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Neutrophils are obligate cells entering lymph nodes shortly after immunization with protein antigens in adjuvants, starting during the first hour and continuing for several days in two distinct waves. Previously, we demonstrated the strong suppressive effects of neutrophils on CD4 T cell and B cell responses, using either neutrophil-depleting antibodies or genetically neutropenic mice. In this study, we find that neutrophils are the major cells controlling the spread of T cell responses to distal lymph nodes. Although in the presence of neutrophils, ~75% of the response was restricted to the draining node, in their absence, most of the response was found in distal nodes. Prostanoids were responsible for the rapid entry of neutrophils into the draining nodes, as well as for the two distinct neutrophil effects: the modulation of the magnitude of the cellular response, and in its spread outside the draining nodes. Neutrophil-produced thromboxane A_2 was the key eicosanoid controlling both effects. Adoptive transfer of neutrophils into mice genetically deficient in neutrophils indicated their role in both. These functions of neutrophils are important in infections and vaccinations with adjuvants where neutrophils are abundant in the initial stages.

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Abbreviations used: HEL, hen egg white lysozyme; INDO, indomethacin; PICO, picotamide; PMA, phorbol myristic acid; qRT-PCR, quantitative RT-PCR.

Neutrophils constitute the first line of defense against pathogens, including extracellular and intracellular bacteria, viruses, fungi, and parasites (Rogers and Unanue, 1993; Pedrosa et al., 2000; Appelberg, 2007; Saitoh et al., 2012). They control infections by killing or inhibiting the growth of the invading microorganisms through their generation of reactive oxygen species and antimicrobial components (Nathan, 2006). For decades, neutrophils were thought to function only in innate immune responses because they are short-lived cells responding to inflammatory stimuli. Emerging evidence suggests that neutrophils also have effects on adaptive immunity, but many of these studies have been done ex vivo, and their relevance to in vivo reactions need to be appraised (van Gisbergen et al., 2005; Zhang et al., 2009; Mantovani et al., 2011; Pillay et al., 2012).

Neutrophils migrate to the lymph nodes after infections or vaccination (Abadie et al., 2005; Maletto et al., 2006; Chtanova et al., 2008; Yang et al., 2010). Little is known about the in vivo function of these cells in the lymph nodes. Previously, we showed the very fast entrance of neutrophils into lymph nodes draining the site of adjuvant injection, and their inhibitory effects on both CD4 and B cell responses (Yang et al., 2010).

Here, we examined the role of neutrophils in modulating the extent and spread of T cell responses after protein immunizations. The phenomenon of "lymph node cell shutdown" was reported years ago: in steady state, the rate of lymph flow and number of cell output in the efferent lymph was constant, but within hours of antigen injection a transitory decrease of lymphocyte exiting the efferent lymph was observed (Hall and Morris, 1962, 1965; Cahill et al., 1976; Hopkins et al., 1981). Prostaglandins were thought to be the critical component in this process (Johnston et al., 1979, 1980; Hopkins et al., 1981), but the cell types responsible for their production was never established. Our findings establish that neutrophils are obligate cells that enter nodes after adjuvant injections, and have a profound effect, both in the extent of the T cells response and in controlling the release of lymphocytes. Thromboxane is the key metabolite responsible for both processes.

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RESULTS

Two waves of neutrophil influx into the draining lymph nodes after immunization

Our previous study showed an early influx of neutrophils into the draining lymph nodes after immunization with proteins in any of three adjuvants (complete or incomplete Freund's adjuvant or alum; Yang et al., 2010). In wave 1, neutrophils appeared in the popliteal nodes by 15 min, reaching peak number within 2 h after immunization. By 24 h, their numbers were barely over the numbers found in unimmunized lymph nodes (Yang et al., 2010). The majority of wave 1 neutrophils accumulated in the cortical sinus and superficial cortex in the popliteal nodes, indicating that they entered via lymphatics (Yang et al., 2010). By histology, neutrophils appeared in the dermis of the footpads within 10 min after immunization, suggesting a pathway from blood to the site of injection, to the lymphatics,

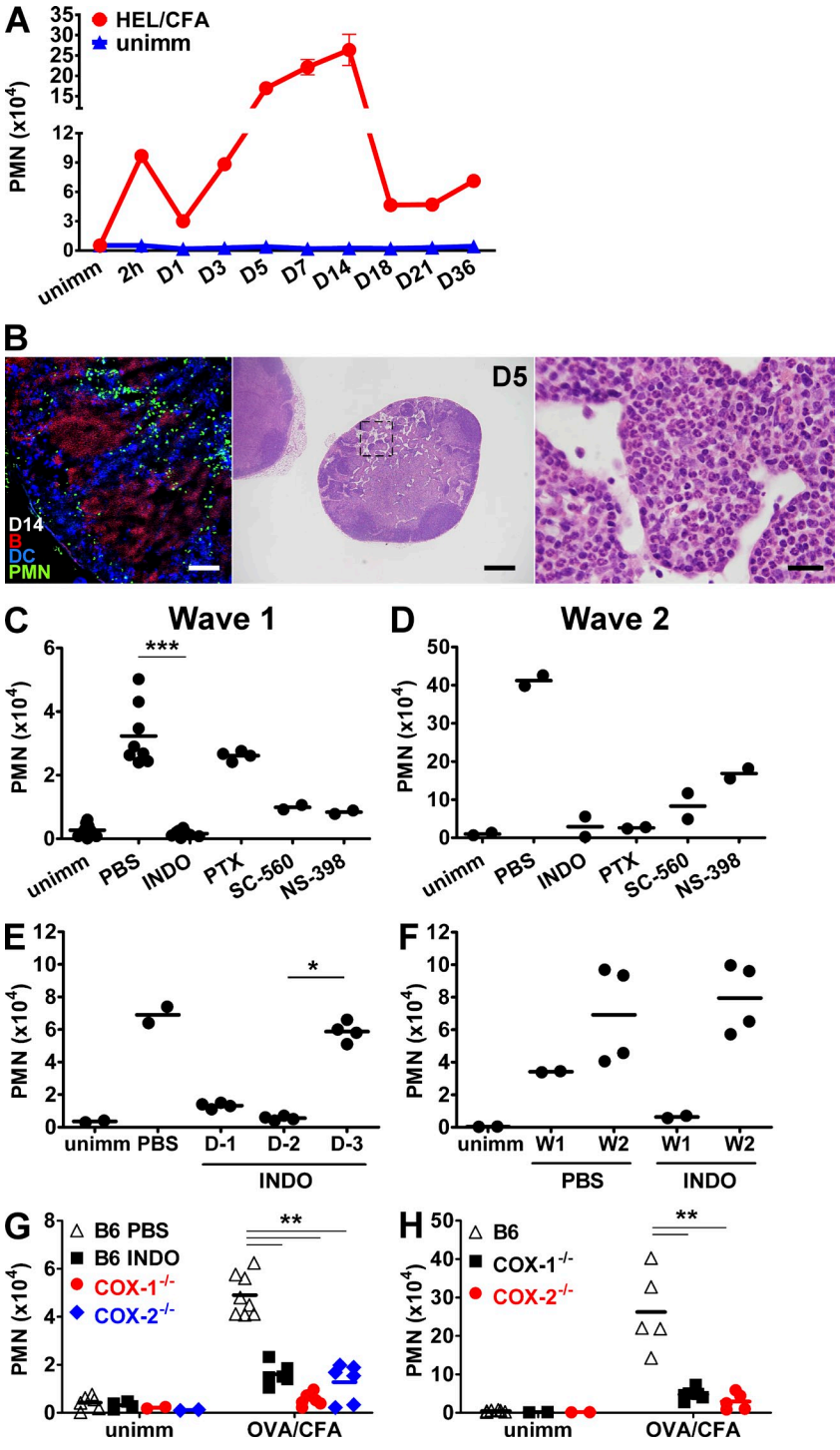

Figure 1. Entry of two waves of neutrophils in the lymph nodes after immunization. (A) B10.BR mice were immunized with 10 nmol HEL/CFA. The numbers of neutrophils in the popliteal nodes at the indicated time after immunization were analyzed by FACS of $CD11b^+$ $Ly\text{-}6G^+$ cells and (B) immunofluorescence and H&E staining. Original magnification in B, ×40 (left); ×4 (middle); ×100 (right). Bars: 200 µm (left); 20 µm (middle); 500 µm (right). (C and D) B10.BR mice were i.p. injected with PBS, 40 µg/mouse indomethacin (INDO), or 500 ng/mouse of PTX 1 d before immunization (C); or 6 d after immunization (D). The number of neutrophils in the popliteal nodes was analyzed by FACS of $CD11b^+$ $Ly\text{-}6G^+$ cells at 2 h (C) or 7 d (D) after immunization. (E) B10.BR mice were i.p. injected with indomethacin at 1, 2, or 3 d before immunization. PBS was given 1 d before immunization. The number of neutrophils in the popliteal lymph nodes was analyzed after 2 h. (F) B10.BR mice were given PBS or indomethacin 1 d before immunization. The entry of wave 1 or wave 2 neutrophils in the popliteal nodes was analyzed by FACS of $CD11b^+$ $Ly\text{-}6G^+$ cells at 2 h (W1) or 6 d (W2) after immunization. (G and H) C57BL/6 wild-type, COX-$1^{-/-}$, or COX-$2^{-/-}$ mice were immunized with 10 nmol OVA/CFA and the number of neutrophils in the popliteal nodes was analyzed by FACS at 2 h (G) or 7 d (H) after immunization. (A and B) Shown are representative data from 3 individual experiments with $n = 2$ in each group; (C–H) pooled results from 2–4 experiments, in which each dot presents data from individual mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

and into the draining lymph nodes. We now find a second wave of neutrophils entering popliteal nodes starting at 3 d after immunization. Different from the fast kinetics of wave 1, the second entry of neutrophils persisted until about 18 d after immunization (Fig. 1 A). In contrast to wave 1, wave 2 neutrophils localized in the cortex and medullary area, suggesting that they entered the lymph nodes via blood (Fig. 1 B).

Neutrophil chemoattractant factors include chemokines, cytokines, complement-derived peptides, and lipid mediators such as leukotriene B4 (Rogers et al., 1994; Forlow et al., 2001; Semerad et al., 2002; Kim et al., 2006; Chou et al., 2010). The early wave 1 neutrophil influx was completely suppressed by injecting the mice with indomethacin before immunization (Fig. 1 C), indicating a requirement for prostanoids in neutrophil recruitment. This entry was resistant to treatment with pertussis toxin (PTX) used to block chemokine receptor signaling (Cyster and Goodnow, 1995; Fig. 1 C). Tested at the seventh day after immunization, the influx of wave 2 neutrophils was sensitive to both indomethacin and PTX (Fig. 1 D). The two waves of neutrophils were independent of each other. The effects of a single intraperitoneal injection of indomethacin lasted for 2 d in vivo and had no effect on the wave 2 entry (Fig. 1, E and F).

COX-$1^{-/-}$ and COX-$2^{-/-}$ mice were examined to determine the role of cyclooxygenase in neutrophil recruitment because they show severe reduction of prostanoids under basal level or after inflammatory stimuli (Langenbach et al., 1995; Morham et al., 1995). Both COX-$1^{-/-}$ and COX-$2^{-/-}$ mice had significantly diminished wave 1 or wave 2 neutrophil entries in the popliteal nodes after immunization (Fig. 1, G and H).

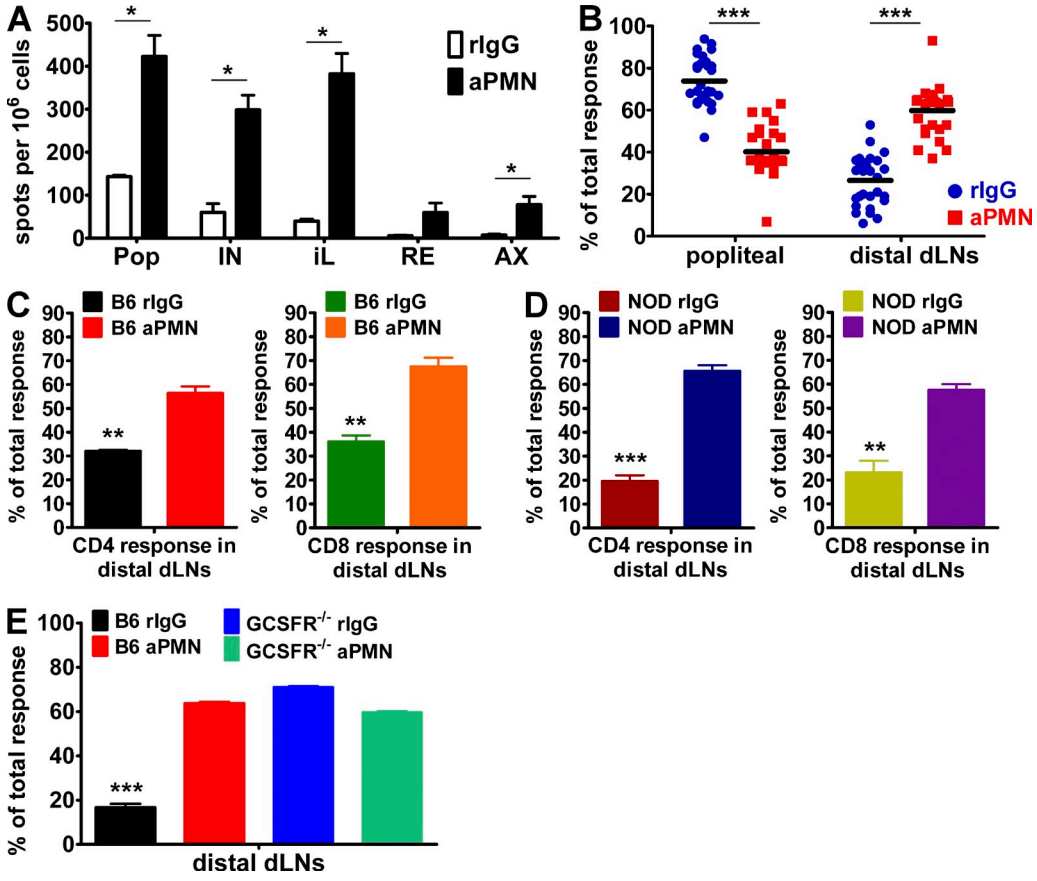

Figure 2. Spread of T cell responses to distal draining lymph nodes in the absence of neutrophils. (A) B10.BR mice were i.p. injected with either neutrophil-depleting antibodies (1A8 aPMN; 1 mg/mouse) or control Ig 1 d before immunization with 10 nmol HEL/CFA. T cell recall responses in individual draining lymph nodes were measured by IL-2 and IFN-γ ELISPOT at day 7 after immunization. Shown are data from 3 individual experiments with $n = 2$ in each group. (B) Pooled results from 10 individual experiments. T cell recall responses by ELISPOT at 7 d after immunization were shown as percentage of total responses analyzed by the number of cytokine-producing cells in popliteal nodes or distal draining lymph nodes, including iliac, inguinal, renal, and axillary nodes. (C and D) CD4 or CD8 T cell recall responses in OVA/CFA immunization in C57BL/6 mice (C); or HEL/CFA immunization in NOD mice (D) were measured at day 7 after immunization by ELISPOT using CD4 or CD8 recall epitopes. (E) C57BL/6 wild-type or G-$CSFR^{-/-}$ mice were i.p. injected with 1A8 Ab or control Ig 1 d before immunization with 10 nmol HEL/CFA. T cell recall response at day 7 was measured by IL-2 ELISPOT shown as the percentage of total responses in distal draining lymph nodes. For statistical analysis in (E), the group of wild-type mice with control Ig was compared with other groups by Mann-Whitney *t* test. Shown are data from 3 (C and D) and 4 (E) individual experiments with $n > 2$ in each group. (A–E) Data were presented as mean ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Pop, popliteal; iL, iliac; IN, inguinal; RE, renal; AX, axillary lymph nodes.

These results were also confirmed by testing COX-1–selective (SC-560) or COX-2–selective (NS-398) chemical inhibitors (Fig. 1, C and D; Dannhardt and Ulbrich, 2001; Loftin et al., 2002). For SC-560, the IC_{50} value of COX-1 is 9 nM and the IC_{50} value of COX-2 is 630 nM. For NS-398, the IC_{50} value of COX-1 is 10^5 nM and the IC_{50} value of COX-2 is 30 nM.

Neutrophils control the spread of T cell responses to distal draining lymph nodes

We had previously examined the immune responses of popliteal nodes that drain the lymph from the footpad immunization (Yang et al., 2010). We now evaluated the magnitude and the spread of the immune responses to distal nodes, which included the inguinal, iliac, renal, and axillary nodes (Harrell et al., 2008). T cell recall responses were measured by IL-2 or IFN-γ ELISPOT at 7 d after immunization with hen egg white lysozyme (HEL) in Freund's adjuvant. Consistent with our previous findings, neutrophil depletion resulted in a consistent 3–4 fold increase in CD4 T cell responses in popliteal nodes. Strikingly, the enhanced responses were even more apparent in all the distal draining nodes: a 3-fold increase in popliteal nodes, a 5-fold increase in inguinal nodes; a 10-fold increase in iliac nodes; an 11-fold increase in renal nodes; and an 11-fold increase in axillary nodes. As a percentage of the total response (i.e., that found in popliteal nodes and the distal nodes) in regular mice, ∼75% of the response was found in the draining popliteal nodes, whereas only 30% was found in the neutrophil-depleted mice (Fig. 2 B). Immunization with ovalbumin or HEL/CFA in B6 or NOD mice, respectively, gave similar results (Fig. 2 B, pooled results), as did testing the CD8 response to an HEL epitope from HEL immunization in NOD mice (Belizaire and Unanue, 2009; Fig. 2, C and D).

The increase in magnitude of the total response, and the spread to the distal nodes were also found in immunized G-CSFR$^{-/-}$ mice, which have very low levels of circulating neutrophils (Liu et al., 1996). Approximately 20% of total T cell responses were found in the draining popliteal node (Fig. 2 E). It is important to note as a control that antibody depletion in G-CSFR$^{-/-}$ mice did not result in enhanced spread of the responses to distal draining nodes, which is an indication of the lack of nonspecific effects of the antibody. In brief, neutrophils influenced not only the magnitude of the lymphocyte response, but also the extent to which it was confined to the local node.

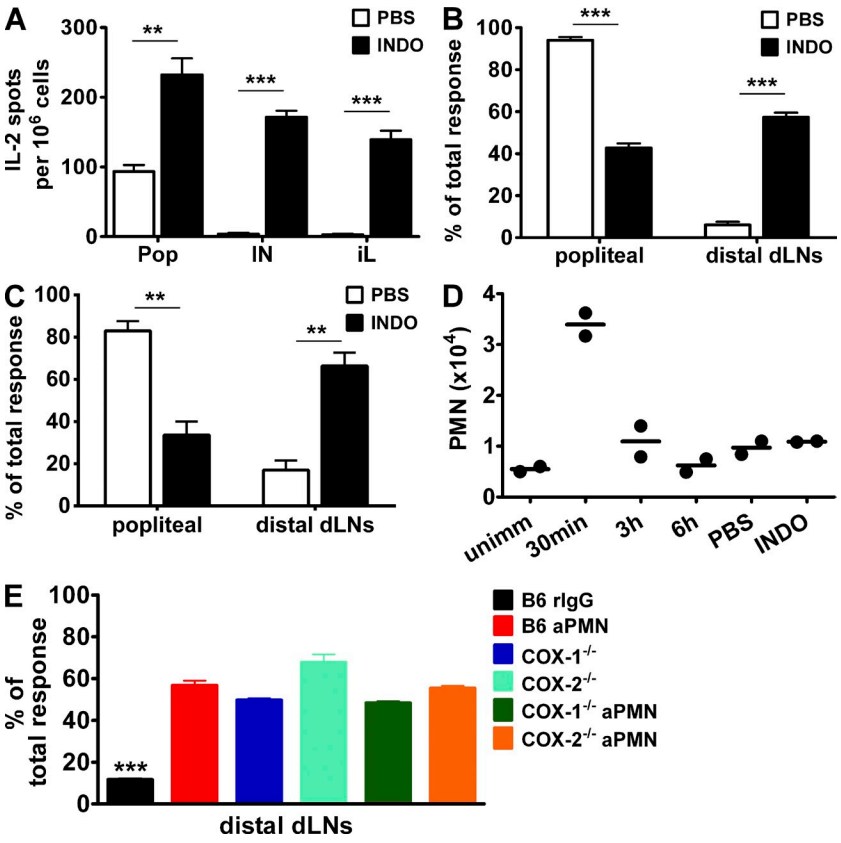

Figure 3. Prostanoids mediate the neutrophil effect on the magnitude and spread of T cell responses. (A and B) B10.BR mice were i.p. injected with PBS or indomethacin (INDO, 40 µg/mouse) 1 d before immunization with 10 nmol HEL/CFA. T cell recall responses were measured by IL-2 ELISPOT at day 7 after immunization. (A) The number of IL-2 spots in individual draining lymph nodes. (B) Percentage of total responses in popliteal nodes or distal draining nodes was calculated by the number of IL-2–producing cells. (C) B10.BR mice were i.p. injected with indomethacin at 3 h after 10 nmol HEL/CFA immunization. T cell recall responses in popliteal nodes or distal draining nodes were measured by IL-2 ELISPOT at day 7 after immunization. (A–C) Shown are data from three individual experiments. (D) B10.BR mice were immunized with 10 nmol HEL/CFA. The number of neutrophils in popliteal nodes was analyzed at 30 min, 3 h, or 6 h after immunization by FACS of CD11b$^+$ Ly-6G$^+$ cells. PBS or indomethacin was given by i.p. injections at 3 h after immunization, and the number of neutrophils was analyzed at 6 h after immunization. Each dot represents a single mouse from one representative experiment. (E) C57BL/6, COX-1$^{-/-}$, or COX-2$^{-/-}$ mice were i.p. injected with 1A8 Ab or control Ig 1 d before 10 nmol OVA/CFA immunization. T cell recall responses in the popliteal nodes or distal draining lymph nodes were measured by IL-2 and IFN-γ ELISPOT at day 7 after immunization. Shown is the percentage of total responses in distal draining lymph nodes analyzed as described. For statistical analysis in E, the group of C57BL/6 mice with control Ig was compared with other groups. (E) Data from 4 individual experiments with $n = 3$ in each group. (A–E) Data were presented as mean ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by Mann-Whitney t test.

Different manipulations evaluated prostanoids as possible mediators of the aforementioned neutrophil actions. First, reducing the wave 1 neutrophil entry by administration of indomethacin before immunization increased both the magnitude of T cell response and its spread to the distal draining nodes (Fig. 3, A and B). Second, injecting indomethacin at 3 h after immunization did not interfere with the wave 1 neutrophil entry, yet resulted in the spread of T cell responses to distal draining nodes (Fig. 3, C and D). Third, both COX-$1^{-/-}$ and COX-$2^{-/-}$ mice showed effects similar to neutrophil depletion in wild-type mice: 50–60% of the total responses in distal draining nodes were comparable to the minimal spread of T cell responses in wild-type mice (~10%). Neutrophil depletion in COX-$1^{-/-}$ and COX-$2^{-/-}$ mice did not enhance the effect (Fig. 3 E).

Prostanoid production from neutrophils

The neutrophil transcriptional activity of *Ptgs-1* and *Ptgs-2* genes encoding COX-1 or COX-2, respectively, was examined by quantitative PCR (qRT-PCR). Bone marrow isolates were practically 100% pure neutrophils (Fig. 4 A). *Ptgs-1*

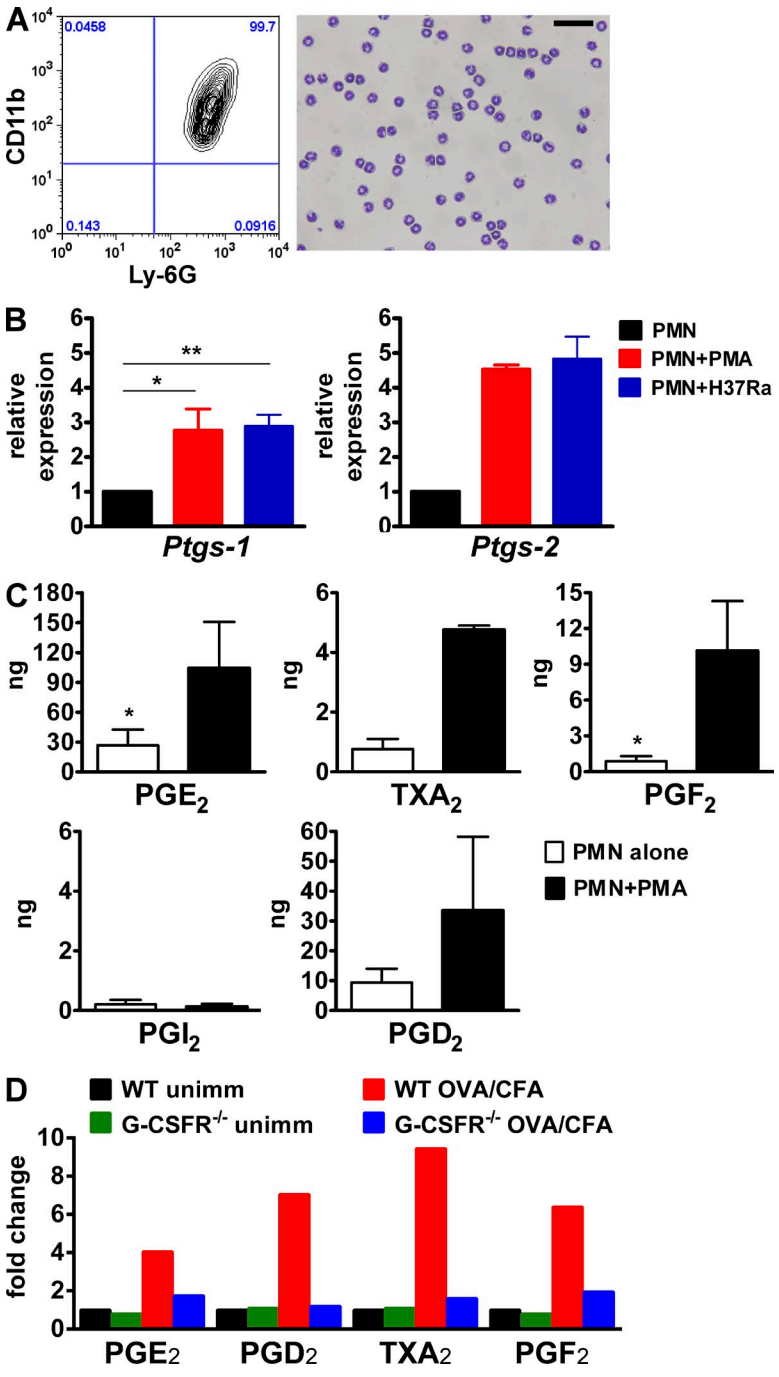

Figure 4. Prostanoid production from neutrophils. Bone marrow neutrophils from B10.BR mice were isolated by Percoll gradient followed with anti–Ly-6G microbeads. (A) Purity of neutrophils was analyzed by FACS of $CD11b^+$ $Ly\text{-}6G^+$ cells or Hema 3 stain for the nuclear morphology. Bar, 50 µm. Shown is one representative experiment. (B) Neutrophils (3×10^5) were stimulated with either PMA (10 µM) for 30 min, or 10mg/ml *M. tuberculosis* H37Ra for 2 h at room temperature. Total RNA was isolated for qRT-PCR analysis of *Ptgs-1* (left) or *Ptgs-2* (right) gene expression. Gene expression was shown relative to neutrophils in PBS alone. Shown are data from 3~5 individual experiments with $n > 2$ each group. (C) Neutrophils (2×10^6) were cultured in PBS with the stimulation by 100 µM of PMA for 2 h at room temperature. In the control group, neutrophils were cultured with PBS alone. Exogenous arachidonic acid (40 µM) was added into the culture as the substrates. After 2 h of incubation, the reactions were stopped by methanol and the supernatants were collected for mass spectrometry analysis. Shown are pool results from three individual experiments. (B and C) Data were shown as mean± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by Mann-Whitney *t* test. (D) Popliteal nodes were harvested from C57BL/6 or G-$CSFR^{-/-}$ mice at 2 h after immunization with 10 nmol OVA/CFA and examined for prostanoid production by mass spectrometry. Shown are fold changes relative to unimmunized C57BL/6 mice. Shown is of one single experiment with $n = 3$ in each group. The level of PGI_2 was undetectable from the samples.

and *Ptgs-2* expression were up-regulated by approximately threefold in phorbol myristic acid (PMA)–stimulated neutrophils, or after their uptake of dead *Mycobacterium tuberculosis*, a component in CFA (Fig. 4 B). Our data confirms the microarray studies in which neutrophils highly increased their transcriptional activity of *Ptgs-1* and *Ptgs-2* in the presence of inflammatory stimuli (Immunological Genome Project Database). Direct examination of prostanoid metabolites by mass spectrometry showed high level of PGE_2, PGD_2, $PGF_{2\alpha}$, and thromboxanes from cultured neutrophils compared with unstimulated neutrophils. The level of PGI_2 remained low after stimulation (Fig. 4 C). We also measured the level of prostanoid metabolites from whole lymph node 2 h after immunization. The levels of PGE_2, PGD_2, $PGF_{2\alpha}$, and thromboxanes was markedly increased in immunized mice but not in G-CSFR$^{-/-}$ mice, which had amounts comparable to unimmunized mice (Fig. 4 D). Thus, neutrophils are a major cellular source of prostanoids in the draining lymph nodes after immunization.

Prostanoids could also be generated by other cells in the node interacting with intranodal neutrophils. For example, uptake of dead neutrophils by macrophages resulted in prostaglandin release (Fadok et al., 1998). We examined the direct contribution of neutrophils by transferring them to G-CSFR$^{-/-}$ mice. Neutrophils injected into the footpad entered the draining nodes even after their in vitro treatment with indomethacin (Fig. 5 A). The magnitude of the T cell response and the spread was markedly decreased after the transfer of neutrophils (Fig. 5 B, 5C). In contrast, indomethacin-treated neutrophils or neutrophils from prostanoid-deficient, COX-1$^{-/-}$, or COX-2$^{-/-}$ mice resulted in the spread of T cell responses to the distal draining nodes (~70% and ~75%, respectively) compared with the findings with wild-type neutrophils (Fig. 5 C). We concluded that neutrophil-derived prostanoids accounted for the effects on the T cell responses.

Thromboxane in the neutrophil-mediated effects

We examined the role of thromboxanes in the two effects mediated by neutrophils. Thromboxanes are potent inducers of vessel contraction, and are also involved in negatively modulating the T cell proliferative responses and in the spread of bacterial infections (Tripp et al., 1987; Kabashima et al., 2003). Injection of picotamide, which inhibits thromboxane synthase and also acts as a receptor antagonist, did not interfere with neutrophil entry in the popliteal nodes compared with indomethacin treatment, indicating that neutrophil entry was induced by prostanoids other than thromboxanes (Fig. 6 A). However, picotamide treatment before or after immunization resulted in an enhancement of the T cell responses in the popliteal nodes as well as the spread into distal draining lymph nodes, effects similar to those resulting from neutrophil depletion or indomethacin treatment (Fig. 6, C–F). In addition, administration of the thromboxane receptor agonist U-46619 to G-CSFR$^{-/-}$ mice reduced the T cell responses and restricted the spread of the T cell responses (Fig. 6, G and H). Transfer

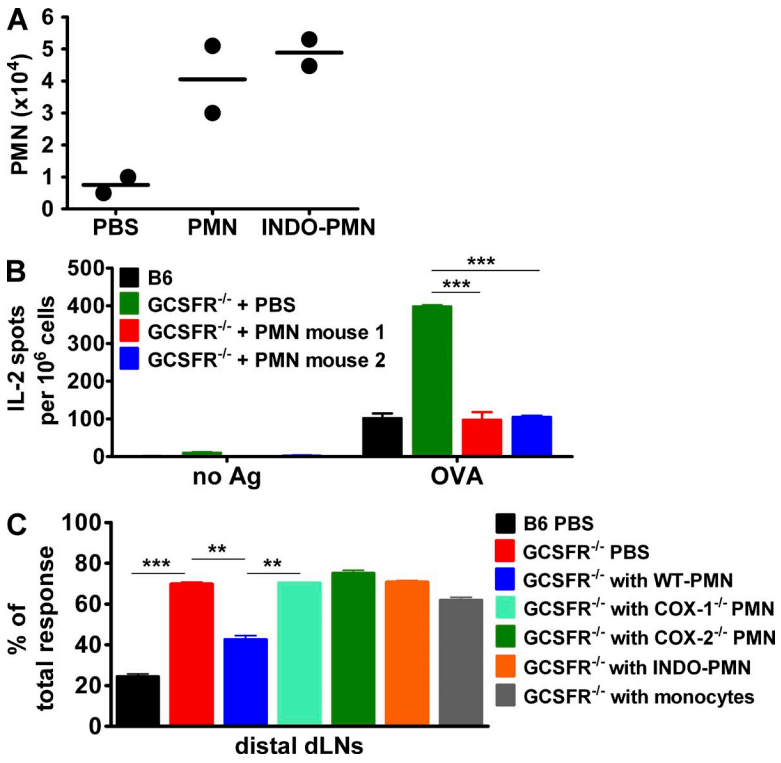

Figure 5. Prostanoids from neutrophils restricted the spread of T cell responses to distal draining lymph nodes. (A) Bone marrow neutrophils (2×10^6) were adoptively transferred into G-CSFR$^{-/-}$ recipients by footpad injection 20 min before immunization with 10 nmol OVA/CFA. In the group of INDO-PMN, neutrophils were preincubated with 0.2 mg/ml indomethacin for 15 min at 37°C before adoptive transfer. Popliteal nodes were harvested at 2 h after immunization. The number of transferred neutrophils was analyzed by FACS of CD11b$^+$ Ly-6G$^+$ cells. Each dot represents one single mouse of a representative experiment. (B) G-CSFR$^{-/-}$ mice were transferred with wild-type bone marrow neutrophils or PBS and immunized with 10 nmol OVA/CFA. Immunized C57BL/6 wild-type mice were used as control group. T cell recall responses in popliteal nodes were measured by IL-2 ELISPOT at day 7 after immunization. Shown is data from 2 individual experiments with $n = 2$ in each group. (C) Bone marrow neutrophils (2×10^6) from C57BL/6 wild-type, COX-1$^{-/-}$ or COX-2$^{-/-}$ mice; bone marrow monocytes from wild-type mice, or in vitro indomethacin-treated neutrophils were transferred into G-CSFR$^{-/-}$ recipients immunized with 10 nmol OVA/CFA. PBS injected wild-type or G-CSFR$^{-/-}$ mice were used as a control group. The percentage of total T cell recall responses in the distal draining nodes was analyzed by ELISPOT at day 7 after immunization. Shown are data from 5 individual experiments with $n = 2$ in each group. (B and C) Data were presented as mean ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by Mann-Whitney *t* test.

of neutrophils pretreated with picotamide in vitro resulted in the spread of the response similar to neutrophil depletion (Fig. 6 B). In sum, pharmacological manipulations point to thromboxanes as the key prostanoid metabolites controlling the magnitude and spread of the T cell responses.

Migration in the absence of neutrophils

We examined the drainage of dye and the trafficking of antigen-specific or nonspecific T cells to the distal nodes in the absence of neutrophils. By injecting Evans blue dye into the footpad, we ascertained that 12 h after immunization with HEL/CFA, most of the dye remained in popliteal nodes with minimal extension to the distal draining lymph nodes (Fig. 7 A). Neutrophil-depleted mice had a noticeable increase into the distal draining nodes (Fig. 7 A).

In different experiments, 3A9 HEL transgenic CD4 T cells were transferred into the footpads of immunized B10.BR or G-CSFR$^{-/-}$ mice. At 3 h after immunization, the majority of the transferred antigen-specific 3A9 T cells remained in the popliteal nodes in both wild-type and G-CSFR$^{-/-}$ recipients

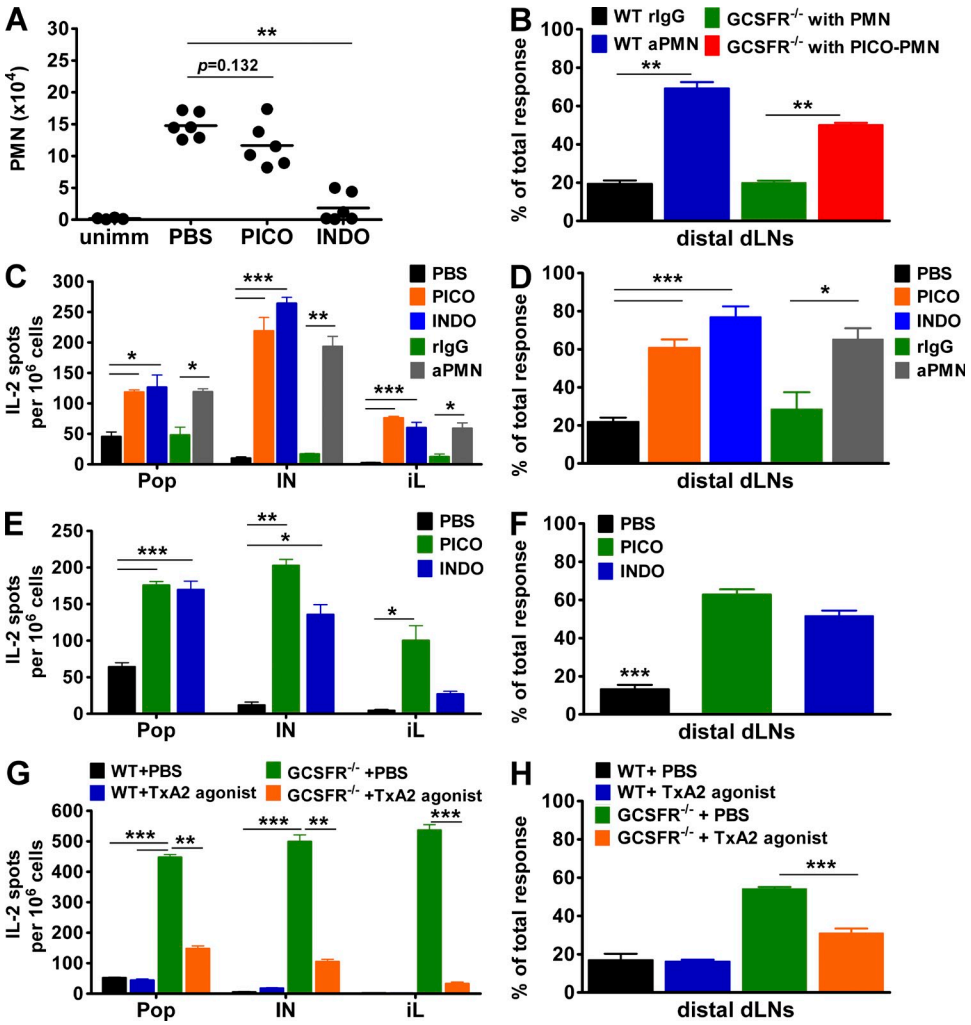

Figure 6. Thromboxane in neutrophil-mediated effects. (A) B10.BR mice were i.p. injected with indomethacin (INDO, 40 µg/mouse) 1 d before immunization or picotamide (PICO, 2.5 mg/mouse) at 2 h before immunization with 10 nmol HEL/CFA. The number of neutrophils in the popliteal nodes was analyzed by FACS of CD11b$^+$ Ly-6G$^+$ cells. Shown is pooled data from three individual experiments. Each dot represents one single mouse. Statistical analysis was processed by Mann-Whitney *t* test. (B) G-CSFR$^{-/-}$ mice were adoptively transferred with bone marrow neutrophils pretreated with picotamide (2 mg/ml for 30 min at 37°C) before immunization with 10 nmol HEL/CFA. Included are results of wild-type mice treated with aPMN antibody. Shown is data from 2 individual experiments with $n = 2$ in each group. (C and D) B10.BR mice were i.p. injected with: 1A8 antibody or control Ig; indomethacin or PBS at 1 d before immunization with 10 nmol HEL/CFA. Picotamide was given at 2 h before immunization. (E and F) B10.BR mice were immunized with 10 nmol HEL/CFA in the footpad. At 3 h after immunization, mice were i.p. injected with indomethacin or picotamide. (G and H) G-CSFR$^{-/-}$ or wild-type mice were immunized with 10 nmol HEL/CFA and i.p. injected with U-46619 (15 µg/mouse) or PBS control. (B–H) T cell recall responses were measured by IL-2 ELISPOT analysis and presented as the number of IL-2 spots (C, E, and G) or the percentage of total responses in the distal draining lymph nodes (B, D, F, and H). (C–H) Shown are data from 3 separate experiments with $n = 2$ in each group. (A–H) Data were presented as mean ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by Mann-Whitney *t* test.

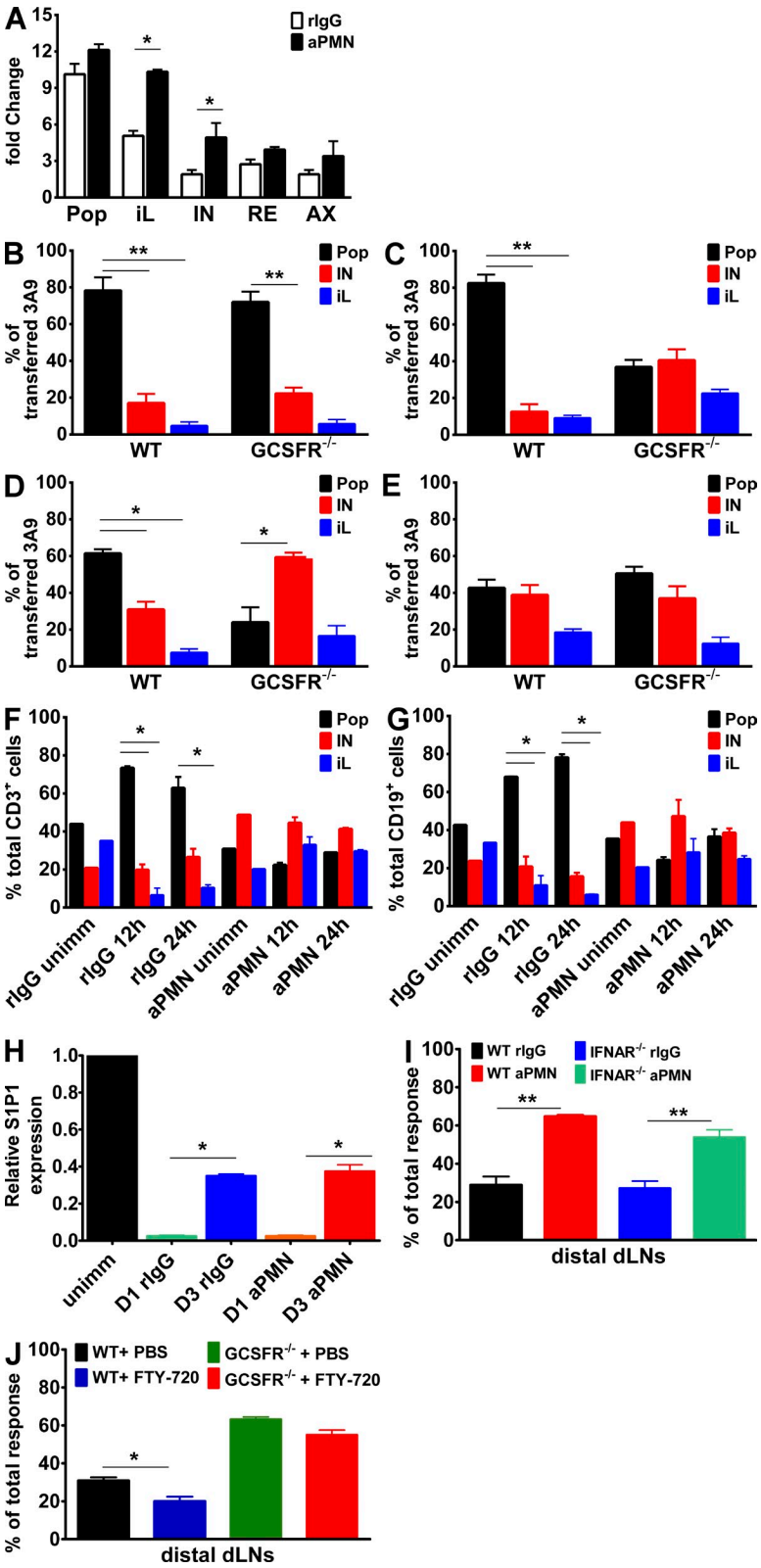

Figure 7. Migration in the draining lymph nodes after neutrophil depletion. (A) B10.BR mice were injected with 10% Evans blue or PBS into the footpad 12 h after immunization with 10 nmol HEL/CFA. Popliteal nodes or distal draining nodes were removed after 30 min of dye injection. The whole lymph nodes were cultured in formamide overnight at 37°C, and the absorbance was measured at 620 nm. Shown is fold change relative to PBS injection. Pop, popliteal; iL, iliac; IN, inguinal; RE, renal; AX, axillary lymph nodes. Shown are data from 3 individual experiments with $n = 2$ in each group. (B–E) Splenic CD4$^+$ T cells (2×10^6) from 3A9 mice were adoptively transferred into the footpad of B10.BR wild-type or G-CSFR$^{-/-}$ recipient mice under B10.BR genetic background. The recipient mice were either immunized with 10 nmol HEL/CFA (B, C) to follow antigen-specific T cells; or injected with CFA alone (D and E) to follow non–antigen-specific T cells. At 3 (B, D) or 22 h (C, E) after cell transfer, the number of 3A9 T cells in individual draining lymph nodes was measured by FACS analysis of CD4$^+$ 1G12$^+$ cells. (B–E) Shown is the percentage of total transferred 3A9 T cells in each individual lymph node. (B–E) Data are from 2 individual experiments with $n = 2$ in each group. (F and G) B10.BR mice were injected with control Ig or 1A8 Ab 1 d before immunization with 10 nmol HEL/CFA. Lymph nodes were harvested at indicated time points. The numbers of CD3$^+$ cells or CD19$^+$ cells were analyzed by FACS. Shown is the percentage of T cells (F) and B cells (G) in the individual nodes. (F and G) Data are from one experiment with $n = 3$ in each group. (H) 3A9 mice were i.p. injected with either 1A8 Ab or control Ig 1 d before 10 nmol HEL/CFA immunization. Total CD4$^+$ T cells were isolated from popliteal nodes at indicated time for mRNA extraction and qRT-PCR. Gene expression of S1P1 was analyzed by fold increase normalized with CD4$^+$ T cells from unimmunized mice. Shown are data from 2 individual experiments with $n = 2$ in each group. (I) T cell recall responses in immunized wild-type or IFNAR$^{-/-}$ mice were measured by IL-2 ELISPOT at day 7 after immunization. Shown are data from 2 individual experiments with $n = 2$ in each group. (J) G-CSFR$^{-/-}$ or wild-type B10.BR mice were i.p. injected with 50 µg/mouse FTY-720 or PBS and immunized with 10 nmol HEL/CFA. T cell recall responses were measured by IL-2 ELISPOT at day 7 after immunization. Data shown are results from 3 individual experiments with $n = 2$ in each group. Identical results were obtained from injecting FTY-720 every 3 d until day 7 after immunization. (I and J) Shown is the percentage of total responses in distal draining lymph nodes. (A–J) Data were presented as mean ± SD. Statistical analysis was processed by Mann-Whitney *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(Fig. 7 B). In neutropenic mice, the transferred 3A9 T cells also localized to the distal draining nodes at 22 h after immunization, whereas most were still retained in the popliteal nodes of wild-type mice (Fig. 7 C). We also examined the trafficking kinetics of non–antigen-specific T cells by transferring 3A9 T cells into recipients injected with CFA that did

not contain HEL. Compared with wild-type mice, nonspecific T cells started to spread to distal draining lymph nodes in both normal and neutropenic mice at 3 h after transfer, albeit more noticeably in the latter mice (Fig. 7 D). At 22 h, the spreading of nonspecific T cells were similar in both wild-type and neutropenic mice (Fig. 7 E). The differences between activated 3A9 (Fig. 7, B and C) and nonactivated 3A9 (Fig. 7, D and E) were likely caused by retention resulting from the HEL-specific interaction with APC. From these results, we concluded that neutrophils had restrictive effects on the spread of both antigen-specific and nonspecific T cells. In the absence of neutrophils, nonspecific T cells already showed early kinetics of the spread by 3 h, compared with antigen-specific T cells, where the spread is evident some hours later. These results reflected our functional studies in which T cell recall responses at day 7 after immunization spread to the distal nodes in the absence of neutrophils (Fig. 2).

We also measured the number of T and B cells in the lymph nodes after immunization with HEL/CFA at 12 or 24 h after immunization. In unimmunized mice, ~40% of total T and B cells resided in popliteal nodes in both control Ig–treated or neutrophil-depleted mice. After immunization of the control Ig–treated mice, the majority of T and B cells were retained in popliteal nodes at 12 or 24 h after immunization. In neutrophil-depleted mice, the spread of T and B cells to the distal draining nodes was observed at 12 h after immunization (Fig. 7, F and G). In total, the data from the experiments in Fig. 7 (B–G) shows that the retention of cells in the node during the first 24 h depends upon the presence of neutrophils.

We also examined the possible influence of neutrophils on sphingosine-1 phosphate (S1P), a lipid that is also involved in the retention of antigen-specific T cells. During an immune response, antigen-specific T cells down-regulate the S1P receptor and $S1P_1$ expression and are selectively retained in the lymphoid organs around 24 h of immunization. At 2–3 d of activation, the T cells reexpress $S1P_1$ and leave the lymphoid organs to return to the circulation (Matloubian et al., 2004; Cyster, 2005). IFN-α/β act as the upstream molecules and regulate $S1P_1$ expression via the action of CD69 upon polyI:C stimulation (Shiow et al., 2006). In this study, we evaluated whether neutrophil entry in the lymph nodes had an impact on S1P or IFN-α/β signaling, therefore interfering with $S1P_1$ expression for T cell egress. Transcriptional activity of $S1P_1$ after HEL immunization was measured by quantitative RT-PCR in T cells from popliteal nodes of 3A9 T cell receptor transgenic mice. In control Ig–treated mice, down-regulation of $S1P_1$ was observed at day 1 after immunization compared with unimmunized mice. At day 3 of immunization, T cells reexpressed $S1P_1$, allowing for their egress (Fig. 7 H; Matloubian et al., 2004). Neutrophil depletion did not alter the expression pattern of $S1P_1$ after immunization, indicating that S1P-$S1P_1$ ligation and neutrophil-mediated lymphocyte retention were independent events (Fig. 7 H). We also examined the neutrophil effect in $IFNAR^{-/-}$ mice. Consistently, neutrophil depletion in wild-type mice resulted in increased spread of T cell responses to the distal draining nodes. This effect was unaffected in $IFNAR^{-/-}$ mice (Fig. 7 I). Blockade of $S1P_1$ signaling pathway by using FTY-720 drug slightly reduced the spread of the response to distal nodes in wild-type mice, assaying the standard response at day 7 of immunization. The drug did not affect the spread of T cell responses to distal nodes in the absence of neutrophils (Fig. 7 J). In brief, the early escape of lymphocytes from the node mediated by neutrophils is independent from the subsequent release of activated T cells by the S1P pathway.

DISCUSSION

There are five findings from these investigations. First, we documented the rapid entrance of neutrophils after adjuvant injection, which was followed by a second wave a few days later. Second, we confirmed our previous findings that neutrophils were strongly suppressive, but we now add that this suppression was modulated by prostanoids. Third, we proved that the entry of neutrophils to the draining node was responsible for the rapid control of lymphocytes egress that takes place after adjuvant administration. Fourth, we established that neutrophil-associated thromboxane was the eicosanoid responsible for both the control of the response and the spread of it. Finally, we documented that the egress of lymphocytes controlled by the S1P pathway is distinct from the neutrophil effects that precede it. In brief, we emphasize the influence of another innate cell, the neutrophil, to the adaptive immune response, which should be placed in two contexts: (1) in infections in which the neutrophil could exerts both positive and negative effects, the end result of which may depend on the infectious agent; and (2) in vaccination. Our findings suggest that controlling the neutrophil, which invariably will follow adjuvant immunization, could well lead to a better systemic response.

Entry

We showed two waves of neutrophils in the draining lymph nodes after immunization. Analysis indicated that these two waves were distinct. The first wave was extremely rapid, depended on prostaglandins (prostanoids other than thromboxane), and was insensitive to PTX treatment, which is widely used to block chemokine receptor signaling (Fig. 1, C and D; and Fig. 6 A). Prostaglandin receptors have been shown to be coupled with PTX-insensitive G proteins (Burch and Axelrod, 1987; Nakahata et al., 1989). Entry was likely by way of the lymphatics, as indicated by the presence of neutrophils in the cortical sinus of the nodes that drain the afferent lymphatics (Yang et al., 2010). The mechanism of this fast entry could involve a mechanical process: the pressure in the compact foot pad resulting from the edema, and the adjuvant injection aided by vasodilatation induced by the prostaglandins, could propel the neutrophil and other cells into the lymphatics. The prostaglandins could derive from vascular or mesenchymal cells or from mast cells in the footpad. The entry was not affected by PTX, in contrast to wave 2.

The second wave persisted for a long period of time and was likely in response to chemokines made in the inflamed

node. Neutrophil migration to tissue sites or lymph nodes involves chemokine ligands to CCR1, CXCR2, and CCR7 (Beauvillain et al., 2011; Mantovani et al., 2011; Sadik et al., 2011). To note, there was strong localization of neutrophils to medullary cords in close relationship to activated B cells; whether neutrophils influence their biology is an issue to consider (Puga et al., 2012).

Inhibitory effects

The findings directly ascribed a role of neutrophils through the release of prostanoids. By adoptive transfer, neutrophil-derived prostanoids had functional effect on the spread of T cell responses (Fig. 5 C). Our results from mass spectrometry demonstrated that neutrophils directly produced prostanoids, but an alternative mechanism by which neutrophils influence prostaglandin production is through production by macrophages after their ingestion of apoptotic neutrophils (Fadok et al., 1998). Using TUNEL staining, we did not find neutrophil-related apoptosis until 6 h after immunization, which suggests that this alternative pathway was a late response. Moreover, blocking neutrophil apoptosis by z-VAD, a cell-permeable pan caspase inhibitor, did not change the spread of T cell responses.

In our previous study, we identified two mechanisms responsible for the modulation of the response. One involved competition for antigen between the neutrophils and the APCs that resulted in a reduction of the antigen available to the latter cells (Yang et al., 2010). A second and perhaps more critical finding involved a reduction on the DC and T cell interaction after the entrance of neutrophils, the molecular basis of which was not identified. Intravital microscopy indicated that the frequency and time of contact between DC and CD4 T cells was highly improved in a neutrophil-depleted situation (Yang et al., 2010). Notably, we found that such interaction did not involve neutrophils directly and, moreover, they took place at a time when the first wave neutrophils had disappeared. Altogethr, our past and present findings point to a late effect of the neutrophil-produced thromboxane. Indeed, a recent report has made the point that thromboxanes affected the response of CD4 T cells (Kabashima et al., 2003). By microscopy, thromboxane induced random movement of T cells that prevented the formation of DC–T cell clusters (Kabashima et al., 2003), findings that are entirely compatible with our in vivo findings. Other findings showed that thromboxane receptor–deficient mice had enhanced contact hypersensitivity (Kabashima et al., 2003) and that thromboxane inhibited class II MHC expression in APCs (Snyder et al., 1982). In *Trypanosoma cruzi* infection, parasite-induced thromboxane production favored parasite survival and transition to chronic state, possibly by modulation of adaptive immune responses (Ashton et al., 2007).

Although neutrophil depletion resulted in enhanced CD4 T cell responses, the extent of recall responses against CD8 epitopes remained unaffected or slightly reduced, suggesting that neutrophils alternatively contributed to CD8 T cell responses while having a suppressive effect on CD4 T cell responses (Yang et al., 2010). It is possible that apoptotic neutrophils containing antigen provided substrate for cross presentation (Tvinnereim et al., 2004). Our results of increased spread of CD8 responses in the distal draining nodes after neutrophil depletion suggested that neutrophils had distinct impact on the magnitude and the dissemination of CD8 T cell responses, and there was a balance between the effects of thromboxane production from neutrophils and the possible role of neutrophil in contribution to CD8 T cell responses.

In our system, we observed similar results in COX-$1^{-/-}$ and COX-$2^{-/-}$ mice (Figs. 1 and 3–5). Although it is generally accepted that COX-1 maintains the homeostasis of prostanoid production while COX-2 is induced in inflammatory settings, this view may be an oversimplification. In the experimental models of tumors and inflammatory diseases, lack of either isoforms gave similar phenotypes (Chulada et al., 2000; Morteau et al., 2000; Tiano et al., 2002). This could be caused by individual cyclooxygenase isoforms that are involved at different stages of diseases, or different prostanoid profiles from COX-$1^{-/-}$ or COX-$2^{-/-}$ mice. We are currently evaluating the profiles from wild-type, COX-$1^{-/-}$, and COX-$2^{-/-}$ neutrophils that may allow us to determine the specific pathway of prostanoid-mediated neutrophil effects.

The role of thromboxanes in the spread of the response

Neutrophils restricted the early spread of T cell responses via the production of thromboxane (Fig. 6), a molecule known to contract lymphatics (Johnston and Gordon, 1981; Sinzinger et al., 1984; Dabney et al., 1991; Quick et al., 2009). Among the arachidonic acid metabolites, lymphatic vessels showed a more sensitive pattern of their contractility in response to thromboxane analogue in vitro (Johnston and Gordon, 1981; Sinzinger et al., 1984). Previous studies of lymph node cell shutdown showed a dramatic rise in prostaglandin production in the efferent lymph after antigen stimulation. While in steady state, the levels of prostanoids remained undetectable (Johnston et al., 1979). The rapid kinetics production (<10 h) was similar to the kinetics of neutrophil entry (Yang et al., 2010). Our study provides evidence that neutrophils are a direct source of prostanoids, and neutrophil-derived thromboxane A_2 is the specific molecule restricting the spread of T cell responses after antigen stimulation.

Our study suggested a distinct but complementary pathway for lymphocyte egress in addition to S1P. In our experiments, neutrophil influx and their control of the spread of T cells took place in a very early time (22 h) after immunization (Fig. 1 A; and Fig. 7, B–E). The lack of influence of FTY-720 in the spread of the response in neutrophil-depleted mice may be explained by the finding that the escape in the absence of neutrophil is a fast process, whereas the retention by the S1P system takes place slowly, ending by about the time that a number of cells have already left the node. In brief, two retention systems are operating in the node, having distinct mechanisms but involving lipid mediators for both.

Neutrophil-derived thromboxane A_2 provides a generalized block, whereas S1P-$S1P_1$ ligation targets the antigen–specific T cells.

MATERIALS AND METHODS

Mice. All mice were bred and maintained under pathogen-free conditions at Washington University in accordance with institutional animal care guidelines. B10.BR and C57BL/6 (B6) mice obtained from The Jackson Laboratory were used as controls. G-CSFR$^{-/-}$ mice under C57BL/6 background were generated and obtained from the laboratory of D. Link (Washington University, St. Louis, MO; Liu et al., 1996). G-CSFR$^{-/-}$ mice were backcrossed to B10.BR (I-A^k) background for 10 generations. COX-1$^{-/-}$ and COX-2$^{-/-}$ mice were under C57BL/6 background and generated by Smithies et al. (Langenbach et al., 1995; Morham et al., 1995). Type 1 IFN receptor–deficient mice (IFNAR$^{-/-}$) under C57BL/6 background were generated by Aguet et al. (Müller et al., 1994). COX-1$^{-/-}$, COX-2$^{-/-}$, and IFNAR$^{-/-}$ were initially obtained from The Jackson Laboratory. IFNAR$^{-/-}$ mice were backcrossed to B10.BR (I-A^k) background for 10 generations.

Antigen. HEL was obtained from Sigma-Aldrich and purified by affinity chromatography to remove ~3% of contaminant proteins. Purified HEL contained <0.1 EU/μg LPS. Ovalbumin was purchased from Worthington Biochemical. Peptides were synthesized by 9-fluorenylmethoxycarbonyl techniques and verified by mass spectrometry: OVA 323–339 (ISQAVHAAHAEINEAGR), OVA 257–264 (SIINFEKL), HEL 11–25(AMKRHGLDNYRGYSL), HEL 23–31(YSLGNWVCA).

Immunization and cellular assays. Mice were immunized with 10 nmol of the proteins and emulsified with Freund's complete adjuvant into the footpad (50 μl per footpad). T cell responses were measured at 7 d after immunization using ELISPOT analysis in which popliteal lymph nodes or distal draining lymph nodes (inguinal, iliac, renal, and axillary nodes) were harvested for single-cell suspension and challenged with individual proteins or peptides in vitro (Harrell et al., 2008). Lymph nodes were digested with Liberase TL (Roche) at 37°C for 30 min to make single-cell suspension. IL-2 and IFN-γ ELISPOT analysis were performed for each individual experiment. Because the findings were similar, most experiments reported only the IL-2 results. To deplete neutrophils, mice were injected i.p. with 1 mg of the Ly-6G specific antibody 1A8 mAb (BioXCell) or with isotype control rat IgG (Sigma-Aldrich) 1 d before immunization. Mice treated with indomethacin received i.p. 2 mg/kg (Sigma-Aldrich); those treated with pertussis toxin (Sigma-Aldrich) received 500 ng, 1 d before immunization. In experiments with FTY-720 (Cayman Chemical), mice were i.p. injected with 50 μg/mouse FTY-720 at the time of immunization. Identical results were obtained from one injection at the time of immunization or injecting FTY-720 every 3 d until day 7 after immunization.

To track lymphatic drainage, 10 μg of Evans blue (Sigma-Aldrich) was injected into the footpad of immunized mice 30 min before lymph nodes were harvested. Individual lymph nodes were incubated in 150 μl formamide (Sigma-Aldrich) for 24 h at 37°C. The supernatant were collected for absorbency at 620 nm to measure dye content in the tissues. Injection of PBS was used as background control.

Adoptive transfer. For neutrophil isolation, the marrow from B10.BR or C57B6 micewas removed from femur and tibia and red blood cells were lysed. Cells were resuspended in 4 ml HBSS without calcium, magnesium, phenol red, and sodium bicarbonate and laid on two-layer Percoll (Sigma-Aldrich) gradient of 80 and 55% diluted in 3 ml HBSS (Sigma-Aldrich) and centrifuged 2,500 rpm for 30 min at room temperature without braking. Neutrophils from the 80 and 55% interface that were harvested for further purification positively selected with anti–Ly-6G MicroBead kit (Miltenyi Biotec). Purity of neutrophil preparation was examined by flow cytometry as well as microscopy (Fig. 4 A) and was close to 100%. Neutrophils were injected into the footpad of G-CSFR$^{-/-}$ recipient mice (2×10^6 cells per mouse; 50 μl per footpad) 20 min before immunization. In some experiments, neutrophils were preincubated with 0.2 mg/ml indomethacin for 15 min or 2 mg/ml picotamide for 30 min at 37°C before transfer (Díaz-González et al., 1995). The entry of transferred neutrophils into popliteal nodes was examined with flow cytometry, as well as functional assay in which T cell response was measured by ELISPOT at day 7 of neutrophil transfer (Fig. 5).

To follow the kinetics of the spread of T cells to distal draining lymph nodes, 2×10^6 splenic T cells from 3A9 mice (T cell receptor transgenic mice recognizing the dominant 48–62 epitope of HEL) were adoptively transferred into the footpad of wild-type or G-CSFR$^{-/-}$ recipients (50 μl per footpad). To follow antigen-specific T cells, recipient mice were immunized with 10 nmol HEL/CFA. To follow non–antigen-specific T cells, recipient mice were injected with CFA alone without HEL antigen. Individual lymph nodes were harvested at indicated time after transfer and the number of 3A9 T cells was analyzed by FACS using 1G12, a specific monoclonal antibody to the 3A9.The data were presented as the percentage of total transferred 3A9 T cell in popliteal, inguinal, or iliac nodes.

Quantitative RT-PCR. For S1P1 qRT-PCR, 3A9 mice were injected i.p. with 1 mg of 1A8 monoclonal antibody (BioXCell, West Lebanon, NH) or with isotype control rat IgG (Sigma-Aldrich) 1 d before 10 nmol HEL/CFA immunization. Total CD4$^+$ T cells were isolated from popliteal lymph nodes at day 1 or 3 of immunization (CD4$^+$ T Cell Isolation kit; Miltenyi Biotec). For *Ptgs-1* and *Ptgs-2* qRT-PCR, bone marrow neutrophils were isolated and stimulated with 10 μM PMA (Sigma-Aldrich) for 30 min at room temperature, or 10 mg/ml *M. tuberculosis* H37Ra (Difco) for 2 h at room temperature. Total RNA was isolated using an RNAqueous-Micro kit according to the manufacturer's instructions (Applied Biosystems). Equal amounts of RNA were used to generate cDNA using the High Capacity Reverse cDNA Transcription kit according to the manufacturer's instructions (Applied Biosystems). 40 ng of cDNA were used per reaction for qRT-PCR analysis, which was performed using SYBR Green kit and ΔΔCT calculations on a StepOnePlus Instrument (Applied Biosystems). The following primers used were used: 18S forward (F), 5′-GTAACCCGTTGAACCCCATT-3′; 18S reverse (R), 5′-CCATCCAATCGGTAGTAGCG-3′; S1P1 forward (F), 5′-ATGGTGTCCACTAGCATCCC-3′; S1P1 reverse (R), 5′-TGCCTGTGTAGTTGTAATGCC-3′; Ptgs-1 forward (F), 5′-AGTGCGGTCCAACCTTATCC-3′; Ptgs-1 reverse (R), 5′-GCAGAATGCGAGTATAGTAGCTC-3′; Ptgs-2 forward (F), 5′-TTCCAATCCATGTCAAAACCGT-3′; Ptgs-2 reverse (R), 5′-CTGGTCAAATCCTGTGCTCAT-3′. 18S was used as the standard.

Microscopy. Immunofluorescence staining was performed by applying fluorescent dye–conjugated antibodies onto frozen sections for 30–60 min at room temperature. For confocal microscopy, images were captured using a Zeiss 510 laser scanning confocal microscope. Histological sections of lymph nodes stained by hematoxylin and eosin were prepared by standard histological techniques.

Prostanoid production by mass spectrometry. Bone marrow neutrophils (2×10^6) were cultured in 200 μl of PBS with 100 μM PMA (Sigma-Aldrich) stimulation at room temperature. After 2 h of incubation at room temperature, 400 μl of methanol was added into the culture to quench the responses. Unstimulated neutrophils were used as a control group, and PBS alone was used for background signal detection. For detection of prostanoids from whole lymph nodes, popliteal lymph nodes were harvested from wild-type C57B/L or G-CSFR$^{-/-}$ mice at 2 h after immunization with 10 nmol OVA/CFA. Lymph nodes were homogenized in 500 μl PBS, and 200 μl methanol was added to quench the responses. Triplicates of each sample group were examined at the Metabolomics Core Facility of Washington University.

Statistical analysis. All measurements were presented as mean ± SD. Statistical analysis was processed by Mann-Whitney testing using Prism 6 software (GraphPad). In all figures, statistical significance of p-values are indicated with asterisks: *****, $P < 0.05$; ******, $P < 0.01$; *******, $P < 0.001$.

All experimental results were done at least twice, and in most instances four times.

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