Basophils Protect against Reinfection with Hookworms Independently of Mast Cells and Memory Th2 Cells

Caspar Ohnmacht and David Voehringer

*J Immunol* 2010; 184:344-350; Prepublished online 2 December 2009;
doi: 10.4049/jimmunol.0901841
http://www.jimmunol.org/content/184/1/344

References

This article cites 37 articles, 16 of which you can access for free at: http://www.jimmunol.org/content/184/1/344.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Basophils Protect against Reinfection with Hookworms Independently of Mast Cells and Memory Th2 Cells

Caspar Ohnmacht and David Voehringer

Hookworms infect several hundred million people worldwide, causing malnutrition, anemia, and growth retardation. Infections generally result in a strong type 2 immune response, but the effector mechanisms that mediate worm expulsion remain poorly characterized. In this study, we determined the role of mast cells and basophils in protective immunity against the murine hookworm, *Nippostrongylus brasiliensis*, during primary and secondary infection. Mast cell–deficient c-Kit/W-sh mice had lower serum IgE levels compared with wild-type mice under steady-state conditions and after *N. brasiliensis* infection. Worm expulsion was delayed during primary but not during secondary infection of c-Kit/W-sh mice, even in the absence of CD4 T cells. However, protective immunity was lost when basophils were depleted before reinfection of c-Kit/W-sh mice. We conclude that basophils play a crucial role for worm expulsion during a memory type 2 immune response independently of mast cells and memory Th2 cells. The Journal of Immunology, 2010, 184: 344–350.

Intestinal nematodes infect ~25% of the human population worldwide, causing major socioeconomic problems mainly in countries in which sanitary standards are low and infection rates are high. Hookworms account for one third of all nematode infections. Efforts to develop effective vaccines against these parasites have not been successful (1). In general, helminths induce a strong type 2 immune response, which is characterized by high IgE levels and increased numbers of Th2 cells, mast cells, eosinophils, and basophils. Although it is well established that IL-4 and IL-13 play a major role in the expulsion of most gastrointestinal helminths, it remains unclear which downstream effector mechanisms are critical in this process (2).

Migratory larvae and adult hookworms may be directly killed by effector cells, such as eosinophils, mast cells, or alternatively activated macrophages (AAMs) (3–5). However, hookworms may also be expelled by a “weep and sweep” mechanism, which involves increased mucus production and smooth muscle contractions, resulting in an inhospitable environment (6). Infections of mice with *Nippostrongylus brasiliensis* or *Heligmosomoides polygyrus* represent two well-established models to study the immune response against hookworms. Worm expulsion during primary infection with *N. brasiliensis* does not require eosinophils or IgE, but is strictly dependent on CD4 T cells and innate IL-4/IL-13–producing cells (2, 7). Interestingly, CD4 T cells are not required for worm expulsion during secondary infection (8). This indicates that non-CD4 T cells that are induced by CD4 T cells during primary infection play an important role in the maintenance of protective immunity against *N. brasiliensis*. We and other investigators showed that eosinophils contribute to immunity during secondary infection (7, 9). Furthermore, AAMs have been shown to protect mice against primary infection with *N. brasiliensis* and secondary infection with *Heligmosomoides polygyrus* (5, 10).

Basophils were shown to be excellent Ag capturing cells after immunization with a model Ag and they can even enhance humoral immune responses, raising the possibility that they can ameliorate their own Ag recognizing capacities by enhancing the production of Ag-specific IgG1 and IgE molecules in plasma cells (11, 12). Furthermore, several recent publications demonstrated that basophils are important APCs driving the differentiation of Th2 cells in allergy models (13–15). Recently, we demonstrated that basophils contribute to the efficient expulsion of *N. brasiliensis* after a primary infection in the absence of Th2 cells (16). In addition, it was shown that basophils are equally important for resolving *Trichinella muris* infection (14). However, their contribution to protective immunity against secondary infection with helminths is not known.

Whether mast cells play an important role during primary or secondary hookworm infections remains unclear. Early studies indicated that mast cells accelerate the expulsion of *N. brasiliensis* (17). These studies were performed with mast cell–deficient c-Ki/W−/− mice, which have additional defects of potential relevance, including severe anemia, sterility, lack of γ/δ T cells in the small intestine, lack of interstitial cells of Cajal, impaired melanogenesis, relatively few neutrophils, and abnormal pacemaker activity in the small intestine (18). These defects can affect the interpretation of experimental results and motivated us to analyze the role of mast cells in immunity against hookworms with a more physiologic model. Most of the defects of c-Ki/W−/− mice are not observed in c-Ki/W-sh mice (19), another mast cell–deficient mouse strain that is available on a C57BL/6 background and, therefore, appears to be a suitable model in which to study the role of mast cells during hookworm infection.

In this study, we determined the role of mast cells and basophils during primary and secondary infection with the hookworm *N. brasiliensis*. Mast cell–deficient c-Ki/W-sh mice were found to express significantly lower levels of IgE and worm expulsion during primary infection was delayed compared with C57BL/6 control mice. Interestingly, rapid worm expulsion during secondary infection occurred in the absence of mast cells and CD4 T cells but was critically dependent on basophils.
Materials and Methods

Mice

Mast cell–deficient c-Kit W-sh mice (19) were obtained from D. Lee (Brigham and Women’s Hospital, Boston, MA). C57BL/6 mice, BALB/c mice, and Stat6 \(^{-/-}\) mice on a BALB/c background (20) were originally obtained from The Jackson Laboratory (Bar Harbor, ME). IL-4/IL-13 \(^{-/-}\) mice (21) were kindly provided by A.N. McKenzie (Medical Research Council, Cambridge, U.K.). Mice were housed according to institutional guidelines and used at 6–12 wk of age. The experiments were approved by the Regierung von Oberbayern.

N. brasiliensis infection

Mice were infected with L3 larvae of *N. brasiliensis*, as described previously (16). Mice were reinfected 4–5 wk after the first infection for analysis of the memory response.

Flow cytometry

Single-cell suspensions were incubated with anti-CD16/CD32 blocking Ab (2.4G2) and stained with PE-labeled anti–c-Kit (2B8; Invitrogen Caltag, Carlsbad, CA), APC-labeled anti-CD49b (HMa2; Invitrogen Caltag), biotinylated anti-IgE (23G3; Southern Biotechnology Associates, Birmingham, AL), PE-labeled anti–Siglec-F (E50–2440; BD Pharmingen, San Diego, CA), PE-labeled anti–Siglec-F (E50–2440; BD Pharmingen), and purpলed anti-CD200R3 (kindly provided by H. Karasuyama, University of Tokyo). FITC–PE–PE-Cy5.5– and APC–labeled streptavidin (Invitrogen Caltag and Southern Biotechnology Associates) was used for detection of biotinylated Abs. PE-labeled goat anti-rat F(ab')2 IgG (H+L) (Invitrogen Caltag) was used for detection of purified rat Abs.

Semiquantitative RT-PCR analysis

RNA was extracted from total lung tissue from two mice per group and RT-PCR was performed, as described previously (16).

ELISA

IgE levels in the serum of naive and infected mice were determined with purified anti-IgE (R35–72; BD Pharmingen, San Diego, CA) for coating and biotinylated anti-IgE (R35–118; BD Pharmingen) for detection. Serum mouse mast cell protease (MMCP)-1 was measured by ELISA according to the manufacturer’s instructions (Moredun Scientific, Midlothian, U.K.).

CD4 T cell and basophil depletion during the memory response in c-Kit\(^{-/-}\) mice

Mice were infected with *N. brasiliensis* and then reinfected after 4–5 wk. Two days before the second infection, CD4 T cells were depleted by a single i.p. injection of 0.4 mg anti-CD4 mAb (GK1.5). Basophils were depleted by a single i.v. injection of 30 μg anti-CD200R3 mAb (Ba103) 2 d before the second infection or by injecting 5 μg anti-FcεRI mAb (MAR-1) twice daily beginning 2 d before the second infection.

Statistics

Statistics were calculated using the two-tailed unpaired Student t test. \(p\) Values \(<0.05\) were considered statistically significant.

Results

Mast cell–deficient c-Kit\(^{-/-}\) mice were used to determine the role of mast cells during primary and secondary infection of mice with the hookworm *N. brasiliensis*. Although adult c-Kit\(^{-/-}\) mice are mast cell deficient, mast cells can be detected in the skin 10 d after birth, suggesting that the “sash” mutation in the upstream c-Kit promoter is not absolutely detrimental for mast cell development (22). Therefore, we determined whether mast cells could be differentiated from bone marrow cells of c-Kit\(^{-/-}\) mice in vitro in the presence of IL-3, a cytokine that was shown to promote the development of mast cell precursors in the absence of the c-Kit ligand (23). Bone marrow cells from wild-type (WT) mice showed
progressive differentiation of c-Kit"FceRI" mast cells over time, whereas this population was completely absent in cultures from c-KitW-sh mice, although we observed a large population of c-Kit"FceRI" cells that might represent potential mast cell precursors (Fig. 1A). Importantly, the development of basophils (c-Kit"FceRI"IL-3Rα"CD49b"CD200R1"CD200R3") was not affected by the "sash" mutation (Fig. 1A, 1B). Because most helminths are strong inducers of mastocytosis in WT mice, we determined whether mast cells could develop in c-KitW-sh mice during infection with the hookworm N. brasiliensis. Analysis of peritoneal lavage and small intestine revealed the complete absence of mast cells in N. brasiliensis–infected c-KitW-sh mice (Fig. 1C) (16). Additionally, we determined the concentration of MMCP-1 in the serum, which is exclusively expressed by mast cells and serves as a marker for mast cell activation (24). As expected, WT mice showed a >60-fold increase in MMCP-1 postinfection with N. brasiliensis. However, we did not detect any MMCP-1 in the serum of naive c-KitW-sh mice and only a marginal increase above the detection limit postinfection with N. brasiliensis (Fig. 1D). In summary we found no evidence for the presence of mast cells in bone marrow–derived mast cell cultures or adult c-KitW-sh mice, even after infection with N. brasiliensis, whereas basophils appeared to develop normally.

Next, we wanted to analyze whether basophils developed normally in vivo. Basophils can be readily identified in the peripheral blood by double-staining for IgE and CD49b. Surprisingly, we could not detect CD49b"IgE" cells in young (5–6 wk old) c-KitW-sh mice, whereas this population was clearly present in WT mice (Fig. 2A, upper panels). However, the transfer of serum from N. brasiliensis–infected WT mice, which contains a high concentration of IgE, was
sufficient to permit the detection of CD49b+IgE+ cells in the peripheral blood of c-Kit\textsuperscript{W-sh} mice (Fig. 2A, lower panel). This indicates that reduced IgE levels, and not the absence of basophils or a defect of these cells to bind IgE via the high-affinity FceRI, was responsible for the observed phenotype. Basophils can also be identified by staining for CD200R3 and CD49b (Fig. 1B). Indeed, a comparable frequency of basophils (CD200R3\textsuperscript{+}CD49b\textsuperscript{+} cells) could be observed in blood and spleen of naive c-Kit\textsuperscript{W-sh} mice using this alternative staining protocol (Fig. 2B). To determine whether c-Kit\textsuperscript{W-sh} mice have a general defect in IgE production, we analyzed basophils in the peripheral blood postinfection with the hookworm \textit{N. brasiliensis}. In contrast to young naive c-Kit\textsuperscript{W-sh} mice, IgE\textsuperscript{+} basophils could be readily detected after \textit{N. brasiliensis} infection (Fig. 2C). The IgE production was substantial, because IgE\textsuperscript{+} basophils could still be detected weeks postinfection, although the IgE level diminished slightly with time (data not shown). All basophils appeared surface IgE\textsuperscript{+}, even long after primary infection; the IgE\textsuperscript{+}B220\textsuperscript{-} population was equivalent to the CD200R3\textsuperscript{+}CD49b\textsuperscript{+} population and vice versa (Fig. 2C). Next, we measured serum IgE levels of naive and \textit{N. brasiliensis}–infected c-Kit\textsuperscript{W-sh} and WT mice and compared them to age-matched WT mice. Indeed, basal levels of IgE were reduced \textasciitilde40-fold in naive c-Kit\textsuperscript{W-sh} mice compared with naive WT mice; although IgE levels increased in both strains postinfection with \textit{N. brasiliensis}, they remained significantly lower in c-Kit\textsuperscript{W-sh} mice (Fig. 2D). Therefore, we conclude that mast cells might be required to increase basal and Ag-induced serum IgE levels.

To further characterize the role of mast cells during the immune response against \textit{N. brasiliensis}, we compared effector cell mobilization, Th2 cell polarization, and worm expulsion of c-Kit\textsuperscript{W-sh} and WT mice. Eosinophilia and basophilia are hallmarks of helminth infections in mice and, therefore, can be used as indicators for an appropriate immune response. Surprisingly, the numbers of basophils and eosinophils were significantly increased in the spleen of \textit{N. brasiliensis}–infected c-Kit\textsuperscript{W-sh} mice compared with WT mice (Fig. 3A). We did not observe this accumulation in the peripheral blood or in the lung, which is one of the main sites of inflammation during infection with \textit{N. brasiliensis}. Importantly, the total number of CD4 T cells was not altered in spleen or lung after infection with \textit{N. brasiliensis}. These findings are consistent with the fact that mast cells are only rarely found in the lung parenchyma (25–27).

Mast cells produce IL-4 and IL-13, two cytokines that are critical for Th2 cell polarization and differentiation of AAMs. To address whether mast cells are involved in Th2 cell polarization in vivo, we analyzed cytokine production by T cells from draining lymph nodes.

**FIGURE 3.** Mast cell deficiency results in increased numbers of eosinophils and basophils in the spleen. A, Naive or \textit{N. brasiliensis}–infected c-Kit\textsuperscript{W-sh} mice (open bars) and age-matched WT mice (shaded bars) were analyzed for effector cell accumulation 10 d postinfection. The upper row shows the percentage of basophils, eosinophils, and CD4 T cells among total peripheral blood leukocytes (PBL). The middle and bottom rows show the total number of these effector cells in the lung and spleen. Basophils were identified as IgE\textsuperscript{+}B220\textsuperscript{-} cells, and eosinophils were identified as Siglec-F\textsuperscript{-}SSChi cells. Plots show pooled results from two independent experiments with a total of 3–7 mice. \( *p < 0.0005; **p < 0.05 \) by the two-tailed unpaired Student \( t \) test. ns, not significant. B, IL-4, IL-5, and IL-13 concentrations in the supernatant of restimulated T cells from mediastinal lymph nodes of infected c-Kit\textsuperscript{W-sh} mice (open bars) or WT mice (shaded bars). The experiment was repeated with similar results. C, Semiquantitive RT-PCR analysis of total lung tissue for markers of AAMs from c-Kit\textsuperscript{W-sh} and WT mice that had been infected 10 d before analysis with \textit{N. brasiliensis}.
of *N. brasiliensis*-infected c-Kit<sup>W-sh</sup> mice. Similar amounts of IL-4, IL-5, and IL-13 were produced from restimulated T cell cultures of WT and c-Kit<sup>W-sh</sup> mice, which suggests that mast cells did not contribute to Th2 cell polarization in this infection model (Fig. 3B). AAMs were recently described to promote immunity against *H. polygyrus* infection (5). Therefore, we analyzed whether mast cells may contribute to the differentiation of AAMs by release of IL-4 or IL-13. We used RT-PCR to determine the mRNA levels of different markers for AAMs in total lung tissue 10 d after infection with *N. brasiliensis*. We found no differences in the expression levels of these markers in c-Kit<sup>W-sh</sup> mice compared with WT mice (Fig. 3C). These results suggest that mast cells (or another c-Kit–dependent pathway) are not required to recruit effector cells to the lung, whereas they seem to limit innate effector cell accumulation in the spleen during infection with *N. brasiliensis*.

Next, we determined whether c-Kit<sup>W-sh</sup> mice could expel *N. brasiliensis* within the same time frame as WT mice. WT mice expelled all worms by day 10, whereas c-Kit<sup>W-sh</sup> mice still contained many worms at that time point. However, worms were readily expelled by day 14 in c-Kit<sup>W-sh</sup> mice (Fig. 4A). Therefore, worm expulsion during primary *N. brasiliensis* infection was delayed but not completely impaired in the absence of mast cells. To determine whether sensitization of mice with serum from *N. brasiliensis*–immune mice would be sufficient to overcome the defect of worm expulsion during primary infection of c-Kit<sup>W-sh</sup> mice, we transferred serum from *N. brasiliensis*-infected WT mice (as a source for *N. brasiliensis*–specific IgE and IgG1) or IL-4/IL-13-deficient mice (as control) into c-Kit<sup>W-sh</sup> mice. Additionally, one group of mice was depleted of basophils. All mice were unable to expel worms by day 10 after infection, which demonstrates that the defect of worm expulsion could not be compensated for by sensitization with serum from previously infected WT mice.

Because the effector mechanisms that operate during secondary infection with the same hookworm might be different compared with a primary infection, we reinfected c-Kit<sup>W-sh</sup> mice 4–5 wk after primary infection and analyzed worm clearance at day 5 postinfection, when worms are usually expelled in WT mice. In addition, we depleted CD4 T cells, basophils, or both 2 d before the secondary infection to uncover their role during a memory response (Fig. 5A, 5B). Nondepleted and CD4 T cell–depleted c-Kit<sup>W-sh</sup> mice showed no defect in worm expulsion, indicating that neither T cells nor mast cells are required for worm clearance during a secondary infection (Fig. 5C). Basophils can be transiently depleted by injecting anti-FcεRI (11) or anti-CD200R3 (28) mAbs. Worm expulsion was severely impaired when basophils were depleted by both basophil-depletion methods (Fig. 5C; *p* < 0.001 by the Student *t* test for control versus basophil-depleted groups). Furthermore, depletion of basophils from *N. brasiliensis*–immune WT mice resulted in impaired worm expulsion (Fig. 5C). This result demonstrates that mast cells that are present in these mice cannot compensate for the lack of basophils, although we

![FIGURE 4. Mast cell deficiency results in delayed primary worm expulsion.](Image)

**FIGURE 4.** Mast cell deficiency results in delayed primary worm expulsion. *A*, c-Kit<sup>W-sh</sup> and age-matched WT mice were infected with *N. brasiliensis*, and adult worms were counted in the small intestine at the indicated time points postinfection. The histograms show pooled results from two independent experiments. *B*, c-Kit<sup>W-sh</sup> mice were reconstituted with serum from *N. brasiliensis*-infected WT or IL-4/IL-13-deficient mice; one group was depleted of basophils by injection of anti-FcεRI Ab. The mice were then infected with *N. brasiliensis* and worm counts were determined on day 9 postinfection.

![FIGURE 5. Basophils, but neither mast cells nor CD4 T cells, are critical for worm expulsion during secondary infection.](Image)

**FIGURE 5.** Basophils, but neither mast cells nor CD4 T cells, are critical for worm expulsion during secondary infection. Mast cell–deficient c-Kit<sup>W-sh</sup> mice or WT mice were infected with *N. brasiliensis* and then reinfected after 4–5 wk. Two days before the second infection, mice were left untreated, depleted of CD4 T cells by anti-CD4 mAb injection, and/or depleted of basophils by anti-CD200R3 or anti-FcεRI mAb injection. Efficiency of depletion of CD4 T cells (*A*) and basophils (*B*) in the blood on day 5 after secondary infection. *C*, Number of adult worms in the small intestine on day 5 after secondary infection. The histograms show pooled results for c-Kit<sup>W-sh</sup> mice from three independent experiments with a total of 3–7 mice per group. The results for WT mice are from one experiment with three nondepleted and four basophil-depleted mice. *p* < 0.001 for all basophil-depleted groups versus nondepleted control mice by the Student *t* test.
cannot exclude that anti-FcεRI treatment interferes with mast cell function of WT mice.

We recently showed that sensitized basophils can release large quantities of IL-4 and IL-13 after encountering secreted substances of *N. brasiliensis* (16). Both cytokines signal via IL-4Rα and Stat6 in nonhematopoietic cells to mediate the primary expulsion of *N. brasiliensis* (26, 29). The requirement of Stat6 for protective immunity during repeated infection has not been described. Therefore, we sought to determine worm expulsion in Stat6-deficient mice that had been infected three times with *N. brasiliensis*. Stat6-deficient mice contained 60–100 worms on day 8 after the third infection, which clearly indicates that protective immunity during repeated infections requires Stat6 (Fig. 6).

**Discussion**

The role of basophils in the protection against helminth infections remains poorly defined. In this study, we demonstrated that basophils are essential for protection against secondary *N. brasiliensis* infection in the absence of mast cells and CD4 T cells, which are both required for efficient worm expulsion during primary infection (8, 30). Recently, we showed that basophils contribute to eosinophilia and worm expulsion during primary infection with *N. brasiliensis* in the absence of Th2 cells (16). However, basophils had to be sensitized in this study with serum from *N. brasiliensis*-infected WT mice to execute their effector functions. Therefore, the arming of basophils through the production of affinity-matured IgE seems central for effective clearance of *N. brasiliensis*. We do not know where and how basophils mediate their protective effect. One possibility is that they rapidly mobilize eosinophils or AAMs, which can kill migrating larvae (5, 7, 9). This scenario means that *N. brasiliensis* larvae are killed in or even before they reach the lung so that they never develop to an adult stage. Alternatively, IL-4 and IL-13 released from activated basophils could induce activation of goblet cells and smooth muscle cells in the small intestine, causing expulsion by a “weep and sweep” mechanism (6). Basophils can rapidly release large amounts of IL-4 during a memory type 2 immune response (31). Because expulsion of *N. brasiliensis* is absolutely dependent on IL-4 or IL-13, it seems likely that basophil-derived IL-4/IL-13 is indeed critical for the rapid elimination of these parasites upon secondary infection. How can we explain that CD4 T cells are essential for worm expulsion during primary infection, whereas long-term protection seems to be provided mainly by basophils, which have a lifespan of only 60 h (16)? Primary infection results in the differentiation of Th2 cells, which induce *N. brasiliensis*-specific IgG1- and IgE-producing plasma cells. Basophils have been shown to express MHC class II molecules and could promote Th2 differentiation in allergy models and a parasite infection model (13–15). Therefore, basophils might prime naïve T cells to become Th2 cells, which then induce class-switch recombination of Ag-specific Igs in B cells. We observed lower serum IgE levels in mast cell–deficient mice, although it remains unclear at present whether a direct interaction between mast cells and B cells is required to maintain normal IgE levels. Despite this phenomenon, mast cells were not required for Th2 cell polarization or AAM differentiation after *N. brasiliensis* infection.

Recently, McCoy et al. (32) demonstrated that polyclonal IgG Abs produced after primary infection with *H. polygyrus* functioned to limit egg production. Comparatively, repetitive infection with this parasite led to the production of affinity-matured *H. polygyrus*-specific IgGs and IgAs, which were essential to prevent adult worm development, even in the absence of CD4 T cells (32). In addition, B cells were shown to be important for the expansion of primary and memory Th2 cells during *H. polygyrus* infection (33). Long-lived plasma cells could be a constant source of Abs so that basophils remain sensitized. This assumption is supported by the observation that sensitized basophils can be isolated from people with a history of filarial infections without recent reencounter of filarial Ags (34). Indeed, long-lived IgG-secreting plasma cells have been identified in the bone marrow where they may constantly produce low amounts of Abs (35, 36). The transfer of serum from repeatedly *N. brasiliensis*-infected rats was shown to protect naïve rats against this helminth (37). Similarly, purified IgE from *Trichinella spiralis*-infected rats was able to provide protection (38). Interestingly, basophils were recently shown to contribute to humoral memory responses, which allows protection from certain bacterial infections (11). Therefore, basophils seem not only to be important as effector cells for providing protective immunity against certain helminth infections, but also as increasing humoral memory responses, which, in turn, leads to the arming of basophils themselves with high-affinity class-switched Abs. Although memory Th2 cells seem to be a critical component for protection against some helminths, efficient vaccination strategies should also focus on the generation of long-lived plasma cells.

The results obtained in this study with a murine hookworm infection model indicate for the first time that basophils may provide important effector functions against repeated infections with hookworms. These results are of particular interest when taking into account the fact that most people suffering from such infections live in endemic regions where repeated infections with hookworms is not unusual. It is hoped that further studies using other helminth infection models and careful analysis of helminth-infected patients will lead to a better understanding of how basophils may execute their beneficial functions in vivo.

**Acknowledgments**

We thank H. Karasuyama (University of Tokyo) for providing the anti-CD200R3 mAb, A. Pullner for excellent technical assistance, and J. Johnson, R. Obst, and T. Brocker for helpful comments on the manuscript.

**Disclosures**

The authors have no financial conflict of interest.

**References**