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Mast Cells Recruited to Mesenteric Lymph Nodes during Helminth Infection Remain Hypogranular and Produce IL-4 and IL-6

Anne Y. Liu,1 Dan F. Dwyer,1 Tatiana G. Jones, Lora G. Bankova, Shiliang Shen, Howard R. Katz, K. Frank Austen, and Michael F. Gurish

Mast cells (MC) and basophils share expression of the high-affinity receptor for IgE (FcεRI) but can be distinguished by their divergent expression of KIT and CD49b. In BALB/c mice, MC lineage cells expressing high levels of FcεRI by flow cytometry were seen only in bone marrow whereas those expressing intermediate levels of FcεRI were present in bone marrow and spleen of naive mice and in mesenteric lymph nodes (mLN) of Trichinella spiralis–infected mice. These FcεRI+KIT+CD49b+ cells had a membrane phenotype similar to i.p. connective tissue-type MC, but were smaller and hypogranular by flow cytometry forward and side scatter profiles, respectively. Consistent with this, they lacked the prominent secretory granules identified by histochemistry and immunodetection for the MC-specific granule proteases that are readily seen in mature jejunal mucosal MC that also are induced by the infection and present at the same time. The concentration of these MC lineage cells in mLN determined by flow cytometry was comparable to that of MC progenitors (MCp) measured by limiting dilution and clonal expansion with maturation. We observed upregulation of IL-4 transcription by MCp in mLN and spleens of helminth-infected 4get mice, and we demonstrated by intracellular cytokine staining production of IL-4 and IL-6 by the mLN MCp in helminth-infected mice. Furthermore, treatment of helminth-infected mice with anti-FcεRI mAb, a protocol known to deplete basophils, also depleted mLN MCp. Thus, this study identifies a hypogranular subset of MCp recruited to mLN by helminth infection that may be an important unrecognized source of cytokines.

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Mast cells (MC) and basophils (Ba) express the high-affinity receptor for IgE and have been appreciated and characterized for their respective effector roles in the Th2 host response to helminth infection (1, 2). Although Ba are detected in bone marrow (BM), spleen, and lymph nodes (LN) by flow cytometry as FcεRI+KIT+CD49b+ cells in various models of immune responses (2–4), the paucity of MC in these tissues has been demonstrated only by histochemical assessment. MC are long lived as connective tissue MC (CTMC) (5) but transient when induced in the intestine as mucosal MC (MMC) (6, 7). Whereas mature MC are not detected in blood and are rare in the parenchyma of BM, LN, and spleen, MC progenitors (MCp) are readily detected in cell suspensions from BM, spleen, and small intestine of naive mice when assayed by limiting dilution and clonal expansion/mutation assays (LDA) (8–11). MCp are recruited to intestine and mesenteric LN (mLN) during Th2-mediated helminth-induced inflammation (12–15) or in response to hyperimmunization (9, 16). Nonetheless, as MCp cannot be detected histochemically, their presence has not been directly assessed in many models of inflammation.

We used the LDA assay with stem cell factor (SCF) and IL-3 as growth and maturation factors to demonstrate the absence of jejunal MCp in mice lacking β2 integrin (11). We further demonstrated that MCp migration from the BM to jejunum is constitutive and requires α4β1 integrin, as revealed by the depletion of jejunal MCp in wild-type (WT) mice treated for a week with mAb to this integrin (11, 17). This constitutive basal homing from BM to intestine provides a dynamic reservoir of MCp in naive mice from which MMC are induced with helminth infection (18). Such basal homing is minimal to lung, but recruitment of MCp to lung parenchyma occurs during Th2-mediated pulmonary inflammation using interdependent adhesion and migration pathways (19, 20). This recruitment precedes the induction of MMC in the trachea and large airways (21). Because MCp reside in tissues of naive mice and can be recruited to peripheral tissues with inflammation, their predominant function has been viewed as providing a reservoir for MMC expansion and attendant secretory functions.

Numerous recent studies have been focused on the role of the Ba in the immune response (reviewed in Ref. 22) and yet the role of the MCp in these studies is rarely considered. Furthermore, the analysis of MCp numbers by the in vitro LDA does not distinguish between the committed FcεRI+KIT+ cells and those earlier multipotent cells capable of entering into the lineage. We turned to flow cytometric analysis without sorting to study the development and distribution of MC and Ba during an inflammatory response, with the goal of identifying both progenitor and more mature...
populations. These studies included mice in the naïve state as well as mice injected locally with papain or infected systemically with the helminth Trichinella spiralis. MC lineage cells and mature Ba resided in BM and spleen of naïve BALB/c mice. Ba could be recruited to local LN with both papain and T. spiralis infection whereas only the infection elicited a LN MC lineage population. Forward scatter (FSC) and side scatter (SSC) analysis by flow cytometry of these FcεRI+KIT+CD49b+ MC revealed that they were small hypogranular cells. The parallel assessment of their numbers by LDA, which requires maturation, indicated that they were MCp at days 6 and 13 of infection. The hypogranular phenotype of these local nodal MCp at day 6 of infection was dramatically different from the appearance of fully granulated intestinal MMC in the same mice at the same time. Moreover, the hypogranular MCp in LN and spleen exhibited IL-4 mRNA that increased during T. spiralis infection in 4get reporter mice. Furthermore, in infected BALB/c mice, this population of MCp could produce IL-4 and IL-6 as determined by flow cytometric detection. Our findings suggest that MCp are not simply a developmental stage, but rather may be a tissue population with independent functions in locations where granulation is not needed or desirable.

Materials and Methods

Mice

Seven- to 18-wk-old BALB/c mice and 4get (C.129-H-4m1Lky/J) mice were obtained from Taconic Farms or The Jackson Laboratory. IL-3+/− mice are maintained in-house at the Dana Farber Cancer Institute (23). The use of mice for these studies was in accordance with institutional guidelines with review and approval by the Animal Care and Use Committee of the Dana Farber Cancer Institute.

Abs

Fluorescently labeled mAb directed against FcεRI (MAR-1), mouse IgE (23G3), CD49b (DX5), B220 (RA3-6B2), CD3 (145-2C11), CD19 (6D5), CD4 (RM4-5), Ly-6G/Ly-6C (Gr-1) (RB6-8C5), TCRβ (H57-597), IL-4 (11B11), IL-6 (20F-3), and TNF-α (MP6-XT22) were obtained from BioLegend (San Diego, CA). Anti-FcεRII/III (2.4G2), anti-KIT (2B8), and anti-β2 integrin (M293) mAb were obtained from BD Biosciences (San Jose, CA). Monoclonal anti-mouse MC protease (mMCP-1) was obtained from R&D Systems (Minneapolis, MN). Anti-mMCP-5 is a rabbit anti-peptide preparation made against a specific peptide (24).

Papain administration

Fifty micrograms of papain (Sigma-Aldrich, St. Louis, MO) in HBSS was injected s.c. into bilateral footpads at a concentration of 1 mg/ml 3 d prior to harvest of popliteal LN (3).

T. spiralis infection

Mice were infected with 450 larvae of T. spiralis by gavage as previously described (6).

Identification of MCp and Ba and intracellular cytokines by flow cytometry

Single-cell suspensions of spleen, BM, and LN were obtained by grinding tissues through 70-μm cell strainers (BD Biosciences) into RPMI 1640, with 10% FCS, l-glutamine, penicillin, streptomycin, gentamicin, HEPES buffer, sodium pyruvate, and 2-ME (Sigma-Aldrich). Peripheral blood was collected by cardiac puncture into syringes containing 100 μl of 0.5 M EDTA. Erythrocytes in spleen and blood were lysed (1–2 min in 0.1 mM EDTA, 2.0 g/l potassium bicarbonate, 16.6 g/l ammonium chloride). Percutaneous cells were collected by peritoneal lavage with 10 ml RPMI 1640. Non-specific mAb uptake was blocked with CD16/32 (2.4G2) (BD Biosciences) for 10 min, and appropriate mAb were added for 30 min. Cells were analyzed on a FACS\textsuperscript{C}anto flow cytometer (BD Biosciences) using FACSDiva acquisition software. FlowJo software (Tree Star, Ashland, OR) was used for data analysis. Positive cells were defined as those having fluorescence intensities >99% of cells incubated with isotype control mAb. For exclusion of other cell types, we used mAb against CD19, CD3, CD4, B220, TCRβ, and Gr-1. In experiments with naïve mice or papain-injected mice, anti-FcεRIα was used to identify FcεRI+ cells. After T. spiralis infection, anti-IgE mAb was used for identification of FcεRI+ cells.

For isolation of MCp from the mLN to examine their morphology, the nodes were removed from T. spiralis–infected mice on day 6 after infection. The single-cell suspensions were fractionated on a 44/67% Percoll gradient and the interface cells were harvested and incubated with biotinylated anti-CD19 and anti-CD3 (BioLegend) for 30 min, after which they were washed and incubated with biotin binder Dynabeads (Invitrogen) for 30 min according to the manufacturer’s protocols. The cells were then placed on a Dynabead magnet (Invitrogen) for 5 min, and the unbound cells were collected and stained with anti-FcεRI, anti-CD117, and anti-CD49b. FcεRI+CD117+CD49b+ cells were collected using a BD FACSAria II (BD Biosciences), affinity to slides using a CytoSpin cytocentrifuge (Thermo Scientific), and stained with either Diff-Quick (Siemens Healthcare Diagnostics) or toluidine blue.

To assess for intracellular cytokine production by MCp, mLN cells were cultured for 1 h in culture media supplemented with IL-3 (1–10 ng/ml) to improve viability and then for 4 h with 1 μg/ml brefeldin A in the same cell culture media. After 5 h in culture, cells were spun down, fixed and permeabilized using a BD Cytofix/Cytoperm kit according to the manufacturer’s directions, and stained for flow cytometry. Production of a specific cytokine was identified as an increase in the mean fluorescence relative to that obtained from the same cells stained with an isotype control mAb in parallel. The mean fluorescence intensity (MFI) index was defined as the MFI from the anti-cytokine mAb minus the MFI from the isotype control and then divided by the MFI from the isotype control.

To develop BM-derived MC (BMMC), BM cells were cultured in complete RPMI with 10 ng/ml each of SCF and IL-3. Nonadherent cells were washed weekly and the BMMC were used after 4–6 wk in culture.

Identification of MCp by limiting dilution and clonal expansion

The LDA was performed as previously described with inclusion of SCF with IL-3 to improve viability and maturation (9, 11). Mononuclear cells (MNC) were obtained by Percoll (Sigma-Aldrich) gradient fractionation of harvested cells by using the 44/67% Percoll interface. After LDA, the original MC progenitor concentration is expressed as the number of MCp per 10⁶ MNC isolated from the tissue.

Histochemical and immunohistochemical evaluation of MC

Tissues and isolated cells were evaluated for mature MC numbers by chloroacetate esterase (CAE) reactivity and immunohistochemistry for the mMCP (21). Briefly, tissue was harvested, fixed in 4% paraformaldehyde overnight, and then embedded in paraffin for evaluation of mMCP expression or in glycol methacrylate for evaluation of CAE reactivity. To quantify the number of mature MC in the LN preparations, cytopsins of ∼200,000 cells per slide were stained for CAE reactivity and counted for CAE+ cells with morphologic characteristics consistent with mature MC.

Ab-mediated cell depletion

Injection of anti-FcεRI mAb (MAR-1 from eBioscience [San Diego, CA] and BioLegend) was performed essentially as described by others (4, 25). Mice were injected with 10 μg MAR-1 i.p. 72, 48, and 24 h prior to T. spiralis infection. In initial studies with naïve mice, this protocol depleted most of the Ba in spleen and BM. In studies of T. spiralis–infected mice, BM, spleen, and LN were harvested at the times noted and analyzed for MC lineage cells by clonal expansion or flow cytometry and for Ba lineage cells by flow cytometry.

Statistical analysis

Data are expressed as the means ± SEM when derived from three or more values. Significance was determined with a two-tailed Student t test where three or more values were available for analysis. Significance was determined with the Mann–Whitney U test when values did not follow a normal distribution. A p value <0.05 was considered significant.

Results

Identification of two Ba and two MC lineage populations in BALB/c BM and a single phenotype for each lineage in peripheral tissues

Earlier studies by our group and others with BM-derived cells cultured in vitro demonstrated that commitment to the MC and Ba lineages was closely followed by de novo expression of the high-affinity IgE receptor detected either by mAb to the α-chain or the
function of hypogranular MC

ability to bind IgE (26–29). Differentiation along the MC lineage was associated with the continued expression of KIT, the receptor for SCF, whereas this receptor was downregulated with Ba lineage development (29). To follow the parallel lineage development of MC and Ba in vivo, we analyzed the non–B, non–T cells (by excluding cells expressing CD19 or CD3) in BALB/c BM for expression of FcεRα and KIT (CD117), as well as CD49b as a Ba marker (30–32). Within the non–B, non–T population there were two contiguous populations of cells positive for both FcεRI and CD49b expression (pentagonal and ovoid gates in Fig. 1A). These cells showed a 10-fold range in the fluorescence intensities of FcεRI expression, and the population of cells expressing the highest levels of FcεRI (ovoid gate in Fig. 1A) had a 10-fold range in expression of CD49b. A third population of cells was negative for CD49b expression and intermediate in FcεRI expression (rectangular gate in Fig. 1A) and was predominantly KIT+ (Fig. 1A, top histogram), suggesting a committed MC lineage identity. The CD49b+ population that expressed intermediate levels of FcεRI (pentagonal gate in Fig. 1A) uniformly lacked KIT expression (Fig. 1A, bottom histogram), suggesting a committed Ba identity. The cells expressing high levels of FcεRI (ovoid gate) with a range of CD49b expression were heterogeneous in their KIT expression (Fig. 1A, middle histogram), suggesting at least two different precursor populations. To further classify these BM cell populations, we reanalyzed the non–B, non–T cells for FcεRI and KIT expression and then assessed CD49b expression (Fig. 1B). We identified a KIT+ population (rectangle gate in Fig. 1B) that was predominantly CD49b+ (Fig. 1B, top dot plot), suggesting it is mostly composed of Ba lineage cells also identified in the oval and pentagonal gates in Fig. 1A. The population of cells expressing high levels of KIT and intermediate levels of FcεRI (Fig. 1B, ovoid) was largely CD49b+ (Fig. 1B, bottom dot plot), compatible with MC commitment. The population of cells with intermediate KIT and high FcεRI expression (Fig. 1B, ovoid gate) was heterogeneous for CD49b, suggesting two different precursor populations (Fig. 1B, middle dot plot). This population made up ~0.9% of non–B, non–T cells in BALB/c BM.

Using the same three cell surface markers, we compared the phenotypes of MC (FcεRI+KIT+CD49b+) and Ba (FcεRI+KIT+CD49b−) within the non–B, non–T cells of BALB/c spleen, blood, and popliteal LN with those in the BM, using injection of papain in the footpads to elicit Ba in popliteal LN (3). In the spleen, there was only a single population of FcεRI+KIT+ cells expressing intermediate levels of FcεRI in both naive and papain-treated mice (Fig. 2A, top panel, rectangular gate). Few or no FcεRI+KIT− cells were detected in blood or popliteal LN of naive and papain-injected mice (Fig. 2A, middle and bottom panels). This CD49b+ population corresponds to the unmarked population of FcεRI+KIT− cells seen in Fig. 2A. There were very few LN Ba in naive mice, but papain administration induced an influx of Ba into LN (Fig. 2B, bottom panels) as previously noted (3). The KIT+ FcεRI+ and KIT−FcεRIhi populations seen in BM were notably undetectable in these peripheral tissues.

Infection with T. spiralis expands MC and Ba lineage populations and recruits them to mLN

To directly study recruitment of MC lineage progenitors to LN as predicted by LDA (12–15), we employed a T. spiralis infection in which tissues were harvested in the middle of (days 6–7) and toward the end of (days 12–14) the acute phase of intestinal infection. In contrast to the studies with papain, we used anti-IgE rather than anti-FcεRI to monitor FcεRI expression during T. spiralis infection to avoid any competing effects of host IgE on the assessment of FcεRI+ cells (33). IgE+ KIT+ MC lineage populations in naive mice were present in BM and spleen but were rare in mLN (Fig. 3A, top). The unmarked IgE+ KIT− Ba population can be seen directly to the left of the gated MCP (Fig. 3A). After T. spiralis infection, there were significant increases in the frequency of BM IgE+ KIT+CD49b+ MC at days 6 and 12 (Fig. 3A, 3B). Furthermore, distinct IgE+ KIT+ MC lineage populations of similar phenotype appeared in spleen and mLN (Fig. 3A). There were concurrent significant rises in these splenic and LN populations on days 6 and 12 (Fig. 3B). Ba populations identified as IgE+CD49b+ cells also significantly expanded in BM on days 6 and 12 (Fig. 3C). Splenic Ba numbers lagged at day 6 but had
a significant increase at day 12. The number of Ba in the mLN was significantly increased at days 6 and 12. In the mLN, MC lineage cells constituted a larger (~2-fold) fraction of the population than Ba at each time point.

**LN MCp assessed by flow cytometry are highly immature and correspond in number to MCp assessed by limiting dilution and clonal expansion**

To determine whether the numbers of lineage-committed MCp in mLN detected directly by their distinct cell surface phenotype in flow cytometry are comparable to the numbers of MCp assayed indirectly ex vivo by LDA, we compared these numbers in naive mice and in mice 13 d after *T. spiralis* infection by both assays using the MNC fraction. There were very few MCp in naive mLN with either assay (55.8 and 56.4 MCp/10⁶ MNC, mean from two experiments, five mice). At 13 d postinfection there were significant increases in the number of MCp detected by flow cytometry (6.2-fold, *p*, 0.001) and by LDA (8.7-fold, *p*, 0.01) (Fig. 4A).

There was no significant difference between the measurements of MCp per million MNC obtained by the two methods in either

**FIGURE 2.** Identification of MC and Ba lineage populations in spleen, blood, and LN of naive and papain-injected BALB/c mice. (A) Three days after papain or PBS injection, MC lineage cells were identified as FcεRI⁺KIT⁺ cells in spleen, blood, and popliteal LN. (B) Three days after papain or PBS injection, Ba lineage cells were identified as non–T, non–B, FcεRI⁺CD49b⁺KIT⁺ cells in spleen, blood, and popliteal LNs. Data are representative of 9–12 mice in three experiments. Numbers indicate the frequency of MCp and Ba as a percentage of the non–T, non–B cells isolated from the indicated tissues. Isotype control values were <5% of the indicated values.

**FIGURE 3.** *T. spiralis*-infected BALB/c mice recruit MC and Ba to mLN and expand spleen and BM lineage pools. (A) Dot plots of BM, spleen, and LN cells from naive and day 12 infected BALB/c mice show the IgE⁺ KIT⁺ MC lineage populations (square gate). Dot plots are representative of data from four experiments. (B) Frequencies of MC lineage populations from naive, day 6, and day 12 postinfection mice are shown as a percentage of total cells isolated from BM, spleen, and LN. (C) Frequencies of Ba lineage populations (IgE⁺ CD49b⁺) in the BM, spleen, and LN as in (B). Data in (B) and (C) represent means ± SEM, *n* = 13 mice per group, four experiments. *p* < 0.05, **p* < 0.001, ***p* < 0.0001.
naive or infected mice. The trend toward higher numbers obtained by LDA may reflect the lower specificity of this assay attributable to participation by myeloid lineage cells that differentiate into MC colonies in the presence of IL-3 and SCF (34).

To assess the maturity of LN MCp detected by flow cytometry, we analyzed the numbers of MCp in the LN obtained by cytometry before and after density gradient fractionation and compared them to the numbers of granulated MC detected by histochemistry. It had been established previously using peritoneal cells obtained by lavage that density gradient isolation of MNC yields a cell preparation enriched for MCp and depleted of mature MC, which sediment in the pellet (17). *T. spiralis*-infected mLN harvested at day 13 postinfection yielded 281 ± 57 MCp (IgE+KIT+) cells per 10^6 LN cells (mean ± SEM, n = 8, two experiments). Histochemical evaluation of granulated MC in these and one additional mLN cell preparation yielded 12.5 ± 3.1 CAE+ MC per 10^6 LN cells (mean ± SEM, n = 11, three experiments). After isolation of the MNC fraction, there were 332 ± 40 MCp per 10^6 MNC by flow cytometry and 7.5 ± 1.0 CAE+ MC per 10^6 MNC cells by histochemistry. Thus, 96 and 98% of the LN cells identified as IgE+KIT+ were histochemically hypogranular MCp before and after isolation of the MNC fraction, respectively.

Most mLN MCp appeared in a low SSC gate, with only a scant few mature MC in a high SSC gate (Fig. 4B). In contrast, most IgE+KIT+ cells recovered from peritoneal lavage appeared to be mature MC in the high SSC gate, with few MCp in the low SSC gate (Fig. 4B). The finding of low granularity for the MCp is consistent with the low density of helminth-induced LN MCp reported by Jarboe et al. (14). When we compared mLN and splenic MCp populations from day 6 after *T. spiralis* infection, these induced MCp were virtually identical in size (FSC profile) and granularity (SSC profile) and greater than the lymphocyte population in these parameters. Of note, these induced MCp were lower in both assessments than were immature BMMC derived with IL-3 and SCF, emphasizing their relative agranularity (Supplemental Fig. 1). Thus, although the recruited LN MCp, splenic MCp, BMMC, and peritoneal MC exhibit the same surface marker phenotype, when identified by flow cytometry, they are different in regard to size (FSC) and granularity (SSC).

In a histological examination of the parenchyma of mLN for MC we used CAE reactivity as well as immunohistochemistry to mMCP-1 and mMCP-5 to detect mature MC and evaluate their protease phenotype. In the intestine after *T. spiralis* infection, mMCP-1 and mMCP-5 identify the induced MMC and the constitutive submucosal MC, respectively (6, 7, 21). The LN parenchyma from naive mice demonstrated virtually no CAE reactivity or immunoreactivity for mMCP-1 or mMCP-5 (data not shown). After *T. spiralis* infection, there was little CAE reactivity in the LN as compared with the distinct MMC staining in the jejunum. There were only rare cells immunoreactive for mMCP-1 or mMCP-5 in parenchyma of mLN (Fig. 4C, top panels) whereas the intraepithelial MMC from the jejunum of the same mice expressed mMCP-1 and the submucosal MC expressed mMCP-5 (Fig. 4C, bottom panels). These observations support the findings...
by flow cytometry that with *T. spiralis* infection, dispersed mLN parenchymal cells harbor very few mature MC in concert with a dominant hypogranular subset of MCp.

To directly visualize the morphology of this recruited subset, we sorted them from the mesenteric nodes of *T. spiralis*-infected mice at day 6 based on their distinct phenotype. After depleting the CD3⁺CD19⁺ cells, the sorted KIT⁺FceRI⁺CD49b⁺ cells were stained with Diff-Quick or toluidine blue. The LN MCp are small cells with a prominent nucleus and a cytosol that contains numerous empty vesicles after staining with Diff-Quick and reveals a few toluidine blue–positive granules (Fig. 4D). In comparison, CTMCs from the peritoneal cavity are large and heavily granulated cells by either Diff-Quik or toluidine blue staining.

**Depletion of MCp by administration of anti–FceRI mAb with *T. spiralis* infection**

Depletion of Ba by systemic administration of anti–FceRI mAb (MAR-1) to assess their role in mouse models of inflammation (3, 4, 25, 32) is based on a protocol by Denzel et al. (33) in which Ba depletion is not accompanied by a change in the number of mature peritoneal MC. To assess the effect of this treatment on MCp, we administered anti–FceRI mAb i.p. to BALB/c mice prior to infection with *T. spiralis* and then enumerated the numbers of MCp in mLN, spleen, and BM. MCp were identified as β2 integrin⁺KIT⁺ cells, a combination that was equivalent to anti-IgE⁺KIT⁺ identification of MCp in isotype-treated infected mice (data not shown). Six days after infection, the β2 integrin⁺KIT⁺ MCp were significantly reduced by ~70% (from 471 to 141 MCp per 10⁶ nucleated LN cells) as compared with isotype control–injected mice (Fig. 5A). The fold reduction was similar when assessed by LDA in which cell numbers were reduced from 860 to 260 MCp per 10⁶ LN MNC (Fig. 5B). In contrast, the numbers of MCp in the BM and spleen at day 6 postinfection were not reduced by this treatment when assessed by flow cytometry or by LDA. The number of β2 integrin⁺KIT⁺ MCp in isotype control mAb versus MAR-1–injected mice in the BM and spleen was 2020 ± 328 versus 2058 ± 157 MCp per 10⁶ BM cells (mean ± SEM, n = 3) and 534 ± 48 versus 837 ± 227 MCp per 10⁶ splenic MNC, respectively (mean ± SEM, n = 3). By LDA, we found 624 ± 63 MCp per 10⁶ MNC in the spleens of isotype control–injected mice versus 750 ± 128 MCp per 10⁶ MNC in the spleens of MAR-1–injected mice (mean ± SEM, n = 6, two experiments).

To address the possibility that the influx of Ba influenced the recruitment of MCp to draining nodes, we evaluated the response in the IL-3⁻/⁻ strain, which had been reported to lack Ba in their mLN by FACS analysis after *Nippostrongylus brasiliensis* infection (35). The number of LN MCp in the IL-3⁻/⁻ versus the BALB/c mice 6 d postinfection with *T. spiralis* was not different (0.70 ± 0.15 versus 0.68 ± 0.05% of non–B, non–T LN cells, respectively; n = 4 mice per genotype in a single experiment) whereas the increase in the number of Ba in these same mice was reduced by 77% (0.03 ± 0.01 versus 0.15 ± 0.05%, respectively). We obtained similar results in a second experiment indicating the independence of MCp recruitment to the draining LN.

**T. spiralis infection increases IL-4 mRNA in mLN MCp of 4get mice**

The infection-driven expansion of hypogranular MCp in the draining LN without the appearance of MMC that are so prominent in the intestine suggests that LN MCp might have a granule-independent function. Because many helminth infections generate a substantial Th2 type inflammation with an increased IgE response implicating IL-4, we examined IL-4 mRNA levels in MCp using IL-4/enhanced GFP (4get) reporter mice. In these mice, an enhanced GFP gene is inserted in the 3’ untranslated region of the endogenous IL-4 locus such that enhanced GFP is transcribed in parallel with IL-4 and thus serves as a marker of IL-4 mRNA levels. In naive 4get mice, BM MCp express very little GFP as measured by MFI (184 ± 10.6), consistent with the findings of Geissner et al. (36), whereas splenic MCp express moderate levels of GFP (76.1 ± 5.06), and peritoneal MC express uniformly high levels of GFP (1789 ± 85.3) (Fig. 6A, 6B, left panels). Very few naive LN MCp were detectable but those seen expressed low GFP levels comparable to naive splenic MCp (Fig. 6A, black histograms). Six days after *T. spiralis* infection, GFP expression increased in MCp in all tissues (Fig. 6A, red histograms). The increase in MFI with infection was significant for the MCp in BM, spleen, and mLN as well as for peritoneal MC (Fig. 6B, left panel). The Ba lineage demonstrated initial baseline GFP expression in BM, spleen, and LN, and with *T. spiralis* infection it significantly increased in the BM and splenic Ba. (Fig. 6A, 6B, right panels). Of note, the hypogranular MCp of spleen and LN increased expression of GFP by 2.5- and 3.6-fold, respectively, whereas the mature granular peritoneal MC increased GFP expression by only 1.3-fold with infection.

**T. spiralis infection induces IL-4 and IL-6 production by mLN MCp of WT BALB/c mice**

We then evaluated IL-4 and IL-6 production in LN MCp by intracellular cytokine staining in BALB/c mice on day 6 after *T. spiralis* infection. The LN MCp of infected mice are detectable but those seen expressed low GFP levels comparable to naive splenic MCp (Fig. 6A, black histograms). Six days after *T. spiralis* infection, infiltration of IL-4 and IL-6 mRNA levels in MCp using IL-4/enhanced GFP (4get) reporter mice. In these mice, an enhanced GFP gene is inserted in the 3’ untranslated region of the endogenous IL-4 locus such that enhanced GFP is transcribed in parallel with IL-4 and thus serves as a marker of IL-4 mRNA levels. In naive 4get mice, BM MCp express very little GFP as measured by MFI (184 ± 10.6), consistent with the findings of Geissner et al. (36), whereas splenic MCp express moderate levels of GFP (76.1 ± 5.06), and peritoneal MC express uniformly high levels of GFP (1789 ± 85.3) (Fig. 6A, 6B, left panels). Very few naive LN MCp were detectable but those seen expressed low GFP levels comparable to naive splenic MCp (Fig. 6A, black histograms). Six days after *T. spiralis* infection, GFP expression increased in MCp in all tissues (Fig. 6A, red histograms). The increase in MFI with infection was significant for the MCp in BM, spleen, and mLN as well as for peritoneal MC (Fig. 6B, left panel). The Ba lineage demonstrated initial baseline GFP expression in BM, spleen, and LN, and with *T. spiralis* infection it significantly increased in the BM and splenic Ba. (Fig. 6A, 6B, right panels). Of note, the hypogranular MCp of spleen and LN increased expression of GFP by 2.5- and 3.6-fold, respectively, whereas the mature granular peritoneal MC increased GFP expression by only 1.3-fold with infection.

**Discussion**

We have addressed the parallel in vivo responses of MC and Ba from BM to blood, spleen, and regional LN using flow cytometry in mice subjected to local or systemic inflammation as compared with naive controls. Unlike other myeloid lineage cells, MC are released from BM as a progenitor, which is devoid of the secretory granules that define the mature tissue-resident cells. Thus, we first established that their cytometric detection and enumeration as FceRI⁺KIT⁺CD49b⁺ MCp corresponded in numbers with their classical assay in tissues by limiting dilution and clonal expansion with maturation to granulated immature MC (34, 37). We then ob-
using either CAE reactivity or immunohistochemistry for the secretory granule proteases whereas those in the LN were not detectable by either measure. Turning to a positive definition of the nodal MCp, we sorted this subset by their lineage phenotype and assessed their morphology on cytopsins. As expected, nodal MCp are small cells with a prominent nucleus and empty cytoplasmic vesicles by Diff-Quik stain. Toluidine blue staining showed a few granules. The contrast to peritoneal MC by size and granularity was marked, again as expected from the FACS study. Thus, even though the nodal MCp had the same membrane phenotype as mature granulated MC such as those in the peritoneal cavity, they are a novel MC subset with small size and little granularity.

Because the MCp recruited to parenchymal tissue of mLN or expanded in spleen with *T. spiralis* infection are granule deficient by cytometric presentation or direct histochemistry, they would not have been directly recognized in prior studies of MC biology. However, their phenotypic definition allows for a dynamic real-time assessment of their accumulation with cytometry. Whereas the jejunum was enriched with mMCP-1+ MMC likely derived from MCp recruited by the *T. spiralis* infection (12, 13), the nodal MCp were hypogranular and remained as such even as they declined in numbers by day 13 when the infection is subsiding and the jejunal MMC are at their peak. Thus, we sought evidence of function in vivo for the hypogranular nodal subset. Exocytosis of preformed secretory granule mediators seemed excluded based on the lack of granules, so we turned to the production of cytokines, which is a well-recognized function for BMMC in response to various agonists (38). Using 4get reporter mice, we found upregulation of IL-4 mRNA during helminth infection that increased to a greater degree in mLN MCp than in granulated mature peritoneal CTMC or in the recruited Ba. Importantly, intracellular cytokine staining demonstrated that MCp of WT BALB/c mice had cytokine-generating function as shown by IL-4 and IL-6 production in most mice at day 6 after *T. spiralis* infection. We did not need to further activate these LN ex vivo to obtain cytokine generation but did provide IL-3 to maintain their viability during their exposure to brefeldin. We have not yet sought to optimize the postinfection time course for MCp cytokine production or surveyed for other cytokines. Rather, our current goal was to demonstrate function for this essentially agranular MC subset prominent in draining LN after *T. spiralis* infection. We appreciate that there is a longer term need to address the role of the MC lineage in afferent as well as the traditional efferent components of adaptive immunity.

Ginsburg and Lagunoff (16) first extrapolated the appearance of MC lineage progenitors in LN of BALB/c mice, using repeated injections of horse serum or hen albumin for systemic sensitization. In their study, LN cells cultured on embryonic fibroblast monolayers differentiated into histochemically defined MC at higher rates from immunized mice, and the dependence of this outcome on Ag restimulation during the culture suggested cytokine-dependent maturation. Subsequently, Crapper and Schrader (9) used a conditioned media to grow immature MC from putative MCp and used a limiting dilution analysis with clonal expansion to define the levels of MCp in BM, spleen, and LN of naive CBA mice as well to show an increase in their number following a local immunization. Guy-Grand et al. (10) used such an assay to indirectly recognize MCp in both the thoracic duct lymph and in the mLN during *N. brasiliensis* infection in BALB/c mice. Jarboe et al. (14) extended these findings to show that putative MCp harvested from mLN were of low density and could be expanded and matured with cytokine culture alone or on a fibroblast monolayer with the latter inducing a histochemical positivity for heparin. Similarly, Rennick et al. (15) demonstrated that IL-4 and IL-10 promoted expansion observed that the size (FSC) and granularity (SSC) of MCp by cytometric assay were more than those observed for lymphocytes and demonstrably less than these parameters for culture-derived immature BMMC. These findings were also confirmed by histochemistry in which MC in the jejunum were readily observed

FIGURE 6. Increased IL-4 transcription in 4get mice and production of IL-4 and IL-6 by LN MCp after *T. spiralis* infection of BALB/c mice. (A) BM, spleen, mLN, and peritoneal lavage (PL) fluid were harvested from naive BALB/c and 4get mice, and from 4get mice on day 6 after *T. spiralis* (*Ts*) infection. The single-cell suspensions were assessed for GFP expression within the MC and Ba lineage populations. Histograms of GFP expression are shown for MC (left) and Ba (right) from each tissue as indicated. Histogram colors represent: gray, naive BALB/c mice; black, naive 4get mice; red, *T. spiralis*-infected 4get mice. Naive LN data are based on very few events. (B) MFI of GFP in the indicated MC (left) or Ba (right) lineage populations from 4get mice represent mean ± SEM, n = 13–14 mice, three experiments. **p < 0.0005, calculated by the Mann–Whitney *U* test. (C) Intracellular cytokine staining for the indicated cytokines in mLN MCp day 6 after infection in a representative BALB/c mouse. The gray lines are the isotype control, and black lines are the indicated cytokine. Histograms are from one mouse whose response was close to the mean shown in (D). (D) MFI index of intracellular cytokine staining for IL-4 and IL-6 in mLN MCp, showing individual values and mean values ± SEM (bars), day 6 postinfection, 13 mice per group, five experiments.
and maturation, respectively, of MCp recruited to mLN with helminth infection and that fibroblast monolayer coculture induced heparin biosynthesis. Thus, the seminal assay of LN MCp from immunized mice by Ginsburg and Lagunoff (16) had components such as added Ag and fibroblast monolayers favoring their maturation to identifiable immature MC. A recent study has suggested that MCp identified by clonal expansion in the LN of helminth-infected mice could be due to the influx of early myeloid progenitors (34). In that study, the authors noted the recruitment of type 2 multipotential progenitors to the LN and their maturation to MC, BA, and macrophages on ex vivo culture. Notably, these prior studies have relied on in vitro culture with MC growth factors to indirectly demonstrate the presence of MC lineage progenitors and this indirect approach does not reveal their relative maturation or purity of their lineage commitment. Our studies provide a direct assay for the MCp and, in concert with LDA, demonstrate that most cells of the MC lineage recruited to the draining LN with helminth infection are committed MCp. However, our findings also point out that the assays of MCp by ex vivo expansion with maturation do not define their hypothgranular in vivo phenotype and apparent failure to mature to MMC within the LN parenchyma.

The number of MC subsets is of course not limited to agranular and granular. For example, within the granular subset, the MMC in the intestine induced by helminth infection express mMCP-1 whereas MMC induced to accumulate in the trachea by OVA sensitization and challenge express not only mMCP-1, a chymase, but also another chymase, mMCP-4, an elastase, mMCP-5, two tryptases, mMCP-6 and mMCP-7, and carboxypeptidase A3 (6, 21). There are also differences in the protease profile of the innate CTMC in intestine and trachea. Taken together, it begins to appear that the particular tissue and its microenvironment to which MCp are recruited can determine whether there is granulation as well as the protease profile of those that become granulated (21, 37). This heterogeneity along with the added contexts of cytokine and eicosanoid generation for a lineage with major innate and adaptive immune–influenced members suggest that subset recognition is a step toward clarifying the complexities of overall MC lineage function.

Because the MCp recruited to parenchymal tissue of LN and spleen with T. spiralis infection were granule deficient by cyometric presentation or histochemistry on cytopsins, they would not have been recognized in studies in which mAb to FcεRI was used to deplete and thereby recognize a BA function. Germaine to this, Giacomin et al. (39) recently evaluated this treatment in C57BL/6 mice infected with T. spiralis and noted a decrease in the mucosal RI was used in normal tissues determined by an in vitro assay: antigen induces parallel increases in the frequency of P cell precursors and mast cells in normal and M. helispiralis infected mice. Thus, the seminal assay of LN MCp from the intestine depends on autologous expression of the chemokine receptor P. A. Koni, and M. F. Gurish. 2005. Constitutive homing of mast cell progenitors to the inflamed lung. J. Immunol. 168: 20478–20483.

References
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Supplemental Data

SUPPLEMENTAL FIGURE 1. Comparison of the forward and side scatter profiles of mesenteric LN and splenic MCp with cultured BMMC. Mesenteric LN and spleen MCp populations from d6 *T. spiralis*-infected mice and 6 wk old BMMC were assessed for forward and side scatter profiles by flow cytometry. The left panel shows LN and spleen MCp and BMMC size as assessed by forward scatter. The right panel shows the granularity as assessed by side scatter from the same 3 populations. The black line is LN MCp, the gray line is spleen MCp and the filled histogram is BMMC.