Mast Cells Regulate the Magnitude and the Cytokine Microenvironment of the Contact Hypersensitivity Response

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The role that mast cells play during contact hypersensitivity (CS) response is unclear because some studies have shown that mast cell-deficient mice have relatively intact CS responses whereas others have shown opposing results. Mast cells secrete a wide range of immunomodulatory mediators and can potentially influence the type of immune response generated in the skin during CS. Therefore, we examined the type of microenvironment generated during CS in both W/W^v mast cell-deficient and wild-type mice in response to different immunizing doses of hapten (oxazolone). The CS response elicited after low-dose oxazolone was significantly diminished in W/W^v mice compared with wild-type mice. Unexpectedly, the CS response elicited in W/W^v mice immunized with high-dose oxazolone was more severe compared with wild-type mice. In addition, after immunization with high-dose oxazolone, the granulocyte infiltrate in W/W^v mice was increased by twofold compared with wild-type mice. A shift in the cytokine milieu toward the expression of type-1 cytokines as well as a significant increase in the local adhesion of neutrophils and CD4 T cells in the microvasculature of the skin was observed after hapten challenge in W/W^v mice immunized with high-dose oxazolone compared with wild-type mice. These results suggest that mast cells can act as regulators and inducers of the inflammatory response depending on immunizing stimulus strength. (*Am J Pathol* 2008, 172:1638–1649; DOI: 10.2353/ajpath.2008.070559)

Contact sensitivity (CS), a form of delayed type hypersensitivity is a prototypical T-cell-mediated skin inflammatory response that occurs after cutaneous exposure to haptens. Re-exposure of the skin to the same sensitizing hapten results in an inflammatory response that is characterized by substantial leukocyte infiltration into tissue and edema formation that peaks throughout 24 to 48 hours. This response is subject to the regulatory effects of cytokines produced by T lymphocytes and is dependent on the recruitment of antigen-specific T cells to the site of hapten challenge.^{1–3} Multiple types of T cells have been shown to play a role in murine CS. In addition to interferon gamma (IFN- γ)- and interleukin (IL)-2-producing CD4 helper T lymphocytes (T_H1 cells), IFN- γ -producing CD8 T cells, $\gamma\delta$ T cells, and IL-4- or IL-10-secreting CD4 T cells (T_H2 cells) also participate in murine CS.^{4–8} Thus, to potentially control hypersensitivity responses it is important to understand the antigen-specific/nonspecific events that are responsible for the recruitment of T-lymphocyte subsets into skin.

The inflammatory response elicited after re-encountering antigen can be separated into two distinct phases, both demarcated by a macroscopic swelling response. The first phase occurs within the first 2 hours after elicitation and is thought to involve the activation of the complement system, degranulation of mast cells, and the release of vasoactive and inflammatory mediators such as histamine, serotonin, and tumor necrosis factor (TNF)- α .³

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This is followed by leukocyte migration into tissue, and generation of a second larger peak of swelling occurring throughout 24 to 48 hours. This late-phase response is dependent on the recruitment of antigen-specific T cells to the site of hapten challenge within the first 2 hours of the response.^{1,2,9} Clearly events that occur within the first 2 hours are important in governing the entire CS response.

As one of the early events in the initiation of CS is thought to be the degranulation of mast cells; it has been hypothesized that mast cells contribute to the initial recruitment of T cells into the skin.^{1,2,10–12} However we have recently shown that C5a acting directly on T cells and not tissue resident cells is responsible for their recruitment during this early phase.¹³ Although, this does not rule out the participation of mast cells during the early phase of CS, the impact of mast cells on T cell and other leukocyte recruitment may also occur at later time points. Indeed, the specific role of mast cells in the elicitation phase of CS is unclear because similar CS protocols have yielded conflicting results.^{11,14,15} Some studies have shown that mast cell-deficient mice have intact or only slightly diminished CS responses compared with their wild-type controls.^{14,15} In fact, the remaining CS response in these mice was reported to be dependent on serotonin released from platelets.¹⁶ In contrast, other studies have shown that mast cells are important in effector cell recruitment into tissue. In these studies, mast cell-deficient mice exhibited reduced tissue swelling and reduced cell recruitment into the skin.^{11,17,18} Slight differences in hapten or diluents have been hypothesized to contribute to disparate results.^{17,18} In line with this, it is interesting to note that in many studies examining edema in the elicitation phase of CS, the role of components such as complement that play a role during the early elicitation phase of CS in augmenting the late elicitation phase are only uncovered if a suboptimal dose of hapten is used to sensitize mice.^{19,20}

In the current study, we have performed a series of experiments to systematically evaluate the role of mast cells in modulating the microenvironment generated during CS. The early and late challenge phases of the CS response were studied in the skin microvasculature of both mast cell-deficient (*W/W^v*) and wild-type mice immunized with either a low or high dose of hapten. Whereas the lower immunizing dose of hapten induced less inflammation in mast cell-deficient mice after subsequent challenge, the higher immunizing dose caused an unexpected worsening of inflammation in mast cell-deficient mice, with increased type-1 cytokine expression.

Materials and Methods

Mice

BALB/c, DO11.10, mast cell-deficient *Kit^W/Kit^{W-v}* (*W/W^v*) mice, and their congenic normal littermates (WBB6F1/J) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in the viral antigen-free

double-barrier unit at the University of Calgary. The protocols used were in accordance with the guidelines drafted by the University of Calgary Animal Care Committee and the Canadian Council on the Use of Laboratory Animals. All mice weighed between 20 and 32 g and were used between 6 and 10 weeks of age.

Oxazolone-Induced CS

For the high hapten dose protocol, mice were sensitized by the topical application of 50 μ l of a 5% oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one; Sigma-Aldrich, St. Louis, MO) in olive oil/acetone vehicle (1:4) to the shaved flank. Five to seven days later, mice received a 10- μ l challenge of 1% oxazolone solution on the ventral aspect of the left ear. The right ear was challenged with 10 μ l of the vehicle control solution (olive oil/acetone alone). The CS response was assessed at 0, 2, 24, or 48 hours after antigen challenge. For the low hapten dose protocol, mice were sensitized with 50 μ l of a 2% oxazolone olive oil/acetone solution applied to the shaved flank and were challenged on the left ear with 0.8% oxazolone olive oil/acetone solution 5 to 7 days later.¹⁷ We also demonstrated in preliminary data that challenge at 1% in this group resulted in identical responses as 0.8%. In some cases after the sensitization protocol described above the CS response was elicited in the mouse flank, with mice receiving a 50- μ l challenge of 1% oxazolone solution to the upper right abdomen of the mouse.

Cell Cultures

Single cell suspensions were made from the draining lymph nodes of 4- or 5-day CS sensitized *W/W^v* mice or WBB6F1/J mice and were enriched for T cells. The remaining cells were irradiated and used as antigen-presenting cells (APCs). Some APCs were haptenized by incubation with a 20 μ mol/L oxazolone solution for 30 minutes whereas others were left untreated.²¹ APCs (1×10^7 cells/ml) were then incubated with T cells (1×10^7 cells/ml) for 72 hours, after which the cell culture supernatant was harvested and immediately frozen at -80°C .

Measurement of Ear Thickness

The thickness of antigen challenged ears was measured using an engineer's dial micrometer (Mitutoyo Co., Aurora, IL). Ear thickness was calculated by subtracting the thickness of vehicle control-challenged right ear from that of the oxazolone-challenged left ear.

Quantitative Enzyme-Linked Immunosorbent Assay (ELISA) for IFN- γ , IL-4, TNF- α , and IL-10 in Ear Homogenates and Cell Culture Supernatants

Three 4-mm punch biopsies were taken from the ears of contact-sensitized mice and were flash-frozen in liquid

nitrogen. Samples were extracted in cold phosphate-buffered saline with a tissue microhomogenizer. A commercially available ELISA kit was used to measure cell culture and tissue homogenate concentrations of IFN- γ , IL-4, TNF- α , and IL-10 (BD Biosciences, Franklin Lakes, NJ). Final concentrations of each cytokine were corrected for the concentration of total protein in each homogenate sample.

Serum IgE Levels

IgE levels were measured from serum derived from WBB6F1/J or W/W^v mice 5 days after sensitization with either a high dose or low dose of oxazolone using the BD OptEIA mouse IgE ELISA set (BD Biosciences).

Histology

Tissue samples were fixed in 10% formalin, processed, and hematoxylin and eosin (H&E)-stained by the Department of Histopathology at the University of Calgary. In addition, slides were stained for granulocytes with a chloroacetate esterase (Leder) stain (Sigma-Aldrich) and analyzed by light microscopy in a blinded manner. Leukocyte numbers were determined by counting the number of positive-stained cells over 10 fields at a magnification of $\times 400$. The mean number of positive cells per field of view was then calculated.

Spinning Disk Confocal Microscopy

Leukocyte-endothelium interactions were studied in the microcirculation of mouse flank skin. Animals were anesthetized by an intraperitoneal injection of a mixture of 10 mg/kg of xylazine hydrochloride (MTC Pharmaceuticals, Cambridge, Canada) and 200 mg/kg of ketamine hydrochloride (Rogar/STB, London, Canada). The right jugular vein was cannulated to administer additional anesthetic and fluorescent dyes.

The microcirculation of the ventral abdominal skin was prepared for microscopy as previously described.²² Briefly, a midline abdominal incision was made extending from the pelvic region up to the level of the clavicle. The skin was separated from the underlying tissue, remaining attached laterally to ensure the blood supply remained intact. The area of skin was then extended over a viewing pedestal and secured along the edges using 4.0 sutures. The loose connective tissue lying on top of the dermal microvasculature was carefully removed by dissection under an operating microscope. The exposed microvasculature was immersed in isotonic saline and covered with a coverslip held in place with vacuum grease.

The flank skin microvasculature was visualized using a spinning disk confocal microscope. Images were acquired with an Olympus BX51 (Olympus, Center Valley, PA) upright microscope using a $\times 20/0.95$ XLUM Plan FI water immersion objective. The microscope was equipped with a confocal light path (WaveFx; Quorum, Guelph, Canada) based on a modified Yokogawa CSU-10 head

(Yokogawa Electric Corporation, Tokyo, Japan). Anti-CD4 fluorescein isothiocyanate (L3T4, 3 μ g/mouse; BD Biosciences) and anti-mouse Ly-6G phycoerythrin (Gr-1, 2 μ g/mouse; BD Biosciences) were injected intravenously into WBB6F1/J and W/W^v mice to image CD4⁺ T lymphocytes and neutrophils, respectively. Both 488- and 561-nm laser excitation (Cobalt, Stockholm, Sweden) were used in rapid succession and visualized with the appropriate long pass filters (Semrock, Rochester, NY). Typical exposure times for both excitation wavelengths were 168 ms. A 512×512 pixel back-thinned electron-multiplying charge-coupled device camera (C9100-13'; Hamamatsu, Bridgewater, NJ) was used for fluorescence detection. Velocity Acquisition software (Improvision, Lexington, MA) was used to drive the confocal microscope. Simultaneous CD4 T lymphocyte and neutrophil rolling and adhesion were assessed in 20 random fields of view in the postcapillary venules of the skin. Rolling leukocytes were defined as those cells moving at a velocity slower than that of erythrocytes within a vessel. Leukocyte rolling flux was determined by counting the number of leukocytes that rolled by a fixed point in the venule for 1 minute. Leukocyte rolling velocity was determined by measuring the time required for a leukocyte to roll along a 100- μ m length of venule. Leukocytes were considered adherent to the venular endothelium if they remained stationary for 30 seconds or longer.

In Vitro Generation of OVA-Specific T_H1 and T_H2 Lymphocyte Populations

CD4⁺ T lymphocytes were isolated from pooled spleens of DO11.10 mice using mouse CD4 (L3T4) Dynabeads per the manufacturer's protocol (DynaL Biotech Inc., Brown Deer, WI). Isolations routinely resulted in greater than 90% purity of CD4⁺ cells. T_H1 and T_H2 cells were generated *in vitro* from antigen-naïve CD4⁺ T cells as described previously.^{23,24} Briefly, purified CD4⁺ T cells were cultured at a ratio of 1:5 (for T_H1) and 1:25 (for T_H2) with irradiated BALB/c splenocytes and 5 μ g/ml of OVA peptide 323-339. The addition of 50 U/ml of IL-12 (R&D Systems, Minneapolis, MN) and 10 μ g/ml of anti-IL-4 (11B11) was used to generate T_H1 cells, whereas 1000 U/ml of IL-4 (R&D Systems), 10 μ g/ml of anti-IL-12 (C17.8), and 10 μ g/ml anti-IFN- γ (XMG1.2) was used to generate T_H2 cells. Optimal conditions for T_H2 generation required that the T_H2 cells underwent a second 7-day period of culture as described above. This culture procedure ensured that the majority of *ex vivo* polarized cells were of a T_H2 phenotype.^{23,24} Recovered cells were washed and transferred at 10^7 cells/recipient mouse. These cells were characterized in great detail for their trafficking characteristics elsewhere.²³⁻²⁵ Their behavior closely mimicked endogenous T_H1 and T_H2 cells. It is noteworthy that the two 7-day culture protocol generated T_H2 cells with functional selectin ligands as reported recently for endogenous T_H2 cells isolated from inflamed tissue.²⁶⁻²⁸

Intravital Microscopy

The mouse ear was used to study T_H1 and T_H2 endothelial interactions in the dermal microcirculation. Animals were anesthetized as described above. The hair on the left ear was removed with a depilatory cream (Nair; Armkel LLC, Princeton, NJ) and the ear gently rinsed with 0.9% normal saline. The animal was placed on a heating pad and rectal temperature was monitored and maintained at 37°C. The left ear was gently placed against the adjustable Plexiglas microscope pedestal, immersed in saline, and covered with a coverslip that was held in place with vacuum grease. A plasma marker, fluorescein isothiocyanate-labeled albumin (100 μ l of 0.5% solution, Sigma-Aldrich) was administered intravenously to aid visualization of the microvasculature. T_H1 and T_H2 lymphocytes were incubated with 5 mg/ml of rhodamine 6G (Sigma-Aldrich) in saline and washed extensively before injection (10^7 cells i.v.) into recipient mouse. Fluorescence was visualized by epi-illumination using 510 and 560 filters.

The ear microvasculature was visualized using an intravital microscope (Axioskop; Carl Zeiss, Thornwood, NY) with a $\times 40$ water immersion objective lens (Weltzlar; E. Leitz, Munich, Germany) with a $\times 10$ eyepiece. A video camera (5100 HS; Panasonic, Osaka, Japan) was used to project images on to a monitor and the images were recorded for playback analysis using a videocassette recorder. One to four dermal venules (20 to 40 μ m in diameter) were selected in each experiment. Leukocyte trafficking parameters were assessed as described above.

In Vitro Generation of Bone Marrow-Derived Mast Cells (BMMCs)

BMMCs were isolated from the femurs and tibias of C57BL/6 mice and were cultured in complete RPMI 1640 medium supplemented with 10 ng/ml of IL-3 and 12.5 ng/ml of stem cell factor. BMMCs were used after a minimum of 6 weeks in culture at $\sim 98\%$ purity as determined by flow cytometry and Wright-Giemsa staining. For reconstitution, BMMCs (1×10^6 in 20 μ l of sterile saline) were injected intradermally into the pinna of both ears of W/W^v mice. As controls some W/W^v mice and wild-type littermates were injected with 20 μ l of sterile saline. Mice were housed for an additional 5 to 6 weeks before being sensitized with oxazolone.²⁹

Statistics

All data are displayed as mean $\pm$ SEM. Data were analyzed using standard statistical analyses (analysis of variance and Student's *t*-test with Bonferroni's correction for multiple comparison where appropriate). Statistical significance was set at $P < 0.05$.

Results

The CS Response in Low Oxazolone Dose-Immunized Mast Cell-Deficient Mice (W/W^v) Is Less Severe than in Wild-Type Mice (WBB6F1/J)

To determine the role of mast cells in mice immunized with a low hapten stimulus we observed the CS elicitation response 0, 2, 24, and 48 hours after hapten challenge in W/W^v and WBB6F1/J mice immunized with a 2% dose of oxazolone. Little to no tissue injury or leukocyte infiltration could be observed in unchallenged ears or 2 hours after hapten challenge in either WBB6F1/J mice or W/W^v mice (data not shown). By contrast, the CS elicitation inflammatory response in WBB6F1/J mice at 24 hours (Figure 1A) and 48 hours (Figure 1B) was characterized by substantial leukocyte infiltrate as well as erosion of the epidermal layer and the formation of microabscesses (highlighted by arrows). However, tissue injury was diminished both 24 (Figure 1C) and 48 hours (Figure 1D) after hapten challenge in W/W^v mice compared to WBB6F1/J mice. W/W^v mice had little to no microabscess formation and a lower degree of leukocyte infiltrate at both time points tested (Figure 1, C and D).

In addition to being profoundly deficient in mast cells, W/W^v mice are also anemic, sterile, and lack melanocytes.³⁰ To confirm that the difference in CS response observed between low oxazolone dose-immunized W/W^v mice and the wild-type was because of the lack of mast cells, we injected bone marrow-derived mast cells (BMMCs) from wild-type mice, exclusively into the pinna of ears of W/W^v mice. This resulted in the local reconstitution of mast cells to $\sim 8.9 \pm 2.5$ cells/mm², significantly lower than the numbers found in wild-type mice (37.9 ± 3.4 cells/mm²). Despite the fewer mast cells, tissue damage as assessed by microabscess formation and leukocyte infiltration in low oxazolone dose-immunized W/W^v BMMC reconstituted mice was restored to levels similar to that observed in wild-type mice 24 (Figure 1E) and 48 (Figure 1F) hours after hapten challenge. This suggests that the diminished late-phase CS response in low oxazolone dose-immunized W/W^v mice is indeed attributable to the lack of mast cells and not other cell types. This response is hapten-dependent because there was no leukocyte infiltration or tissue injury in vehicle control-treated ears 24 hours after hapten challenge in wild-type (Figure 1G) or W/W^v mice (data not shown).

Tissue edema was not different between the two mouse strains during the early phase of the CS challenge response (2 hours) (data not shown) or at 24 hours after hapten (Figure 1H). However, at a later time point, tissue swelling was significantly decreased in W/W^v mice compared to that seen in WBB6F1/J mice (Figure 1H). By comparison, BMMC reconstitution of W/W^v mice restored the ear swelling CS response to that seen in wild-type mice (Figure 1H). The reduction in ear swelling and in tissue injury during the CS response in W/W^v mice suggests that after a low immunizing dose of ox-

Figure 1. Elicitation response in WBB6F1/J, W/W^v, and W/W^v + BMMC mice immunized with a low dose of oxazolone. H&E staining of oxazolone-treated WBB6F1/J (A, B), W/W^v (C, D), and W/W^v BMMC reconstituted (E, F) mice 24 and 48 hours after challenge, respectively. Epidermal microabscess formation is indicated by arrows. G: H&E staining of vehicle-treated WBB6F1/J mice 24 hours after challenge. H: Ear thickness was assessed at 0, 24, and 48 hours after hapten challenge. Total number of leukocytes (I) and the differential leukocyte counts (J, K) were performed on ester-stained histology sections and are expressed as number of infiltrated leukocytes/mm². A total of four animals were completed at each time point. Experiments were repeated three times with reproducible results (**P* < 0.05 relative to WBB6F1/J mice, ***P* < 0.05 relative to W/W^v + BMMC reconstituted mice). Scale bars = 220 μm. Original magnifications, ×100 (A–F); ×400 (I–K).

azolone, mast cells play a role in generating the inflammatory response.

Counting the number of leukocytes that infiltrated the tissue revealed that the total number of leukocytes present in the extravascular tissue of low oxazolone dose-immunized W/W^v mice was significantly decreased compared to WBB6F1/J mice and W/W^v BMMC reconstituted mice at 24 and 48 hours after hapten challenge (Figure 1I). Although the total level of leukocyte infiltration

at 48 hours was significantly increased in WBB6F1/J and W/W^v BMMC reconstituted mice from that seen at 24 hours, no further increase in number of leukocytes was observed in W/W^v mice at 48 hours (Figure 1I). The reduction in leukocyte recruitment at 24 hours and 48 hours in W/W^v mice compared with WBB6F1/J mice and W/W^v BMMC reconstituted mice was attributable to a significant reduction in both granulocyte and mononuclear cell infiltration (Figure 1, J and K). Overall, these

Figure 2. Tissue cytokine microenvironment after elicitation in mice immunized with a low dose of oxazolone. ELISAs for IL-4 (A), TNF- α (B), IFN- γ (C), and IL-10 (D) were performed on ear homogenates from unchallenged and 24-hour and 48-hour challenged WBB6F1/J and W/W^v mice. BD, below detection limits. A total of four animals were completed at each time point (* $P < 0.05$ relative to baseline mice).

observations are consistent with other groups showing that mast cells contribute to inflammatory responses particularly in controlling neutrophil recruitment during the CS response.^{11,17}

Despite a difference in tissue damage and leukocyte infiltration between low oxazolone dose-immunized W/W^v, WBB6F1/J mice, and W/W^v BMMC reconstituted mice there was no difference in ear homogenate cytokine expression profile after CS elicitation (Figure 2). IL-4 levels were not different from baseline levels at 24 or 48 hours after hapten challenge in all strains of mice (Figure 2A). Interestingly, detectable TNF- α levels in the tissue

were not present until 48 hours after hapten challenge. However, there was no significant difference in the expression levels of TNF- α between W/W^v, WBB6F1/J mice, and W/W^v BMMC reconstituted mice at this time point (Figure 2B). IFN- γ levels were significantly elevated in all strains of mice at 24 hours but were back to baseline levels at 48 hours (Figure 2C). IL-10 was not found in ear homogenates at detectable levels in either W/W^v, WBB6F1/J mice, or W/W^v BMMC reconstituted mice at any time point tested (Figure 2D). Thus challenge of mice that have been sensitized with a low dose of oxazolone results in nonrobust cytokine expression in the challenged ear with no discernable difference between mast cell-deficient mice and their wild-type controls. There was no difference in tissue T_H1 or T_H2 cytokine production in the presence or absence of mast cells.

The CS Response in High Oxazolone Dose-Immunized Mast Cell-Deficient Mice (W/W^v) Is More Severe than in WBB6F1/J Mice

To determine the role of mast cells in mice immunized with a high hapten stimulus we observed the CS elicitation response 0, 2, 24, and 48 hours after hapten challenge in W/W^v and WBB6F1/J mice immunized with a 5% dose of oxazolone. Gross tissue injury generated after challenge in wild-type mice immunized with a high dose of oxazolone (Figure 3) was not different from that generated in wild-type mice immunized with a low dose of oxazolone (Figure 1) but the role of mast cells was vastly different. In contrast to results observed in mice immunized with a low dose of oxazolone, late-phase tissue injury was markedly worse in high oxazolone dose-immunized W/W^v mice compared with wild-type mice and W/W^v BMMC reconstituted mice at 24 hours and 48 hours after hapten challenge. W/W^v mice displayed histological evidence of increased microabscess formation (arrows) around hair follicles and increased macroscopic ulceration of the epidermis (Figure 3, C and D) compared to WBB6F1/J mice (Figure 3, A and B) and W/W^v BMMC reconstituted mice (Figure 3, E and F). This was observed at 24 hours after oxazolone challenge but the differences were more striking at 48 hours after oxazolone challenge. Similar to results seen in mice immunized with a low dose of oxazolone, tissue edema was not different between high oxazolone dose-immunized W/W^v and wild-type mice during the early phase of the CS response (2 hours) (data not shown), nor at 24 hours after hapten (Figure 3G). Interestingly, at a later time point, tissue swelling was significantly increased in W/W^v mice compared to wild-type mice (Figure 3G). This suggests that the CS elicitation response is exacerbated in high oxazolone dose-immunized W/W^v mice compared to their wild-type controls. BMMC reconstitution of W/W^v mice restored the ear swelling CS response to that seen in wild-type mice (Figure 3G).

To determine the numbers of cells that entered the tissue after challenge of the high oxazolone dose-immunized WBB6F1/J and W/W^v mice, we examined H&E-stained tissue sections. Little to no tissue injury or leuko-

Figure 3. Elicitation response in WBB6F1/J, W/W^v, and W/W^v + BMMC mice immunized with a high dose of oxazolone. H&E staining of oxazolone-treated WBB6F1/J (**A**, **B**), W/W^v (**C**, **D**), and W/W^v BMMC reconstituted mice (**E**, **F**) 24 and 48 hours after challenge, respectively. **G**: Ear thickness was assessed at 0, 24, and 48 hours after hapten challenge. Total number of leukocytes (**H**) and the differential leukocyte counts (**I**, **J**) were performed on esterase stained histology sections and are expressed as number of infiltrated leukocytes/mm². A total of four animals were completed at each time point. Experiments were repeated twice with reproducible results (**P* < 0.05 relative to WBB6F1/J mice 48 hours CS, [#]*P* < 0.05 relative to W/W^v + BMMC reconstituted mice 48 hours CS). Scale bars = 220 μm. Original magnifications, ×100 (**A–F**); ×400 (**H–J**).

cyte infiltration could be observed in unchallenged ears or 2 hours after hapten challenge in either WBB6F1/J mice, W/W^v mice, or W/W^v BMMC reconstituted mice (data not shown). Twenty-four hours after hapten challenge resulted in substantial leukocyte recruitment into the extravascular tissue, although there was no difference in the total number of leukocyte infiltration between W/W^v, WBB6F1/J, and W/W^v BMMC reconstituted mice at this time point (Figure 3, **H** and **I**). However, by 48 hours after hapten challenge, W/W^v mice had a 1.4-fold increase in leukocyte infiltration

per field of view compared with WBB6F1/J and W/W^v BMMC mice at this time point (Figure 3**H**). The significant increase in leukocyte infiltration in W/W^v mice over wild-type mice was attributable to a substantial increase in granulocyte infiltration into the skin (Figure 3**J**). Overall, these observations indicate that in high oxazolone dose-immunized mice the CS elicitation response to oxazolone was certainly not diminished and was actually enhanced and qualitatively different in the absence of mast cells, at the very late phase of the CS response.

Figure 4. Tissue cytokine microenvironment after elicitation in mice sensitized with a high dose of oxazolone. ELISAs for IL-4 (A), TNF- α (B), IFN- γ (C), and IL-10 (D) were performed on ear homogenates from unchallenged, 24-hour and 48-hour challenged WBB6F1/J and W/W^v mice. BD, below detection limits. A minimum of four animals was completed at each time point (* $P < 0.05$ relative to baseline CS, # $P < 0.05$ relative to WBB6F1/J 48 hours CS).

A Type-1 Cytokine Bias in the Skin of Mast Cell-Deficient Mice in Response to a High Dose of Oxazolone

To further evaluate the environment generated during the CS response in high oxazolone dose-immunized mice, the cytokine milieu in ear homogenates was examined from baseline (unchallenged) as well as 24-hour and 48-hour posthaptan-challenged WBB6F1/J, W/W^v mice, and W/W^v BMMC reconstituted mice. Cytokine levels in untreated ears were not different between the three mouse strains (Figure 4). In addition, cytokine levels

within vehicle (olive oil/acetone)-challenged ears 24 and 48 hours after vehicle challenge were not found to be different from baseline levels in each strain of mouse tested (data not shown). This suggests that the difference in leukocyte infiltration between WBB6F1/J and W/W^v mice is not attributable to an underlying difference in cytokine milieu at baseline conditions and that changes in cytokine milieu are hapten-dependent.

The cytokine profile observed in the challenged ears of high oxazolone dose-immunized WBB6F1/J mice was completely different to that observed in low oxazolone dose-immunized WBB6F1/J mice even though tissue damage and leukocyte recruitment profile was similar between the two protocols. WBB6F1/J mice displayed elevated levels of the prototypical Th2 cytokine, IL-4, at both 24 and 48 hours after hapten challenge compared with baseline levels (Figure 4A). W/W^v mice also had significantly elevated levels of IL-4 present in ear homogenates compared to baseline levels at both time points tested, however by 48 hours IL-4 levels were 1.5-fold lower compared to levels in WBB6F1/J mice. The IL-4 levels present in W/W^v BMMC reconstituted mice were significantly elevated at 24 hours of CS but were back to baseline levels 48 hours after hapten challenge. The levels of the proinflammatory cytokine, TNF- α were also significantly increased compared to baseline levels in all strains of mice at both time points (Figure 4B). Interestingly, the levels of TNF- α in W/W^v mice were twofold higher compared with WBB6F1/J mice and W/W^v BMMC reconstituted mice at the 48-hour time point. IFN- γ levels were only marginally elevated over baseline levels in WBB6F1/J mice at the 24-hour and 48-hour time points and the IFN- γ levels were not different in the W/W^v mice or W/W^v BMMC reconstituted mice (Figure 4C). Interestingly, the regulatory cytokine IL-10 was only found to be present at detectable levels at 48 hours in WBB6F1/J mice, but was not present at detectable levels in W/W^v mice at any time points tested (Figure 4D). Overall these data suggest that the CS response generated in high oxazolone dose-immunized wild-type mice results in the expression of inflammatory cytokines (TNF- α) with an increased expression of T_H2 cytokines (IL-4). In addition, regulatory cytokines (IL-10) expressed at later time points may act to down-regulate the CS response in wild-type mice. In comparison, high oxazolone dose-immunized W/W^v mice displayed elevated levels of T_H1 cytokine (TNF- α) with diminished levels of T_H2 cytokine (IL-4) and no IL-10 after challenge.

To test whether the difference between the CS cytokine microenvironments generated in W/W^v mice and WBB6F1/J mice is attributable to the initial generation of different populations of type-1 and type-2 lymphocytes during the sensitization phase of CS, the cytokine production by hapten restimulated T cells obtained from low-dose- and high-dose-sensitized W/W^v and WBB6F1/J mice were examined (Figure 5). Changing the dose of oxazolone used to sensitize the different mouse strains did not alter the profile of cytokines produced from restimulated T cells from W/W^v or WBB6F1/J mice (data not shown). However significant differences in the cytokine profile produced from the sensitized T cells were ob-

Figure 5. Cytokine production patterns of hapten-stimulated sensitized T cells from WBB6F1/J and W/W^v mice (high dose). ELISA for IL-4 (A), IFN-γ (B), TNF-α (C), and IL-10 (D) were performed on cell culture supernatants of hapten-stimulated T cells derived from WBB6F1/J and W/W^v mice 5 days after hapten sensitization (high dose). Dotted line represents cytokine levels seen from unstimulated sensitized cells. Experiments were repeated twice with reproducible results.

served between the two strains of mice (data shown for the high oxazolone dose of stimulation). Restimulation of cells extracted from W/W^v mice had a reduced ability to produce IL-4 (Figure 5A) and IFN-γ (Figure 5B) compared to wild-type cells. However, these cells had a significantly increased ability to produce TNF-α (Figure 5C) compared with wild-type cells. Both cell types produced IL-10 to a similar degree (Figure 5D). Thus, mast cells may influence the profile of T cells generated in lymph nodes. Different sensitization protocols resulted in a slight but significant difference between total IgE levels in wild-type mice. Low-dose oxazolone sensitization resulted in IgE levels of 62 ± 14 pg/ml versus high-dose oxazolone sensitization IgE levels of 98 ± 13 pg/ml. IgE levels in mast cell-deficient mice were not different in response to low- or high-dose sensitizing hapten (135 ± 12 pg/ml).

Mast Cell Modulation of Neutrophil Recruitment as Well as CD4 T-Cell Recruitment during the Elicitation Phase Is Dependent on the Immunizing Dose of Hapten

To more closely examine the impact of mast cells on the recruitment of different populations of leukocytes during the CS response, we used spinning disk confocal microscopy with rapid automatic filter switching to simultaneously track endogenous neutrophil and CD4 T-lymphocyte rolling and adhesion in the skin microvasculature. For this, we observed leukocyte-endothelial interactions in the dermal microvasculature of the mouse flank rather than the ear to increase vessel visibility. The 24-hour CS response was followed in the flank skin of WBB6F1/J mice or W/W^v mice sensitized by a low or high hapten dose (Figure 6). Similar to the CS elicitation response observed in the ear, the CS elicitation response in the flank skin of high or low hapten dose-immunized W/W^v mice resulted in either an exacerbation of the response or a dampening of the response compared to wild-type mice (data not shown). Fluorescently labeled antibodies (anti-mouse CD4-fluorescein isothiocyanate and anti-mouse Gr-1 phycoerythrin antibodies) were administered into WBB6F1/J mice or W/W^v mice to directly visualize the

simultaneous trafficking patterns of endogenous CD4 T lymphocytes and neutrophils (Figure 6). Approximately 15 to 20 rolling neutrophils for every rolling CD4 T cell were observed in both WBB6F1/J mice and W/W^v mice at both sensitizing doses (Figure 6, A, B, E, and F). The

Figure 6. Gr-1⁺ neutrophil and CD4 T lymphocyte interactions in the post-capillary venules of WBB6F1/J and W/W^v mice 24 hours after hapten challenge. The total number of rolling neutrophils (A, E) and rolling CD4⁺ T lymphocytes (B, F) were assessed throughout several fields of view in WBB6F1/J and W/W^v mice immunized with either a low or high hapten dose. The total number of adherent neutrophils (C, G) and adherent CD4⁺ T lymphocytes (D, H) were assessed throughout several fields of view in WBB6F1/J and W/W^v mice immunized with either a low or high hapten dose. A minimum of four animals were completed at each time point (**P* < 0.05 relative to WBB6F1/J mice).

numbers of rolling neutrophils and CD4 T cells was not different between strains of mice at either sensitizing dose of hapten (Figure 6, A, B, E, and F).

A different profile of adherent neutrophils and CD4 T cells was observed between wild-type and W/W^v mice in response to different sensitizing oxazolone doses. Neutrophil adhesion per field of view was significantly decreased in low-dose-immunized W/W^v mice compared to wild-type mice (Figure 6C). However, the numbers of CD4 T-cell adhesion were not different between the two strains of mice (Figure 6D). This defect in neutrophil adhesion was also observed at the early 2-hour CS time point (data not shown). These data correlate with decreased neutrophil accumulation observed in the skin 24 and 48 hours after challenge in low oxazolone dose-immunized W/W^v mice and suggests that at this dose of sensitization, mast cells may contribute mainly to neutrophil adhesion. In comparison, both neutrophil and CD4 T-cell adhesion were increased by twofold in high oxazolone dose-immunized W/W^v mice compared to wild-type mice (Figure 6, G and H). These data suggests that in high oxazolone dose-immunized wild-type mice, mast cells may act to dampen both neutrophil and lymphocyte recruitment into the skin at 24 hours after oxazolone challenge that translates to less injury at 48 hours after challenge.

Given that increased endogenous CD4 T-cell adhesion as well as differences in cytokine production in the tissue was observed in W/W^v mice compared to wild-type mice only with a high dose of oxazolone immunization, we investigated whether this resulted in changes to the receptiveness of the microenvironment for different T-cell subsets. Intravital microscopy was used to observe the vascular endothelial interactions of ova-specific CD4⁺ lymphocytes polarized to either a T_H1 or T_H2 phenotype in either W/W^v or wild-type mice 24 hours after hapten challenge.

The polarized ova-specific T_H1 lymphocytes and T_H2 lymphocytes rolled equally well on the endothelium of W/W^v and wild-type mice 24 hours after hapten challenge (data not shown). However, the pattern of adhesion of these ova-specific T_H1 and T_H2 lymphocyte populations was significantly different in high oxazolone dose-immunized W/W^v mice compared with wild-type mice. T_H1 lymphocyte adhesion was significantly enhanced in W/W^v mice compared with wild-type mice, with a fourfold increase in T_H1 lymphocyte adhesion over that seen in wild-type mice (Figure 7A). Conversely, T_H2 lymphocyte adhesion was significantly increased in wild-type mice by

almost sixfold compared with adhesion in W/W^v mice (Figure 7B). These observations suggest that the microenvironment in wild-type mice after induction of CS is more conducive for T_H2 lymphocyte recruitment whereas the microenvironment generated in mast cell-deficient mice is more conducive to T_H1 lymphocyte recruitment.

Discussion

Mast cells are a key cell type that respond early within the CS response and contain a wide variety of proinflammatory mediators that can influence the recruitment of effector cells. To better understand the role of mast cells in the CS response we have compared the local microenvironment generated in challenged mast cell-deficient mice and their congenic controls after immunization with two different hapten concentrations. Our results show that the challenge response in low oxazolone dose-immunized W/W^v mice was decreased compared with their wild-type controls. This was specifically attributable to a reduction in granulocyte (neutrophil) recruitment during this response as previously reported.¹⁷ Interestingly, challenge of high oxazolone dose-immunized W/W^v mice resulted in a more severe late-phase CS inflammatory response than their wild-type controls. W/W^v mice displayed increased histological tissue damage characterized by increased leukocyte infiltrate with a predominance of neutrophils present in the tissue. In addition elicited responses showed a twofold increase in the recruitment of neutrophils and CD4 T lymphocytes observed in the postcapillary venules of high oxazolone dose-immunized W/W^v mice compared to wild-type mice. The cytokine response in these immune-challenged mice was also altered with increased type-1 cytokines present at later time points compared with levels elicited in the wild-type controls. These data suggest that after a strong immunizing dose of hapten, mast cells can act to down-regulate the ensuing CS response, whereas they facilitated elicitation of responses after a low immunizing dose of oxazolone.

Different groups have examined CS responses in wild-type versus mast cell-deficient mice with mixed results. Slight differences in hapten or diluents have been proposed to contribute to disparate results found with CS responses in mast cell-deficient mice.^{17,31} Indeed, in the current study we have shown that there are different requirements for mast cells during CS depending on the strength of the immunizing dose. With a low dose of hapten used at sensitization and challenge we saw re-

Figure 7. T_H1 and T_H2 lymphocyte recruitment at 24 hours after hapten challenge in mice immunized with a high dose of hapten. *In vitro* generated ova-specific T_H1 and T_H2 cells were fluorescently labeled and injected intravenously into high oxazolone dose-immunized WBB6F1/J and W/W^v mice at 24 hours of CS challenge. T_H1 adhesion (A) and T_H2 adhesion (B) are shown in both mouse strains. A minimum of three animals was completed at each time point (**P* < 0.05 relative to WBB6F1/J mice).

sults similar to several groups who have shown that the CS response is significantly diminished in mast cell-deficient mice.^{11,17,18} Despite no changes in the types of cytokines detectable in the local tissue between wild-type and W/W^v mice in response to a low dose of hapten, W/W^v mice have significantly reduced tissue injury, with reduced granulocyte infiltrate. These results are similar to that observed by Biedermann and colleagues¹⁷ who have suggested that mast cells can orchestrate the type of cell (ie, granulocytes) infiltrating into tissue through the chemokines they produce (MIP-2). By contrast, the challenge response that is elicited after a high dose of immunizing oxazolone was found to be relatively worse in mast cell-deficient mice, with increased type-1 cytokines still present at later time points. This suggests that mast cells may play a key role in initiating the inflammatory response that occurs after a low immunizing stimulus and dampening the response with a strong immunizing stimulus (ie, bi-directional role according to the immunizing dose). With both sensitizing hapten doses no difference in ear swelling between mast cell-deficient mice and wild-type mice was observed until the 48-hour time point. Thus in studies in which CS is evaluated solely by measuring ear swelling at the 24-hour time point, differences between animal strains might be missed.

In the current study, we have shown that mast cells can regulate the trafficking of leukocytes within the postcapillary venules of the skin during CS. A similar number of CD4 T cells were seen to adhere in the high-dose sensitization protocol as the low-dose sensitization protocol in wild-type mice. However, a significant increase in the CD4 T-lymphocyte adhesion was observed in mast cell-deficient mice after the high-dose sensitization protocol. Similarly neutrophil recruitment was exacerbated in these mice. These differences were observed at 24 hours after hapten challenge at a time in which the cytokine profile present in the ears of wild-type and mast cell-deficient mice are similar, as such we hypothesize that mast cells may modulate the recruitment of different cell types via a different mechanism such as chemokine release. In the high oxazolone dose-immunized mice, the increase in leukocyte recruitment within the postcapillary venules of mast cell-deficient mice at 24 hours correlates with later increased tissue injury at 48 hours of CS.

T_H1 and T_H2 lymphocytes differentially regulate the outcome of an immune response through the types of cytokines they produce. T_H1 cells are mainly involved in cell-mediated inflammatory responses (including delayed-type hypersensitivity reactions) whereas T_H2 cells support antibody production and mediate allergic and anti-parasitic reactions.^{32,33} As such, understanding the mechanisms that can control the type of response generated during an inflammatory response remains an important area of research. In the current study the typical proinflammatory T_H1 cytokine, TNF- α , and the typical T_H2 cytokine, IL-4, were both present in the challenged ears of contact-sensitized mice initiated by a high immunizing dose of oxazolone. However, there was a definite skew toward production of TNF- α over IL-4 in the absence of mast cells. In addition, the microenvironment generated in mast cell-deficient mice was more conducive to the recruitment of *in vitro* polarized

ova-specific T_H1 cells compared to T_H2 cells. Given that the differential trafficking patterns of polarized T_H1 and T_H2 cells has been purported to be attributable to different chemokine receptor expression, differences in tissue chemokine expression between W/W^v mice and wild-type mice also likely exist.^{27,28,34}

In our current study it was not possible to directly attribute the change in cytokine milieu in the challenged ears of high hapten dose-immunized mice to a change in the type of T cell recruited. Indeed, a similar pattern of cytokine production was observed by sensitized cells derived from mast cell-deficient mice compared to wild-type mice after hapten restimulation to that observed in the tissue of the respective mouse strains. Lymphocytes from sensitized mast cell-deficient mice produced lower levels of IL-4 but higher levels of TNF- α compared to wild-type cells. This was not dependent on the immunizing dose of hapten. This suggests that mast cells may contribute to the initial generation of antigen-specific T lymphocytes during the CS response.^{31,35} As such increased tissue levels of TNF- α in mast cell-deficient mice may reflect the general increase in CD4 T-cell recruitment observed in these mice.

It is becoming clear that antigen-specific antibodies are required for the initiation of the elicitation phase of CS response.³ It is possible that they may have additional roles in CS apart from their role in the initial recruitment of CS-initiating T cells into the skin.^{36–38} It is interesting to speculate that one contributing factor for differences in mast cell participation in the CS response could be levels of circulating antibodies generated in response to different strengths of sensitizing stimulus. Indeed, we have shown that low-dose versus high-dose sensitization in wild-type mice leads to a slight but significant increase in the circulating levels of total IgE. It has recently been shown that IgE can prime mouse mast cells to increase mRNA levels for certain mast cell mediators and therefore one could speculate that different levels of mast cell priming may occur in the response to different sensitization doses.^{18,31}

An interesting aspect of this study was the observation that IL-10 was produced in wild-type mice immunized with high but not low hapten doses. Moreover, high-dose immunization of mast cell-deficient mice also failed to produce IL-10. Recently, while the current article was in preparation, Grimaldeston and colleagues³⁹ revealed that mast cell-deficient mice exhibited exacerbated and prolonged CS responses compared to controls. Differences between mice were only revealed starting from 72 hours after hapten challenge. Mast cell-derived IL-10 was shown to be responsible for the resolution of the late phase CS response. The participation of IgG1 antibodies rather than IgE was shown to contribute to mast cell production of IL-10 and it was suggested that the induction of hapten-specific IgG1 response may be a mechanism to limit the extent and duration of the CS response.³⁹ Our current study confirms the role for mast cells in the down-regulation of the CS response and show that that changes in cytokine microenvironment and cell recruitment start as early as 24 hours after hapten challenge.

In summary, our data support the hypothesis that mast cells are tunable regulators of the immune response.³¹

We reveal a role for mast cells both as regulators and inducers of the inflammatory response depending on the strength of the immunizing stimulus. With a strong immunizing stimulus, mast cells play a regulatory role by inducing a late-phase type-2 inflammatory response after hapten challenge, regulating the recruitment of lymphocytes from the mainstream of blood.

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