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IL-17 Promotes Neutrophil Entry into Tumor-Draining Lymph Nodes following Induction of Sterile Inflammation

Craig M. Brackett,* Jason B. Muhitch,* Sharon S. Evans,* and Sandra O. Gollnick*,†

Blood-borne neutrophils are excluded from entering lymph nodes across vascular portals termed high endothelial venules (HEVs) because of lack of expression of the CCR7 homeostatic chemokine receptor. Induction of sterile inflammation increases neutrophil entry into tumor-draining lymph nodes (TDLNs), which is critical for induction of antitumor adaptive immunity following treatments such as photodynamic therapy (PDT). However, the mechanisms controlling neutrophil entry into TDLNs remain unclear. Prior evidence that IL-17 promotes neutrophil emigration to sites of infection via induction of CXCL2 and CXCL1 inflammatory chemokines raised the question of whether IL-17 contributes to chemokine-dependent trafficking in TDLNs. In this article, we demonstrate rapid accumulation of IL-17–producing Th17 cells in the TDLNs following induction of sterile inflammation by PDT. We further report that nonhematopoietic expression of IL-17RA regulates neutrophil accumulation in TDLNs following induction of sterile inflammation by PDT. We show that HEVs are the major route of entry of blood-borne neutrophils into TDLNs through interactions of t-selectin with HEV-expressed peripheral lymph node addressin and by preferential interactions between CXCR2 and CXCL2 but not CXCL1. CXCL2 induction in TDLNs was mapped in a linear pathway downstream of IL-17RA–dependent induction of IL-1B. These results define a novel IL-17–dependent mechanism promoting neutrophil delivery across HEVs in TDLNs during acute inflammatory responses.

Sterile inflammation refers to inflammation that occurs in the absence of pathogens and is observed following ischemia–reperfusion, chemotherapy, radiation, and other anticancer modalities (1). Like pathogen-induced inflammation, sterile inflammation is characterized by a rapid infiltration of neutrophils into the site of damage. Recent studies showed that neutrophils can also accumulate in inflamed lymph node (LNs) following induction of both pathogen-mediated (2–5) and sterile (6) inflammation. However, the mechanisms controlling neutrophil entry into inflamed LNs remain unclear.

Neutrophil migration into inflamed extralymphoid tissue is mediated by the chemokines CXCL2 (MIP-2) and CXCL1 (KC) through their interaction with CXCR2 (7). CXCL2 and CXCL1 expression is regulated by the proinflammatory cytokine IL-17 (8). The IL-17 family of cytokines consists of six members: IL-17A–F (9). The biological effects of IL-17A, hereafter referred to as IL-17, are mediated through IL-17RA, the receptor subunit necessary for signal transduction (10). IL-17 signaling leads to enhanced mRNA transcription and translation in mesangial cells (12), and production of IL-8 (human homolog of murine CXCL2) in human lung endothelial cells (13).

IL-17 induction of CXCL2 expression is augmented by IL-1B (14). IL-1B is a potent proinflammatory cytokine that also was shown to play a critical role in controlling neutrophil recruitment (1). IL-1B regulates endothelial cell expression of adhesion molecules that support recruitment of neutrophils (15) and enhances CXCL2 expression (16). Recruitment of neutrophils to sterile inflammation of the liver is dependent on IL-1B and CXCL2 (17).

Migration of naive and central memory lymphocytes in LNs is orchestrated via a multistep adhesion cascade that begins with tethering/rolling along specialized postcapillary venules termed “high endothelial venules” (HEVs) (18). Tethering and rolling are mediated by HEV-expressed peripheral LN addressin (PNAd) and t-selectin expressed on the lymphocyte (19, 20). Transition to firm arrest during homeostatic recirculation is mediated by the interaction of lymphocyte CCR7 with the chemokine CCL21 and subsequent LFA-1 binding to ICAM-1 and ICAM-2 (21), which have redundant functions during steady-state lymphocyte trafficking across HEVs (22).

Neutrophils were initially thought to be excluded from LNs because, although they can engage HEV-borne PNAd through interaction with t-selectin expressed on their cell surface, they do not express CCR7 (18, 19). Recent studies showed that neutrophils could enter inflamed LNs via the afferent lymphatics (2, 3) following infection and that entry is mediated by pathogen-induced upregulation of CCR7 on neutrophils (5). It is unclear whether neutrophils use a similar mechanism to gain entry into LNs following sterile inflammation.

Previous studies (6) by our group showed that neutrophils play a pivotal role in the induction of antitumor immunity following treatment of tumors with photodynamic therapy (PDT). PDT is a U.S. Food and Drug Administration–approved anticancer therapy used to cure early-stage disease and achieve palliation of advanced disease (23). PDT of tumors induces a sterile inflammatory response that is characterized by systemic release of proinflammatory cyto-

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Abbreviations used in this article: CTO, CellTracker Orange CMTMR; DAMP, danger associated molecular pattern; HA, hemagglutinin; HEV, high endothelial venule; HPPH, 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a; LN, lymph node; PDT, photodynamic therapy; PLN, peripheral lymph node; PNAd, peripheral lymph node addressin; S1P, sphingosine 1-phosphate; TDLN, tumor-draining lymph node.

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kines (24), increased systemic neutrophilia (25), and rapid migration of neutrophils into the tumor (26) and tumor-draining LNs (TDLNs) (6). The inflammatory response generated by PDT contributes to the overall efficacy of treatment (26). Induction of neutrophilia and migration of neutrophils into tumor and TDLNs are critical for generation of adaptive immunity following PDT (6). Long-term tumor control by PDT in patients is dependent upon induction of antitumor immunity (27).

Our results show that increased entry of neutrophils into TDLNs following induction of sterile inflammation by PDT is regulated by IL-17:IL-17RA. We also report that expression of IL-17RA by the nonhemato poetic compartment is required for the migration of neutrophils in TDLNs following induction of sterile inflammation. Neutrophil entry into TDLNs preferentially occurs across HEVs and is supported by IL-17–induced expression of CXCL2 but not CXCL1. Our data further provide evidence that IL-17 increases the production of IL-1β, which, in turn, enhances expression of CXCL2 that provides a molecular switch for increased entry of neutrophils into TDLN HEVs. These findings describe critical differences in the regulation of neutrophils in inflamed LNs via pathogen-mediated and sterile induction of acute inflammation. Although pathogen-mediated inflammation increases neutrophil entry into inflamed LNs through afferent lymphatics, sterile inflammation modifies the chemokine availability, allowing neutrophil recruitment in TDLNs through the blood vascular pathways.

Materials and Methods

Animals and tumor system
Pathogen-free BALB/c mice were obtained from the National Cancer Institute: C.B-17SCID-Bg-Cmkr2tm1Wnn (C57/129x10(5)) mice were obtained from The Jackson Laboratory. IL17ra(−/−) and BALB/c mice were given two doses of 6-Gy radiation 24 h apart. Six hours after the second dose of radiation, the mice were rescued with 106 bone marrow cells injected i.v. The mice were allowed to reconstitute the hematopoietic compartment for 9 wk prior to use.

In vivo Ab treatments
For chemokine- and cytokine-neutralization studies, 100 μg of the following Abs were administered i.v. immediately before PDT: anti-CXCL1 (R&D Systems; clone 48415), anti-CXCL2 (R&D Systems; clone 40605), anti-IL-1β (R&D Systems; clone 30311), or anti-IL-17A (24). For l-selectin (Pharmingen; clone MEL-14) and PNA (Pharmingen; clone MECA-79) neutralization, 100 μg of Ab were injected i.v. immediately prior to PDT.

Total RNA extraction
TDLNs were harvested at the indicated time points and frozen in TRIzol reagent. Total mRNA was extracted according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). Briefly, tissue was homogenized in TRIzol reagent and extracted with chloroform. The RNA was precipitated using isopropanol and washed once with ethanol. The RNA pellet was dried and reconstituted in Molecular Grade Water.

Short-term homing assays and immunofluorescence histology
Quantification of neutrophil trafficking was performed essentially as described previously (28, 29). Briefly, an enriched population of neutrophils (~70% Ly-6G−) was isolated from bone marrow by FACS-based flow sorting based on their characteristic size and granularity. These cells were labeled with CellTracker Orange CMTCM (Molecular Probes) and adoptively transferred i.v. into tumor-bearing mice 3 h after PDT treatment. TDLNs (axillary and brachial) and peripheral lymph nodes (PLNs; inguinal and popliteal) were harvested 30 min later from either PDT-treated or HPPH-only–treated mice and embedded in optimum cutting temperature compound (Sakura Finetek). Both endogenous and adoptively transferred neutrophils were identified by immunofluorescence staining of 9-μm cryosections that were fixed in methanol/acetone (3:1) for Ly-6G (Pharmingen; clone 1A8, 68 μg/ml). Expression of adhesion molecules was determined by performing intravascular staining by injecting 20 μg of purified mAb specific for ICAM-1 or ICAM-2 (Pharmingen; clone 3E2 or 3C4, respectively) in the tail vein. TDLNs were harvested 20 min later and embedded in optimum cutting temperature compound. Tissues were counterstained for PNA (Pharmingen; clone MECA-79, 20 μg/ml) and CD31 (AbD Serotec; clone 2H8, 5 μg/ml). After washing, the primary Abs were detected by fluorochrome-conjugated (fluorescein, rhodamine, or aminomethylcoumarin acetate) secondary Abs from Jackson Immunotechnical (West Grove, PA). Digital images were captured by investigators blinded to sample identity using an Olympus BX50 upright fluorescence microscope equipped with a SPOT RT camera (Spectra Services); all images were captured with identical exposure times and settings. The number of Ly-6G+ cells (with or without CTO) cells associated with CD31+ vessels was quantified in at least nine fields (area of field = 0.34 mm2), unless the size of the TDLN restricted the number of fields. Images were analyzed with ImageJ software to determine the relative fluorescence staining intensity of hundreds of vessels across 10 fields for PNA, CD31, ICAM-1, and ICAM-2.

Quantitative real-time PCR
The SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit was purchased from Invitrogen (Carlsbad, CA) and used according to the manufacturer’s protocol. For each reaction, 0.5 μg total RNA was used, and primers specific for murine IL-17 (100 nM), CXCL2 (200 nM), and GAPDH (100 nM) were used. Primer sequences are as follows: IL-17A forward: 5′-TTC CAG AAG GCC CTC AGA CTA C-3′ and reverse 5′-AAC CCA GG CCA TTA CTC AAT ACC CTT-3′; CXCL2 forward: 5′-GAA ACG CAA GGC TCA CTG A-3′ and reverse 5′-AAT CCA TGG CTC AAT TTT CTC CCG-3′; and GAPDH forward: 5′-ACG CCA AAT TCA ACG GCA CAG TCA-3′ and reverse 5′-TGG GGG CAT CCG GAG AAG G-3′. The samples were run on an ABI 7900 real-time instrument (Applied Biosystems, Carlsbad, CA) with a dissociation curve. The analysis was performed using SDS 2.3 software (Applied Biosystems) to obtain the Ct values for IL-17, CXCL2, and GAPDH. ΔCt was determined by subtracting the GAPDH Ct value from either the IL-17 or CXCL2 Ct value.
The relative amount of IL-17 or CXCL2 mRNA is reported and was calculated as $2^{-\Delta\Delta C_t}$.

**Determination of CXCL1, CXCL2, and IL-1β protein expression**

TDLNs were harvested at the indicated time points and flash frozen on dry ice. Total protein lysate was generated as previously described (30). Briefly, tissue was homogenized in Celllytic MT containing protease inhibitor mixture (both from Sigma, Saint Louis, MO). Cellular debris was removed by centrifugation. The protein concentration of the supernatant was determined using the Bio-Rad Protein Assay (Hercules, CA). ELISA kits specific for murine CXCL1, CXCL2, and IL-1β (R&D Systems) were used according to the manufacturer’s instructions. The results are expressed as pg/µg total protein.

**Statistical evaluation**

All measured values are presented as mean ± SEM. The nonpaired Student t test with the Welch correction was used for comparison between groups in all experiments. In all cases, significance was defined as $p \leq 0.05$.

**Results**

IL-17 regulates accumulation of neutrophils in TDLNs following induction of sterile inflammation by PDT

To determine whether the induction of sterile inflammation by PDT modulates TDLN expression of IL-17, quantitative real-time PCR with IL-17–specific primers was performed using RNA collected from TDLNs of Colo26-HA tumor–bearing mice. IL-17 message levels increased significantly within 4 h following PDT and returned to baseline levels by 8 h (Fig. 1A).

Potential sources of IL-17 within the TDLNs were examined using cell type–specific markers and intracellular staining for IL-17. The major IL-17–producing cells in the TDLNs following PDT appear to be Th17 cells (CD3⁺CD4⁺RORγt⁺ IL-17⁺). Th17 cells increased significantly within 2 h of PDT (Fig. 1B). Inmate immune cells, such as macrophages and γδ T cells, which are resident populations of LNs, can produce IL-17 in response to inflammation (31). IL-17–producing γδ T cells were not detected within the TDLNs before or after PDT. A minor population of IL-17–producing macrophages (CD11b⁺Ly6C⁻) was present prior to PDT, but this population did not change following PDT (data not shown). Based on these results, we characterized the regulation of neutrophil entry into TDLNs by IL-17 4–8 h post the induction of sterile inflammation by PDT.

To determine whether IL-17 regulates increased neutrophil accumulation in TDLNs following induction of sterile inflammation by PDT, the presence of these leukocytes was measured by flow cytometry in TDLNs of BALB/c and Il17ra⁻/⁻ mice. Neutrophils were defined as cells expressing CD11b, high levels of Ly-6G, and Ly-6C but lacking expression of F4/80 and CD11c (32); the identity of these cells as neutrophils was confirmed morphologically (6) (data not shown). The number of neutrophils in TDLNs of BALB/c mice increased significantly within 4 h of treatment (Fig. 1C). The accumulation of neutrophils in TDLNs of Il17ra⁻/⁻ mice following PDT was substantially reduced compared with accumulation in BALB/c TDLNs. Similar results were obtained when IL-17A was neutralized immediately prior to PDT by Abs (Fig. 1D), indicating that IL-17 activity was required during the acute phase of sterile inflammation induced following PDT. IL-17 also regulated accumulation of neutrophils in TDLNs following treatment of a second mouse model: EMT6 (Supplemental Fig. 1A). PDT induces systemic neutrophilia (23), which could account for the presence of neutrophils in the TDLNs. However, equivalent increases in the number of systemic neutrophils were detected following PDT in both BALB/c and Il17ra⁻/⁻ mice, suggesting that improved entry of neutrophils into the TDLNs involves an active mechanism of entry regulated by IL-17, rather than passive mechanisms due solely to neutrophilia (Supplemental Fig. 1B).

**FIGURE 1.** Increased expression of IL-17 by Th17 cells regulates entry of neutrophils into TDLNs following induction of sterile inflammation by PDT. (A) BALB/c Colo26-HA tumor–bearing mice were subjected to PDT. TDLNs were harvested at the indicated time points post-PDT to measure IL-17 mRNA by quantitative RT-PCR. The amount of IL-17 message was normalized to GAPDH and is reported as IL-17 mRNA copies. Each group contains a total of six mice; the data represent two independent experiments. (B) BALB/c Colo26-HA tumor–bearing mice were subjected to PDT. TDLNs were harvested at the indicated time points post-PDT, and a single-cell suspension was generated. The absolute number of Th17 cells in the TDLNs was analyzed by flow cytometry and is defined as CD3⁺CD4⁺RORγt⁺ IL-17⁺. Each group contains a total of six mice; the data represent two independent experiments. (C) Il17ra⁻/⁻ or BALB/c mice bearing Colo26-HA tumors were subjected to PDT. (D) BALB/c mice bearing Colo26-HA tumors were treated with 100 µg anti–IL-17A or isotype control Abs i.v. immediately before PDT. In (C) and (D), TDLNs were harvested at the indicated time points, and a single-cell suspension was generated. The absolute number of neutrophils in the TDLNs was analyzed by flow cytometry and is defined as CD11b⁺Ly-6G⁺Ly-6C⁻F4/80⁻CD11c⁻. Each group contains a total of at least nine mice, and the experiment was repeated twice. Data are mean ± SEM. *$p < 0.05$, versus 0 h, **$p < 0.05$, versus BALB/c (C) or isotype control Ab (D).
To ascertain whether the contribution of IL-17 to neutrophil accumulation in TDLNs is unique to PDT or occurs in other sterile inflammatory settings, turpentine was injected intratumorally into Colo26-HA tumors grown in BALB/c and Il17ra−/− mice. Like PDT, intratumoral injection of turpentine results in tumor cell necrosis and acute inflammation characterized by rapid neutrophil infiltration and release of IL-6 (6, 26). There is no difference between the acute tumor response of BALB/c and Il17ra−/− mice (data not shown). The number of neutrophils increased markedly in TDLNs within an hour of turpentine injection (Supplemental Fig. 1C). Significantly fewer total neutrophils were detected 2 h after turpentine injection in TDLNs of Il17ra−/− mice compared with BALB/c mice. Thus, it appears that secretion of IL-17 by Th17 cells in TDLNs contributes to neutrophil accumulation in TDLNs following induction of sterile inflammation.

Nonhematopoietic expression of IL-17RA regulates neutrophil accumulation in TDLNs post-PDT

Both nonhematopoietic and hematopoietic cells express IL-17RA (33). Bone marrow chimeras were generated to investigate whether expression of IL-17RA by either hematopoietic or nonhematopoietic cells regulates accumulation of neutrophils in TDLNs following sterile inflammation (Fig. 2). Colo26-HA tumor–bearing chimeras were treated with PDT, and neutrophil accumulation in TDLNs was examined. Neutrophil accumulation was similar in TDLNs of chimeras expressing IL-17RA only in nonhematopoietic cells (Il17ra−/− → BALB/c) and control chimeras (BALB/c → BALB/c). In contrast, significantly fewer neutrophils accumulated in TDLNs of chimeras lacking IL-17RA expression in nonhematopoietic cells (BALB/c → Il17ra−/−), which was similar to the accumulation observed in Il17ra−/− → Il17ra−/− chimeras. These results demonstrate that expression of IL-17RA by nonhematopoietic stromal cells regulates accumulation of neutrophils in TDLNs following induction of sterile inflammation.

HEVs of TDLNs are the primary portal of entry for neutrophils following PDT treatment

Neutrophil entry into TDLNs following PDT-induced sterile inflammation might occur through three vascular ports: afferent lymphatics functioning as conduits for migration from tumor sites, as reported during infection (5); inflamed blood vessels within TDLNs; and postcapillary HEVs in TDLNs. To distinguish among these possibilities, we took a two-pronged approach, as outlined in Fig. 3A. First, the spatial distribution of endogenous Ly-6G+ neutrophils relative to vessels was quantified 3.5 h post-PDT in TDLN cryosections. Vessels were counterstained for the panendothelial CD31 adhesion molecule that demarks flat-walled vessels (i.e., afferent and efferent lymphatics and inflamed blood vessels) and HEVs (34). HEVs were distinguished morphometrically based on their cuboidal endothelium and confirmed, in all cases, to be PNA+ in adjacent serial sections. Additionally, we tracked the destination of an enriched population of CTO-labeled neutrophils following i.v. transfer in PDT-treated mice. Quantification at 30 min post-adoptive transfer allowed for definitive evaluation of the frequency of CTO/Ly-6G+ neutrophil interactions exclusively with blood vessels (CD31+ flat-walled vessels or HEVs), because this time period is too short for leukocytes to emigrate from peripheral tissues via afferent lymphatics (29).

The data shown in Fig. 3B and 3C indicate that constitutive accumulation of endogenous neutrophils within the parenchyma of TDLNs or PLNs is low following administration of the HPPH photosensitizer alone, consistent with reports of minimal homeostatic trafficking of neutrophils to LNs (29). A marked increase in the localization of endogenous neutrophils upon PDT treatment occurred in the HEV-rich paracortical region exclusively in TDLNs, whereas homing in distal PLNs remained low. Improved trafficking in TDLNs following PDT was accompanied by a modest increase (p < 0.07) in the association of endogenous neutrophils with flat-walled vessels, including the subcapsular sinus (Fig. 3B, 3C, Supplemental Fig. 2), which is indicative of influx via afferent lymphatics and/or egress through effenter lymphatics (35, 36). However, these cells represented a relatively minor proportion (<10%) of total vessel-associated neutrophils.

In sharp contrast, PDT-induced sterile inflammation caused a profound increase in the number of neutrophils associated with HEVs. Notably, >90% of vessel-associated endogenous neutrophils interacted with HEVs. These findings were substantiated in short-term (30 min) homing assays in which >95% of adoptively transferred neutrophils detected in TDLNs interacted selectively with HEVs (Fig. 3B, 3D). Moreover, trafficking of endogenous neutrophils in TDLNs was strongly inhibited by Ab neutralization of prototypical homing receptors required for binding to HEVs (i.e., leukocyte-expressed t-selectin and HEV-presented PNAd) (Fig. 4A, 4B). The density of TDLN HEVs remained constant following PDT (average number of HEVs/field in HPPH control and PDT-treated mice, 5.5 ± 0.95 and 5.4 ± 0.41, respectively; n = 2 mice), indicating that the intrinsic binding activity of endothelial cells lining HEVs was enhanced. The increase in HEV adhesion could not be explained by increases in the intravascular density of molecules required for transient tethering/rolling interactions (PNAd) or firm arrest (ICAM-1, ICAM-2), or transendothelial migration (CD31) as determined by quantitative immunofluorescence histology (Fig. 4C, Supplemental Fig. 3). Additionally, t-selectin expression was not increased on neutrophils recovered from TDLNs after PDT (Supplemental Fig. 3). Taken together, these data strongly implicate HEVs as the major route of neutrophil entry into TDLNs following induction of sterile inflammation by PDT and point to chemokine-dependent adhesion as the molecular switch that converts HEVs to an active site of neutrophil recruitment.

Increased neutrophil entry into TDLNs depends on CXCR2 and CXCL2 but not CXCL1

Neutrophils are equipped with trafficking molecules necessary for binding PNAd and ICAM-1/2 (i.e., t-selectin and LFA-1, respectively), although they normally fail to extravasate across HEVs because they cannot initiate CCR7/CCL21-dependent transition
from rolling to firm arrest within vessel walls (35). Beauvillain et al. (5) demonstrated that neutrophils acquire CCR7 and gain access to LNs through the afferent lymphatics in response to pathogen-mediated inflammation. However, neutrophils that accumulate in TDLNs following induction of sterile inflammation by PDT express CXCR2 but only minimal levels of CCR7 (Supplementary Fig. 3). Furthermore there is no significant change in the levels of homeostatic chemokines, CCL19/21, expressed in the TDLNs post-PDT (data not shown). These observations suggested that chemokines other than CCL19/21 are responsible for neutrophil accumulation following PDT treatment.

FIGURE 3. TDLN HEVs support increased neutrophil accumulation following PDT treatment. (A) A single-cell suspension of neutrophils was fluorescently labeled with CTO (red) and adoptively transferred into treated mice, as shown. (B) LN tissue sections were stained with anti-CD31 (green) and anti-Ly6G (blue). Endogenous (blue) and adoptively transferred Ly6G+ (pink) cells were counted following short-term (0–30 min) homing assays. Representative photomicrographs of control (HPPH only; left panel) and PDT-treated (right panel) LNs are shown. Scale bars, 50 μm. Quantification of endogenous Ly6G+ cells (C) and adoptively transferred Ly6G+ neutrophils (D). Serial sections were stained to confirm that CD31-expressing cubic cells also expressed the HEV-specific marker PNAd. Data (mean ± SEM) are for at least nine fields, unless the size of the TDLN restricted the number of fields, in individual mice and are representative of three independent experiments. *p < 0.05. PLN refers to the TDLN stroma.

FIGURE 4. Neutrophils gain entry to TDLNs following induction of sterile inflammation through HEV interactions. TDLNs were harvested at the indicated time points post-PDT. A single-cell suspension was generated and analyzed by flow cytometry for the number of neutrophils/TDLN in the presence of 100 μg isotype control or 100 μg anti-L-selectin Abs (A) or 100 μg isotype control or 100 μg anti-PNAd Abs (B). (C) Immunofluorescence staining of TDLNs for PNAd and ICAM-1 was performed as described in Materials and Methods. The numerical value represents the mean fluorescence intensity. Each group contained a minimum of nine mice, and the experiment was repeated twice. Data are mean ± SEM. *p < 0.05, versus 0 h, #p < 0.007, versus isotype.
IL-17-mediated neutrophil entry into TDLNs following induction of sterile inflammation.

CXCR2 mediates neutrophil migration into nonlymphoid tissue at sites of inflammation (7). To determine whether neutrophils were using CXCR2 to enter TDLNs, neutrophil accumulation in TDLNs was measured in Cxcr2−/− mice following the induction of sterile inflammation. The lack of CXCR2 expression significantly reduced the number of neutrophils in TDLNs following PDT (Fig. 5A). The chemokines CXCL2 and CXCL1 are ligands for CXCR2 (37). Injection of Abs specific to CXCL2 immediately prior to induction of sterile inflammation by PDT significantly reduced the number of neutrophils in TDLNs at 4 and 8 h post-PDT (Fig. 5B). In contrast, neutralizing CXCL1 reduced the accumulation of neutrophils in TDLNs only at 8 h post-PDT (Fig. 5C). Collectively, these results suggest that entry of neutrophils into TDLNs following induction of sterile inflammation is mediated by ligation of CXCR2 with CXCL2.

IL-17 regulates CXCL2 expression in TDLNs post-PDT

IL-17:IL-17RA interaction is critical for CXCL2/CXCL1 expression following pathogen infection (8). To determine whether IL-17 also controls CXCL2/CXCL1 expression following induction of sterile inflammation, expression of CXCL2/CXCL1 in TDLNs following PDT was assessed. The expression of CXCL2 mRNA (Fig. 6A) and protein (Fig. 6B) profoundly increased in TDLNs of Colo26-HA tumor–bearing BALB/c mice post-PDT. In sharp contrast, expression of CXCL2 mRNA and protein in TDLNs of Il17ra−/− mice was largely unchanged post-PDT.

In contrast to the regulation of CXCL2 mRNA in TDLNs by IL-17 post-PDT, minimal differences were observed in CXCL1 mRNA and protein expression in TDLNs of BALB/c and Il17ra−/− mice following PDT (Supplemental Fig. 4). CXCL1 protein levels in Il17ra−/− TDLNs were 1.4-fold lower than in BALB/c mice at the 4-h time point. In comparison, CXCL2 levels were 4-fold lower at

**FIGURE 5.** Increased neutrophil entry into TDLNs is supported by CXCR2 and CXCL2 but not CXCL1. (A) Colo26-HA tumor–bearing Cxcr2−/− or BALB/c mice were subjected to PDT, and the number of neutrophils/TDLN was determined by flow cytometry at the indicated time points. (B and C) TDLNs were harvested at the indicated time points post-PDT, and a single-cell suspension was generated. The number of neutrophils/TDLN was analyzed by flow cytometry in the presence of 100 µg anti-CXCL2 Abs (B) or 100 µg anti-CXCL1 Abs (C) given i.v. immediately prior to PDT. Each group contained a minimum of nine mice, and the experiment was repeated twice. Data are mean ± SEM. *p < 0.02, versus 0 h, †p < 0.02, versus isotype or BALB/c.

**FIGURE 6.** IL-17 regulates induction of CXCL2 in TDLNs post-PDT. Il17ra−/− or BALB/c Colo26-HA tumor–bearing mice were subjected to PDT. TDLNs were harvested at the indicated time points post-PDT to measure expression of CXCL2 mRNA or protein. (A) Qualitative real-time PCR was performed, and the amount of CXCL2 message was normalized to GAPDH and is reported as CXCL2 mRNA copies. (B) An ELISA kit (R&D Systems) specific for murine CXCL2 was used, and the amount of CXCL2 protein/µg total protein is reported. Each group contained a minimum of nine mice, and the experiment was repeated twice. Data are mean ± SEM. *p < 0.05, versus 0 h, †p < 0.05, versus BALB/c.

IL-17–regulated IL-1β expression enhances CXCL2 expression and accumulation of neutrophils in TDLNs postinduction of sterile inflammation by PDT

IL-1β synergizes with IL-17 to augment the expression of CXCL2 (14). Therefore, we sought to determine the contribution of IL-1β to the regulation of CXCL2 expression and neutrophil entry into TDLNs by IL-17 post-induction of sterile inflammation by PDT. Although the expression of IL-1β in TDLNs of tumor-bearing BALB/c and Il17ra−/− mice significantly increased following PDT (Fig. 7A), induced levels of IL-1β were significantly less in Il17ra−/− mice compared with BALB/c mice. This result suggests that IL-17 contributes to the regulation of IL-1β in the TDLNs post-PDT. To determine whether IL-1β regulates CXCL2 expression in TDLNs, neutralizing Abs against IL-1β were administered prior to the induction of sterile inflammation by PDT (Fig. 7B). Neutralizing IL-1β partially inhibited CXCL2 expression in TDLNs, suggesting that IL-1β regulates CXCL2 expression. To ascertain the contribution of IL-17 and IL-1β to the accumulation of neutrophils in TDLNs following induction of sterile inflammation, IL-17 or IL-1β, either alone or in combination, were neutralized with Abs immediately before PDT treatment (Fig. 7C). Blocking either IL-17 or IL-1β significantly reduced the entry of neutrophils into TDLNs post-PDT. Although blocking IL-1β had a greater effect on the entry of neutrophils into TDLNs than did blocking IL-17, the difference was not significant. Blocking both IL-17 and IL-1β resulted in no further reduction in the entry of neutrophils into TDLNs than did blocking either alone. Taken together, these results suggest that, rather than acting in synergy, IL-17 acts upstream of IL-1β to enhance CXCL2 expression and support entry of neutrophils into TDLNs via HEV following the induction of sterile inflammation (Figure 8).
FIGURE 7. Regulation of IL-1β by IL-17RA enhances CXCL2 expression and neutrophil access to TDLNs post-induction of sterile inflammation. (A) Il17ra<sup>−/−</sup> or BALB/c Colo26-HA tumor-bearing mice were subjected to PDT. TDLNs were harvested at the indicated time points post-PDT, and IL-1β protein expression was measured by ELISA (R&D Systems). The amount of IL-1β protein/µg total protein is reported. (B) Colo26-HA tumor–bearing BALB/c mice were treated i.v. immediately prior to PDT. TDLNs were harvested at the indicated time points to measure CXCL2 protein expression by ELISA (R&D Systems). The amount of CXCL2 protein/µg total protein is reported. (C) Colo26-HA tumor–bearing BALB/c mice were treated i.v. with 100 µg isotype control or 100 µg anti–IL-1β Abs either alone or in combination, immediately prior to PDT. TDLNs were harvested at the indicated time points post-PDT, and a single-cell suspension was generated. The number of neutrophils was determined by flow cytometry. Each group contained a minimum of nine mice, and the experiment was repeated twice. Data are mean ± SEM. *p < 0.04, versus 0 h. †p < 0.04, versus isotype or BALB/c.

Discussion

The paradigm has been that neutrophils are normally excluded from gaining entry to LNs via HEVs (18); thus, their contribution to controlling adaptive immune responses has been largely overlooked. In this article, we report that the induction of sterile inflammation by PDT increases the entry of neutrophils into TDLNs. Our results demonstrate that entry of neutrophils into TDLNs during sterile inflammation occurs via HEV portals and is dependent upon IL-17–mediated induction of CXCL2. The effects of IL-17 on CXCL2 are dependent upon IL-1β, which is enhanced by IL-17. To our knowledge, this is the first demonstration that IL-17 regulates IL-1β expression in TDLNs post-induction of sterile inflammation. These findings support a model for neutrophil entry into TDLNs following induction of sterile inflammation whereby increased expression of IL-1β by IL-17 leads to the induction of CXCL2, which acts as a molecular switch that converts HEVs to active sites of neutrophil recruitment (Fig. 8).

Immunohistochemical analysis of PDT-treated TDLNs revealed an extremely high density of neutrophils within the paracortex region, a HEV-rich region of LNs. PDT leads to an increase in the overall size of TDLNs (6), which results in an overall increase in the number of HEVs available for trafficking. PDT also increases the intrinsic binding properties of HEVs, as is evident by the findings that endogenous and adoptively transferred neutrophils exhibit increased association with individual HEVs following treatment. Thus, it appears that modulation of neutrophil numbers in TDLNs following induction of sterile inflammation is due to a combination of increased LN size and enhanced adhesion within the HEVs.

The majority of studies demonstrating that neutrophils can access inflamed LNs during infection suggested that migration occurs via the lymphatics (2, 4, 5) because although neutrophils can use L-selectin to roll along HEVs, firm adhesion and extravasation are prevented by the lack of activating chemokines (19). Recent work by Beauvillain et al. (5) showed that neutrophils acquire CCR7 and access inflamed LNs through the afferent lymphatics during pathogen-induced inflammation. Our study indicates that the majority of neutrophils that enter TDLNs through HEV portals following induction of sterile inflammation are CCR7<sup>low</sup>. Mechanistic studies revealed that, during sterile inflammation, HEVs undergo a molecular switch involving CXCR2 engagement by CXCL2 that enables blood-borne neutrophils to gain access to TDLNs. Blocking of CXCL2 or the use of CXCR2-deficient mice virtually eliminated neutrophil accumulation in the TDLNs. These results support the conclusion that neutrophil migration to TDLNs following induction of sterile inflammation by PDT is unique. The distinction between the mechanisms of neutrophil entry into LNs triggered by sterile and pathogen-induced inflammation is further supported by the lack of increase in neutrophil expression of CCR7 or LN expression of CCL19/21 following the induction of sterile inflammation. Furthermore, previous studies (19) showed that the lymphatic endothelium does not express either PNAd or
IL-17–mediated neutrophil entry into TDLN

ICAM-1. There is one report (38) demonstrating that the lymphatic endothelium expresses CXCL2 mRNA; additional support for the lack of involvement of CCR7 in neutrophil migration during sterile inflammation could be obtained by examining migration in CCR7-deficient mice. However, these mice are only available on the B6 background, which is incompatible with the CT26 tumor model.

IL-17 has no effect on the expression of CCR7 by neutrophils (5). However, IL-17 was shown to augment the migration of neutrophils in response to CCL19/21 in the presence of GM-CSF (5). Systemic levels of GM-CSF increase following PDT (23, 39), and neutrophils can enter LNs via CCR7-mediated migration through the lymphatics (5). Additionally, sphingosine 1–phosphate (SIP) can enhance neutrophil transendothelial migration (40), and inhibition of SIP blocks the IL-23/IL-17–mediated neutrophil migration from the blood into tissue (41). SIP is expressed in LNs, and its expression increases during inflammation (41). We observed a minor population of neutrophils that appeared to enter TDLNs via afferent lymphatics or an alternative manner. Thus, it is possible that, although our data indicate that neutrophils primarily gain access to TDLNs following induction of sterile inflammation via IL-17/CXCL2–mediated migration across HEV portals, minor populations of neutrophils enter the TDLN via the lymphatics through IL-17–mediated increases in CCR7 or via SIP-mediated transendothelial migration.

The lack of i-selectin downregulation post-migration of neutrophils through HEVs is surprising. Previous reports showed that neutrophils shed i-selectin upon activation and that rolling neutrophils have reduced expression of i-selectin (42–45). i-selectin shedding is initiated by cell activation and the subsequent induction of apoptosis (44, 46). However, activated neutrophils can re-express i-selectin following activation-induced downregulation and prior to cell death (47–49). Therefore, it is possible that the i-selectin expression observed in our study is a result of the molecule being re-expressed following activation-induced downregulation.

Neutrophil accumulation in TDLNs post-PDT was not completely suppressed in Ill7ra -/− mice or by blocking Abs to IL-17A, suggesting that other IL-17R subunits or isoforms of IL-17 may be involved. IL-17RC, which forms a heteromeric complex with IL-17RA, may also regulate neutrophil entry into TDLNs post-PDT. Although the relative contributions of each cytokine receptor subunit to downstream signaling have remained elusive, IL-17RC has a higher affinity for IL-17F than IL-17A (9). Ho et al. (50) demonstrated that, in vitro, IL-17C does not require IL-17RA for IL-17-dependent signaling in HEK293T cells and, in vivo, IL-17RC acts indistinguishably from IL-17RA for IL-17–dependent responses. Therefore, it is possible that IL-17F signals through IL-17RC expressed on the stromal elements of the TDLNs independently of IL-17RA and contributes to CXCL2 expression and neutrophil accumulation in TDLNs post-PDT.

PDT induces a sterile inflammatory response within the treated tumor (23). Treatment results in rapid apoptosis of tumor cells induced by the generation of singlet oxygen. The primary apoptotic response is replaced by secondary necrosis due to an overwhelming of the clearance response. Direct tumor cell death is accompanied by vascular damage and induction of acute inflammation that contribute to overall tumor destruction. The acute inflammatory response within tumors is characterized by rapid neutrophil infiltration and systemic release of proinflammatory cytokines, including IL-6. A similar response is observed within the tumor bed upon injection of turpentine (6, 24, 26).

Our studies suggest that Th17 cells are the primary source of IL-17 following PDT, with significant increases in these cells detected in TDLNs within 2 h of PDT. The rapid accumulation of IL-17–expressing CD3+CD4+RORγT+ cells following PDT suggests that these cells may be natural Th17 cells. Natural Th17 cells acquire the ability to produce IL-17 in the thymus; conventional Th17 cells require further stimulation in peripheral tissue to produce IL-17 (51, 52). Natural and conventional Th17 cells share similar phenotypes (CD3+CD4+, RORγT+) and express similar cytokines (IL-17 and IL-22). Both cell types mediate host protection during inflammation through recruitment of neutrophils.

The distinct mechanisms by which neutrophils access the LNs following sterile inflammation and pathogen-induced inflammation indicate that neutrophil migration into LNs depends on the inflammatory stimuli. Both PDT and infection result in acute inflammation. PDT-generated sterile inflammation is triggered by release of danger associated molecular patterns (DAMPs), endogenous factors that normally are sequestered intracellularly from dying cells. In contrast, pathogen-generated acute inflammation is initiated by small molecular sequences conserved by pathogens called pathogen associated molecular patterns. The release of DAMPs from necrotic cells following PDT induces a sterile inflammatory response characterized by an increase in the expression of IL-1β (53), a known regulator of CXCL2 expression (54), and marked enhancement of neutrophil recruitment in TDLNs. Thus, the sterile inflammatory response generated by PDT is poised to support the entry of neutrophils into TDLNs.

The mechanism by which IL-17 regulates IL-1β is unclear. Secretion of IL-1β is a two-step process that involves production of pro–IL-1β and cleavage of pro–IL-1β into its active form (1). The production of pro–IL-1β is dependent upon NF-κB activation (55). IL-17RA (9) and TLR signaling (56) both activate NF-κB. Thus, the production of pro–IL-1β may be due to IL-17–dependent and -independent mechanisms. Cleavage of pro–IL-1β into its biologically active form occurs following activation of the NALP3 inflammasome (55). NALP3 can be activated by a variety of endogenous mediators, including reactive oxygen and extracellular ATP, which are released during sterile inflammation (57, 58). Pro–IL-1β can also be processed by neutrophil serine proteases (59). Both NALP3 and elastase expression/activity can be increased by IL-17 (60, 61).

Intriguingly, our findings demonstrate that, although IL-17 regulates the expression of CXCL2 in the TDLNs post-PDT, expression of CXCL1 is largely independent of IL-17. The mechanism of regulation for these chemokines in the TDLNs post-induction of sterile inflammation remains unclear. IL-17 activates NF-κB, a hallmark transcription factor associated with the induction of inflammation (62). DNA elements that bind NF-κB in the promoters of IL-17 target genes are required for IL-17–induced gene expression (63). Sequence analysis identified three NF-κB DNA binding sites within the CXCL1 promoter region, whereas the CXCL2 promoter region contains an IRF DNA binding site upstream of two NF-κB sites (64). This critical difference in the promoter regions suggests that, in addition to the NF-κB–signaling pathway, CXCL2 gene expression is controlled by the TRIF pathway. TRIF is an adapter protein that mediates signal transduction downstream of TLR3 and TLR4. TLRs were shown to affect the efficacy of chemotherapy (65) and PDT (66). It was reported that IL-17 additively cooperates with TLR4 ligands to augment the expression of neutrophilic chemokines (67). Therefore, cancer therapies that result in tumor cell death may initiate the release of DAMPs that bind TLR4 and lead to downstream signaling through TRIF, resulting in binding of IRF to the CXCL2 promoter. Activated NF-κB by IL-17 would also bind to the CXCL2 promoter. Cooperation between the binding of IRF and NF-κB to their respective DNA binding elements in the CXCL2 promoter would enhance CXCL2 gene transcription.
Our results show that, although CXCL2 and CXCL1 are both induced in TDLNs post-sterile inflammation, only CXCL2 is required for the entry of neutrophils into TDLNs. Call et al. (68) reported that, although acute inflammation increased the expression of both CXCL2 and CXCL1 in the infected tissue, CXCL2 was limited to the inflamed tissue, whereas CXCL1 was found systemically in the circulation. CXCL2 is considered an inducible inflammatory chemokine that is thought to mediate stress- or injury-induced neutrophil migration (69). The sequestration of CXCL2 in inflamed TDLNs may be explained by its ability to be presented on the HEVs; however, it remains unclear whether this occurs. A recent report (70) demonstrated an intravascular chemokine gradient of CXCL2 on the venular endothelium of the cremaster muscle, which directed neutrophil crawling. This finding supports the possibility that CXCL2 is capable of being presented on the HEVs through a process termed transcytosis, leading to chemokine activation of neutrophils and transendothelial migration across HEVs into TDLNs. This scenario would not be unprecedented, because Palfman et al. (71) reported that inflammatory chemokines are capable of undergoing transcytosis and becoming expressed on HEVs.

The TDNL is the site of T cell activation. Neutrophils were shown to influence T cell activation through secretion of chemokines and granule proteins that recruit monocytes and dendritic cells (72), activation of dendritic cells via cell-to-cell contact and secretion of TNF-α (73, 74), and secretion of IFN-γ that stimulates monocytes and T cell differentiation (72). We showed that neutrophils are required for PDT efficacy (26) and that migration of neutrophils to TDLNs enhances the generation of antitumor immunity (6). The precise mechanism by which neutrophils contribute to PDT efficacy and the generation of antitumor adaptive immunity is under investigation. However, the findings reported in this article suggest that sterile inflammation alters the microenvironment to permit neutrophil entry into TDLNs via a previously unrecognized delivery mechanism across HEV portals of entry.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Figure 1. Neutrophils are found in the TDLNs of EMT6 tumors post PDT and post turpentine. (A) *Il17ra<sup>−/−</sup>* or BALB/c mice bearing EMT6 tumors were subjected to PDT. TDLNs were harvested at the time points indicated and a single-cell suspension was generated and analyzed by flow cytometry for the number of neutrophils per TDLN. (B) *Il17ra<sup>−/−</sup>* or BALB/c mice bearing Colo26-HA tumors were subjected to PDT. Blood was harvested at the indicated time points, rbc were lysed and the number of systemic neutrophils was determined by flow cytometry. (C) *Il17ra<sup>−/−</sup>* or BALB/c mice bearing Colo26-HA tumors were given 30 μL
turpentine or PBS intra-tumorally. The number of neutrophils was determined by flow cytometry as described in A. Error bars represent mean ± SEM. *P<0.0009 to 0h; #P<0.02 to BALB/c.

Supplementary Figure 2. Neutrophils are sparsely found within subcapsular sinus of TDLNs. A representative photomicrograph of a PDT TDLN section is shown. Endogenous neutrophils were detected within the subcapsular sinus and high endothelial venules (HEVs) by immunofluorescence staining 3h following PDT treatment. The section is stained with anti-CD31 Ab (green; clone 2H8, 5 μg/mL, AbD Serotec) and anti-Ly6G Ab (blue; clone 1A8, 68 μg/mL, PharMingen). Note that localization of endogenous Ly6G⁺ neutrophils in the subcapsular sinus is a relatively rare event, as indicated by overall quantification (Figure 3C) in which >90% of neutrophils are associated with HEVs post PDT. Scale bar represents 50 μm.
Supplementary Figure 3. PDT does not alter ICAM-2 or CD31 expression on TDLN HEVs or L-selectin or CCR7 expression on neutrophils. Colo26-HA tumor-bearing BALB/c mice
were subjected to PDT. (A) Intravascular staining was performed for ICAM-2 (clone 3C4, PharMingen) by injection of 20 μg of purified antibodies 20 minutes prior to euthanization. (A and B) TDLNs were harvested twenty minutes later and embedded in OTC. Tissues were counterstained for PNAd (clone MECA-79, 20 μg/mL, PharMingen) or CD31 (clone 2H8, 5 μg/mL, AbD Serotec). After washing, the primary antibodies were detected by fluorochrome-conjugated (fluorescein, rhodamine, or AmCa) secondary antibodies from Jackson ImmunoResearch (West Grove, PA). Digital images were captured by investigators blinded to sample identity with an Olympus BX50 upright fluorescence microscope equipped with a SPOT RT camera (Spectra Services); all images were captured with identical exposure times and settings. Representative images are shown; the numerical values present in the upper left corner represent MFI for staining of HEVs. (C) The expression of L-selectin on neutrophils in the TDLN was determined by flow cytometry. A representative histogram is shown for L-selectin on neutrophils at 4h post PDT (left panel) and the MFI values for L-selectin expression are reported (right panel). TDLNs were harvested 8h after PDT and single-cell suspensions were generated. The expression of CCR7 on neutrophils (D) or dendritic cells (E) was analyzed by flow cytometry; neutrophils are defined as CD11b+Gr1hiCD11c- expressing cells. Dendritic cells are defined as CD11b+CD11c+ expressing cells. Representative histograms are shown. Open histograms represent staining with isotype control antibodies; filled histograms represent staining with anti-CCR7 antibodies.

Supplementary Figure 4. IL-17RA partially regulates KC expression in the TDLN post PDT. Il17ra−/− or BALB/c Colo26-HA tumor-bearing mice were subjected to PDT. TDLNs
were harvested at the indicated time points to measure CXCL1 mRNA by qRT-PCR or protein by ELISA. (A) qRT-PCR was performed and the amount of CXCL1 message was normalized to GAPDH. (B) The amount of CXCL1 per μg total protein is reported. Error bars represent mean ± SEM. *P<0.02 to 0h; #P<0.02 to BALB/c.