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CCL17 Controls Mast Cells for the Defense against Filarial Larval Entry

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Filarial parasites have to trespass many barriers to successfully settle within their mammalian host, which is equipped with mechanical borders and complex weaponry of an evolved immune system. However, little is known about mechanisms of early local events in filarial infections. In this study, bone marrow-derived dendritic cells not only upregulated activation markers CD40 and CD80 upon in vitro stimulation with filarial extracts, but also secreted CCL17, a chemokine known to be produced upon microbial challenge. Mice deficient for CCL17 had an up to 4-fold higher worm burden compared with controls by day 10 of infection with the murine filaria Litomosoides sigmodontis. Also, numbers of mast cells (MCs) invading the skin and degranulation were significantly increased, which was associated with enhanced vascular permeability and larval establishment. This phenotype was reverted by inhibition of MC degranulation with disodium cromoglycate or by blockade of histamine. In addition, we showed that CCL17-mediated vascular permeability was dependent on the presence of Wolbachia endosymbionts and TLR2. Our findings reveal that CCL17 controls filarial larval entry by limiting MC-dependent vascular permeability.

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Medast cells (MCs) are in close proximity to epithelial surfaces such as in the skin, respiratory and gastrointestinal systems, allowing prompt interaction with environmental agents. As a result of the observation that parasites can induce strong IgE-mediated MC activation, they have been implicated in the defense against parasitic infections, which is mediated by adaptive immune responses. However, the precise role of MCs in vivo is yet largely unclear. Increased numbers of mucosal MCs are often observed in tissues affected by gastrointestinal helmint infections. Indeed, functional MCs are required for the expulsion of Trichinella spiralis (1, 2), whereas defense against Heligmosomoides polygyrus (3) and Nippostrongylus brasiliensis (4) does not appear to be dependent on MCs (5). Different to parasites residing in the gut lumen, the function of MCs in response to tissue-dwelling filarial nematodes in primary infection has not been analyzed. Onchocerca volvulus, the filarial species that causes river blindness, affecting 37 million people in Africa, resides in s.c. nodules in humans and attracts MCs in high numbers. As opposed to live filariae within the onchocercomata, dead filariae induced significantly enhanced MC enrichment (6). The rare hyperreactive clinical manifestation of onchocerciasis (sowda), characterized by strong immune responses and low number of established worms, clearly correlates with strong mastocytosis (7). Therefore, in human onchocerciasis, the localization and frequency of MCs are inversely correlated to vitality and productivity of the worms and thus on the release of O. volvulus Ags. However, it has not yet been investigated whether MCs take part in the early defense against filariae, determining the fate of the parasite after entering its mammalian host. In the murine model of filariasis with Litomosoides sigmodontis infection, it is possible to follow the infection route from larval entry to full establishment. In this model, it has been shown that the extent of susceptibility is determined during the first days, with only a proportion of infectious L. sigmodontis stage 3 (L3) surviving and developing into adults (8). The worm load, which is maximal by day 10, stays constant for the subsequent 20 d (8). Innate mechanisms must therefore exist that allow only some of the incoming larvae to migrate and develop into adults.

Members of the chemokine superfAMILY are known to be involved in innate immunity. This also applies to the thymus and activation-regulated chemokine (TARC/CCL17), which is produced by dendritic cells (DCs) upon microbial challenge (9). It has previously been observed that CCL17 expression is high in human PBMC from filaria-infected individuals (10). However, localization and function of CCL17 in filarial infections remain unclear.

Using L. sigmodontis infection, we report that deficiency of CCL17 results in a higher worm load, accompanied by a higher number of skin MCs. MC function is essential for local vascular permeability, and blockade of MC granule release or histamine signaling prevents the more effective establishment of worms in the host seen in CCL17-deficient mice. A unique feature of many
filarial species is the mutualistic symbiosis with Wolbachia endobacteria. These endobacteria can activate the TLR system (11–13) mainly through TLR2 and induce innate inflammatory responses. CCL17-mediated MC numbers and vascular permeability were indeed dependent on the presence of Wolbachia. Our study provides evidence for an essential role for CCL17 during innate immune response against infective L3 larva to control MC-derived vascular permeability and thereby helminth infection.

Materials and Methods

Animal maintenance/injections

Six- to 8-wk-old female C3H/HeN and background-matched CCL17-deficient (E/E) and heterozygous (E/+), mice (9) and TLr2−/− (14) mice, were maintained under specific pathogen-free conditions, according to animal welfare guidelines. “E” denotes replacement of CCL17 by hCCL17 (eGFP). As indicated, mice were daily treated i.p. (8 mg/kg body weight) with the MC stabilizer disodium cromoglycate (cromolyn) dissolved in 0.9% NaCl (Sigma-Aldrich) starting 6 d before infection until mice were sacrificed. In vivo treatments used anti-CCL17 Ab (MAB529, administered i.p. at 20 μg/mouse on days −1, 1, 3, and 5; R&D Systems). Anti–IL-10R Ab (clone 1B1.3a, provided by Dr. M. D. Taylor, University of Edinburgh) and anti-CDC25 (clone PC61) were administered i.p. at a concentration of 1 mg on days −1 and 3. For inhibition of histamine signaling, 400 μg pyrilamine (HR1 antagonist) and 800 μg cimetidine (HR2 antagonist) were administered 1 h prior to and 2 h postinfection (p.i.) as well as once per day for 5 consecutive days, as described previously (15). At least five mice were used for each group.

Generation of bone marrow–derived DCs

Bone marrow cells were removed from femurs and tibias of CCL17+/+ mice by flushing the bones with PBS and filtered through a nylon mesh. Cells were centrifuged and 5×106/ml were plated in RPMI 1640 medium containing 10% FCS, 1% L-glutamine, and 50 μg/ml gentamicin, supplemented with 20% supernatant of GM-CSF–transfected X63Ag8 cells. Medium was replaced 3 d after the start of the culture. At day 8, DC1c cells were isolated by magnetic bead separation with the MACS system (Miltenyi Biotec). The purity of DCs was typically >95%. Cells were washed and plated at a density of 2.5×105/well and stimulated with the respective Ags for 24 h.

Parasites and infection

 Infective L3 larvae were transmitted through the bite of the vector mite Ornithonyssus bacoti (16), termed “natural infection” in the following sections. For a “localized” infection mites were collected in the lid of a 1.8-ml Eppendorf tube, which was fixed to the skin surface. On average, 10 infected mites could be observed to take their blood meal on a circle with a diameter of 0.5 cm. L3 without Wolbachia were harvested from mites that had acquired a blood meal from jirds treated with doxycycline for 4 wk, with the consequence that microfilariae, which the mites picked up were Wolbachia deficient (16). Parasites were harvested at 10, 20, and 30 d.p.i from the thoracic cavity and counted as described previously (17). For Ag preparation, worms were rinsed in sterile PBS before being mechanically minced. Insoluble material was removed by centrifugation of 300×g for 10 min at 4°C. Protein concentrations of crude extracts were determined using the Advanced Protein Assay (Cytoskeleton). All procedures were conducted under sterile conditions.

Skin RNA extraction, cDNA synthesis, and real-time PCR

Skin tissues were homogenized in TRIzol reagent 2×30 s at 6800 rpm in a Precellsy 24 (Peqlab) using 2.8-mm steel beads. RNA was extracted with TRIzol reagent (Invitrogen). Genomic DNA was removed from RNA samples by DNase digestion. cDNA was generated using Omniscript-RT kit (Qiagen), following the manufacturer’s protocol. Expression levels of IL-4, IL-5, IL-10, IL-12p35, IL-17a, CCL5, and thymic stromal lymphopoietin were quantified by real-time PCR using a Rotor-Gene RG-3000 (Corbett Research). Real-time PCR was performed in 20 μl of master mix containing 2 μl 10× PCR buffer, 0.2 mM 2′-deoxynucleoside 5′-triphosphates, 0.2 μM Sybr Green I (1/1000 in DMSO), 0.5 μU Hotstar Taq polymerase (Qiagen), and 2 μl cDNA template. Primer sequences are given in Supplemental Table 1. Reaction conditions were 15 min at 95°C, followed by 45 cycles of 15 s at 94°C, 20 s at 58°C, and 20 s at 72°C. Specific mRNA expression levels were normalized to β-actin mRNA expression levels.

Histology

Peripheral and mediastinal lymph nodes, lung, and skin of the lumbar area of mice were prepared for confocal microscopy of eGFP expression as described previously (9). Cystostat results were analyzed at given time points after natural infection by confocal microscopy (×400) for eGFP expression of cells. For MC evaluation, the skin at the site of L3 inoculation was isolated 6 h p.i., and formalin-fixed paraffin sections were stained with toluidine blue. The total number of mast cells in the epidermis, dermis, and hypodermis was counted on 20 successive, complete fields of a light microscope (×1000) per section and were averaged from 10 sections of each skin sample, which were separate from each other by 4-μm sections that were not analyzed. Mast cells were classified as non-degranulated or degranulating (18). The examiner (J.K.F.) was blinded with regard to the nature of the mouse groups.

Flow cytometry and cytokine ELISA

The following mAbs against mouse Ags were used as PE or allophycocyanin conjugates for analysis of BMDCs or cell composition in the thoracic cavity fluid: CD4 (3/23), CD8 (53-6.7), CD11b (M1/70), CD11c (HL3), CD40 (3/23), CD80 (16-10A1), CD4 (RM4), CD8a (53-6.7), CD25 (PC61), CD45R/B220 (RA3-6B2), CD11b (M1/70), or CD11c (HL3). Surface staining with mAb, acquisition, and analyses were performed as described previously (20, 21). For eGFP expression of reporter mice, organs were collagenase/DNase digested and CD11c+ cells sorted using MACS (Miltenyi Biotec). For surface staining PE-conjugated MHC class II (55114.15.2) and PE-Cy7–conjugated CD11c (HL3) Abs were used. Flow cytometric analysis was performed using FACSanto running Diva software (BD Biosciences). Cytokines in the thoracic cavity fluid were quantified by ELISA, using standard protocols (DuoSet; R&D Systems).

Statistics

Comparison between the groups of single experiments in case of parametrical data was done using the Student t test or ANOVA and Tukey’s multiple comparison tests. In case of nonparametrical data, Kruskal–Wallis or Mann–Whitney U test was used. Data in all graphs are presented as mean ± SD or median and range. Statistics were performed with GraphPad Prism 5 software or SPSS. Significant values are indicated as follows: *p<0.05, **p<0.01, and ***p<0.001.

Results

Early expression of CCL17 following filarial infection

DCs are among the first immune cells that filarial parasites encounter when entering the skin. Their key role is to ingest, sense, and process Ag to efficiently activate adaptive immunity, but also to recruit immune cells to the site of Ag encounter (e.g., by secretion of chemokines). We found that after stimulation with filarial extract (LsAg), bone marrow–derived DCs (BMDCs) upregulated the activation markers CD40 and CD80 compared with unstimulated controls and secreted significant amounts of IL-6 and CCL17 upon stimulation with LsAg (Fig. 1).

To investigate the in vivo expression of CCL17, mice were infected with the rodent filaria L. sigmodontis, an established model for filarial diseases (8, 17, 22). Using a fluorescence (eGFP)-based in vivo reporter system (9), in which an eGFP cassette was inserted into the endogenous murine ccl17 locus, we show that CCL17 is expressed in murine skin (Fig. 2A) as early as 6 h post filarial infection, but not in naive mice. Also, in the draining lymph node and lung CCL17 expression was increased over time. These eGFP-producing cells have been previously identified as CD11c+ DCs (9). Consistently, quantification of CCL17+CD11c+ cells revealed their presence at higher percentages in the draining lymph nodes at 6 h p.i. as compared with naive mice, which
peaked at day 5 p.i. (Fig. 2B). Lungs contained these DCs at enhanced numbers at 1 d p.i. compared with the uninfected stage or 6 h p.i. (Fig. 2C). These organs are along the migratory pathway of infective larvae, their final destination being the thoracic cavity. CCL17+ DCs were not present in the spleen (data not shown), according to the lack of CCL17 expression in the spleen upon bacterial challenge (9).

CCL17 confers limitation of worm establishment early in infection

To analyze the relevance of CCL17 as functional player in filariasis, we observed parasitological and immunological consequences during the course of a natural infection with L. sigmodontis in CCL17+/+ heterozygous and CCL17−/−-deficient mice (“E” denotes replacement of CCL17 by eGFP). At day 10 p.i., when the filariae have reached the thoracic cavity, an up to 4 times higher parasite load in CCL17−/− mice compared with CCL17+/+ mice was observed (Fig. 3A, Supplemental Fig. 1). This difference was maintained throughout day 30 p.i., being in line with several earlier observations of L. sigmodontis infection that the worm levels, which are established by day 10, do not change over the next 20 d (8). Furthermore, anti-CCL17 Ab treatment in heterozygous (+/+) and homozygous (E/E) control mice did not differ with regard to parasite loads (Fig. 4D).

Cytokines and cellular responses in CCL17−/− mice during filarial infection

To investigate whether there are different filaria-driven immune responses in the presence or absence of CCL17, the cellular composition and cytokine responses in the thoracic cavity were analyzed at day 10, 20, and 30 p.i. Of the cell types analyzed, no major differences were observed, except for slight but significant elevation of CD4+CD25+ T cells. Numbers of other cells such as CD4+ and CD8+ T cells and DCs were unaltered in both groups (Supplemental Table II). Analysis of concentrations of the cytokines IL-5, IL-10, IL-12, and IFN-γ in the thoracic cavity fluid did not reveal Th1/Th2 imbalance, except a small increase in IL-10 (Supplemental Table III). In the mediastinal lymph nodes, there was no alteration in the cellular composition between CCL17-deficient and control mice at day 10 p.i. (Supplemental Table IV). Upon Ag specific restimulation of mediastinal lymph node cells, cytokine production was at the limit of detection and did not
parasite loads in CCL17E/+ mice treated with cromolyn (gray bars) or left untreated (black bars). Numbers of degranulating MCs were counted in the skin of cromolyn-treated or untreated mice 6 h after natural infection (Fig. 3). Mice were inoculated with Evans blue and extravasation into the surrounding tissue was measured (19). Aside from the expected change in OD when injecting Evans blue compared with noninjected mice, we found that vascular permeability was significantly enhanced in CCL17E/E as well as in anti-CCL17-treated CCL17E/+ mice compared with untreated CCL17E/+ mice. 

CCL17 inhibits filarial induced activation of MCs in the skin

Within the repertoire of host cells remaining in proximity to epithelial surfaces, such as the skin, MCs are particularly frequent. However, the role of MCs in primary filarial infection is unclear. We analyzed the number and status of skin MCs in CCL17E/E mice and heterozygous littermates after either natural infection with L3-containing mites or a mock infection using mites without L3, the latter being used to control for the vector bite itself. MCs were grouped as either nondegranulating or degranulating (18). As shown in Fig. 5A, a slight increase in the number of total skin MCs could be observed following exposure of mice to mites without L3 in both CCL17E/E and CCL17E/+ mice. In contrast, a significant increase was observed after exposure to L3-containing mites in CCL17E/E mice but not in heterozygous controls. The number of degranulating MCs was also significantly higher in CCL17E/E mice after exposure to L3-containing mites (Fig. 5C). This difference was more profound after a localized natural infection where mites were allowed to bite only on a circular field with a diameter of 0.5 cm (Fig. 6C, left pair of bars, see also below).

Equivalent results were also observed in heterozygous mice treated with anti-CCL17 Abs. Ab treatment resulted in increased numbers of total MCs (Fig. 5D) and degranulating MCs (Fig. 5F) similar to those seen in CCL17-deficient mice after natural challenge with infective larvae (Fig. 5C).

Vascular permeability is enhanced in CCL17 deficient mice after L3 challenge

MCs reside in close association with blood vessels and nerves at the sites that are most likely to be exposed to pathogens. Many of the mediators that are released by MCs cause alterations in selective cellular recruitment and vascular function (5).

We investigated whether deficiency of CCL17 could modify the permeability of endothelial cells. After s.c. injection of medium or LsAg (Fig. 7A), mice were inoculated with Evans blue and extravasation into the surrounding tissue was measured (19). Aside from the expected change in OD when injecting Evans blue compared with noninjected mice, we found that vascular permeability was significantly enhanced in CCL17E/E as well as in anti-CCL17-treated CCL17E/+ mice compared with untreated CCL17E/+ mice.
controls. Changes in vascular permeability were also induced in CCL17-deficient mice by infective larvae, either when injected (Fig. 7B) or when transmitted by their natural vector in a localized infection (Fig. 7C).

These data suggest a new mechanism whereby L3 induces vascular permeability, probably to facilitate their migration by entering the lumen of vessels situated along this pathway.

**Inhibition of MCs reverts vascular permeability and parasite loads**

To prove that the increased parasite load observed in CCL17E/E mice is essentially dependent on the increased number of degranulating MCs and the ensuing elevated vascular permeability either in the skin or at other sites along the migratory pathway of L3 toward the pleural space, we inhibited MC degranulation by injection of disodium cromoglycate (cromolyn). It is hypothesized that cromolyn induces conformational changes of moesin that is involved in the regulation of a functional association between the cell surface and the cytoskeleton in MCs, thereby inhibiting the secretion of granules (23).

**FIGURE 5.** Increased numbers of MCs are present in the skin of CCL17E/E or anti-CCL17-treated mice after natural infection. Skin from the lumbar area of CCL17E/+ (white bars), CCL17E/E (black bars), and anti-CCL17-treated mice (gray bars) was removed 6 h following natural infection with either L3-containing mites (A–F) or mock infection using mites without L3 (A–C). The total number of toluidine-blue stained MCs was counted (A, D), and MCs were subdivided into non-degranulating (B, E) and degranulating MCs (C, F). The number of MCs is given per 20 successive, complete fields of a light microscope (×1000) per section, averaged from 10 sections per skin sample as described in Materials and Methods. Depicted are results of one of two independent consistent infection experiments and the mean ± SD of MCs. Data were analyzed using ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 6.** A–C, Wolbachia are essential for increased vascular permeability and MC degranulation. Wolbachia-containing (LsAg) or -depleted worm extract (LsAg Wb-depl.) was inoculated s.c. into the lumbar area of CCL17E/+ mice; CCL17E/E mice received LsAg. Vascular permeability was measured by Evans blue leakage into the tissue (A). Data represent one of two consistent experiments. **p < 0.01 (ANOVA). CCL17E/E (C) and CCL17E/E (D) mice underwent natural dispersed (B) or localized (C) infection with mites harboring Wolbachia-containing or -depleted L3 larvae (see Materials and Methods). Numbers of degranulating skin MCs were counted after toluidine-blue staining. Data of one of two consistent experiments are shown as mean ± SD and were analyzed using ANOVA. D–F, Larval establishment in CCL17-depleted mice is dependent on TLR2. Compared with CCL17E/+ mice, vascular permeability after inoculation of 10 μg LsAg (C), number of degranulating MCs (E), and parasite numbers 10 d p.i. (F) are elevated in CCL17E/E mice or in CCL17E/+ mice treated with anti-CCL17 Abs but not in anti-CCL17 treated TLR2−/− mice. *p = 0.0122 (ANOVA). *p < 0.05, **p < 0.01, ***p < 0.001.
blue following cromolyn treatment (Fig. 4B), arguing that inhibition of MC degranulation by cromolyn reduced vascular permeability to the level in CCL17E/− mice. Even more important, cromolyn treated CCL17- deficient mice no longer displayed increased numbers of parasites in the thoracic cavity (Fig. 4C, thus showing reversion to the phenotype of heterozygous CCL17E/+ mice. Treatment of CCL17E/+ mice with cromolyn did not alter parasite loads (Supplemental Fig. 2A).

To further underscore the role of MCs for parasite establishment in CCL17-deficient mice, we blocked the activity of histamine, the main MC product released during degranulation, in vivo. Administration of antagonists for histamine receptors 1 (pyrilamine) and 2 (cimetidine) reverted the increased parasite loads of CCL17-deficient mice to the levels of both homozygous (+/+ and heterozygous (E/+ control mice (Fig. 4D, Supplemental Fig. 2B).

These data conclusively suggest that the increase in MCs in CCL17-deficient mice is of functional relevance for vascular permeability and parasite establishment.

Vascular permeability and MC degranulation are dependent on Wolbachia via TLR2 stimulation

Most filarial nematode species have the unique feature that they contain endosymbiotic bacteria of the genus Wolbachia, which are essential for development of L3 into adult worms and for fertility and survival of the adults (11, 24, 25). Therefore, we investigated whether Wolbachia endosymbionts are involved in MC activation and consequently increased vascular permeability. First, we injected LsAg or LsAg devoid of Wolbachia into the skin, followed by injection of Evans blue. Only the extract containing Wolbachia Ags was able to increase vascular permeability in CCL17E/− mice (Fig. 6A). Next, it was of interest whether this would also hold true for infective L3. Therefore, the effect of normal, Wolbachia-containing L3 on the induction of MC degranulation was compared with that of L3 generated in vectors that had ingested MF from animals treated with the Wolbachia-depleting drug doxycycline, as described previously (Ref. 16, see also Materials and Methods). Infective L3 were either injected (Fig. 6B) or transferred by mites in a localized infection (Fig. 6C).

These data reveal that in CCL17E/E mice, the number of degraded MCs in comparison with Wolbachia-containing L3, whereas in heterozygous mice, MC degranulation was not significantly affected by the presence or absence of Wolbachia (compare the white bars in Fig. 6B, 6C). Taken together, these results indicate that Wolbachia are involved in MC degranulation and increased vascular permeability.

Because several publications could link activation of the immune system by Wolbachia with TLR2 (12, 13, 26), we set out to test whether the observed dependency on Wolbachia for the induction of vascular permeability was due to interaction with TLR2. Therefore, we compared the effects of anti-CCL17 Ab treatment on CCL17E/−/− mice, where this treatment results in elevated parasite loads (Figs. 3B, 6F) and increased vascular permeability (Fig. 6D), to TLR2-deficient mice on the same background (C3H).

After injection of LsAg followed by Evans blue, we found that only genetically deficient CCL17E/−/− and anti–CCL17-treated CCL17E/+ mice displayed increased vascular permeability. In contrast, anti–CCL17-treated TLR2−/− mice did not show permeability changes (Fig. 6D). The number of degranulating MCs was not increased in these mice after natural infection with L3 by the insect vector (Fig. 6E). Importantly, these results were associated with the parasitological outcome: as expected, CCL17E/−/− and anti–CCL17-treated CCL17E/+ mice contained significantly higher numbers of parasites 10 d p.i. However, TLR2−/− mice when given anti-CCL17 Abs, did not display elevated parasite loads (Fig. 6F), indicating that signaling via TLR2 is dispensable for the increased MC function after CCL17 blockade. These data show that control of vascular permeability and parasite loads by CCL17 acts in a TLR2 dependent manner.

Discussion

Early defense mechanisms against entry of filarial parasites are poorly documented. In particular, the role of chemokines has not been addressed. The data presented in this study show that CCL17 is involved in early immune responses limiting filarial parasite invasion into the host. Genetic deficiency of CCL17 or, equivalently, administration of anti–CCL17 Abs, results in MC accumulation, their degranulation, and increased vascular permeability. This mechanism is dependent on the presence of Wolbachia endosymbionts in infective L3 larvae and promoted by TLR2-dependent signaling. To our knowledge, they link CCL17, MCs, histamine, and control of filarial infection for the first time.

It is known that L3, after transmission into the skin of mice, migrate via the lymphatics (27–29) into the pleural space. Accordingly, CCL17 expression was detected not only upon artificial stimulation of BMDCs but also in CD11+ DCs in the skin, in the draining lymph nodes and the lung after natural filarial infection. Genetic deficiency or Ab depletion of CCL17 resulted in increased
parasite loads, indicating a major role for CCL17 in parasite containment.

CCL17 is known to be produced by DCs to recruit activated and memory T cells to the site of infection. However, we did not observe differences in effector cell composition at day 10 p.i., neither in the thoracic cavity nor in the draining lymph node. This also applied for cytokine production, except for slight differences in CD4+CD25+ T cells and IL-10 production in the thoracic cavity. In murine filariasis, regulatory T cells expand in numbers at the site of infection and are observed in the skin draining lymph nodes or coelomic cavities as early as day 7 p.i. (21, 30). However, in CCL17+/+ mice, neither IL-10 neutralization nor depletion of CD25+ T cells prior to infection altered the parasitological outcome, in accordance with previous results showing that anti-CD25 Abs altered the parasite loads only during infection, but did not affect initial larval establishment (21). Together with the findings that neither cellular composition nor production of Th1/Th2 cytokines differed between CCL17+/+ and CCL17−/− mice, our data suggest that induction of effector or regulatory cells is not the major mode of action of CCL17 in early murine filarial infections.

This is also in accordance with earlier observations that differences in parasite survival were obvious already at day 10, and it has been shown that the proportion of worms that establish themselves in the host following the entry of infective L3 is determined during the first days (i.e., at times before adaptive immune responses can control the immune reaction (29, 31, 32)).

MCs are frequently located in proximity to epithelial surfaces, such as the skin or the lung, ideally placed to carry out their function in host defense. After natural transmission of L3 by the vector, but also after injection of parasite extract (LsAg), we detected MCs at higher numbers per se and among those higher proportions of degranulating MCs in the skin of CCL17+/+ mice. Because bites from mites that did not carry L3 failed to induce MC accumulation and degranulation, this rules out that vector saliva plays a major role in the activation of MCs. A similar inhibitory effect of CCL17 on human cord blood MC migration has been reported: CCL5-induced migration of human cord blood-derived MCs was suppressed by CCL17 (33). Conversely, in transgenic mice overexpressing CCL17, lower numbers of MCs as well as of lymphocytes and neutrophils were found in the skin of mice after a single challenge with oxazolone (34). Considering CCL5 as a possible chemoattractant for MCs, CCL5 gene expression was equivalently elevated in both CCL17+/+ and CCL17−/− mice. This suggests that CCL17 restricts CCL5-induced MC accumulation in the skin following exposure to L3, thus leading to the elevated numbers of MCs and facilitation of degranulation in the absence of CCL17. These data add to the recognition of CCL17 not only as chemoattractant but also as limiting factor for cell migration to maintain the homeostasis at pathogen-exposed interfaces.

The influx of MCs detected by histology appears to be rather minor; however, this could be due to the failure to detect MCs that are already fully degranulated, or to the nature of a natural infection. We have established a way to localize the bite of mites to a limited area of the skin, which resulted in a higher ratio of degranulating MCs between infected CCL17−/− and control mice in a localized compared with a dispersed natural infection (2.9 versus 1.6; compare Figs. 6C and 5C). The parasite infection differs considerably from skin inflammation models in that the parasite trespasses the skin within one day following infection, trying to avoid skin irritation, whereas in other models inflammatory triggers are administered over days or weeks (35, 36).

In addition to MCs located in the skin, these cells could also play an important role for the facilitation of larval migration at other sites (e.g., lymphatics or lungs), which the parasites encounter on their route toward the pleural space, where the juvenile and adult worms reside. To address the function of those MC comprehensively, we carried out functional blockade of MCs and the histamine signaling pathway.

As a consequence of recruitment and activation, degranulating MCs release a magnitude of substances, among them many molecules that are involved in vascular permeability, such as histamine and granymes. To identify MC function in the context of early filarial infection, we pharmacologically blocked MC function by administration of the MC stabilizer cromolyn. Indeed, cromolyn treatment reversed all phenotypes investigated (i.e., MC degranulation, vascular permeability, and parasite loads). MC inhibition had no effect on parasite recovery in CCL17+/+ mice, suggesting that there is a threshold of degranulation below which filarial recovery is not modified (Supplemental Fig. 2).

Additional studies using histamine receptor antagonists in vivo point toward histamine, the main vasodilatory molecule secreted by MCs, because inhibition of the mediator of the effects seen in CCL17-deficient mice, was able to revert the parasitological phenotype (Fig. 4D). Vascular permeability is a common feature of inflammation, which is associated with leukocyte extravasation, increased interstitial fluid, and consequently, increased lymphatic drainage; this elevated flow into the lymphatic vessels may explain the greater entry of larvae inside the lymphatic vessels.

Prototypic MC activation is induced by cross-linking of FcRb-bound IgE. Recent studies show that activation of murine MCs by TLR2 or TLR4 ligands acts synergistically with IgE-mediated activation, because administration of TLR ligands can augment airway sensitization in mice. It has been suggested that this process involves TLR-dependent activation of DCs (reviewed in Ref. 37). It is however unclear, whether degranulation itself can be induced via TLR directly. Some reports showed that TLR2 ligands are able to induce degranulation directly (38, 39), but a clear mechanism cannot be defined yet, because such responses have not been observed consistently in all studies (40, 41). At this point it should be noted that MCs exhibit some variation in phenotype according to anatomical location, species and culture conditions.

Wolbachia endosymbionts present in most filarial species (42) and also in L. sigmodontis provide ligands for TLR2 (and TLR6). We show that vascular permeability after injection of Ag and MC numbers after transmission of L3 via the natural vector is markedly reduced in the absence of Wolbachia in a TLR2 dependent manner.

Our data demonstrate that CCL17 inhibits filaria-induced MC activation/function in genetically modified or anti–CCL17-treated, mice. In particular, higher numbers of MCs in CCL17-deficient mice were accompanied by more degranulating MCs. This was associated with increased vascular permeability in CCL17-deficient mice. Both MCs and vascular permeability could be causatively linked to the parasitological phenotype seen in CCL17-deficient mice, as inhibition of MC degranulation by cromolyn or administration of histamine-receptor antagonists reverted worm establishment. These observations were monitored within the first barrier that the parasite has to encounter, suggesting the skin to be a site for MC action. However, the parasite will also trespass other organs such as the lung on its way to the pleural space. This was not differentiated in this study and constitutes interesting further studies.

In conclusion, this study suggests a new molecular mechanism for filarial worm migration and establishment by MC-mediated increased vascular permeability. This is counterregulated by CCL17 expression of the host, implicating an important role for CCL17 in early responses against a skin-invading pathogen.
Disclosures

The authors have no financial conflicts of interest.

References


19. Th17, CCL17 CONTROLS FILARIAL LARVAL ESTABLISHMENT
**Supplementary Figure 1:** (A,B) Parasite numbers are increased in CCL17<sup>E/E</sup> compared to CCL17<sup>E/+</sup> mice from day 10 throughout day 30. Data represent two additional experiments (see also Fig. 1). Data were analysed using ANOVA.

**Supplementary Figure 2:** Parasite loads in CCL17<sup>E/E</sup> mice were reversed by inhibition of MC degranulation with disodium cromoglycate (A) or histamine-receptor antagonists (B). Mice were infected with *L. sigmodontis* and parasite loads were determined 10 days post natural infection. Data show additional experiments (see Figure 6) and are shown as mean ± SD and were analysed using ANOVA.

**Supplementary Figure 3:** Parasite loads in CCL17<sup>E/E</sup> mice are unaltered by depletion of CD25<sup>+</sup> cells (A) or IL-10 (B). Mice were infected with *L. sigmodontis* and parasite loads were determined 10 days post natural infection. Mice were treated with either αCD24 or αIL-10R antibodies. Data show one representative experiment of two and are shown as mean ± SD. They were analysed using ANOVA.
Supplementary Table 1: *Forward and reverse primers used for mRNA expression profile of skin tissue*.

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>ß-actin</td>
<td>5’ AGA GGG AAA TCG TGC GTG AC 3’</td>
<td>5’ CAA TAG TGA TGA CCT GGC GGT 3’</td>
</tr>
<tr>
<td>IL-12p35</td>
<td>5’ AAC CAG CAC ATT GAA GAC CTG 3’</td>
<td>5’ ACA GGG TCA TCA TCA AAG ACG 3’</td>
</tr>
<tr>
<td>IL-10</td>
<td>5’ GGT TGC CAA GCC TTA TCG GA 3’</td>
<td>5’ ACC TGC TCC ACT GCC TTG CT 3’</td>
</tr>
<tr>
<td>IL-4</td>
<td>5’ TGT ACC AGG AGC CAT ATC CAC 3’</td>
<td>5’ GTT CTT CGT TGC TGT GAG GAC 3’</td>
</tr>
<tr>
<td>IL-5</td>
<td>5’ AGC ACA GTG GTG AAA GAG ACC TT 3’</td>
<td>5’ TCC AAT GCA TAG CTG GTG ATT T 3’</td>
</tr>
<tr>
<td>CCL5</td>
<td>5’ TTG CAG TCG TGT TTG TCA CTC 3’</td>
<td>5’ CAG GGA AGC TAT ACA GGG TCA 3’</td>
</tr>
<tr>
<td>IL-17A</td>
<td>5’ CCG CAA TGA AGA CCC TGA TA 3’</td>
<td>5’ TCC CTC TTC AGG ACC AGG AT 3’</td>
</tr>
<tr>
<td>TSLP</td>
<td>5’ ATC GAG GAC TGT GAG AGC AAG 3’</td>
<td>5’ TTG TGC CAT TTC CGT AGT ACC 3’</td>
</tr>
</tbody>
</table>

* Primer concentration used for all PCRs was 300nM in combination with 4,5μM MgCl\textsubscript{2}. For CCL5-PCR, 200nM primers and 2,5μM MgCl\textsubscript{2} were used.
Supplementary Table 2: Accumulation of T- and B-lymphocytes and APCs (% of living cells) in the thoracic cavity of C3H/HeN CCL17<sup>E/+</sup> and CCL17<sup>E/E</sup> mice infected with <i>L. sigmodontis</i> 10, 20 and 30 days p.i.

<table>
<thead>
<tr>
<th></th>
<th>Day 10 p.i.</th>
<th>Day 20 p.i.</th>
<th>Day 30 p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CCL17&lt;sup&gt;E/+&lt;/sup&gt;</td>
<td>CCL17&lt;sup&gt;E/E&lt;/sup&gt;</td>
<td>&lt;i&gt;p&lt;/i&gt;-value</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>6.15 ± 4.16</td>
<td>7.93 ± 0.61</td>
<td>0.99</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt;</td>
<td>2.88 ± 2.87</td>
<td>1.73 ± 0.92</td>
<td>0.86</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;/25&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.43 ± 0.75</td>
<td>2.88 ± 0.55</td>
<td>0.02</td>
</tr>
<tr>
<td>B cells</td>
<td>38.0 ± 11.9</td>
<td>26.7 ± 5.3</td>
<td>0.23</td>
</tr>
<tr>
<td>Macrophages</td>
<td>37.18 ± 13.56</td>
<td>52.37 ± 7.98</td>
<td>0.11</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>4.15 ± 1.49</td>
<td>4.67 ± 1.1</td>
<td>0.63</td>
</tr>
</tbody>
</table>

CCL17<sup>E/E</sup> and CCL17<sup>E/+</sup> mice were naturally infected with <i>L. sigmodontis</i>. Cells were isolated from the thoracic cavity fluid 10, 20 and 30 days after infection and stained for CD4, CD8, F4/80, CD11c and CD19 and analysed by FACS. Values represent mean ± SD. Data from one representative experiment out of two are shown. Data were analysed using student’s t-test.
Supplementary Table 3: Cytokine production in the thoracic cavity of CCL17<sup>E/E</sup> and CCL17<sup>E/+</sup> mice after infection with <i>L. sigmodontis</i>

<table>
<thead>
<tr>
<th></th>
<th>Day 10 p.i.</th>
<th>Day 20 p.i.</th>
<th>Day 30 p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CCL17&lt;sup&gt;E/+&lt;/sup&gt;</td>
<td>CCL17&lt;sup&gt;E/E&lt;/sup&gt;</td>
<td>p-value</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.13 ± 0.05</td>
<td>0.12 ± 0.04</td>
<td>0.77</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.04 ± 0.05</td>
<td>0.09 ± 0.15</td>
<td>0.54</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.02 ± 0.04</td>
<td>0.01 ± 0.02</td>
<td>0.56</td>
</tr>
<tr>
<td>IL-12 p70</td>
<td>0.13 ± 0.17</td>
<td>0.11 ± 0.06</td>
<td>0.82</td>
</tr>
</tbody>
</table>

CCL17<sup>E/E</sup> and CCL17<sup>E/+</sup> mice were naturally infected with <i>L. sigmodontis</i>. IFN-γ, IL-5, IL-10 and IL-12p70 (ng/ml) was measured in the thoracic cavity fluid 10, 20 and 30 days after infection. Values represent mean ± SD. Representative data from one experiment out of two are shown. Data was analysed using student’s t-test.
Supplementary Table 4: Cell numbers of T- and B-lymphocytes and APCs in mediastinal lymph nodes (cell number/ LN) of C3H/HeN CCL17E/+ and CCL17E/E mice infected with L. sigmodontis 10 days p.i.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>CCL17E/+</th>
<th>CCL17E/E</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ (x10^3)</td>
<td>190 ± 72</td>
<td>142 ± 40</td>
<td>0.180</td>
</tr>
<tr>
<td>CD8+ (x10^3)</td>
<td>87 ± 36</td>
<td>70 ± 19</td>
<td>0.589</td>
</tr>
<tr>
<td>CD4+/25+ (x10^3)</td>
<td>15 ± 6</td>
<td>10 ± 4</td>
<td>0.093</td>
</tr>
<tr>
<td>B-cells (x10^3)</td>
<td>73 ± 35</td>
<td>75 ± 28</td>
<td>0.818</td>
</tr>
<tr>
<td>Macrophages (x10^3)</td>
<td>23 ± 29</td>
<td>26 ± 14</td>
<td>0.485</td>
</tr>
<tr>
<td>CD11c+ (x10^3)</td>
<td>32 ± 20</td>
<td>18 ± 11</td>
<td>0.132</td>
</tr>
<tr>
<td>CD11c+ MHC class II+ eGFP+</td>
<td>1125 ± 964</td>
<td>1730 ± 763</td>
<td>0.093</td>
</tr>
</tbody>
</table>

CCL17E/E and CCL17E/+ mice were naturally infected with L. sigmodontis. Mediastinal lymph nodes were Collagenase/ DNase I-digested 10 days after infection and isolated cells were stained for CD4, CD8, CD25, F4/80, CD11c, MHC class II and CD19 and analysed by FACS. Values represent mean ± SD. Data from one representative experiment out of three are shown. Data were analysed using student’s t-test.
Supplementary Fig. 1
Supplementary Fig. 3

A

B