100:305–309
27. Warner JA, Peters SP, Lichtenstein LM, Hubbard W, Yancey KB, Stevenson HC, Miller PJ, MacGlashan DW Jr: Differential release of

mediators from human basophils: differences in arachidonic acid metabolism following activation by unrelated stimuli. J Leukoc Biol 1989, 45:558–571

28. Lie WJ, Homburg CH, Kuijpers TW, Knol EF, Mul FP, Roos D, Tool AT: Regulation and kinetics of platelet-activating factor and leukotriene C4 synthesis by activated human basophils. Clin Exp Allergy 2003, 33:1125–1134

29. Kanaoka Y, Urade Y: Hematopoietic prostaglandin D synthase. Prostaglandins Leukot Essent Fatty Acids 2003, 69:163–167

30. Lazarus M, Kubata BK, Eguchi N, Fujitani Y, Urade Y, Hayaishi O: Biochemical characterization of mouse microsomal prostaglandin E synthase-1 and its colocalization with cyclooxygenase-2 in peritoneal macrophages. Arch Biochem Biophys 2002, 397:336–341

31. Ugajin T, Kojima T, Mukai K, Obata K, Kawano Y, Minegishi Y, Eishi Y, Yokozeki H, Karasuyama H: Basophils preferentially express mouse mast cell protease 11 among the mast cell tryptase family in contrast to mast cells. J Leukoc Biol 2009, 86:1417–1425

32. Hida S, Yamasaki S, Sakamoto Y, Takamoto M, Obata K, Takai T, Karasuyama H, Sugane K, Saito T, Taki S: Fc receptor gamma-chain, a constitutive component of the IL-3 receptor, is required for IL-3-induced IL-4 production in basophils. Nat Immunol 2009, 10:214–222

33. Urade Y, Ujihara M, Horiguchi Y, Igarashi M, Nagata A, Ikai K, Hayaishi O: Mast cells contain spleen-type prostaglandin D synthetase. J Biol Chem 1990, 265:371–375

34. Urade Y, Eguchi N: Lipocalin-type and hematopoietic prostaglandin D synthases as a novel example of functional convergence. Prostaglandins Other Lipid Mediat 2002, –69:375–38268

35. Murakami M, Naraba H, Tanioka T, Semmyo N, Nakatani Y, Kojima F, Ikeda T, Fueki M, Ueno A, Oh S, Kudo I: Regulation of prostaglandin E2 biosynthesis by inducible membrane-associated prostaglandin E2 synthase that acts in concert with cyclooxygenase-2. J Biol Chem 2000, 275:32783–32792

36. Tanioka T, Nakatani Y, Semmyo N, Murakami M, Kudo I: Molecular identification of cytosolic prostaglandin E2 synthase that is functionally coupled with cyclooxygenase-1 in immediate prostaglandin E2 biosynthesis. J Biol Chem 2000, 275:32775–32782

37. Tanikawa N, Ohmiya Y, Ohkubo H, Hashimoto K, Kangawa K, Kojima M, Ito S, Watanabe K: Identification and characterization of a novel type of membrane-associated prostaglandin E synthase. Biochem Biophys Res Commun 2002, 291:884–889

38. Urade Y, Hayaishi O: Prostaglandin D2 and sleep regulation. Biochim Biophys Acta 1999, 1436:606–615

39. Murakami M, Nakashima K, Kamei D, Masuda S, Ishikawa Y, Ishii T, Ohmiya Y, Watanabe K, Kudo I: Cellular prostaglandin E2 production by membrane-bound prostaglandin E synthase-2 via both cyclooxygenases-1 and -2. J Biol Chem 2003, 278:37937–37947

40. Hara S, Kamei D, Sasaki Y, Tanemoto A, Nakatani Y, Murakami M: Prostaglandin E synthases: understanding their pathophysiological roles through mouse genetic models. Biochimie 92:651–659

41. Reddy ST, Tiano HF, Langenbach R, Morham SG, Herschman HR: Genetic evidence for distinct roles of COX-1 and COX-2 in the immediate and delayed phases of prostaglandin synthesis in mast cells. Biochem Biophys Res Commun 1999, 265:205–210

42. Obata K, Mukai K, Tsujimura Y, Ishiwata K, Kawano Y, Minegishi Y, Watanabe N, Karasuyama H: Basophils are essential initiators of a novel type of chronic allergic inflammation. Blood 2007, 110:913–920

43. MacGlashan DW Jr, Peters SP, Warner J, Lichtenstein LM: Characteristics of human basophil sulfidopeptide leukotriene release: releasability defined as the ability of the basophil to respond to dimeric cross-links. J Immunol 1986, 136:2231–2239

44. Nguyen KL, Gillis S, MacGlashan DW Jr: A comparative study of releasing and nonreleasing human basophils: nonreleasing basophils lack an early component of the signal transduction pathway that follows IgE cross-linking. J Allergy Clin Immunol 1990, 85:1020–1029

45. Yahara H, Satoh T, Miyagishi C, Yokozeki H: Increased expression of CRTH2 on eosinophils in allergic skin diseases (letter to the editor). J Eur Acad Dermatol Venereol 2010, 24:75–76

46. Ito Y, Satoh T, Takayama K, Miyagishi C, Walls AF, Yokozeki H: Basophil recruitment and activation in inflammatory skin diseases. Allergy 2011, 66:1107–1113

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

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We established a diphtheria toxin (DT)-based conditional deletion system using *Il4* 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 com-

pounds 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 cysteine 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, Kit$^{W-sh/W-sh}$ 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-FcεRIα (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 Mcpt8Cre 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′ untranslated 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) (22) 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 construct. IE (+311 to +3,534) and 3′UTR (+6,231 to +10,678) fragments were isolated from a mouse YAC clone (catalog no. 95022; Research Genetics, Huntsville, AL) 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 -TCRβ (H57-597) Abs (all from BD Biosciences, San Jose, CA) and anti-B220 (RA3-6B2), CD8a (53-6.7), IgM (eB121-15F9) and -Gr1 (RB6-8C5) (eBioscience, San Diego, CA), and anti-CD200R3 (Ba91) Abs (23) were used for FACS analysis. Anti-DNP IgE mAb (Sigma Aldrich, St Louis, MO) was used for cross-linking of basophils. PMA (Sigma Aldrich) and ionomycin calcium salt (Sigma) were 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 (SPI-Bio) 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 (6HD5). 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 IGELb4 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–Giemsa 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 × 10^5 cells) were restimulated with OVA (10 μg/ml) for 48 h in the presence of irradiated syngeneic spleen cells (5 × 10^5 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 (RA3-6B2), anti–Thy1.2-biotin (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
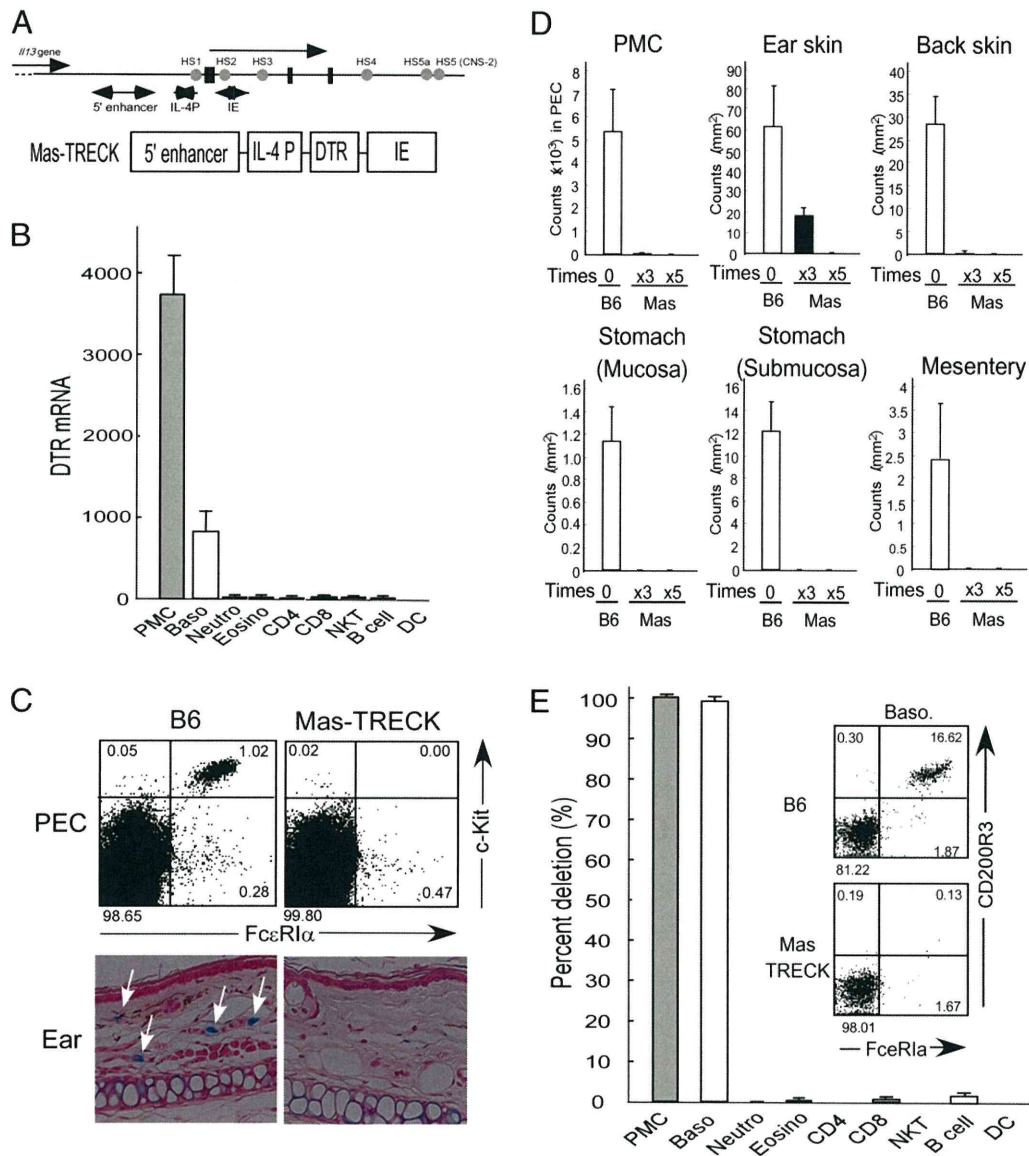
FIGURE 1. Deletion of MCs and basophils after DT treatment in Mas-TRECK mice. *A,* The mouse *Il4* locus, *cis*-acting elements, and structure of the transgene used to generate Mas-TRECK mice. *B,* PMCs, basophils, neutrophils, and eosinophils were prepared from bone marrow cells, and CD4+ T cells, NK cells (NK1.1+TCR-β−), NKT cells (NK1.1+TCR-β+), 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+FcεRIα+) 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.

mesentery (Fig. 1*D*). 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. 1*B,* 1*E*). 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. 2*A*). We generated three independent lines (lines 1–3) of Bas-TRECK mice. Intraperitoneal injection with DT (500 ng/mouse) resulted in complete deletion of

DX5+ FcεRIα+ CD200R3+ basophils both in bone marrow and in the peripheral blood of lines 1 and 3 but not line 2 (Fig. 2*B,* 2*C*). However, lines 1 and 2 showed unexpected deletion of peritoneal MCs (PMCs), indicating that line 3 was most useful for further experiments (Fig. 2*D*). We next examined expression of DTR mRNA among various cell types isolated from bone marrow of line 3. DTR was highly expressed in basophils but not in MCs, neutrophils, eosinophils, T and B cells, or DCs (Fig. 2*E*). Thus, the specificity of the DT effect in Bas-TRECK mice was consistent with the expression profile of DTR mRNA. There was no effect on MCs, neutrophils, eosinophils, or T cell populations in DT-treated line 3 mice (Fig. 2*F–H*).

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$^+$CD200R3$^+$ FcεRIα$^+$) were analyzed both in bone marrow (BM) and peripheral blood (PB). *C*, B6 and the three Bas-TRECK Tg lines (1–3) were treated with DT, as described in *B*, and basophils (DX5$^+$CD200R3$^+$ FcεRIα$^+$) in bone marrow (BM) were analyzed by flow cytometry. Percentage (*left panel*) and cell number (*right panel*) of DX5$^+$CD200R3$^+$FcεRIα$^+$ cells. *D*, Bone marrow-derived basophils (Baso; DX5$^+$ CD200R3$^+$FcεRIα$^+$) and PMCs (c-Kit$^+$FcεRIα$^+$) 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^{int}Siglec-F$^+$), and neutrophils (Gr-1^{hi}Siglec-F$^-$) in bone marrow, as well as CD4$^+$ and CD8$^+$ 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^2 (*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.
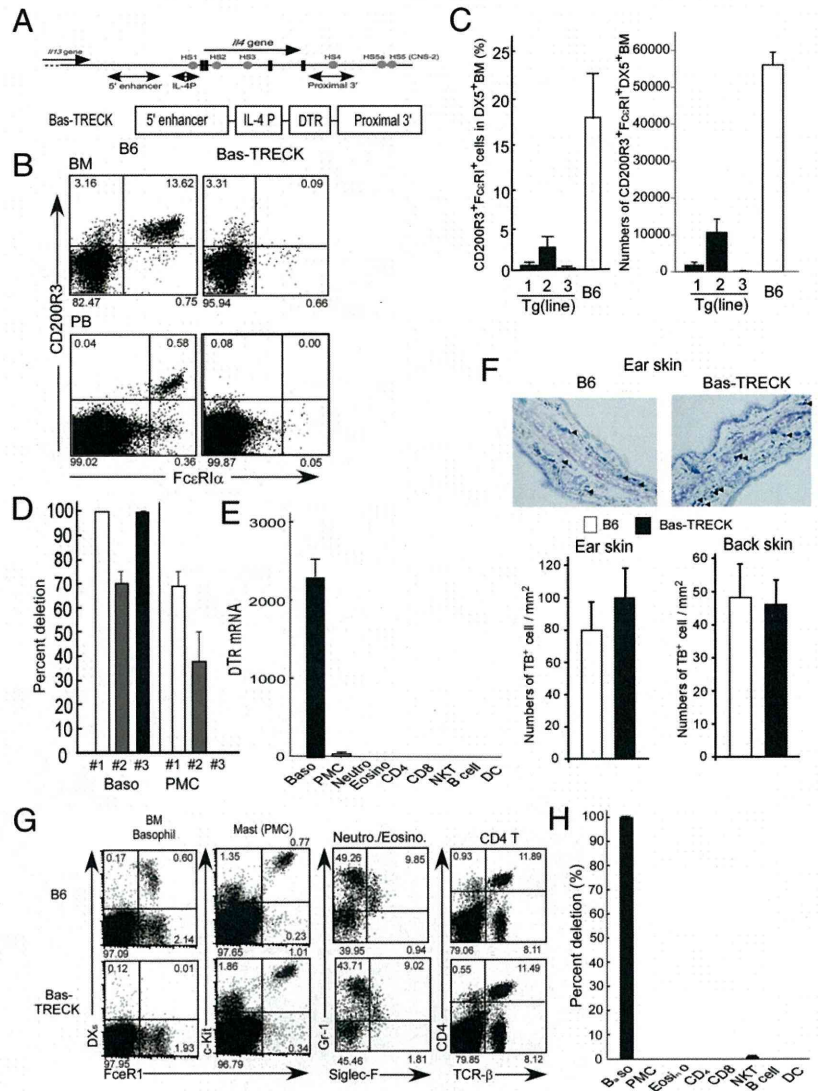
We also examined the effect of DT treatment on Th1/Th2 differentiation and natural helper cells in Mas-TRECK and Bas-TRECK mice. The lymphoid fraction was isolated from adipose tissue of DT-treated Mas-TRECK and Bas-TRECK mice; both lines showed a comparable proportion of natural helper cells (c-Kit$^+$ CD25$^+$ lymphoid cells) (Supplemental Fig. 1*A*). Moreover, DT treatment did not have any effect on development of Th1/Th2 cells derived from splenic CD4 T cells of Mas-TRECK and Bas-TRECK mice (Supplemental Fig. 1*B*). 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$^+$ PMCs and DX5$^+$ 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 crosslinking 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. 3*A*).

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 (eotaxin, 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. 3*B*), 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. 3*C*). A similar reduction was observed in Kit$^{W-sh/W-sh}$ 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

FIGURE 3. *A*, IL-4 production by MCs and basophils derived from Mas-TRECK and Bas-TRECK mice. The c-Kit$^+$ peritoneal cells and DX5$^+$ bone marrow cells were isolated as PMCs and basophils from B6, Mas-TRECK, and Bas-TRECK mice. Cells were cultured in the presence of DT (300 ng/ml) for 12 h to delete MCs or basophils. PMCs were stimulated with PMA (50 ng/ml) + ionomycin (500 nM), and DX5$^+$ basophils were stimulated with anti-DNP IgE mAb (10 μg/ml) and DNP-OVA (10 ng/ml) for 24 h. IL-4 was measured by ELISA. *B*, B6, Mas-TRECK, and Bas-TRECK mice were treated with DT using the standard deletion protocol. After 24 h, 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 (eotaxin, RANTES, MIP-1α, MIP-1β, and MCP-1) were measured by a Bio-Plex cytokine-assay system. *C*, Histamine concentration in blood samples from B6, Mas-TRECK, Bas-TRECK, and Kit$^{W\_sh/W\_sh}$ mice was assessed by ELISA. Mice were treated with 250 ng of DT for 5 consecutive days for B6 and Mas-TRECK mice and with 500 ng of DT for Bas-TRECK mice. Blood samples were obtained 24 h after the last DT injection. Data represent mean and SEM of 13 mice/group. *p < 0.01.
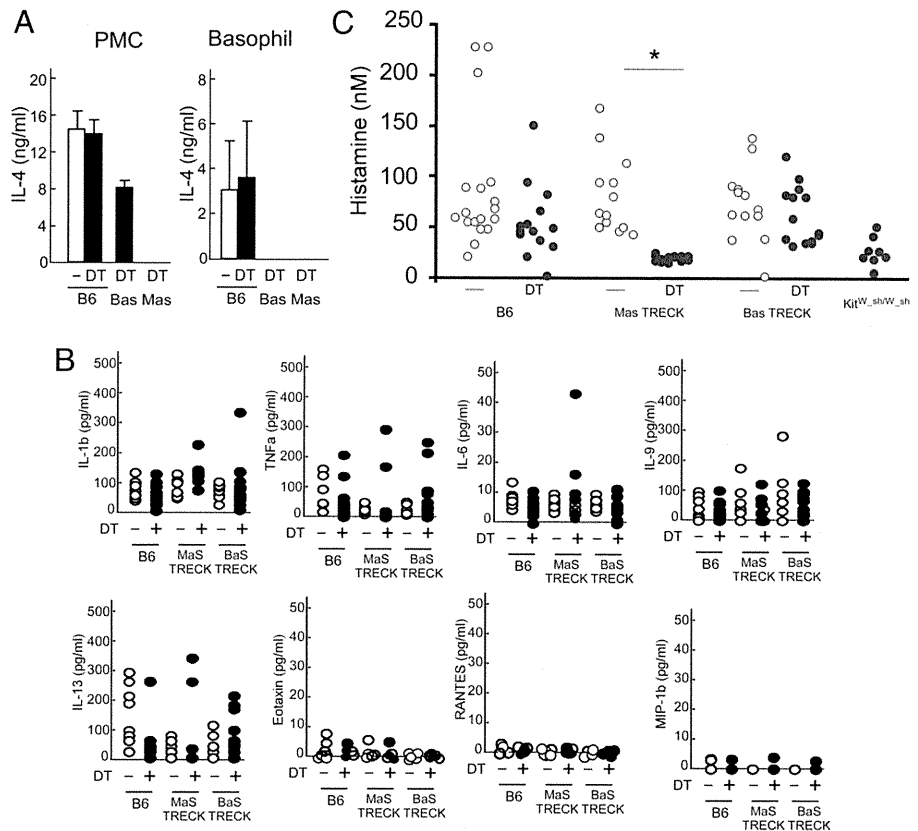
measurement of dye leakage at the challenge site. Although the control B6 and Bas-TRECK mice displayed a clear PCA reaction, Mas-TRECK mice showed no signs of dye leakage in the ear (Fig. 4*A*). To examine IgE-mediated passive systemic anaphylaxis, Mas-TRECK and Bas-TRECK mice were injected i.v. with 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. 4*B*).

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 such increase (Fig. 4*C*). 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$^{W\_sh/W\_sh}$ 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. 5*A*). 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. 5*B*). However, these mice showed a remarkable reduction in serum histamine levels (Fig. 5*C*), and Ag-stimulated BAL-derived cells produced significantly less histamine (Fig. 5*D*). Interestingly, IL-4, but not other Th2 cytokines (IL-5 and IL-13), was also significantly reduced (Fig. 5*D*). 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. 6*A*). 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. 6*A*).

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
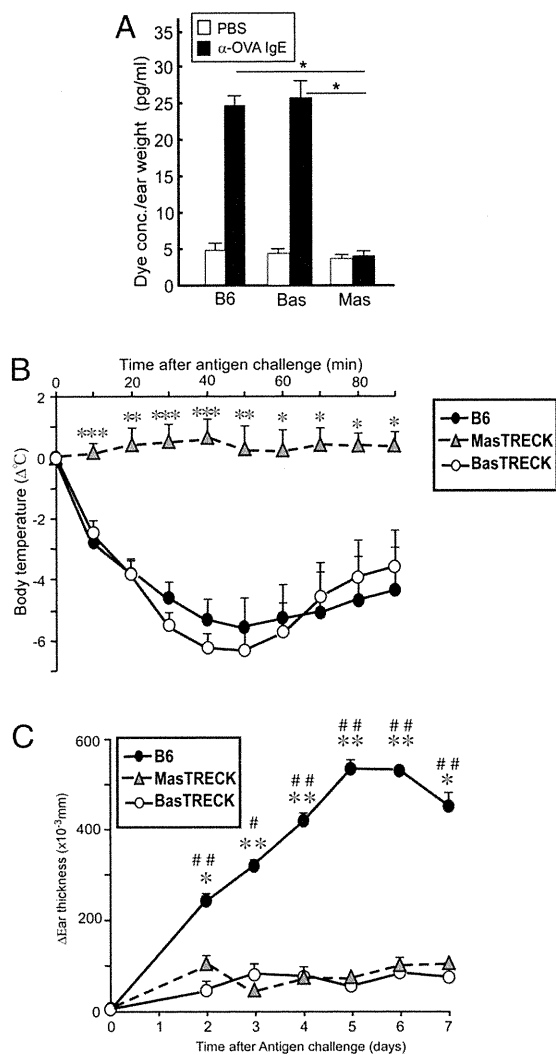
FIGURE 4. *A*, PCA reaction in Mas-TRECK and Bas-TRECK mice. The day after the last DT treatment, mice were injected s.c. with anti-OVA IgE (left ear) or PBS (right ear). On the following day, an OVA and Evans blue dye solution was injected i.v. After 30 min, the mice were sacrificed to measure the concentration of dye accumulating in the ears. Dye concentration was measured at OD 620 nm and normalized to a dye reference based on ear weight. Data represent mean and SEM of four independent experiments. $*p \leq 0.01$. *B*, IgE-mediated anaphylaxis in Mas-TRECK and Bas-TRECK mice. Mice were treated with DT, as described in *A*, and injected i.v. with anti-IgE mAb. Rectal temperature was measured every 10 min. Data represent mean and SEM of four individual mice. $*p < 0.05$ B6 versus MaS TRECK, $**p < 0.01$ B6 versus MaS TRECK, $***p < 0.001$ B6 versus MaS TRECK. *C*, IgE-CAI in Mas-TRECK and Bas-TRECK mice. Mice received DT, as described in *A*. Thereafter, they were injected i. v. with TNP-specific IgE mAb. One day later, the mice were injected s.c. with TNP-OVA, and ear swelling was measured for 7 d. Data represent mean and SEM of four individual mice. $^{\#}p < 0.01$ B6 versus BaS TRECK, $^{\#\#\#}p < 0.001$ B6 versus BaS TRECK, $*p < 0.01$ B6 versus MaS TRECK, $**p < 0.001$ B6 versus MaS TRECK.

IgE response, which is normally controlled by IL-4. Mice were immunized with OVA in alum adjuvant during DT treatment at 3-d intervals, and we measured total and OVA-specific Ab responses (Fig. 6*B*). Before immunization, there were no differences in the basal level of serum IgE between B6 and Bas-TRECK mice. Upon challenge with OVA/Alum, both WT and Bas-TRECK mice showed equivalent total IgE and OVA-specific (IgE, IgG1, and IgG2c) Ab levels, clearly indicating that basophils are dispensable for the development of a systemic IgE response.

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. 6*C*). 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. 6*D*). 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. 5*B*, 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 Kit^{W-sh}/Kit^{W-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
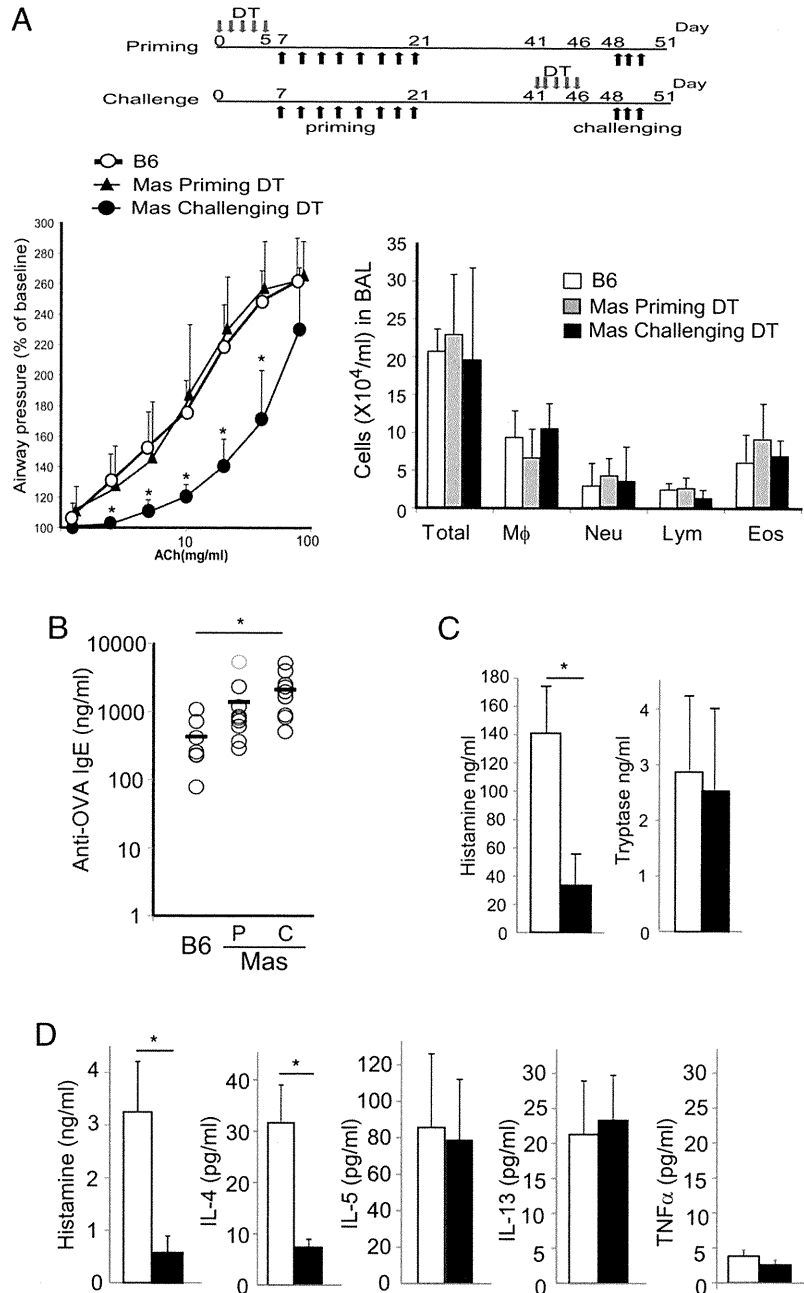
FIGURE 5. AHR in Mas-TRECK mice treated with DT. *A*, B6 and Mas-TRECK mice were immunized by i.p. injection of soluble OVA at 2-d intervals for 2 wk (priming) and treated with OVA three times (challenge). DT treatment was carried out at two points before either priming or challenge. The OVA-immunized mice were challenged with OVA by inhalation, and acetylcholine-dependent AHR was measured. 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. *$p < 0.01$. *B*, Serum was obtained at day 51 from B6 and Mas-TRECK mice that were treated with DT in primed (P) and challenge (C) phases. OVA-specific IgE titer was measured by ELISA. *$p < 0.05$. *C*, Blood samples were obtained at day 7 from B6 and Mas-TRECK mice that were treated with DT in the challenge phase. Histamine and tryptase concentration were measured by ELISA. *$p < 0.01$. *D*, Cells were isolated from BAL fluid at day 51 from B6 and Mas-TRECK mice that were treated with DT in the challenged phase and stimulated with OVA. After 48 h, culture supernatant was obtained, and histamine IL-4, IL-5, IL-13, and TNF-α were measured by ELISA. Data represent mean and SEM of six individual mice. *$p < 0.01$.

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 $Kit^{W/Wv}$ and $Kit^{W\text{-}sh/W\text{-}sh}$, 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 $Kit^{W\text{-}sh/W\text{-}sh}$ mouse, although the non-MC abnormalities are less severe than in $Kit^{W/Wv}$ 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 $Kit^{W\text{-}sh/W\text{-}sh}$ 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 $Tnf^{-/-}$ mice failed to rescue the AHR in
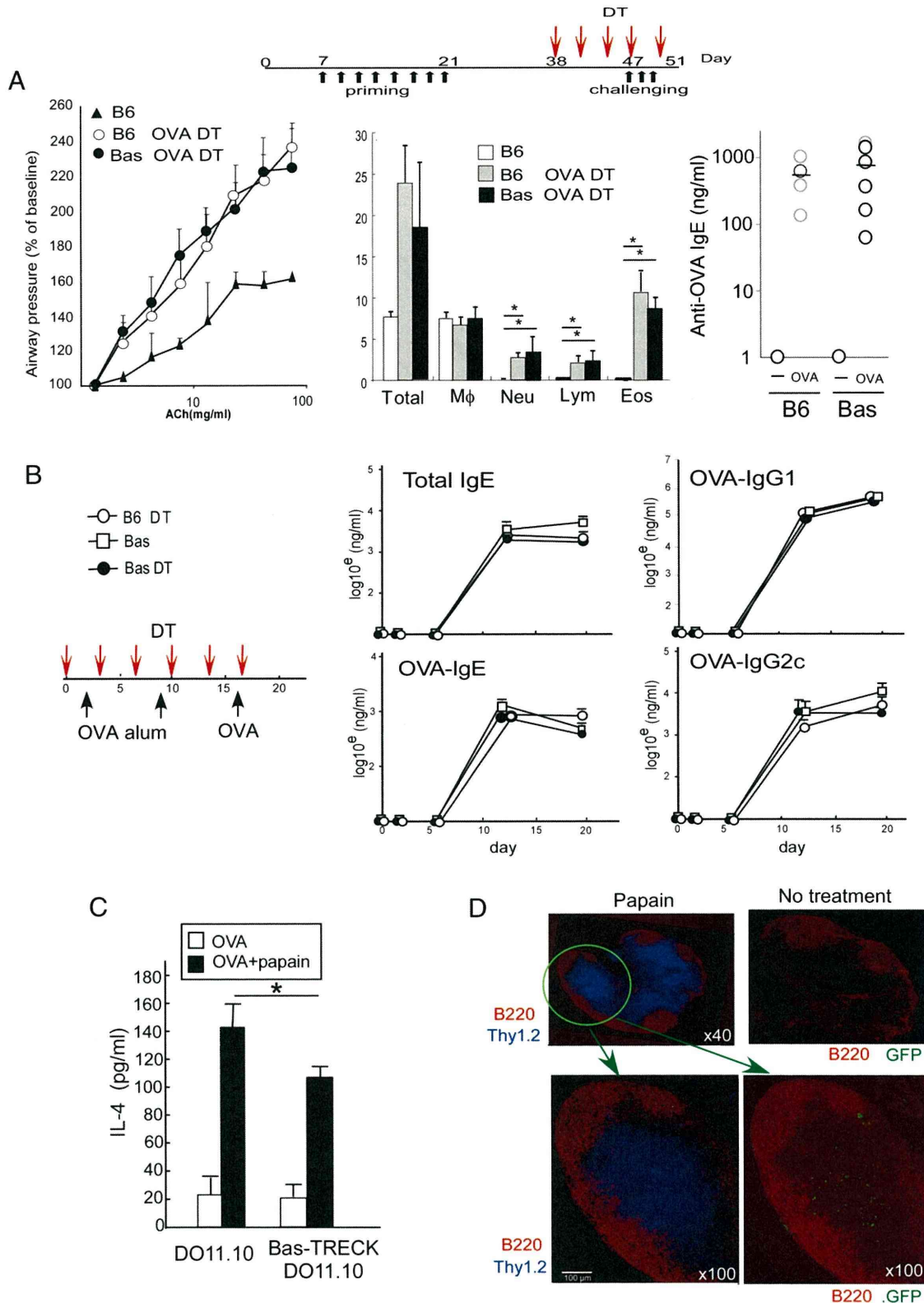
FIGURE 6.   AHR in Bas-TRECK mice treated with DT. *A, Left* and *top panels*, B6 (○) and Bas-TRECK mice (●) were immunized by i.p. injection of soluble OVA at 2-d intervals for 2 wk and challenged with OVA three times (black arrows). The unprimed B6 (▲) and OVA-immunized mice (○, ●) were challenged 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*)
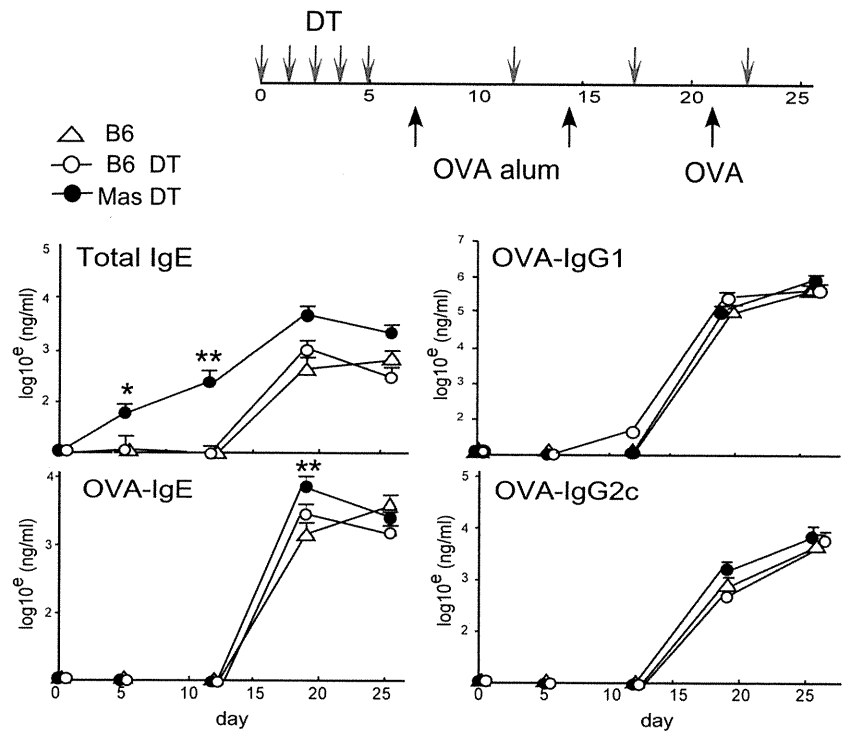
FIGURE 7. IgE production in Mas-TRECK mice treated with DT. B6 and Mas-TRECK mice were injected with DT (250 ng) five times and then treated with DT (100 ng) at 7-d intervals at the time points indicated (↓) (*top panel*). Mice were immunized with OVA in alum on days 7 and 14 and boosted with OVA in 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. $*p \leq 0.05$, $**p \leq 0.01$.

Kit$^{W-sh/W-sh}$ 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 re-

cent 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$^{W-sh/W-sh}$ 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

Disclosures

The authors have no financial conflicts of interest.

References

1. Galli, S. J. 2000. Mast cells and basophils. *Curr. Opin. Hematol.* 7: 32–39.
2. Kitamura, Y. 1989. Heterogeneity of mast cells and phenotypic change between subpopulations. *Annu. Rev. Immunol.* 7: 59–76.
3. Metcalfe, D. D., D. Baram, and Y. A. Mekori. 1997. Mast cells. *Physiol. Rev.* 77: 1033–1079.
4. Karasuyama, H., K. Mukai, K. Obata, Y. Tsujimura, and T. Wada. 2011. Nonredundant roles of basophils in immunity. *Annu. Rev. Immunol.* 29: 45–69.
5. Karasuyama, H., K. Mukai, Y. Tsujimura, and K. Obata. 2009. Newly discovered roles for basophils: a neglected minority gains new respect. *Nat. Rev. Immunol.* 9: 9–13.
6. Sokol, C.L., Barton, G.M., Farr, A.G. & Medzhitov, R. 2008. A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat. Immunol.* 9: 310–318.
7. Sokol, C. L., N. Q. Chu, S. Yu, S. A. Nish, T. M. Laufer, and R. Medzhitov. 2009. Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. *Nat. Immunol.* 10: 713–720.
8. Ohnmacht, C., C. Schwartz, M. Panzer, I. Schiedewitz, R. Naumann, and D. Voehringer. 2010. Basophils orchestrate chronic allergic dermatitis and protective immunity against helminths. *Immunity* 33: 364–374.
9. Mukai, K., K. Matsuoka, C. Taya, H. Suzuki, H. Yokozeki, K. Nishioka, K. Hirokawa, M. Etori, M. Yamashita, T. Kubota, et al. 2005. Basophils play a critical role in the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells. *Immunity* 23: 191–202.
10. Kitamura, Y., S. Go, and K. Hatanaka. 1978. Decrease of mast cells in W/Wv mice and their increase by bone marrow transplantation. *Blood* 52: 447–452.
11. Galli, S. J., and Y. Kitamura. 1987. Genetically mast-cell-deficient W/Wv and Sl/Sld mice. Their value for the analysis of the roles of mast cells in biologic responses in vivo. *Am. J. Pathol.* 127: 191–198.
12. Grimbaldeston, M. A., C. C. Chen, A. M. Piliponsky, M. Tsai, S. Y. Tam, and S. J. Galli. 2005. Mast cell-deficient W-sash c-kit mutant Kit W-sh/W-sh mice as a model for investigating mast cell biology in vivo. *Am. J. Pathol.* 167: 835–848.
13. Denzel, A., U. A. Maus, M. Rodriguez Gomez, C. Moll, M. Niedermeier, C. Winter, R. Maus, S. Hollingshead, D. E. Briles, L. A. Kunz-Schughart, et al. 2008. Basophils enhance immunological memory responses. *Nat. Immunol.* 9: 733–742.
14. Perrigoue, J. G., S. A. Saenz, M. C. Siracusa, E. J. Allenspach, B. C. Taylor, P. R. Giacomin, M. G. Nair, Y. Du, C. Zaph, N. van Rooijen, et al. 2009. MHC class II-dependent basophil-CD4$^+$ T cell interactions promote T(H)2 cytokine-dependent immunity. *Nat. Immunol.* 10: 697–705.
15. Wada, T., K. Ishiwata, H. Koseki, T. Ishikura, T. Ugajin, N. Ohnuma, K. Obata, R. Ishikawa, S. Yoshikawa, K. Mukai, et al. 2010. Selective ablation of basophils in mice reveals their nonredundant role in acquired immunity against ticks. *J. Clin. Invest.* 120: 2867–2875.
16. Brown, M. A., and J. Hural. 1997. Functions of IL-4 and control of its expression. *Crit. Rev. Immunol.* 17: 1–32.
17. Min, B., M. Prout, J. Hu-Li, J. Zhu, D. Jankovic, E. S. Morgan, J. F. Urban, Jr., A. M. Dvorak, F. D. Finkelman, G. LeGros, and W. E. Paul. 2004. Basophils produce IL-4 and accumulate in tissues after infection with a Th2-inducing parasite. *J. Exp. Med.* 200: 507–517.
18. Voehringer, D., T. A. Reese, X. Huang, K. Shinkai, and R. M. Locksley. 2006. Type 2 immunity is controlled by IL-4/IL-13 expression in hematopoietic non-eosinophil cells of the innate immune system. *J. Exp. Med.* 203: 1435–1446.
19. Mohrs, M., K. Shinkai, K. Mohrs, and R. M. Locksley. 2001. Analysis of type 2 immunity in vivo with a bicistronic IL-4 reporter. *Immunity* 15: 303–311.
20. Yagi, R., S. Tanaka, Y. Motomura, and M. Kubo. 2007. Regulation of the Il4 gene is independently controlled by proximal and distal 3′ enhancers in mast cells and basophils. *Mol. Cell. Biol.* 27: 8087–8097.
21. Saito, M., T. Iwawaki, C. Taya, H. Yonekawa, M. Noda, Y. Inui, E. Mekada, Y. Kimata, A. Tsuru, and K. Kohno. 2001. Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. *Nat. Biotechnol.* 19: 746–750.
22. Kubo, M., J. Ransom, D. Webb, Y. Hashimoto, T. Tada, and T. Nakayama. 1997. T-cell subset-specific expression of the IL-4 gene is regulated by a silencer element and STAT6. *EMBO J.* 16: 4007–4020.
23. Kojima, T., K. Obata, K. Mukai, S. Sato, T. Takai, Y. Minegishi, and H. Karasuyama. 2007. Mast cells and basophils are selectively activated in vitro and in vivo through CD200R3 in an IgE-independent manner. *J. Immunol.* 179: 7093–7100.
24. Rudolph, A. K., P. D. Burrows, and M. R. Wabl. 1981. Thirteen hybridomas secreting hapten-specific immunoglobulin E from mice with Iga or Igb heavy chain haplotype. *Eur. J. Immunol.* 11: 527–529.
25. Seki, Y., H. Inoue, N. Nagata, K. Hayashi, S. Fukuyama, K. Matsumoto, O. Komine, S. Hamano, K. Himeno, K. Inagaki-Ohara, et al. 2003. SOCS-3 regulates onset and maintenance of T(H)2-mediated allergic responses. *Nat. Med.* 9: 1047–1054.
26. Yamaizumi, M., E. Mekada, T. Uchida, and Y. Okada. 1978. One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. *Cell* 15: 245–250.
27. Park, S. J. et al. 2001. Promoted expression of mast cell-specific proteases in IgE-dependent passive cutaneous anaphylaxis responses. *Clin. Chim. Acta* 314: 231–236.
28. Zhou, J. S., W. Xing, D. S. Friend, K. F. Austen, and H. R. Katz. 2007. Mast cell deficiency in Kit(W-sh) mice does not impair antibody-mediated arthritis. *J. Exp. Med.* 204: 2797–2802.
29. Thomas, W. R., and J. W. Schrader. 1983. Delayed hypersensitivity in mast-cell-deficient mice. *J. Immunol.* 130: 2565–2567.
30. Yu, M., M. Tsai, S. Y. Tam, C. Jones, J. Zehnder, and S. J. Galli. 2006. Mast cells can promote the development of multiple features of chronic asthma in mice. *J. Clin. Invest.* 116: 1633–1641.
31. Finkelman, F. D., M. E. Rothenberg, E. B. Brandt, S. C. Morris, and R. T. Strait. 2005. Molecular mechanisms of anaphylaxis: lessons from studies with murine models. *J. Aller. Clin. Immunol.* 115: 449–457; quiz 458.
32. Tsujimura, Y., K. Obata, K. Mukai, H. Shindou, M. Yoshida, H. Nishikado, Y. Kawano, Y. Minegishi, T. Shimizu, and H. Karasuyama. 2008. Basophils play a pivotal role in immunoglobulin-G-mediated but not immunoglobulin-E-mediated systemic anaphylaxis. *Immunity* 28: 581–589.
33. Kobayashi, T., T. Miura, T. Haba, M. Sato, I. Serizawa, H. Nagai, and K. Ishizaka. 2000. An essential role of mast cells in the development of airway hyperresponsiveness in a murine asthma model. *J. Immunol.* 164: 3855–3861.
34. Kung, T. T., D. Stelts, J. A. Zurcher, H. Jones, S. P. Umland, W. Kreutner, R. W. Egan, and R. W. Chapman. 1995. Mast cells modulate allergic pulmonary eosinophilia in mice. *Am. J. Respir. Cell Mol. Biol.* 12: 404–409.
35. Masuda, T., H. Tanaka, M. Komai, K. Nagao, M. Ishizaki, D. Kajiwara, and H. Nagai. 2003. Mast cells play a partial role in allergen-induced subepithelial fibrosis in a murine model of allergic asthma. *Clin. Exp. Allergy* 33: 705–713.
36. Williams, C. M., and S. J. Galli. 2000. Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice. *J. Exp. Med.* 192: 455–462.
37. Ogawa, K., O. Kaminuma, H. Kikkawa, R. Kameda, K. Ikezawa, M. Suko, H. Okudaira, K. Akiyama, and A. Mori. 1999. Primary role of CD4$^+$ T cells and supplemental role of mast cells in allergic pulmonary eosinophilia. *Int. Arch. Allergy Immunol.* 120(Suppl. 1): 15–18.
38. Brusselle, G. G., J. C. Kips, J. H. Tavernier, J. G. van der Heyden, C. A. Cuvelier, R. A. Pauwels, and H. Bluethmann. 1994. Attenuation of allergic airway inflammation in IL-4 deficient mice. *Clin. Exp. Allergy* 24: 73–80.
39. Nogami, M., M. Suko, H. Okudaira, T. Miyamoto, J. Shiga, M. Ito, and S. Kasuya. 1990. Experimental pulmonary eosinophilia in mice by *Ascaris suum* extract. *Am. Rev. Respir. Dis.* 141: 1289–1295.
40. Takeda, K., E. Hamelmann, A. Joetham, L. D. Shultz, G. L. Larsen, C. G. Irvin, and E. W. Gelfand. 1997. Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice. *J. Exp. Med.* 186: 449–454.
41. Duttlinger, R., K. Manova, T. Y. Chu, C. Gyssler, A. D. Zelenetz, R. F. Bachvarova, and P. Besmer. 1993. W-sash affects positive and negative elements controlling c-kit expression: ectopic c-kit expression at sites of kit-ligand expression affects melanogenesis. *Development* 118: 705–717.
42. Yamazaki, M., T. Tsujimura, E. Morii, K. Isozaki, H. Onoue, S. Nomura, and Y. Kitamura. 1994. C-kit gene is expressed by skin mast cells in embryos but not in puppies of Wsh/Wsh mice: age-dependent abolishment of c-kit gene expression. *Blood* 83: 3509–3516.
43. Nakae, S., L. H. Ho, M. Yu, R. Monteforte, M. Iikura, H. Suto, and S. J. Galli. 2007. Mast cell-derived TNF contributes to airway hyperreactivity, inflammation, and TH2 cytokine production in an asthma model in mice. *J. Allergy Clin. Immunol.* 120: 48–55.
44. Koarai, A., M. Ichinose, S. Ishigaki-Suzuki, S. Yamagata, H. Sugiura, E. Sakurai, Y. Makabe-Kobayashi, A. Kuramasu, T. Watanabe, K. Shirato, et al. 2003. Disruption of L-histidine decarboxylase reduces airway eosinophilia but not hyperresponsiveness. *Am. J. Respir. Crit. Care Med.* 167: 758–763.
45. Cowden, J. M., J. P. Riley, J. Y. Ma, R. L. Thurmond, and P. J. Dunford. 2010. Histamine H4 receptor antagonism diminishes existing airway inflammation and dysfunction via modulation of Th2 cytokines. *Respir. Res.* 11: 86.
46. Blümchen, K., K. Gerhold, I. Thorade, C. Seib, U. Wahn, and E. Hamelmann. 2004. Oral administration of desloratadine prior to sensitization prevents allergen-induced airway inflammation and hyper-reactivity in mice. *Clin. Exp. Allergy* 34: 1124–1130.
47. Neumann, D., S. Beermann, and R. Seifert. 2010. Does the histamine H4 receptor have a pro- or anti-inflammatory role in murine bronchial asthma? *Pharmacology* 85: 217–223.
48. Yoshimoto, T., K. Yasuda, H. Tanaka, M. Nakahira, Y. Imai, Y. Fujimori, and K. Nakanishi. 2009. Basophils contribute to T(H)2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to CD4$^+$ T cells. *Nat. Immunol.* 10: 706–712.
49. MacGlashan, D., Jr. 2008. IgE receptor and signal transduction in mast cells and basophils. *Curr. Opin. Immunol.* 20: 717–723.
50. Kawakami, T., and S. J. Galli. 2002. Regulation of mast-cell and basophil function and survival by IgE. *Nat. Rev. Immunol.* 2: 773–786.