PRESENCE OF NEUTROPHIL BEARING ANTIGEN IN LYMPHOID ORGANS OF IMMUNE MICE

**Short title:** OVA-FITC⁺ neutrophils in secondary lymphoid organs

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This work was supported by grants from the Agencia Córdoba Ciencia, Secretaría de Ciencia y Técnica de la Universidad Nacional de Córdoba, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP # 02962). M.C.P.P., V.G.M. and A.S.R. are career members of CONICET. D.O.A. and M.V.L. are recipients of graduate fellowships from CONICET.

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**Word Count:** 4943

**Abstract word count:** 134

**Scientific categories:** Immunobiology
ABSTRACT

Neutrophils play a crucial early role during the innate response, but little is known about their possible contribution when an adaptative immune response is installed. A robust neutrophilia and a Th1 immune response are present after immunization with Complete Freund’s Adjuvant (CFA). We show that when FITC-labeled OVA was injected into the footpad of OVA/CFA immunized mice, the main OVA-FITC⁺ cells recruited in draining popliteal lymph nodes (LN) were neutrophils, with most of them arriving at the LN by means of lymphatic vessels. The development of this OVA-FITC⁺ neutrophil influx requires an immune response against OVA. The OVA-FITC⁺ neutrophils present in LN displayed mainly intracellular TNF-α and their depletion resulted in an increase in the specific IL-5 levels. These data provide new evidence about the role played by neutrophils in vivo in adaptative immunity.
INTRODUCTION

It is generally known that neutrophils (Ne) play a key role in innate immune defence. They are present in the bloodstream, and help in recognizing and neutralizing microorganisms. By being able to adhere to the blood vessel walls at sites of tissue damage, they transmigrate through endothelial cells to the infection site, where they are stimulated to phagocytose and kill microorganisms. This accumulation of leukocytes forms the first step in immune surveillance and plays a key role in innate immunity to infection. On the other hand, in recent years some reports have described the involvement of Ne in directing T-cell polarisation to the type of pathogens occurring during an infection process. However, it is still unclear whether this is a common phenomenon in other situations, such as in models with inflammation but without infection.

In previous studies, we have observed differences in the behavior of the immune system, in terms of intensity and quality of humoral and cellular responses, depending on the adjuvant used. In the case of Complete Freund’s Adjuvant (CFA), a Th1 immune response was induced, and in recent reports we have observed a high hemopoiesis in the spleen, along with an expansion of myeloid CD11b+ cells and an important neutrophilia when CFA was used as the adjuvant. However, this scenario was not present when synthetic oligodeoxynucleotides containing CpG motifs and Bordetella pertussis were used as adjuvants. Therefore, taking into account that there was an increase in the number of neutrophils in the blood, we decided to explore the possible contribution of Ne in this model of immune inflammation when an immune response is taking place. With this objective, we injected the antigen into the footpad of previously immunized animals, and analyzed the cells involved in antigen capture in the skin and lymphoid organs. Interestingly, we found that not only did Ne uptake antigen in skin, but also that they...
comprised the main population of cells bearing the antigen in lymphoid organs. The influx of Ne was present only if there was a specific immune response occurring, in which case they drained popliteal lymph nodes (LN) through the lymphatic vessels, and remained there for at least 48 hours, before undergoing apoptosis. Moreover, Ne in lymph nodes mainly expressed TNF-α and contributed to the quality of the established secondary immune response. Our results emphasize the involvement of Ne as cells not only involved in innate immune responses, but also in adaptative ones.
MATERIALS AND METHODS

Mice
Female 8- to 12-week-old BALB/c mice were used. Mice were originally obtained from the Comisión Nacional de Energía Atómica (CNEA), Argentina. The Institutional Experimentation Animal Committee (authorization # 15-01-44195) approved animal handling and experimental procedures.

Immunization
Mice were s.c. immunized on the base of the tail and in the neck region on days 0, 15 and 30 with 0.5 ml of albumin, chicken egg (OVA), Keyhole Limpet Hemocyanin (KLH) or PBS emulsified in CFA (60µg OVA or KLH/per animal/per dose) (OVA/CFA, KLH/CFA or PBS/CFA). OVA and CFA were both from Sigma-Aldrich Argentina S.A. (Buenos Aires, Argentina). KLH was obtained from Pierce Biotechnology (Rockford). Mice were injected in the hind footpad with 0.1 mg of OVA labeled with FITC (OVA-FITC; mol wt 45,000; Molecular Probes, Eugene, OR), KLH was conjugated with FITC following a previously described protocol, PBS or immune complexes.

Immune complex formation
To produce OVA-anti OVA antibody complex, 50 µl of OVA-FITC (0.75 mg/ml) was pre-incubated for 30 min at 37°C with 100µl of plasma from OVA/CFA mice (diluted 1:2 in sterile PBS). To eliminate free antigen, we used Microcon centrifugal filters (Millipore, Billerica, USA).
Cell preparation

Samples of blood were collected by periorbital bleeding. Lymph nodes and spleen were collected in RPMI 1640 medium supplemented with 2% heat-inactivated FCS (Natocor, Córdoba, Argentina). Erythrocytes from spleen and peripheral blood were removed by RBC lysing buffer (Sigma-Aldrich). A single cell suspension of lymph nodes and spleen was prepared by gently pressing the organs through a stainless-steel mesh. In all cases, cell viability was >95%, as determined by Trypan Blue dye exclusion. In some experiments, LN were treated for 45 minutes at 37°C with 400 U/ml collagenase D and 50 µg/ml DNase I (Roche Diagnostics GmbH, Mannheim, Germany) in RPMI 1640 medium. After inhibition of collagenase activity with 6mM EDTA in PBS, cell suspension of lymph nodes was obtained as indicated above.

Cell cultures

Cell suspensions were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 50 µM 2-ME (Sigma-Aldrich) and gentamicin (40 µg/ml) (Schering-Plough S.A., Lomas del Mirador, Argentina) at 37°C in a 5% CO₂ humidified incubator. Cell suspensions were cultured with medium alone or with OVA (100 µg/ml).

Antibody assays

Specific antibodies against OVA were determined by ELISA. Briefly, 96-well flat-bottom plates were coated and incubated overnight at 4°C with OVA (1µg/well) in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6). Plates were then blocked with PBS containing 0.5%
gelatin for 1 hour at 37°C. After washing, the plates were incubated for 1 hour at 37°C with plasma samples diluted in PBS with 0.05% Tween containing 0.5% gelatin. For IgG detection, plates were incubated with HRP-conjugate anti-mouse IgG (Sigma-Aldrich). Plates were examined on a Bio-Rad Model 450 microplate at 490 nm after incubation with H2O2 and o-phenylenediamine.

**Cytokine-specific ELISA**

Levels of IFN-γ and IL-5 were measured in culture supernatants by capture-ELISA following instructions from the manufacturer (BD Pharmingen, San Diego, CA). The following antibodies were used for coating and detection respectively: R4-6A2 and XMG1.2 clones for anti-IFN-γ; TRFK5 and TRFK4 clones for anti-IL-5. Values of IFN-γ and IL-5 were calculated by reference to a standard curve constructed by assaying serial dilutions of the respective murine standard cytokines.

**Flow cytometry**

The cells were first incubated with anti-CD32/CD16 (clone 2.4G2) for 30 minutes. Then, the cells were stained on the surface for 30 minutes with the corresponding phycoerytrin (PE)- or biotin-conjugated antibodies. When biotin-conjugate antibody was used, a third step involving a 30 minute incubation was performed with strepavidin-Cy-chrome. Surface staining was performed in Hanks’ balanced salt solution with 0.1% albumin bovine, 0.1% sodium azide and 0.005 mM Na3EDTA at 4 °C. Cells were analyzed on a Cytoron Absolute
cytometer (Ortho Diagnostic System, R, NJ) and the data processed using WinMDI 2.8 software (http://facs.scripps.edu. Copyright © by Joseph Trotter).

To detect intracellular cytokines, cells (1.5 x 10^6/ml) were incubated in RPMI medium 10% FCS containing 4.8 µM monensin (Sigma-Aldrich) for 3-5 hours at 37°C in a moist atmosphere of 5% CO₂ in air. After this time period, cells were washed and stained with anti-Gr-1 antibody as described above. Cells were then fixed with 4 % paraformaldehyde for 20 minutes at 4 °C, and permeabilized with Perm/Wash solution (BD Pharmingen) for 30 minutes at room temperature. Intracellular cytokines were detected by addition of a specific antibody diluted in Perm/Wash solution for 60 minutes at 4 °C. After final washes with Perm/Wash solution, cells were analyzed by flow cytometry.

Antibodies against murine CD3 (145-2C11), CD19 (1D3), CD11b (M1/70), Gr-1 (Ly-6G and Ly6-C) (RB6-8C5), I-Ad/I-Ed (2G9), CD40 (HM40-3), CD80 (16-10A-1), CD86 (GL1), CD11c (HL3), B220 (RA3-6B2), Ly-6G (1A8), TNF-α (MP6-XT22), IL-4 (11B11), IL-12 (C15.6), IFN-γ (XMG1.2), matching isotype controls and strepavidin-Cy-chrome were purchased from BD Pharmingen. Antibody anti-F4/80 (A3-1) was purchased from Caltag (Burlingame, CA). Isotype controls were obtained from BD Pharmingen or eBioscience, Inc.

Apoptosis assay

For apoptotic cell detection propidium iodide staining was performed to analyze the hypodiploid DNA peak as described previously by Nicoletti et al. Briefly, 1 x 10^6 cells were fixed overnight in 500 µl 70% ethanol at 4 °C. Cell pellets were gently resuspended in 500 µl hypotonic fluorochrome solution (50 µg/ml propidium iodide in 0.1% sodium...
citrate plus 0.3% nonidet P-40) and kept at 4 °C for 18 hours. After that, cells were analyzed as indicated above.

**Morphologic analysis of cells by cytospin**

Cell suspensions diluted in 10% FCS RPMI 1640 medium were centrifuged on microscope slides in a Cytospin apparatus (Shandon, Cheshire, UK) and stained with DAPI (0.1 µg/ml, Molecular Probes) or May-Grünwald-Giemsa (Merck, Darmstadt, Germany).

**Fluorescent microscopy**

LN and skin (footpad) were inserted into OCT embedding compound (Miles, Elkhart, IN) and snap-frozen in liquid air. Eight-µm/thick cryostat sections of tissues were sliced, air-dried, and fixed in citrate-acetone-formaldehyde at room temperature for 1 minute. Next, samples were rehydrated in PBS and mounted on glycerine. These sections and cytospin slides were observed using a fluorescence microscope. In some cases cytospin slides were examined with a Carl Zeiss LSM5 Pascal laser-scanning confocal microscope equipped with an argon/helium/neon laser and a 40X objective (Carl Zeiss, Inc.). Images were obtained and processed with the Zeiss LSM image software and Adobe Photoshop 5.0.

**Neutrophil depletion**

To deplete Ne, an anti-mouse Gr-1 antibody (RB6-8C5) was used. Mice received 0.5 mg/mouse anti-Gr-1 antibody or control rat IgG i.p. on days 37, 38, 39, 40 and 41 post first immunization. This treatment resulted in >98% depletion of Ne (Gr-1<sup>high</sup> CD11b<sup>high</sup>). On day 40, mice were injected with OVA-FITC into the footpad, sacrificed 48 hours later, had
their LN removed and the effect of Ne depletion on the immune response to OVA was evaluated.

**Statistical analysis**

The data were analyzed using a one-way ANOVA followed by Tukey’s post-test for multiple comparisons. We used the Student’s t-test on two groups. All data were considered statistically significant if $p$ values were $<0.05$. 
RESULTS

Analysis of antigen-capturing cells in lymph organs from immune mice

CFA is an adjuvant that induces an inflammatory state with a corresponding increase in neutrophils in the blood, when used with an antigen or with PBS. To analyze the possible contribution of neutrophils during the course of the immune response, we injected animals with the antigen for which they had previously been immunized, and examined the cells involved in antigen-capture in lymphoid organs, using the antigen labeled with FITC dye (OVA-FITC). To this purpose, unimmunized, OVA/CFA-immunized or PBS/CFA-immunized mice were challenged with OVA-FITC in the footpad. In peripheral blood and spleen we found OVA-FITC+ cells in OVA/CFA immunized mice (Figure 1A). In unimmunized or PBS/CFA-immunized mice injected with OVA-FITC, the percentage of OVA-FITC+ cells was very low (data not shown). In order to analyze the antigen-capturing cells in the lymphoid organ that drained the site of injection, we removed the LN. A cell population was found with a forward and side-scatter higher than that of the lymphocytes appearing in LN from OVA/CFA-immunized mice (Figure 1B). This population did not appear in either unimmunized or in PBS/CFA mice injected with OVA-FITC. Also, these cells were not encountered in OVA/CFA-immunized mice when they were injected with PBS instead of OVA-FITC. Interestingly, the proportion of these cells increased according to the number of OVA/CFA immunizations. Similar results were obtained when experiments were done with uncoupled OVA (data not shown). Thus, in blood as well as in lymphoid organs, after an antigen exposure, there were OVA-FITC+ cells with a higher side scatter than lymphocytes.
**Most OVA-FITC^+ cells in popliteal lymph nodes are neutrophils**

We then characterized the OVA-FITC^+ cells in LN. We found that most OVA-FITC^+ cells were CD11b\text{high} (Figure 2A), with very few OVA-FITC^+ cells being CD11c\text{+} (1 ± 0.5 %) or CD3\text{+} (3 ± 1 %) and with F4/80\text{high} cells not being found at all (supplemental Figure 1). Also, few OVA-FITC^+ cells expressed CD19 (Figure 2A), and considering that these cells fell within the normal lymphocyte forward scatter/side scatter gate, these cells were probably antigen-specific B cells. The population of OVA-FITC^+ CD11b\text{high} CD19\text{neg} cells had high side-scatter and they were located in the gate indicated in Figure 1B, suggesting that these cells were not lymphocytes. The discovery of CD11b^+ cells in LN after antigen injection was not surprising, taking into account that the integrin CD11b is expressed in macrophages and in some dendritic cell subsets, and it is widely recognized that these cells are able to take up antigen in the periphery and to transport it to draining lymph nodes. However, most of these OVA-FITC^+ CD11b\text{high} cells were negative for MHC class II (Figure 2A). Further phenotypic analysis showed that these OVA-FITC^+ CD11b\text{high} cells were F4/80\text{low} (20%) and CD11c\text{neg}, excluding the presence of macrophage and dendritic cells in this OVA-FITC^+ CD11b\text{high} cell population (Figure 2B). Moreover, these cells did not express co-stimulatory molecules such as CD86, CD80 and CD40.

We then analyzed the expression of Gr-1 (Ly-6G and Ly-6C) and found that most OVA-FITC^+ cells strongly expressed Gr-1 (Figure 3A) suggesting that they were Ne. In addition, Figure 3B shows that all OVA-FITC^+ Gr-1\text{high} cells are Ly-6G\text{high}. Furthermore, in cytospin preparations of LN we observed abundant cells that displayed the characteristic polylobed nuclei of Ne, while in PBS injected animals the majority of cells showed the typical nuclei
of mononuclear cells (Figure 3C). OVA-FITC was detected inside cells with polylobed nuclei as is shown by DAPI staining (Figure 3Di-ii). Confocal microscopy revealed that OVA-FITC was located in the cytoplasm of Ne which had had the surface stained by anti-Gr-1 antibody (Figure 3Diii-Civ), confirming the results observed by FACS, and excluding the possibility of cell doublets in FACS analysis. On the whole, these observations indicated that in OVA/CFA-immunized mice, most OVA-FITC$^+$ cells present in LN were Ne.

**Influx of OVA-FITC$^+$ Ne in LN increases with the number of immunizations and requires an antigen-specific response.**

During the course of the immunization we observed an increase in the percentage as well as in the mean fluorescence intensity (MIF) of OVA-FITC$^+$ Ne after the first, second or third immunizations (Figure 4A). A 2.8-fold increase in the influx of Ne occurred between days 15 and 30, and a 3.3 fold increase was presented between days 30 and 40. This increase in the influx of Ne into LN was not associated with a corresponding increase in the levels of Ne in peripheral blood during the immunization course (Figure 4B). On the other hand, in LN from unimmunized or PBS/CFA-immunized mice, where no antibodies against OVA were present, very few OVA-FITC$^+$ cells were detected (Figure 1B). We then studied whether OVA-specific antibodies can mediate Ne influx. First, we studied the kinetics and strength of OVA specific IgG responses in immunized OVA/CFA mice (Figure 4C). On day 30, OVA-antibody levels were significantly higher than those seen on day 15, and similar levels were found between days 30 and 40. Second, unimmunized and PBS/CFA immunized mice were injected in the footpad with soluble OVA-FITC or with OVA-FITC immune complexes, and six hours later the presence of OVA-FITC$^+$ cells was analyzed in
LN (Figure 4D). We encountered a higher number of OVA-FITC⁺ cells in mice injected with immune complexes than in those injected with soluble antigen, in both groups of animals. Furthermore, in PBS/CFA-immunized mice, the number of OVA-FITC⁺ cells after immune complex injections was higher than that in unimmunized mice, probably due to the higher number of Ne in blood (52 ± 2 % in PBS/CFA vs. 20 ± 5 % in unimmunized mice) or to the higher inflammatory state in PBS/CFA-immunized mice.

To test whether this Ne influx needs an antigen-specific immune response, mice were immunized with OVA/CFA or KLH/CFA and subsequently injected with OVA or KLH. A Ne population was detected in mice immunized and injected with the same antigen, whereas this population was very low in LN of mice injected with the unrelated antigen (Figure 4E). Also, in PBS/CFA immune mice injected with OVA, very few Gr-1⁺ cells were observed. Taken together, these results showed that the development of this Ne influx requires the presence of an antigen-specific response.

Localization and kinetics of neutrophil influx at the site of inoculation and in popliteal lymph nodes

In LN we observed that independent of the number of immunizations carried out, 100% of Gr-1<sup>high</sup> cells were OVA-FITC⁺ six hours after antigen injection. We then asked ourselves whether Ne took up the antigen in the periphery and then migrated to the LN, or whether the antigen arrived alone at the LN where it was then taken up by Ne. In order to answer this question, we analyzed by FACS the kinetics of Ne influx in LN over different short time periods. We found that 2 hours after antigen injection, all Gr-1<sup>high</sup> cells in LN were OVA-FITC⁺ (Figure 5A). The presence of OVA-FITC⁺ Ne in LN suggests that Ne are in
fact capturing antigen in the skin, and then migrating to LN. If this were not the case we would find Gr-1\textsuperscript{high}/OVA-FITC\textsuperscript{+} cells as well as Gr-1\textsuperscript{high}/OVA-FITC\textsuperscript{neg} cells.

In order to analyze the possibility that Ne take up OVA-FITC in skin, we performed kinetic analysis in hind footpads sections (site of inoculation). OVA-FITC\textsuperscript{+} cells were found in the dermis as little as 2 hours after the OVA-FITC injection, and their number increased 6 hours after injection, as Ne infiltrated the skin (Figure 5B). The number of Ne in skin in PBS/CFA immunized mice was comparable to that of OVA/CFA immunized mice, but few of them were OVA-FITC\textsuperscript{+} and migrated to LN, strongly indicating that uptake of antigen in skin is a pre-requisite for the migration of Ne to LN. We then analyzed the distribution of Ne in LN sections. Figure 5C shows that 2 hours after injection, OVA-FITC\textsuperscript{+} cells mainly infiltrated the subcapsular sinus, whereas 6 hours after injection they filled the cortex. The fact that after a short time we observed a predominant proportion of Ne under the capsule, suggests that Ne had arrived at LN via the lymphatics. To confirm this route, mice immunized with OVA/CFA were injected with OVA-FITC into the right footpad, and six hours after LN draining, the right and left footpad were removed. Figure 5D shows that only 1\% of Ne were observed in the contralateral LN. Thus, migration was restricted to the ipsilateral LN, supporting the idea that Ne had reached the LN via afferent lymphatics.

We also performed a kinetic analysis of Ne influx in LN sections over longer time periods (Figure 5E). Twenty-four hours after antigen injection, there was still an important number of Ne in LN although they were no longer OVA-FITC\textsuperscript{+}. This result was confirmed by FACS analysis (after 24 hours there were 17\% of Ne but only 0.8\% of them were OVA-FITC\textsuperscript{+}). At 48 hours, Ne started to disappear from the LN, and at 72 hours there were no more Ne.
As 72 hours after OVA injection Ne had disappeared from LN, we asked ourselves whether the resolution of this acute inflammation involved programmed cell death of invading inflammatory cells. Twelve hours after the injection of antigen we observed that only 1% of Ne in LN had a hypodiploid DNA content whereas after 18 hours this figure was 22%, indicating that the number of apoptotic Ne increased over time. Very few Ne with hypodiploid DNA were detected in blood from OVA/CFA mice injected with OVA at these times (Figure 5F).

In summary, Ne influx in LN was rapid (2 hours after a challenge) and transient (48 hours). Since we did not observe OVA-FITC\(^+\) cells 24 hours after a challenge, we hypothesize that the Ne arrive at the LN between 2 and 6 hours later, then Ne become OVA-FITC\(^-\), remaining in LN up to 48 hours after an injection. This inflammatory state was resolved 72 hours after injection, and death by apoptosis could have been one of the mechanisms that contributed to its resolution.

**Neutrophils present in LN mainly expressed TNF-\(\alpha\) and their depletion resulted in an antigen-specific increase in IL-5 levels**

The preceding results show that upon injection of OVA in OVA/CFA immunized mice, a population of Ne arrived at the LN after an uptake of OVA, probably in the form of immune complexes. We investigated whether Ne could have contributed to the immune response by secreting cytokines. By evaluating the \textit{in vivo} production of TNF-\(\alpha\), IFN-\(\gamma\), IL-12 and IL-4 by Ne, we found that Ne present in LN exhibited a positive cytoplasmic staining for TNF-\(\alpha\), and a slight expression of IFN-\(\gamma\) and IL-12 (Figure 6A). This profile of cytokine expression was similar for Ne present in blood (Figure 6B) whereas Ne from
OVA/CFA mice injected with PBS showed lower levels of cytokines, especially TNF-α. We then assessed the effect of Ne on the OVA-specific T-cell response by removing in vivo Ne with anti-Gr1 treatment. Mice depleted of Ne were then injected with OVA-FITC, and two days later LN cells were challenged in vitro with OVA. Flow cytometric analysis demonstrated that administration of anti-Gr1 in OVA/CFA-immunized mice was effective in Ne depletion, as 6 hs (Figure 6C) or 48 hs (Figure 6D) after antigen injection, there were no Gr-1<sup>high</sup> cells found in the LN. LN cells from Ne-depleted mice secreted five times more IL-5 than LN cells from IgG-treated mice, whereas no difference was found in terms of INF-γ secretion between both mice groups (Figure 6E). In summary, we have shown that after an antigen challenge, and in the presence of specific antibodies, Ne are recruited into the secondary lymphoid organs, principally secreting TNF-α, and were able to modify the cytokine balance in OVA-specific T cell responses.
DISCUSSION

In this work, we observed that soon after an antigen challenge in OVA/CFA-immunized mice, not only did OVA-FITC⁺ Ne appear at the injection site, but there was also an influx of Ne into secondary lymph organs, where they represented the main OVA-FITC⁺ cells. Although neutrophils have been generally accepted not to be involved in adaptative immune responses, over the last few years some reports have appeared that describe Ne trafficking from peripheral tissue to LN \(^2,15,16\). In the present work, we analyzed the uptake of antigen by Ne, their arrival into LN, and their participation as immunomodulatory cells in immune mice.

On the basis of the classical tenet that uptake and transport of antigens from the skin to lymph nodes is a function of dendritic cells and macrophages \(^12,13\), our discovery about there being an important OVA-FITC⁺ Ne influx into the LN was really unexpected. Ne were the main OVA-FITC⁺ cells in the LN, since we detected few OVA-FITC⁺ dendritic and B cells, and no OVA-FITC⁺ macrophages in LN. In agreement with this, other authors very recently observed that the granulocytes were central microorganism-host cells in draining lymph nodes \(^16,17\). Abadie et. al. showed that bacillus Galmette-Guerin (BCG) shuttles from tissue to lymph nodes via Ne \(^16\). Little is known about the factors that determine which kind of cell can take up antigens in situ, but it is probable that it may be influenced by the microenvironment. Recently, we found no OVA-FITC⁺ CD11b⁺ cells in LN from mice immunized with OVA plus synthetic oligodeoxynucleotide containing CpG motifs or OVA plus *Bordetella pertussis* \(^7\). Our data and these of Abadie \(^16\), suggest that the selection of Ne as cells that take up antigens occurs in response to certain inflammatory signals such as BCG vaccines (containing both live and dead bacilli) or CFA (oil
containing inactive bacilli) revealing the great complexity in the relationship between the immune milieu and the cells involved in the uptake of antigens.

Regarding the influx of OVA-FITC+ Ne in LN, 2 hours after injection of OVA-FITC into the footpad, we observed that OVA-FITC+ Ne were localized in the subcapsular space, principally in the ipsilateral LN suggesting that Ne had reached the LN via afferent lymphs. In addition, we showed that most of the Ne did not take OVA-FITC to the LN, but instead arrived loaded with OVA-FITC from the skin. Nevertheless, our data do not exclude the possibility that some antigens could arrive at LN as free OVA-FITC. In agreement with our results, it has been recently shown in mice vaccinated with BCG strains that Ne migrate via afferent lymphatics to lymphoid tissue. At present, the molecular mechanisms by which Ne home in on LN via lymphatic vessels has not been explored, and subsequent experiments are needed to fully understand it. In addition, we have observed OVA-FITC+ Ne in spleen and blood after antigen injections in the skin. Probably, some OVA-FITC reaches the blood from where it is taken up by Ne and later arrives at the spleen, a secondary lymphoid organ specialized in the generation of immune responses against antigens carried by the blood.

In our model, OVA uptake by Ne is critically dependent on the presence of an OVA specific immune response, taking into account that PBS/CFA mice have Ne in skin but do not have OVA-FITC+ cells in the skin or in LN. Furthermore, injection of OVA-antibody complexes into these mice produces the same results observed in OVA/CFA mice injected with OVA. Although we suggest that the presence of specific antibodies is a pre-requisite for Ne to be able to reach LN, we do not discard the possibility that other signals present during the immune response may also be involved. It is well known that the recruitment of Ne into inflamed tissues is mediated by selectin. However, Coxon et al. demonstrated that
the interaction of immune complexes with Fcγ-RIII may mediate early Ne recruitment in immune complex-mediated inflammations\textsuperscript{18,19}. In our model, we found that in PBS/CFA-immunized mice which did not have OVA-antibody complexes, but showed an increased number of Ne in blood similar to that of OVA/CFA-immunized mice, recruitment of Ne in skin was comparable to that observed in OVA/CFA-immunized mice. Therefore, the recruitment of Ne in skin is independent of the presence of antigen-antibody complexes. However, the uptake of an immune complex seems to be a requirement for Ne to be able to reach LN.

Ne have been shown to influence the priming of Th1 responses, releasing immunoregulatory cytokines and chemokines\textsuperscript{20}. This effect can be direct by providing a Th1 cell cytokine environment by the production of IL-12\textsuperscript{5,21,22}, or indirect through interactions with dendritic cells\textsuperscript{23-25}. In numerous reports it has been shown that Ne triggered by different stimulus are able to produce TNF-α\textsuperscript{23,24,26-28}. Here, we show that \textit{in vivo} Ne display intracellular TNF-α in LN. A recent report described that TNF-α from a Ne-conditioned medium triggers maturation of dendritic cells\textsuperscript{24}. Therefore, in our model it is probable that TNF-α produced by Ne in LN facilitates the maturation of dendritic cells, with these cells then being the ones that participate in the support of a Th1 response. Related to this, it has been previously demonstrated that Ne promote Th1 or Th2 polarization of T cell responses in different experimental models of infection\textsuperscript{2-5,29-31}. Also, van Gisbergen et. al. demonstrated \textit{in vitro} that dendritic cells stimulated by Ne induce Th1 polarization\textsuperscript{25}. Accordingly, a relevant finding of our study is that in the absence of Ne in OVA/CFA mice there was an increase in the OVA-specific IL-5 secretion in LN. Our data has demonstrated for the first time a direct correlation between Ne loaded with immune
complex and the outcome of T cell responses, supporting a new concept of Ne as cells that could help to maintain a Th1 response in the secondary phase of the immune response. The presence of Ne in the absence of an infection addresses the question about its importance during inflammatory diseases where immune complexes coexist with Ne. Of special interest are several reports from the laboratory of Matthys P. et. al., who demonstrated that the development of arthritis in collagen/CFA-immunized mice is determined by CD11b+ myeloid cells. In addition, Wipke et al demonstrated that Ne play a critical role in inflammatory processes in the joint lesion. Further studies need to be addressed exploring the role played by neutrophils not only as effector cells, but also as messenger cells during inflammatory processes.

In summary, the findings presented herein demonstrate that in the presence of an established immune response, Ne can capture antigen in the periphery, transport it to lymphoid organs, and provide help in sustaining the immune response. The findings described here have a particular importance for the use of Ne as a potential target in the latest therapies for inflammatory diseases.
ACKNOWLEDGMENTS

The authors would like to thank native speaker Dr. Paul Hobson, who revised the manuscript.
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FIGURE LEGENDS

Figure 1. Analysis of antigen-capturing cells in lymphoid organs from immune mice
Unimmunized, OVA/CFA-immunized and PBS/CFA-immunized mice were injected with OVA-FITC or PBS into the footpad, and 6 hours later blood, spleen and LN were processed for flow cytometry. (A) The histograms show % of OVA-FITC$^+$ cells in blood and spleen from OVA/CFA-immunized mice injected with OVA-FITC (black line) or PBS (grey area). (B) Upper panels show forward and side light scatter profiles of LN cells. The lower panel shows % of OVA-FITC$^+$ LN cells in OVA-FITC-injected (black line) or in PBS-injected (grey area) mice from the total cell population. A graph showing side light scatter profile of OVA-FITC$^+$ cells is also included. Data shown in parentheses depict MFI. One typical experiment of four performed is shown (n: 3-4 mice per group).

Figure 2. Surface phenotype of OVA-FITC$^+$ cells
Unimmunized and OVA/CFA-immunized mice (day 40) were injected with OVA-FITC into the footpad, and 6 hours later LN and spleen were obtained and processed by flow cytometry. (A) Density Plot graphs show CD19 and CD11b expression vs. green fluorescence in OVA-FITC injected mice. The histograms show staining for MHC-II of the gated population; the percentage of cells with a fluorescent intensity over the marker (black line), corresponding to the upper limit of control background staining (grey area) is indicated. (B) Histograms show in black lines the expression of the indicated markers in OVA-FITC$^+$ CD11b$^{\text{high}}$ LN cells and the grey shaded area denote the expression of the same marker in whole LN cells. The grey lines represent cells stained with an isotype-
matched control antibody. One typical experiment of four performed is shown (n: 3-4 mice per group).

**Figure 3. OVA-FITC+ cells in lymphoid organs are mainly neutrophils**

OVA/CFA-immunized mice were injected in the footpad with OVA-FITC, and 6 hours later lymphoid organs were obtained. (A) Density Plot graphs show Gr-1 expression vs green fluorescence. (B) Gr-1<sup>high</sup> OVA-FITC<sup>+</sup> cells show high levels of Ly-6G. (C) May-Grünwald-Giemsa stained cytospin preparations of LN from OVA-FITC, or PBS injected, OVA/CFA immunized mice. Arrows indicate Ne. (D) LN from OVA/CFA-immunized mice injected with OVA-FITC: i-ii (original, X600) typical polylobed nucleus of OVA-FITC<sup>+</sup> Ne in cytospin preparation stained with DAPI alone (in blue) (i) or DAPI plus anti-Gr-1 labeled with Alexa Fluor 546 (red) (ii); iii-iv (original, X400) confocal microscopy: OVA-FITC<sup>+</sup> cells (green) and Gr-1<sup>+</sup> cells (red) as well as a merged image are shown. The figures C and Di-ii were obtained with conventional microscopy. One typical experiment of four performed is shown (n: 3-4 mice per group).

**Figure 4. Influx of OVA-FITC<sup>+</sup> Ne in LN increases with the number of immunizations and requires an antigen-specific response**

(A-C) Mice were immunized with OVA/CFA on days 0, 15 and 30. (A) After the first (day 15), second (day 30) or third (day 40) immunization, mice were injected with OVA-FITC (▲) or PBS (■), and 6 hours later LN were obtained and the percentages of OVA-FITC<sup>+</sup> Ne were measured by flow cytometry. Each point represents the mean ± SD. (B) The percentage of Ne in smears of peripheral blood stained with May-Grünwald-Giemsa was
evaluated on days 15, 30 and 40. Each point represents the mean ± SD. The value of Ne in peripheral blood from unimmunized mice was 20 % ± 5. (C) Plasma from individual immune mice was assayed for anti-OVA IgG by ELISA on days 15, 30 and 40. Each point represents the mean of specific antibody titers (log_{10}) ± SD. IgG antibody titers were calculated as the reciprocal of the last plasma dilution that yielded an A_{490} above that of the double-mean value of preimmune plasma. (D) OVA-antibody complexes or soluble OVA-FITC were injected in unimmunized or PBS/CFA-immunized animals. Six hours after injection, LN cells were counted and fluorescent cells were analyzed by flow cytometry. Results are expressed as the mean number of OVA-FITC^ + Ne/LN ± SD. (E) OVA/CFA-immunized or KLH/CFA-immunized mice (day 40) were injected in the footpad with OVA or KLH. As the control, a group of mice were immunized with PBS/CFA and injected (day 40) with OVA. After 6 hours, LN were obtained and the cells were stained with anti-Gr-1 and analyzed by flow cytometry. The percentage of Ne (Gr-1^{high}) is indicated (black line); the grey area corresponds to cells stained with isotype-matched control antibody. One typical experiment of four performed is shown (n: 3-4 mice per group).

**Figure 5. Localization and kinetics of neutrophil influx in the site of inoculation and in LNs.**

Mice immunized with OVA/CFA or PBS/CFA were injected with OVA-FITC in the footpads on day 40. (A) Kinetics of Ne (Gr-1, black line) in LN at different short time periods (10 min, 20 min, 30 min or 2 hours), evaluated by FACS. The percentage of Ne (Gr-1^{high}) is also indicated with the grey area corresponding to control background staining. (B) Kinetics of OVA-FITC^ + or Ne influx in skin section evaluated by microscopic analysis (X400). The data are expressed as
number of cells/field. At least 20 and 40 fields of each smear were evaluated. (C) Localization of OVA-FITC\(^+\) cells in LN. The left panels show fluorescent microphotographs of LN sections 2 hours (upper panels) or 6 hours (lower panels) after OVA-FITC injection in the footpad of OVA/CFA-immunized mice. The right panels show the corresponding hematoxylin/eosin-stained tissue sections at X250 and at higher magnification (X1000). Arrows indicate Ne. SS, subcapsular sinuses; C, cortex. (D) Gr-1\(^\text{high}\) cells (black line) from ipsilateral and contralateral LN 6 hours after OVA-FITC injection in OVA/CFA immune mice, processed by FACS. The grey area corresponds to control background staining (E) Kinetics of OVA-FITC\(^+\) cells or Ne influx at longer time periods in LN sections evaluated by microscopic analysis (X400). The data are expressed as number of cells/field. At least 20 and 40 fields of each smear were evaluated. (F) Analysis of apoptosis of Ne in LN or blood obtained 12 or 18 hours after OVA-FITC injection in the footpad of OVA/CFA immune mice. Cells were stained with FITC-anti-Gr-1 antibody and propidium iodide and then analyzed by flow cytometry. The percentage of cells with hypodiploid DNA in the gate of Gr-1\(^+\) cells is indicated in each panel. One typical experiment of three performed is shown (n: 3-4 mice per group).

Figure 6. Neutrophils present in LN mainly expressed TNF-\(\alpha\) and their depletion resulted in an antigen-specific increase in IL-5 levels

(A-B) Mice immunized with OVA/CFA were injected with PBS or OVA, and 6 hours later LN cells and peripheral blood were obtained and analyzed for intracellular cytokines. Cells were stained with anti-TNF-\(\alpha\), anti-INF-\(\gamma\), anti-IL-12, anti-IL-4 (black line) or an isotype-matched control antibody (grey area) (histograms gated on Gr-1\(^\text{high}\) cells). One typical experiment of three performed is shown. (C-D) OVA/CFA-immunized mice were treated
with isotype control (undepleted) or RB6-8C5 (Ne depleted) antibody. On day 40 (C) (6 hours after injection of OVA) and on day 42 (D) (48 hours post-injection to OVA), LN cells were processed for flow cytometry. Density plots are representative of three-four mice analyzed. (E) OVA/CFA-immunized mice treated with isotype control (undepleted) or RB6-8C5 (Ne depleted) antibody were injected on day 40 in footpad with OVA, 48 hours later LN cells were removed and then cultured for 72 hours with 100 µg/ml of OVA (3.5 x 10^5 cells/200 µl/well). The supernatants from triplicate cultures were pooled and cytokine content measured in triplicate by capture ELISA. *p<0.005 compared with undepleted mice. One typical experiment of three performed is shown (n:3-4 mice per group).
FIGURES

Figure 1

(A) Blood

Spleen

(B) popliteal lymph node

Unimmunised

OVA:CEA immunised

PBS:CEA immunised

FSC

SSC

OVA:FITC

OVA:FITC

OVA:FITC
Figure 2

(A) Injected with OVA-FITC

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(B) CD19

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Figure 3

(A) Injected with OVA-FITC

Unimmunized  OVA/CFA immunized

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(B) [Graph showing G-1 (Ly-6G and Ly-6C) vs. OVA-FITC and Ly-6G]

(C) OVA/CFA immunized

<table>
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(D) i, ii, iii, iv, v

Green

Red

Overlay
Figure 4

(A) Graph showing % CTLA-4+ Tim-3+ cells.

(B) Graph showing % Be7+ cells.

(C) Graph showing % F4/80+ cells.

(D) Bar graph comparing OVA/DTU-induced Tim-3+ cells.

(E) Graph comparing OVA/CTA-induced and unimmunized mice.
Figure 5

(A) Kinetics of neutrophil influx in skins

(B) Kinetics of neutrophil influx in popliteal lymph nodes

(C) Images of neutrophils at different time points

(D) Comparison of neutrophil influx in popliteal lymph nodes between OVA/CTA and PBS/CTA

(E) Kinetics of neutrophil influx in popliteal lymph nodes

(F) Neutrophil influx in blood at different time points
Figure 6

(A) popliteal lymph node
GYAC/N vaccine injected with GVA

(B) blood
GYAC/N vaccine injected with FBI
GYAC/N vaccine injected with GVA

(C) popliteal lymph node

(D) popliteal lymph node

(E) Blood

Ferritin (ng/mL)

IL-5 (pg/mL)

Elastase (IU/mL)

Neutrophil depletion
Presence of neutrophil bearing antigen in lymphoid organs of immune mice

Belkys A Maletto, Andrea S Ropolo, Diego O Alignani, Miriam V Liscovsky, Romina P Ranocchia, Victor G Moron and Maria C Pistoresi-Palencia