Mast cell deficiency in Kit<sup>W-sh</sup> mice does not impair antibody-mediated arthritis

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We previously reported that joint swelling, synovial thickening, and cartilage matrix depletion induced by the injection of anti-collagen monoclonal antibodies and lipopolysaccharide (LPS) in BALB/c mice are increased in the absence of inhibitory leukocyte immunoglobulin (Ig)-like receptor B4 (LILRB4; formerly gp49B1) in a neutrophil-dependent manner. Because both mast cells and neutrophils express LILRB4, we sought a mast cell requirement with mast cell-deficient mouse strains, but unexpectedly obtained full arthritis in Kit<sup>W-sh</sup> mice and full resistance in Kit<sup>W/W-v</sup> mice. Kit<sup>W-sh</sup> mice were indeed mast cell deficient as assessed by histology and the absence of IgE/mast cell-dependent passive cutaneous anaphylaxis in the ear and joint as well as passive systemic anaphylaxis. Deletion of LILRB4 in Kit<sup>W-sh</sup> mice exacerbated anti-collagen/LPS-induced joint swelling that was abolished by neutrophil depletion, establishing a counterregulatory role for LILRB4 in the absence of mast cells. Whereas blood neutrophil levels and LPS-elicited tissue neutrophilia were equal in Kit<sup>W-sh</sup> and Kit<sup>+</sup> mice, both were impaired in Kit<sup>W/W-v</sup> mice. Although both strains are mast cell deficient and protected from IgE-mediated anaphylactic reactions, their dramatically different responses to autoantibody-mediated, neutrophil-dependent immune complex arthritis suggest that other host differences determine the extent of mast cell involvement. Thus, a conclusion for an absolute mast cell role in a pathobiologic process requires evidence from both strains.
both Lihb4+ and Lihb4− mice in the KitW−/KitW− background. The ability to detect an effect of mast cell deficiency in KitW+/KitW− mice but not KitW−/KitW− mice suggests that conclusions about absolute mast cell dependence in multicomponent disease models such as mAb-mediated arthritis require confirmation in a mouse strain that is sufficient for the other key cellular elements.

RESULTS AND DISCUSSION

Mast cell deficiency in KitW−/KitW− mice does not prevent anti-collagen/LPS–induced arthritis

When KitW+/KitW− mice and mast cell–sufficient Kit+ mice were injected with 2 mg of anti-collagen and 25 μg LPS 3 d later, joint swelling was detected in both strains on day 5, was maximal by day 6 with clinical scores of 9, and diminished to the baseline level by day 14 (Fig. 1 A). Furthermore, there were no significant differences in Kit− and KitW−/KitW− mice at day 7 in synovial thickness, cartilage matrix depletion, and synovial neutrophilia in ankle joints as assessed histologically (55 ± 5.4 vs. 52.7 ± 6.1 μm; 22.6 ± 2.2 vs. 17.0 ± 2.8% depletion, and 19.0 ± 6.1 vs. 21.2 ± 7.4 neutrophils/unit area; P = 0.8, 0.1, and 0.8, respectively; n = 9). Induction of less joint swelling by reducing the anti-collagen dose to 0.5 mg resulted in peak clinical scores on day 7 in Kit+ and KitW−/KitW− mice of 2.3 ± 0.9 and 2.7 ± 0.9 (n = 5; P = 0.8), respectively, indicating that no effect of mast cell deficiency was uncovered even at the lower limit of clinical detection. Because we had expected a mast cell contribution based on studies reported in mast cell–deficient KitW+/KitW− mice in the arthritis model induced with anti-GPI Abs (5), we evaluated our protocol in that strain and its Kit− control. When WBB6F1–Kit+ mice were injected with 4 mg of anti-collagen and either 25 or 50 μg LPS, they developed joint swelling on day 5 and attained maximal clinical scores of 5 and 6.8, respectively, by day 7, whereas mast cell–deficient KitW+/KitW− mice did not exhibit joint swelling (Fig. 1 B).

Histologic and functional evidence of mast cell deficiency in KitW−/KitW− mice

We confirmed that our KitW−/KitW− mice were mast cell deficient by histologic and functional criteria at the age range at which arthritis was induced. As determined in tissue sections stained for chloroacetate esterase (CAE) activity, there were essentially no mast cells in the synovium and subsynovium of the ankle joint, tongue, spleen, liver, kidney, heart, or intestine of 5–9-wk-old naïve KitW−/KitW− mice, and the number of mast cells in the ear skin and the dermis adjacent to the subsynovium of the ankle joint was 1.7 and 3.4%, respectively, of the number in Kit+ mice (n = 4–7). Estimates of the number of mast cells in the ear and back skin of 10-wk-old KitW−/KitW− mice have ranged from 6–10% of that of Kit+ mice, as assessed by alcian blue (6) or toluidine blue staining (7) of tissue sections, to not detectable in the ear skin by methylene blue staining or CAE activity (8). Kit+ but not KitW−/KitW− mice exhibited passive cutaneous anaphylaxis (PCA) reactions in the ear and paw as measured by extravasation of Evans Blue dye–bearing protein (Fig. 2), indicating that the KitW−/KitW− mice are functionally deficient in dermal mast cells. Finally, to exclude a pool of functional mast cells at any site, we subjected KitW−/KitW− and Kit+ mice to passive systemic anaphylaxis induced by i.v. injection of anti–mouse IgE. Four out of four Kit+ mice became moribund, whereas there was no clinical response in four out of four KitW−/KitW− mice. Thus, the KitW−/KitW− strain is indistinguishable from the Kit+/KitW− strain with regard to mast

Figure 1. Anti-collagen/LPS–induced joint swelling in KitW+/KitW− and KitW−/KitW− mice and their respective WT strains. Mice were injected i.v. with 2 mg of a mixture of four anti-collagen mAbs on day 0 and i.p. with 25 (A and B) or 50 (B) μg LPS on day 3. A score reflecting visual assessment of joint swelling in the paw, wrist, and ankle (described in Materials and methods) of each leg was assigned, and the four scores for each mouse were summed to yield the clinical score. Data are expressed as mean ± SEM; n = 6 from two experiments (days 0–7), n = 3 from one experiment (days 8–14) [A], n = 4 from one experiment with 25 μg LPS, and n = 4 from one experiment with 50 μg LPS (B).

Figure 2. IgE–PCA in mast cell–deficient and –sufficient mice. Mice were injected i.d. in one ear or s.c. in one paw with 25 and 75 ng of mouse monoclonal IgE anti-DNP, respectively, and were injected in the other ear or paw with saline. After 20 h, the mice were injected i.v. with 100 μg DNP-HSA and Evans blue. After 0.5 h, mice were killed, their ears and paws were excised, and the Evans blue was extracted with formamide and quantified by spectrophotometry. Data are expressed as mean ± SEM; n = 7–9 from two experiments (ear) and n = 5 from three experiments (paw).
cell deficiency by morphological and IgE-dependent functional criteria. The functionally equivalent mast cell deficiency of KitW-/- and Kit+/KitW-/- mice had already been observed in models of antigen-induced pulmonary inflammation and venom detoxification, with the latter reflecting the ability of mast cell carboxypeptidase A to degrade sarafotoxins in venom (9–11).

Basal and LPS–induced elevation of circulating and tissue neutrophils differs in mast cell–deficient strains

Because neutrophils are essential for the generation of joint swelling and tissue pathology in the anti-collagen/LPS and anti-GPI models (1, 12), we quantified the number of peripheral blood neutrophils in KitW/KitW-/- and Kit+/KitW-/- mice and their respective Kit+ control strains before and 24 h after i.p. injection of 25 μg LPS, the dose used in the anti-collagen/LPS model. The concentration of peripheral blood neutrophils in 7–9-wk-old naive KitW/KitW-/- mice was only one third of that in WBB6F1-Kit+ mice (Fig. 3). LPS induced a significant threefold increase in the concentration of peripheral blood neutrophils in both of these strains, and the level reached in KitW/KitW-/- mice was again only one third of that in WBB6F1-Kit+ mice. Because there was no arthritis in the KitW/KitW-/- strain, we compared the LPS–elicited efflux of peripheral blood neutrophils into the ear skin of KitW/KitW-/- and WBB6F1-Kit+ mice. 24 h after intradermal (i.d.) injection of 50 μg LPS, there were significantly fewer neutrophils in the peripheral blood of KitW/KitW-/- mice compared with WBB6F1-Kit+ mice (0.68 ± 0.074 vs. 1.5 ± 0.15 × 10^5 cells per μl, respectively; n = 9–11; P = 0.0002) and recruited into the ears (5.8 ± 0.8 vs. 13.3 ± 1.7 neutrophils per unit length, respectively; n = 11–12; P = 0.008). Our data confirm and extend the findings of Chervenick and Boggs reported in 1969 (13), which indicated that the concentration of peripheral blood neutrophils in naive 3-mo-old KitW/KitW-/- mice (the youngest mice examined) was a significantly lower 31% of that of WBB6F1-Kit+ mice, and reached ~80% of normal at 6 mo. The number of BM neutrophils in Kit+/KitW-/- mice was a significantly lower 52–64% of that of WBB6F1-Kit+ mice from 3 to 6 mo of age. Even in older KitW/KitW-/- mice with near normal peripheral blood neutrophil levels, the increase in neutrophils 6 h after i.p. injection of 5 μg LPS was only ~60% of that of WBB6F1-Kit+ mice (13). Naive KitW+/KitSl-d mice that are on the WCB6F1 background and are stem cell factor deficient (14) also have a BM and peripheral blood neutropenia (15).

In contrast with Kit+/KitW-/- mice, the concentration of neutrophils in the blood of naive 7–9-wk-old Kit+/kit-/- mice was not different than that of Kit+ controls (Fig. 3). Injection of 25 μg LPS i.p. induced comparable increases in the concentration of peripheral blood neutrophils after 24 h in Kit+ and Kit+/kit-/- mice, respectively (Fig. 3). 24 h after i.d. injection of 50 μg LPS, there was no significant difference in the number of neutrophils in the blood of Kit+ and Kit+/kit-/- mice (2.0 ± 0.21 vs. 1.9 ± 0.21 × 10^5 cells per μl, respectively; n = 3–4) or migration of neutrophils into the ears (13.9 ± 1.2 vs. 12.2 ± 3.5 neutrophils per unit length, respectively; n = 6–8; P = 0.7). Hence, unlike Kit+/KitW-/- mice, Kit+/kit-/- mice exhibit the same constitutive blood level and LPS–induced tissue recruitment of neutrophils as their Kit+ controls. The cumulative findings suggest a role for the stem cell factor–Kit interaction in neutrophil development in certain genetic backgrounds and/or when mutations in that ligand–receptor pair are located within the molecules themselves (14, 16) and hence are global (i.e., Kit+/KitW-/- and Kit+/kitSl-d mice in the rather unusual WBB6F1 and WCBF1 backgrounds, respectively). In contrast, an alteration upstream of the Kit gene (17, 18) is associated with a selective deficiency of expression in mast cells and probably not in myeloid progenitors that populate the neutrophil lineage (i.e., Kit+/kitSl-d mice in the C57BL/6 background). The neutropenia is not the only difference in the two strains, as Kit+/KitW-/- but not Kit+/kit-/- mice have a macrocytic anemia and deficiency in γδ T cells (7, 19), whereas both strains have deficiencies in melanocytes (20) and interstitial cells of Cajal (7). Because the absence of mast cells in Kit+/kitSl-d mice resulted from a 2-cM inversion upstream of the Kit gene in C3H/HeJ × 101/H F1 mice (19, 21), it is possible that the retention of certain alleles from the F1 strain even after backcrossing into C57BL6 mice contributes to the phenotypic differences of Kit+/kitSl-d mice compared with Kit+/KitW-/- mice.

Splenich, but not BM, myeloid hyperplasia in Kit+/kit-/- mice

As part of a general histologic assessment for tissue mast cells, we noted that the spleens of Kit+/kit-/- mice as compared with their Kit+ controls had a significant 2.4-fold greater number of CAE+ cells with myeloid morphology (Fig. 4 A) that had a primarily subcapsular localization and included a significantly greater percentage of immature forms (68 ± 7% and 16 ± 4%, respectively; n = 3; P = 0.004). Induction of synovitis with anti-collagen/LPS caused an increase in the splenic CAE+ cells in both strains that reached a significantly greater level in the Kit+/kit-/- mice (Fig. 4 A). As measured by flow cytometry,
dispersed splenocytes from naive Kit+ and KitW-sh mice were 7 ± 2 and 32 ± 7% Gr-1+, respectively (n = 4; P = 0.01; representative histograms are presented in Fig. 4 B), consistent with the greater number of CAE+ myeloid cells in KitW-sh mice. In contrast, there was no difference in the number of CAE+ cells in the BM of Kit+ and KitW-sh mice (64 ± 26.5 and 70 ± 1.2 cells per high power field, respectively; n = 4). There was also no difference in the number of BM cells recovered from the femurs and tibias of Kit+ and KitW-sh mice (5.4 ± 1.5 and 5.8 ± 1.4 × 107 cells/mouse, respectively; n = 3) or in the percentage of Gr-1+ cells in the BM (72 ± 8 and 77 ± 5%, respectively; n = 3–4). Because the BM of KitW-sh mice lacked these changes, and the blood level and recruitment of neutrophils in the joints and ears of KitW-sh mice was the same as in Kit+ mice with no increase in morphologically immature myeloid cells, there was no evidence that the splenic dysmyelopoiesis was a factor in the normal response to anti-collagen/LPS-induced arthritis in mast cell–deficient KitW-sh mice. Indeed, there was no difference in Kit+ and KitW-sh mice at day 7 of the anti-collagen mAbs/LPS model in the number of peripheral blood neutrophils (1.9 ± 0.7 and 1.5 ± 0.5 × 107/μl, respectively; n = 4; P = 0.6) and neutrophils per unit area in the synovium (19.6 ± 6.8 and 21.2 ± 7.4 cells/unit area, respectively; n = 9; P = 0.8) and joint space (5.8 ± 1 and 8.4 ± 2.6 cells/unit area, respectively; n = 9; P = 0.3) at day 7.

**Inflammatory arthritis is exacerbated in Lilrb4+/KitW-sh mice**

The full inflammatory arthritis in KitW-sh mice afforded the opportunity to assess the potential contribution of LILRB4 in the absence of mast cells. The clinical scores in the Lilb4+/KitW-sh and Lilb4+/KitW-sh on day 7 reached values of 6.1 ± 1.4 and 8.7 ± 1.6, respectively, and the entire time course of clinical scores in Lilb4+/KitW-sh mice was significantly greater than in Lilb4+/KitW-sh mice (P = 0.03; Fig. 5). Histologic analysis of the synovial thickness in the talo-tibial articulation of the ankle joint at day 7 revealed a trend toward greater thickness in Lilb4+/KitW-sh mice compared with Lilb4+/KitW-sh mice (71.0 ± 10 vs. 52.7 ± 6.1 μm, respectively; n = 9; P = 0.1; the measurements were made in the same experiments that provided the comparison of Kit+ and KitW-sh mice noted above). Moreover, as assessed by digital image analysis of the loss of staining with safranin O, there was a significant approximately twofold greater depletion of cartilage matrix at day 7 in Lilb4+/KitW-sh mice compared with Lilb4+/KitW-sh mice (32.2 ± 6.3 vs. 17.0 ± 2.8%; n = 9; P = 0.04). Hence, the exacerbation of these characteristics in the absence of LILRB4 proceeds without a mast cell influence, compatible with a central role for neutrophils.

To establish an absolute neutrophil requirement in the LILRB4–sufficient and –deficient KitW-sh strains, mice were injected i.p. with anti–Gr-1 on days 2 and 4 of the anti-collagen/LPS protocol, as done previously with Lilb4+ and Lilb4– mast cell–sufficient mice on the BALB/c background (1). Treatment with anti–Gr-1 depleted peripheral blood neutrophils by >95% compared with untreated mice and suppressed clinical scores in both Lilb4+/KitW-sh (0 ± 0 vs. 6.0 ± 3, respectively) and Lilb4+/KitW-sh mice (0 ± 0 vs. 9.0 ± 0) at day 7 (n = 2–5 mice per genotype).

In addressing the discrepancy as to why the KitW-sh but not KitW/KitW-v mast cell–deficient strain is fully susceptible to anti-collagen/LPS-induced arthritis, we confirmed an old observation that KitW/KitW-v mice are neutropenic and mobilize blood neutrophils poorly (13). Although there are also deficiencies in other lineages in KitW/KitW-v mice that are not present in KitW-sh mice, we have focused on the neutrophil because of its prominence in the model. This has recently been highlighted in studies of arthritis induced with Ab to GPI in which clinical and tissue pathology was interrupted when the number of neutrophils fell due to blockade of their continuous recruitment from blood by local generation or action of leukotriene B4 with inhibitors of 5-lipoxygenase or...
the BLT₁ receptor (22, 23). The finding that a critical concentration of neutrophils must be achieved to control the growth of bacteria in tissue (24) may be relevant to the requirement for presence of a sufficient concentration of neutrophils to induce inflammation and tissue pathology in the arthritis models. That the absence of the control receptor LILRB4 in mast cell–deficient KitW−/- mice augments the clinical and tissue pathology in the anti-collagen/LPS model again supports the importance of neutrophils as opposed to mast cells, but does not exclude a contribution by other cell types lacking the receptor. Our findings do not question a role for adaptively transferred WT BM–derived mast cells in uncovering the arthritic potential of mAb in the KitW+/KitW+ strain as shown in the anti–GPI model (5), but rather suggest that the limitation of other cell types, such as neutrophils, allows recognition of a mast cell contribution. Indeed, engraftement of mast cells into KitW+/KitW+ mice corrects a deficiency in the influx of peritoneal neutrophils in response to i.p. injection of peptidoglycan (25). Naïve WBB6F1–Kit+ mice have six times the number of peritoneal mast cells as C57BL/6–Kit+ mice, and 24 h after cecal ligation and puncture have 20 times the number of peritoneal neutrophils (26). The propensity for elevated numbers of mast cells and neutrophils in WBB6F1–Kit+ mice combined with the neutropenia in mast cell–deficient KitW+/KitW+ mice may magnify the contribution of mast cells to neutrophil–dependent models of inflammation in this strain compared with the C57BL/6 strain. Hence, our findings suggest that a potential role for mast cells is not always critical, and that the availability of a second mast cell–deficient strain allows separation of absolute and relative roles for mast cells in disease models.

MATERIALS AND METHODS

Mice. Liliation4+/− mice on a mixed 129/BALB/c background (3) were backcrossed to B6 mice five times, and for the last four of those backcrosses, mice with the greatest number of B6 alleles were selected by microsatellite analyses (Charles River Laboratories). The resulting backcrossed Liliation4+/− mice were intercrossed, and their homozygous progeny were bred in parallel to yield Liliation4−/− and Liliation4−/+ mice. KitW−/+ mice, backcrossed at least 10 times to the B6 background, were obtained via D. Lee (Brigham and Women’s Hospital, Boston, MA) from P. Wolters and G. Caughey (University of California, San Francisco, San Francisco, CA) (27), who obtained them from P. Besmer (Memorial Sloan-Kettering Cancer Center, New York, NY). The mice were bred with Liliation4+ mice to yield Liliation4+/+KitW−/+ mice, which were intercrossed to produce Liliation4−/+ KitW− and Liliation4−/+ KitW− mice. 7–9-wk-old male mice were used for experiments. Mice were maintained in a specific pathogen-free barrier facility at the Dana-Farber Cancer Institute, and the studies were approved by the Animal Care and Use Committee.

Induction of proliferative synovitis. Mice were injected i.v. with a mixture of anti–type II collagen mAbs (Chemicon International Inc.), followed 3 d later by an i.p. injection of LPS (Escherichia coli O111:B4; Chemicon) (1, 28) at the doses indicated in the Results. Because the intensity of clinical pathology obtained with different batches of anti-collagen varied, doses were chosen that provided an average peak clinical score (described below) of at least 5.

Clinical and histologic analyses. A clinical score for joint swelling in each leg was assigned according to the following scale: 0, no detectable swelling; 1, swelling in metatarsal phalange joints, an individual phalanx, or local edema; 2, swelling localized to either the dorsal or ventral surface of a paw; 3, swelling on all aspects of a paw (1). The sum of the four scores was defined as the clinical score for each mouse. Synovial thickness was measured in histologic sections of the talo-tibial articulation of the ankle joint stained for CAE activity as described previously (1). Cartilage matrix depletion was measured in sections stained with Safranin O as described previously (1), except that the areas of cartilage matrix depletion within the cartilaginous regions were outlined in digital photomicrographs, converted to pixels with ImageJ software (Image Processing and Analysis in Java developed by the National Institutes of Health), and expressed as a percentage of the total cartilaginous region. Quantification of LPS–induced neutrophilia was performed in ear sections stained for CAE activity (2).

IgE-dependent anaphylaxis. For PCA in the ear, mice were injected i.d. in one ear with 25 ng IgE anti-DNP (SPE-7; Sigma-Aldrich) and with an equal volume of saline (20 μl) in the other ear. 20 h later, mice were injected i.v. with 100 μg DNP-HSA (30–40 moles DNP/HSA; Sigma-Aldrich) in 100 μl of 1% Evans Blue dye. After 0.5 h, mice were killed, ear tissue was obtained with a 6-mm diameter punch, dye was extracted from the tissue by incubation in 200 μl of formamide at 58°C for 48 h, the tissue was pulsed by centrifugation, and the dye in the supernatant was quantified spectrophotometrically (3, 29). For PCA in the paw, mice were injected s.c. in one hind paw with 75 μg IgE anti-DNP and in the other hind paw with an equal volume (10 μl) of saline. Mice were challenged with antigen and Evans blue, killed, and the paws were collected from the fur line and cut into small pieces. The dye was extracted as for PCA in the ear except that an extraction volume of 1 ml was used. For IgE–dependent systemic anaphylaxis, mice were injected i.v. with 40 μg of rat anti–mouse IgE (R35-72; BD Biosciences).

Peripheral blood neutrophil counts and Ab–mediated depletion. Blood was collected, and leukocyte counts and differentials were determined with an automated cell counter as described previously (1). For neutrophil depletion, mice were injected i.p. with rat anti–Gr-1 mAb or rat IgG2b isotype control mAb (1, 12).

Flow cytometry. Spleens were disaggregated mechanically to liberate splenocytes, and the cells were incubated for 10 min at room temperature with ACK buffer (155 mM NH₄Cl, 10 mM KHCO₃, 111 μM dethox EDTA in Milli-Q water) to lyse erythrocytes, centrifuged, and resuspended at 10⁶ cells/ml in HBA buffer (calcium- and magnesium-free HBSS containing 0.1% [wt/vol] BSA and 0.02% [wt/vol] sodium azide). The spleen cells were incubated for 30 min at 4°C with a saturating concentration of PE–labeled anti–Gr-1 or an isotype control mAb (1, 12).

Statistical analyses. Data are expressed as mean ± SEM unless otherwise noted. Differences in single measurements between groups of mice were assessed with Student’s unpaired, two-tailed t test. Differences in the time courses of clinical scores were assessed with ANOVA. p-values of <0.05 were defined as statistically significant.

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