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Neutrophils Influence the Level of Antigen Presentation during the Immune Response to Protein Antigens in Adjuvants

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Neutrophils modulated Ag presentation following immunization with Ags in CFA or IFA or alum. The neutrophils had an important negative role in the CD4 T cell and B cell responses to three protein Ags: hen egg white lysozyme, OVA, and listeriolysin O. In their absence (by depleting with Abs for only the first 24 h, or using genetically neutropenic mice), the cellular responses increased several-fold. The CD8 response was not affected or slightly decreased. Competition for Ag between the presenting cells and the neutrophils, as well as an effect on the response to Ag-bearing dendritic cells (DCs), was documented. Neutrophils entered the draining lymph nodes rapidly and for a brief period of several hours, localizing mainly to the marginal sinus and superficial cortex. There they established brief contact with DCs and macrophages. Moreover, neutrophils imprinted on the quality of the subsequent DC–T cell interactions, despite no physical contact with them; by intravital microscopy, the clustering of Ag-specific T cells and DCs was improved in neutropenic mice. Thus, neutrophils are obligate cells that briefly enter sites of immunization and set the level of Ag presentation. A brief depletion may have a considerably positive impact on vaccination. The Journal of Immunology, 2010, 185: 2927–2934.

Neutrophils are essential effector cells in acute infections associated with a wide number of microorganisms. They are well established as the major effectors in infections with extracellular bacteria, but they also play a role in controlling infections with intracellular bacteria, viruses, fungi, and parasites (1–3). Neutrophils kill or inhibit the growth of many of these organisms through their generation of reactive oxygen species (ROS) and/or microbicidal peptides (4). Whether neutrophils during infection influence T cell responses independent of their microbicidal effect has been difficult to firmly establish because of the ensuing infection. Although with most infectious diseases neutrophil depletion results in their exacerbation, in a few instances improvement has been shown (5), suggesting a regulatory role (6–9). As an example, in the Listeria monocytogenes model in which neutrophils have a major effect on the course of the infection, evidence exists that their depletion affected the degree of CD8 T cell priming (10). It is known, mostly by ex vivo experiments, that neutrophils migrate to the lymph nodes (11, 12), release cytokines and intracellular enzymes (13–16), degrade mRNAs, that neutrophils migrate to the lymph nodes (11, 12), re-

Materials and Methods

Mice

All mice were bred and maintained under pathogen-free conditions at Washington University (St. Louis, MO), in accordance with institutional animal care guidelines. C57BL/6 (B6), B10. BR, and C.B-17 mice (H-2 haplotype) were obtained from The Jackson Laboratory (Bar Harbor, ME). B10.BR mice having NOX2−/− or iNOS−/− were generated from B6 mutant mice (21). C57BL/6, G-CSFR−/− mice were generated and obtained from the laboratory of Daniel Link (Washington University). LysM-eGFP were generated by Faust et al. (22) and CD11c-eYFP by Lindquist et al. (23). All mice were crossed to B10.BR background. LysM-eGFP/CD11c-eYFP double reporter mice were the F1 heterozygote. B10.BR mice bearing a membrane form of hen egg white lysozyme were generated in our laboratory (24). C.B-17 mice were originally purchased from Taconic Farms (Germantown, NY) and maintained at the animal facility of Washington University.

Ag

Hen egg white lysozyme (HEL) was obtained from Sigma-Aldrich (St. Louis, MO) and purified by affinity chromatography to remove <3% of contaminant proteins. Purified HEL contained <0.1 EU/lg LPS. Recombinant listeriolysin O (LLO) protein was generated and purified as previously described (25). OVA protein was from Worthington Biochemical (Lakewood, NJ). Peptides were synthesized by 9-fluorenylmethoxycarbonyl techniques and verified by mass spectrometry: HEL:48–62 (DGSTYDGILQINSRW), HEL:20–35 (YRGYSLGNWVCAAKFE), HEL:31–47 (AAKFESNFTQATRNRT), HEL:114–129 (RCKGTVDQAWIRGRL), HEL:114–129 (RCKGTVDQAWIRGRL), LLO:91–99 (GYKDGNFYI), LLO:188–201 (RWNEKMQAYPNNVS), OVA:323–339 (ISQAVHAAHAEINEAGR) and OVA:257–264 (SINFEKL).

Immunization and cellular assays

Mice were injected i.p. with 250 μg RB6-8C5 (2) or 1A8 (BioXCell, West Lebanon, NH) mAbs or with isotype control rat IgG (Sigma-Aldrich) before immunization. Protein Ag was emulsified with CFA or IFA or alum and injected into the footpad. T cell responses were measured, usually at day 7 of immunization, using ELISPOT analysis in which popliteal lymph nodes were harvested for single-cell suspension and challenged with individual proteins or peptides in vitro.

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Abbreviations used in this paper: DC, dendritic cell; HEL, hen egg white lysozyme; LLO, lysteriolysin O; mHEL, membrane form of hen egg white lysozyme; ROS, reactive oxygen species.

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For Ag presentation assays, popliteal lymph nodes were harvested and digested with Liberase TL (Roche, Basel, Switzerland) at 37°C. Single-cell suspension was negatively selected with anti-CD90.2 magnetic beads (Miltenyi Biotec, Auburn, CA) and used as a base for the following treatment: DC was separated with anti-CD11c magnetic beads; B cells were separated by anti-CD19 magnetic beads; macrophages were obtained by excluding CD3+, CD11c+, and CD19+ cells. Each single fraction was fixed with 1% paraformaldehyde and tested on T cell hybridoma 3A9 reactive to HEL 38-52.

In experiments transferring APCs, B10. BR, or transgenic mice expressing a membrane form of HEL (hmHEL) were i.p. injected with 10 μg Flt3 ligand for 3 consecutive days. At day 8, spleens were harvested and digested with collagenase D (Sigma-Aldrich) for single-cell suspension. DCs were enriched with anti-CD11c magnetic beads. For macrophages, splenic cells were negatively selected with anti-CD11c magnetic beads, followed by positive selection with anti-CD11b magnetic beads. Cells were stimulated with 10 ng/ml LPS for 1 h at 37°C, followed by extensive washing. Cells (5 × 10^9 – 1 × 10^10) were transferred into the footprint of neutrophil-depleted or -undepleted B10. BR recipient mice. After 3 h of cell transfer, IFA was injected into the footprint to induce neutrophil migration. T cell response was measured by ELISPOT at day 7 of cell transfer.

**Serum Ab assay**

The serum Abs were detected by ELISA. ELISA plates (Nunc, Roskilde, Denmark) were precoated with 10 μg/ml HEL or peptides in sodium bicarbonate buffer (pH = 8.8) overnight at 4°C. Plates were blocked with 1% BSA for 1 h at room temperature and washed with 0.05% Tween 20. Serial diluted serum was added and incubated for 2 h. After washing, secondary Ab (goat anti-mouse IgG-peroxidase 1:5000) was incubated for 1 h and the plates were developed with 1 mM 2-amino-bis(3-ethybenzthiazoline-6-sulfonic acid) in citrate buffer with 0.03% H2O2 (Roche). The absorbency was measured at 405 nm.

**Microscopy techniques**

Popliteal lymph nodes were embedded with Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) on dry ice, and 5-μm frozen sections were obtained using a Cryostat (Microm, Walldorf, Germany). Immunofluorescence staining was performed by applying fluorescent dye-conjugated Abs onto the sections for 30–60 min at room temperature. Images were captured at 4× or 10× magnification using an Olympus (Melville, NY) BX51 microscope. For confocal microscopy, images were captured using a Zeiss (Oberkochen, Germany) 510 laser scanning confocal microscope. Histological sections of lymph nodes stained by H&E were prepared by standard histological techniques.

For autoradiography studies, HEL was labeled with 125I (Amersham Biosciences/GE Healthcare Biosciences, Uppsala, Sweden), using the chloramine-T method to the activity of 5.7 × 10^9 cpm/μg. B10. BR mice were injected with 130 μg 125I-HEL (7.4 × 10^9 cpm/μg) in CFA into the footpad for 1 h. Individual cell subsets from popliteal lymph nodes were prepared as described above. The radioactivity of each cell subset was determined using an automatic γ counter (PerkinElmer, Wellesley, MA).

For autoradiography analysis, cells were spun onto slides that were then coated with NTB liquid emulsion (Eastman Kodak, Rochester, NY), dried, and exposed at 4°C for variable periods. Development was performed with Kodak D19 developer, followed by fixation (Eastman Kodak). Cells were counterstained with the Hema3 stain set (Fisher Scientific, Middletown, VA). In different experiments, the localization of HEL in the lymph nodes was carried out using HEL labeled with HiLyte Fluor 555, using a protein labeling kit (AnaSpec, Fremont, CA).

For imaging of DC–T cell interactions in intact lymph nodes by two-photon microscopy, splenic T cells isolated from 3A9 mice (CD4 T cell Isolation Kit, Miltenyi Biotec) were labeled with 10 mM red CMTPX (Invitrogen Molecular Probes, Carlsbad, CA) at 37°C for 30 min and transferred i.v. into LysM-eGFP/CD11c-eYFP mice 18 h before 10 nmol HEL/IFA immunization. Popliteal lymph nodes were harvested at 3–10 h after immunization, stabilized on a plastic coverslip, and placed in a flow chamber and maintained at 37°C by perfusion with warm media, bubbled with a mixture of 95% O2 and 5% CO2. Images were acquired using an Olympus BX51WI fluorescence microscope equipped with a 20× objective (Olympus) controlled by ImageWarp software (BitFlow, Woburn, MA). Chameleon Ti:Sapphire laser (Coherent, Santa Clara, CA) was used to excite the sample at 890 nm. For detection, eGFP was collected below 510 nm (viewed as cyan), eYFP was collected between 510 and 560 nm (viewed as green), and CMTPX was collected above 560 nm (viewed as red). Tissue volumes of 200 × 220 × 50 μm were captured every 27 s by acquiring 21 sequential 2.5-μm Z-steps, each with 0.5-s averaging to increase signal contrast. Typically, recordings lasted between 30 and 40 min.

Multidimensional rendering and three-dimensional cell tracking were performed using Imaris software (Bitplane AG, Zurich, Switzerland). Automated tracking was manually checked for accuracy. DC–T cell contact analysis was performed manually on three to five individual videos acquired from independent experiments. At least 100 cells in each single field were counted. DC–T cell contacts were observed in three-dimensional images in which two cells were tightly apposed, meaning there were no black pixels between the cells. The total number of DCs and T cells, as well as the number of DC–T cell contacts, was counted every 5 min in the videos.

**Statistical analysis**

All measurements were presented as mean ± SD. Statistical analysis was processed by the Mann-Whitney U test, using Prism 5.02 software (Graph-Pad, San Diego, CA). In all figures, the statistical significance of p values < 0.05 is indicated by asterisks.

**Results**

**Influx of neutrophils into draining lymph nodes after immunization**

Administering the proteins in any of the three adjuvants (CFA, IFA, or alum) resulted in neutrophils already appearing in the draining lymph node by 15 min after immunization, reaching their peak number within an hour, and then decreasing. By 24 h, their presence was barely more than the normal number found in lymph nodes. The presence of neutrophils at the early times was readily documented by standard histological sections. The majority of neutrophils accumulated in the cortical sinus and in the superficial cortex of the draining lymph nodes, not in the distal lymph nodes. Fig. 1A shows the results of immunization with HEL in CFA, with the lymph nodes examined by immunofluorescence. HEL, the Ag most extensively examined, accumulated in the cortical sinus, mainly visible in the macrophages lining the sinus (Fig. 1B).

The presence of neutrophils in the cortical sinuses indicated that they entered by way of different lymphatics, pointing to a pathway from blood to the site of adjuvant injection to the lymphatics and into the draining node.

Quantitation by flow cytometric analysis also confirmed an early wave of neutrophil migration in the draining lymph nodes (Fig. 1C). (The extent of infiltration was 40–50% less when immunizing with IFA or alum, compared with CFA (data not shown).) By confocal microscopy, during 2 h after immunization, many neutrophils contacted and surrounded DCs (Fig. 1D).

The dynamics of neutrophils and DCs in intact lymph nodes was examined in LysM-eGFP/CD11c-eYFP dual mice (22, 23) by two-photon microscopy. In the immunized lymph node at the cortical sinus, but much less in the underlying superficial cortex, the neutrophils and DCs were in contact during the first 2 h post immunization (Fig. 1E and Supplemental Video 1). In these areas, multiple neutrophils swarmed around a single DC and made primarily brief cell contacts, frequently moving from one DC to another within a limited time of contact (Supplemental Video 2), ranging from 1 to 3 min. These findings of neutrophils swarming on DCs are similar to those from Robey’s group, who examined lymph node postinfection with Toxoplasma gondii (11).

**T cell and Ab responses after neutrophil depletion**

Effects of the early neutrophil infiltrate were examined in mice depleted of neutrophils with Abs, or in G-CSF–gene-deficient mice, which are neutropenic. The neutrophil-depleting Abs, either RB6-8C5 or 1A8, were administered to mice immunized with the proteins in adjuvants. An i.p. injection with either of the Abs resulted in ~90% depletion of neutrophils, but did not affect other cell types from peripheral blood or popliteal lymph nodes (data not shown). The mAb RB6-8C5 reacts primarily with Ly6G+ cells (i.e., neutrophils) but also has weak reactivity with Ly6C,
found in some monocytes (26, 27). The Ab 1A8 is Ly6G specific (28). Results with either Ab were identical. Most experimental manipulations compared both Abs.

Many of the experiments examined the response to HEL. At different doses, depletion of neutrophils consistently enhanced by 100% or more the CD4 T cell responses measured by IL-2 or IFN-γ ELISPOT. (Fig. 2A summarizes the various experiments; most of them show the IL-2 response.) The increase in CD4 T cell response was found when HEL was incorporated in CFA (i.e., water in oil with dead Mycobacteria, as in Fig. 2B) or when given just in water or in oil (IFA) or in alum (Fig. 2C, 2D, respectively). Enhancement was also found in assaying the proliferation of lymphocytes to HEL (data not shown).

The increase in CD4 T cell responses was found among the major HEL peptides previously identified (Fig. 2). In mice expressing the class II MHC molecule I-Ak, the CD4 T cell response to HEL is equally focused, mainly on four peptides expressed in very different amounts by APCs (24, 29, 30). For example, the chemically dominant peptide centered on the 52–60 sequence was highly expressed, ~200-fold more than a minor peptide centered on the 20–35 sequence, yet the number of T cells was the same for each. In seeking an explanation to this result, we considered whether neutrophils could affect the relative response to the different epitopes. This was not the case, as all responses were increased about the same extent after neutrophil depletion. Analysis of a pool of 25 experiments showed the increase in T cell response to each epitope after neutrophil depletion to be as follows: 2.9-, 2.7-, 3.2-, and 2.3-fold increases for HEL:48–62, 20–35, 114–129 and 31–47, respectively. Peptides HEL:70–88 and HEL:97–112, which are very weak epitopes, are increased 2-fold after neutrophil depletion (data not shown). In two experiments, mice immunized with the HEL:48–61 peptide also
had a 2- and 2.5-fold increase in the ELISPOT response after neutrophil depletion. This result indicates that enhancement of the HEL response in neutropenic mice was not related to an effect of neutrophils on HEL processing. The involvement of neutrophils in CD4 T cell priming also reflected negatively on the memory response. Mice were treated with the neutrophil-depleting Ab, immunized with HEL, and rested for 2 wk, after which a boost was given of HEL in IFA and the ELISPOT response was evaluated at the third week of immunization. In two experiments the response of the Ab-treated mice was increased by 150 and 200% over the control mice.

The impact of neutrophils was evaluated in the response to OVA and LLO, tested by ELISPOTs on CD4 or CD8 epitopes. In OVA immunization, in vitro restimulation with the OT-II epitope OVA:323–339 resulted in enhanced CD4 T cell responses after neutrophil depletion in all six experiments. However, the extent of recall responses against the OT-I epitope OVA:257–264 (SIINFEKL) remained unaffected (in three of six experiments) or reduced slightly by ∼20–30% (in three of six experiments) compared with the control Ig-treated mice (Fig. 2E, 2F). Similar results were shown in LLO immunization in C.B-17 mice. Production of both IL-2 and IFN-γ by CD4 T cells was significantly enhanced after neutrophil depletion in the six experiments; however, the CD8 T cell response was enhanced only in one, whereas the remaining five showed a slight reduction (Fig. 2G, 2H).

In addition to examining mice depleted of neutrophils by Abs, G-CSFR−/− deficient mice also were tested in their response to HEL. G-CSF is critical in stimulating granulopoiesis (31, 32) mediated through interaction with its receptor (G-CSFR) (33). Decreased levels of circulating neutrophils are found in G-CSFR−/− deficient mice (34). A higher CD4 T cell response to HEL was found in G-CSFR−/− deficient mice, compared with wild-type mice treated with control Ig or neutrophil-depleting Ab. As would be expected, neutrophil-depleting Ab given to G-CSFR−/− deficient mice did not result in further enhancement, an important result indicating that in their absence, the neutrophil-depleting Ab had no effect (Fig. 3A). Thus, examining G-CSFR−/− deficient mice confirmed the findings of an enhanced CD4 T cell response by neutrophil depletion with Ab.

In addition to the first early wave of neutrophil influx in the draining lymph nodes after immunization with adjuvant, there was a second wave of neutrophils at day 3 of immunization (Fig. 3B). However, the enhancing effect of neutrophil depletion was restricted to the first 24 h: Their depletion by Abs after the first day of immunization did not change the magnitude of the response (Fig. 3C). Thus the negative effect of neutrophils was limited to the initial period after immunization when Ag capture and presentation events are prominent.

Whether the immunosuppression by neutrophils resulted from the release of ROS or NO by the neutrophils was evaluated. ROS and NO might diffuse into the neighboring T cells or APCs and affect their response (21, 35). NADPH oxidase- and iNOS-deficient mice depleted of neutrophils still showed an enhanced T cell response (Fig. 3D), implying that neither ROS nor NO was involved in neutrophil suppression of T cell responses. Enhancement of the response was also found in μMT mice, indicating that B cells were not involved (Fig. 3E). In experiments not shown, we discarded the involvement of IL-10 because the enhanced HEL response after neutrophil depletion was not affected in IL-10−/− deficient mice or by neutralization by Abs to IL-10R.

The Ab response to HEL was examined after neutrophil depletion. The peak time of the response was at the second week after immunization. An increase in titer was found after neutrophil depletion in all three experiments (Fig. 4A–C). The isotypes of serum Abs were IgG1, IgG2b, and IgG3 in either treated or control untreated mice. We had found in experiments that most of the anti-HEL response was directed to conformational determinants of HEL, whereas the Ab response to HEL peptides was weak or not detectable (C.-W. Yang and E.R. Unanue, unpublished observations). In the case of HEL-immunized mice not treated with the anti-neutrophil Abs, there was a very weak response to the chemically dominant 48–62 peptide, although consistently all mice responded to the 114–129 peptide (Fig. 4D–F). The responses to the 48–62 and 114–129 peptides were enhanced in the neutrophil-depleted mice. In only one of three mice did we find a small response to the 20–35 and 31–47 peptides in neutrophil-depleted mice (Fig. 4F).

**Ag presentation after neutrophil depletion**

Competition for HEL between neutrophils and APCs could explain the enhancement of T cell responses by neutrophil Ag presentation. Ag presentation was examined in lymph node APCs from untreated and neutrophil-depleted mice at different time points after immunization with HEL. The cells were isolated, fixed, and then tested for their content of HEL peptide–MHC complexes by the response of a T cell hybridoma (3A9) to the dominant epitope 48–62 peptide, although consistently all mice responded to the 114–129 peptide (Fig. 4D–F). The responses to the 48–62 and 114–129 peptides were enhanced in the neutrophil-depleted mice. In only one of three mice did we find a small response to the 20–35 and 31–47 peptides in neutrophil-depleted mice (Fig. 4F).

**FIGURE 3.** Neutrophil effect in mice deficient in G-CSFR, ROS, NO, or B cells. G-CSFR−/− (A), NOX2−/−, iNOS−/− (D), or μMT (E) mice were immunized with 10 nmol HEL in CFA; groups were treated with either control Ig or neutrophil-depleting Ab, as indicated. B, B10.BR mice were injected with either control IgG or neutrophil-depleting Ab 1 d before immunization with 10 nmol of HEL in CFA. Popliteal lymph nodes were harvested for single-cell suspension, and the number of neutrophils (CD11b+ Ly-6G+) was determined by FACS analysis. (n = 4 in each group). C, B10.BR mice were injected with neutrophil-depleting Ab on the indicated day. The number of cells producing IL-2 was determined by ELISPOT at day 7 after immunization. The data represent mean ± SD for a representative experiment from five individual experiments. Data analysis was processed by the Mann-Whitney U test, *p < 0.05.
The increase in presentation after neutrophil depletion occurred in both CD11c+ and CD11c− cells. In control Ig-treated mice, the major APCs for HEL were CD11c+ cells (Fig. 5D), in agreement with our previous findings (37). The relative enhancement was particularly evident among the CD11c− cells (Fig. 5D) and, specifically, the macrophages (CD11c− CD19−) (Fig. 5E). Ag presentation in B cells was low in both neutrophil-depleted and -undepleted mice (Fig. 5F). Together these results indicate that there was a reduction in the content of the HEL peptide–MHC complex by APCs when neutrophils infiltrate the lymph nodes.

Examination of the number of cells in the lymph nodes bearing HEL confirmed the functional results. About 4% of isolated DCs contained HEL by autoradiography examination when 125I-HEL was administered to the mice with CFA (Fig. 5G, 5H). This number approximately doubled in the neutrophil-depleted group (Fig. 5H). This number was found in CD11b+ cells isolated from the nodes. However, the number of autoradiograph grains per APC was similar in both neutrophil-depleted and control groups. The autoradioactivity count of DCs increased by 2.4-fold after neutrophil depletion (627 cpm/10⁶ cells in the control Ig-treated group versus 1488 cpm/10⁶ cells in the neutrophil-depleted group). In control mice, 10% of the neutrophils contained HEL (Fig. 5H).

**Effect of neutrophils on Ag-bearing APCs**

Aside from reducing the amount of HEL available to APCs, neutrophils could also interfere with the presentation by APCs already bearing HEL. To functionally test whether neutrophils were affecting Ag presentation by Ag-bearing cells, DCs or macrophages from transgenic mice in which HEL was expressed on the cell membrane (mHEL) (24) were transferred into neutrophil-depleted or -undepleted mice. The CD4 T cell response was evaluated in conditions where neutrophil migration into the lymph node was induced by injection of IFA (without HEL). At day 7 of cell transfer, mHEL DCs or macrophages elicited a CD4 T cell response in control Ig-treated recipient mice. In neutrophil-depleted mice, mHEL DCs or macrophage transfer resulted in ~100% enhanced CD4 T cell response (Fig. 6). These results demonstrated that neutrophils had a negative effect also by influencing the presentation of Ag-bearing DCs and macrophages.

**Dynamic imaging of DC–T cell interactions in the absence of neutrophils**

Examining lymph nodes by two-photon microscopy substantiated these results. In these experiments, LysM-eGFP/CD11c-eYFP dual transgenic CD4 T cells recognizing the dominant 48–62 epitope of HEL (3A9) were adoptively transferred with labeled transgenic CD4 T cells into neutrophil-depleted or -undepleted mice. The CD4 T cell response was evaluated in conditions where neutrophil depletion (627 cpm/10⁶ cells in the control Ig-treated group versus 1488 cpm/10⁶ cells in the neutrophil-depleted group). In control mice, 10% of the neutrophils contained HEL (Fig. 5H).

**FIGURE 4.** Ab responses after neutrophil depletion. Control Ig or neutrophil-depleting Ab-treated B10.BR mice were immunized with 10 nmol of HEL in the various adjuvants, as indicated in A–C. At day 14 of immunization, sera were collected for ELISA analysis of Ab response. D–F show anti-peptide responses from three individual experiments (n = 8–10). Sera from the various groups were pooled and titrated against an anti-HEL standard; the titer in μg/ml of sera in control versus anti-neutrophil Ab-treated mice were as follows: in A, 9.9 versus 57.6; in B, 16.3 versus 64.2; and in C, 3.7 versus 55.5. Data analysis was processed by the Mann-Whitney U test. *p < 0.05.

**FIGURE 5.** Ag presentation after neutrophil depletion. B10.BR mice were immunized with 10 nmol HEL in CFA after injection of control Ig or neutrophil-depleting Ab. Popliteal lymph nodes were harvested at 3 h (A), 16 h (B), and 24 h (C–F) after immunization. Single-cell suspensions from whole lymph nodes (A–C) or indicated cell subsets separated by MACS (D–F) were fixed with 1% paraformaldehyde and tested on fixed LN cell number. Data were obtained from three to six individual experiments, and shown is one representative result presented as mean ± SD (n = 5–10). *p < 0.05.
and immunized with HEL. Pilot experiments established that 6–10 h later interactions between the T cells and the DC were prominent. This was a time subsequent to the fast influx of neutrophils. DC–T cell interaction was observed in the cortical areas of draining lymph nodes, just below the cortical sinus (Fig. 7A). At these times, in control mice not given the neutrophil-depleting Abs, neutrophils were rarely found next to or attached to the DCs or to the DC–T cell clusters. DC–T cell contacts were found in both treated and untreated mice, but they were markedly improved in neutrophil-depleted mice. T cell velocity was similar in both control Ig and neutrophil-depleting Ab-treated mice (Fig. 7E); however, T cells showed more biased movement in the absence of neutrophils (Fig. 7F). The number of DC–T cell contacts increased ∼150% after neutrophil depletion (Fig. 7C). The contact period also increased (Fig. 7B, 7D). Together these results indicated improved DC–T cell interactions after neutrophil depletion, suggesting that in the normal mouse, neutrophils affect or imprint the DC–T cell interaction without having a direct physical blockade of it.

Discussion

In brief, in a conventional in vivo immunization system a strong negative role for neutrophils was found in the adaptive CD4 T cell and B cell responses. The negative effect of neutrophils was shown by depleting with either of two Abs or in neutropenic mice lacking the G-CSFR. Very rapidly after immunization, neutrophils migrated into the draining lymph nodes, by all indications through the lymphatics draining the site of adjuvant inoculation. The very fast migration suggests the recruitment of neutrophils from blood and the marginal pool, but the mechanisms involved and the initiating molecules need identification. The point is that the neutrophil early response was an obligate component of the inflammation triggered by adjuvants, including not only the water in oil preparations but also those with alum.

The immunosuppressive effect of neutrophils was restricted to the first 24-h entry, pointing to an effect on presentation of the Ag. First, it was clear that the level of HEL entering the APC system was in part controlled by neutrophils, pointing to a competition for HEL between them and APCs. In the absence of neutrophils, more APCs contained HEL, correlating with enhanced presentation. The dampening effect of neutrophils was more marked in macrophages, perhaps because of anatomical considerations, in that the major site of accumulation of neutrophils was in the cortical sinus where macrophages took up the entering Ag (38, 39).

Aside from Ag competition, neutrophils affected presentation by both DCs and macrophages already having peptide–MHC complexes. Notably, in different experimental setups, T cell contacts with DCs in the superficial cortex were enhanced in the neutrophil-depleted mice, yet there was no evidence of a physical interaction with neutrophils that could have blocked the interaction. Neutrophils mostly surrounded DCs in the cortical sinus and superficial cortex during the first hours following immunization (11). In culture, evidence was provided of DC–neutrophil interactions mediated by SIGN-R1 (18). These findings suggest that neutrophils leave an imprint on the DC–lymphocyte interaction, the nature of which we are now examining. This result could have one or more explanations. There could be a reduction in key Ag-presenting molecules resulting from initial interactions between DCs and neutrophils that preceded those between DCs and T cells; a mechanism such as trogocytosis could be involved. In the cortical sinus, the contacts of neutrophils likely involved migratory DCs moving in

**FIGURE 6.** Effect of neutrophils on Ag-bearing APCs. Splenic DCs or macrophages were isolated from mHEL or B10.BR mice by MACS and transferred into the footpad of neutrophil-depleted or -undepleted B10.BR recipients. At 3 h after cell transfer, IFA was injected into the footpad to induce neutrophil migration. CD4 T cell response was measured by IL-2 ELISPOT analysis at day 7 of cell transfer. Shown are representative data from seven individual experiments presented as mean ± SD (n = 8). Data analysis was processed by the Mann-Whitney U test. \( *p < 0.05 \).

**FIGURE 7.** Improved DC–T cell interaction in the absence of neutrophils. LysM-eGFP/CD11c-eYFP mice were adoptively transferred with CMTPX-labeled 3A9 transgenic T cells and immunized with 10 nmol HEL/IFA in the footpad. Popliteal lymph nodes were removed for two-photon microscopy. A and B, Contacts between DCs (green) and T cells (red) were visualized in the cortex area 6–10 h post immunization. Arrows indicate the areas of DC–T cell contact. Scale bars, 20 \( \mu \)m (A) and 10 \( \mu \)m (B). Statistical analysis of three to five videos acquired from individual mice (C–F). Nondraining lymph nodes were used as the no Ag control. Three-dimensional tracking of T cells was performed using Imaris software (E, F). Cell contact analysis was performed manually (C, D). Shown are representative (A, B) or pooled (C–F) data from three individual experiments (n = 6–10). Data analysis was processed using the Mann-Whitney U test and presented as mean ± SD (C–F).
from the immunization site. In the superficial cortex, an involvement with resident DCs should also be taken into account. Whether the resident DC or the migratory DC is more affected by neutrophils was not evaluated. Noteworthy are the results in Fig. 6: Experiments transferring HEL-bearing APCs indicated a negative effect by neutrophils. We are also considering an influence on the biology of DCs by neutrophil-released molecules, and have at this time eliminated ROS, NO, and IL-10. Lipid mediators or other cytokines need to be evaluated. Whether the content of HEL per DC explains the result is unlikely, based on the autoradiograph experiment, which indicated more Ag-bearing APCs but about the same content of HEL per cell.

Negative effects by neutrophils aside from those on Ag presentation are not evident from these results. The finding of a narrow time limit in which neutrophils participated (i.e., the first hours) suggests that late effects, such as those involving neutrophil apoptosis and their uptake by phagocytes, are not major in the framework of these responses (40). Whether there is redistribution of other cells in the lymph nodes in the absence of neutrophils (41) is under evaluation.

One final point is that the CD8 T cell response was not enhanced, or was affected only to a small degree, confirming that the presentation of soluble Ags in vivo for CD4 and CD8 T cells involves distinct cellular intermediates and pathways. Evidence has been presented that protein Ags in neutrophils can eventually enter the class I processing pathways, most likely via cross-presentation following their apoptotic death (10, 42–45). Some of these studies are affected only to a small degree, confirming that the presentation of HEL per DC explains the result is unlikely, based on the autoradiograph experiment, which indicated more Ag-bearing APCs but about the same content of HEL per cell.

Whether there is redistribution of other cells in the lymph nodes in the absence of neutrophils (41) is under evaluation.

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References


Online supplemental material (video 1 and 2)

Title: Dynamic imaging of neutrophils and DC in draining lymph nodes

For in situ tracking DC and neutrophil interaction, LysM-eGFP/CD11c-eYFP mice were immunized with 10nmole HEL/IFA in the footpad and popliteal lymph nodes were removed for two-photon microscopy. Contacts between DC (green) and neutrophils (blue) were visualized in the cortical sinus at 2 hours post-immunization. 3D time-lapse microscopic images (200x220x50μm tissue volume) were acquired every 27 seconds. The movies are ~7 minutes and 13 minutes in duration for video 1 and video 2, respectively. These videos are related to Figure 1E.