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## Extracellular Cyclophilins Contribute to the Regulation of Inflammatory Responses<sup>1</sup>

Kamalpreet Arora<sup>\*</sup>, William M. Gwinn<sup>\*</sup>, Molly A. Bower<sup>\*</sup>, Alan Watson<sup>\*</sup>, Ifeanyi Okwumabua<sup>\*</sup>, H. Robson MacDonald<sup>†</sup>, Michael I. Bukrinsky<sup>\*</sup>, and Stephanie L. Constant<sup>\*,2</sup>

<sup>\*</sup>Department of Microbiology and Tropical Medicine, George Washington University, Washington, D.C. 20037 <sup>†</sup>Ludwig Institute for Cancer Research, Lausanne Branch, University of Lausanne, Epalinges, Switzerland

### Abstract

The main regulators of leukocyte trafficking during inflammatory responses are chemokines. However, another class of recently identified chemotactic agents is extracellular cyclophilins, the proteins mostly known as receptors for the immunosuppressive drug, cyclosporine A. Cyclophilins can induce leukocyte chemotaxis *in vitro* and have been detected at elevated levels in inflamed tissues, suggesting that they might contribute to inflammatory responses. We recently identified CD147 as the main signaling receptor for cyclophilin A. In the current study we examined the contribution of cyclophilin-CD147 interactions to inflammatory responses *in vivo* using a mouse model of acute lung injury. Blocking cyclophilin-CD147 interactions by targeting CD147 (using anti-CD147 Ab) or cyclophilin (using nonimmunosuppressive cyclosporine A analog) reduced tissue neutrophilia by up to 50%, with a concurrent decrease in tissue pathology. These findings are the first to demonstrate the significant contribution of cyclophilins to inflammatory responses and provide a potentially novel approach for reducing inflammation-mediated diseases.

Leukocyte trafficking and recruitment are critical processes in host immune surveillance as well as for inflammation-mediated pathology. The main regulators of leukocyte trafficking are chemokines, a family of chemoattracting cytokines. Another, less appreciated, class of chemotactic agents is cyclophilins, a group of highly abundant cellular proteins, mostly known as receptors for the immunosuppressive drug, cyclosporine A (CsA).<sup>3</sup> Cyclophilin A (CyPA) is a ubiquitously distributed intracellular protein belonging to the cyclophilin family. However, during the course of inflammatory responses, CyPA is released into extracellular tissue spaces by both live and dying cells (1–3). Indeed, elevated levels of CyPA have been reported in several different inflammatory diseases, including sepsis, rheumatoid arthritis, and vascular smooth muscle cell disease (2,4,5). In the case of rheumatoid arthritis, a direct correlation between levels of CyPA and the number of neutrophils in the synovial fluid of rheumatoid arthritis patients was reported (5). *In vitro* studies have shown that extracellular CyPA is a potent chemoattractant for human monocytes, neutrophils, eosinophils, and T cells (6–8).

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<sup>2</sup>Address correspondence and reprint requests to Dr. Stephanie L. Constant, George Washington University, Ross Hall 738, 2300 Eye Street NW, Washington, D.C. 20037. mtmslc@gwumc.edu.

#### Disclosures

The authors have no financial conflict of interest.

<sup>3</sup>Abbreviations used in this paper: CsA, cyclosporine A; ALI, acute lung injury; BAL, bronchoalveolar lavage; CyPA, cyclophilin A.

Furthermore, CyPA can induce a rapid inflammatory response, characterized by neutrophil influx, when injected in vivo (1).

Previous studies have shown that the primary binding site for extracellular cyclophilins is cell surface heparans (9,10). However, we recently identified CD147, a type I transmembrane protein belonging to the immunophilin family, as the main signaling receptor for extracellular CyPA and have shown that the presence of CD147 is essential for cyclophilin-mediated chemotaxis and signaling to occur in human leukocytes (6). Based on all the above findings, we have proposed that CyPA-CD147 interactions might contribute directly to leukocyte recruitment during inflammatory responses and therefore might contribute to the pathogenesis of certain inflammatory diseases (11). Although several studies have explored CyPA-CD147 interactions using in vitro approaches (6,8), the physiological and pathological significance of these interactions has not been investigated. Thus, in the current study we have examined CyPA-CD147 interactions in vivo after the induction of an inflammatory response. Specifically, we used a mouse model of acute lung inflammation that closely resembles acute lung injury (ALI) in humans. The model is characterized by increased capillary permeability, interstitial and alveolar edema, and an influx of circulating inflammatory cells, predominantly neutrophils, into lung tissues and airways after intranasal administration of LPS (12). Our findings demonstrate that CyPA-CD147 interactions do indeed contribute to the recruitment of neutrophils into tissues during an inflammatory response. Furthermore, we show that blocking these interactions significantly inhibits neutrophil influx into inflamed tissues, providing a potentially novel therapeutic approach for the alleviation of inflammation-mediated diseases.

## Materials and Methods

### Animals

Male C57BL/6 mice, 8 wk or older, were used to obtain peritoneal neutrophils. For all other studies, female C57BL/6 mice, 6–8 wk old, were used. All mice were purchased from the National Cancer Institute animal facility. All studies were reviewed and approved by the institutional animal care and use committee at George Washington University Medical Center.

### Abs and reagents

Anti-mouse CD147 was purified from the RL73.2 hybridoma (13). The rat IgG2a hybridoma (HB-189) was obtained from American Type Culture Collection and was used as a source of isotype control mAb. Both Abs were purified by the National Cell Culture Center. FITC-conjugated F(ab')<sub>2</sub> anti-rat IgG was purchased from Jackson ImmunoResearch Laboratories. PE-conjugated anti-mouse GR-1 was purchased from BD Biosciences. Anti-human cyclophilin A mAb (cross-reacts with murine cyclophilin A) was obtained from U.S. Biological, and HRP-conjugated antirabbit secondary Ab was purchased from Amersham Biosciences. LPS (*Escherichia coli* serotype 055:B5) and FMLP were obtained from Sigma-Aldrich. Human recombinant CyPA, which differs from mouse CyPA by only one amino acid residue, was purchased from Calbiochem. The non-immunosuppressive CsA drug, SDZ-NIM811, was a gift from B. Willi (Novartis Pharma, Vienna, Austria). The drug powder was dissolved in 100% ethanol and sequentially diluted into 50%, then 20%, ethanol, followed by a 1/1 dilution in PBS.

### Analysis of CD147 expression on leukocytes

Levels of CD147 expression were compared on different leukocyte and neutrophil populations by FACS analysis. Leukocytes were stained with 1 µg of anti-mouse CD147 or isotype control mAb, followed by FITC-conjugated anti-rat IgG for detection. In some experiments, PE-conjugated anti-GR-1 was added after the second step.

## Neutrophil enrichment

Circulating neutrophils were enriched from mouse peripheral blood collected by cardiac puncture. The collected blood was treated with ACK lysing buffer (Quality Biological) to remove RBC. Peritoneal neutrophils were isolated from the peritoneal cavity of mice 3 h after an i.p. injection of 1 ml of 3% thioglycolate broth. The isolated peritoneal cells were washed three times in cold PBS and were overlaid onto a sequential gradient of Histopaque-1007 and -1119 (Sigma-Aldrich). The enriched (>80%) neutrophils were collected from the interface of the two Histopaque layers. Neutrophils from inflamed tissue were obtained from minced lung tissue 18 h after intranasal delivery of 2 µg of LPS (see below). Lung tissue cells were treated with ACK lysing buffer to remove RBC, yielding a >60% enriched population of neutrophils.

## Chemotaxis assays

All chemotaxis assays were conducted using peritoneal neutrophils. The assays were set up using 48-well modified Boyden chambers (Neuro Probe) with the two compartments separated by a 5-µm polycarbonate membrane (NeuroProbe). Neutrophils ( $10^4$  cells/well) in RPMI 1640 culture medium supplemented with 1% BSA were added to the upper compartments, whereas media containing different dilutions of recombinant CyPA or FMLP (to provide a positive control for chemotactic responses) or medium alone were added to the lower wells. For all studies, we used a dose of  $10^{-7}$  M FMLP, which in our hands induces optimal neutrophil migration. For blocking experiments, different doses of anti-CD147 mAb, isotype control mAb, or CsA drug were included in both the upper and lower compartments. The chambers were incubated at 37°C in 5% CO<sub>2</sub> for 45 min, after which the membrane was removed, the nonmigrated cells were scraped off, and the membranes were stained with Wright-Giemsa (CAMCO) to discriminate neutrophils. A chemotactic index was calculated for each test well by dividing the number of cells counted for that well by the number of cells counted in medium wells. In previous studies, we and others have confirmed that the observed leukocyte migration induced by extracellular cyclophilins is due to chemotaxis and not to an increase in chemokinesis (1,6–8).

## Regimens for inducing LPS-induced ALI and its inhibition

For the induction of acute lung inflammation, C57BL/6 mice were given 2 µg of LPS in 50 µl of PBS intranasally under light anesthesia (halothane). In some experiments, control groups of mice received 50 µl of PBS alone. For the in vivo blocking experiments using anti-CD147, groups of mice received i.p. injections of 100 µg of anti-CD147 or isotype control Ab on days 1 and 0 before intranasal delivery of LPS. For the in vivo blocking experiments using SDZNM811, mice were given an i.p. injection of drug (20 mg/kg body weight) or diluent alone; 10 h later, the mice were given a second i.p. dose, followed immediately by intranasal delivery of LPS. In some experiments, groups of mice were given a combination of the two treatments, with the same dosages and kinetics as those used for the individual treatments. All animals were killed 18 h after intranasal delivery of LPS by exposure to carbon dioxide.

## Bronchoalveolar lavage (BAL) and tissue harvesting

After the animals were killed, lung airway cells were collected from individual mice by BAL, in which a cannula was inserted into the trachea, and three 1-ml washes of cold PBS were infused in and out of the airways. Lungs were then perfused via the right ventricle with 20 ml of ice-cold PBS. Perfused lungs were finely chopped and incubated at 37°C for 20 min in 5 ml of a digestion mixture consisting of Click's medium (BioSource International), 5% FCS, 150 U/ml collagenase type IV (Worthington Biochemical), and 20 µg/ml DNase I (ICN Biomedicals). Digested tissues were then pushed through a metal strainer, followed by a nylon mesh screen, to generate single-cell suspensions. Both BAL cells and lung tissue suspensions were treated with ACK lysing buffer to remove RBC and then counted. Individual cell

suspensions were stained with PE-conjugated anti-GR-1 to establish the numbers of neutrophils present after the different regimens.

### Western blot analysis of BAL fluid

For Western blot studies, a single 1-ml ice-cold PBS wash was used for the BAL procedure. BAL fluids were then cleared of leukocytes by centrifugation and pooled within experimental groups of mice. Equivalent volumes (20  $\mu$ l) of each pool were separated by SDS-PAGE under reducing conditions. A lysate of HEK293T cells was used as a positive control for the detection of CyPA. Separated proteins were then transferred onto an Immun-Blot polyvinylidene difluoride membrane (Bio-Rad). After blocking in 10% milk, the presence of CyPA was detected by incubating membranes in rabbit anti-human CyPA mAb, followed by HRP-conjugated, anti-rabbit secondary Ab. Detection was conducted using ECL (Amersham Biosciences).

### Histology

The lungs were first perfused with 20 ml of ice-cold PBS via the right ventricle, then 1 ml of 10% formalin was infused into the trachea to inflate and fix the lung tissue. The trachea was tied off using suture thread, and the fixed lungs were isolated and stored in 10% formalin until further processing. Fixed organs were embedded in paraffin, and 5- $\mu$ m sections were cut and stained with H&E to identify changes in cell composition (Histoserv).

## Results

### CD147 is expressed on murine neutrophils

Although CD147 expression is well characterized on human leukocytes, little is known of its expression on murine cells. In the current study the expression of CD147 on PBL of C57BL/6 mice was examined by flow cytometric analysis. As shown in Fig. 1A, CD147 was readily detected on all three major subsets of leukocytes, with the highest levels expressed by granulocytes. Focusing our analysis on neutrophils, the elevated level of CD147 was confirmed to be present on both circulating neutrophils as well as neutrophils obtained from tissues, including the peritoneal cavity and inflamed lung tissue (Fig. 1B).

### Murine neutrophils migrate in response to CyPA in vitro

To test the capacity of murine neutrophils to migrate in response to CyPA, in vitro chemotaxis assays were conducted using Boyden chambers. As shown in Fig. 2A, murine neutrophils readily migrated in response to recombinant CyPA. The response was dose dependent and demonstrated the characteristic bell-shaped curve typical of other chemotactic factors. Based on these data, a 100 ng/ml concentration of CyPA was used for subsequent studies. Having established the capacity of CyPA to induce chemotaxis of murine neutrophils, we next confirmed that this response was CD147 dependent. In initial studies testing different doses of anti-CD147 mAb (data not shown), we determined that a 25  $\mu$ g/ml dose provided maximal inhibition of CyPA-mediated migration. Indeed, this dose of anti-CD147 mAb inhibited CyPA-dependent neutrophil chemotaxis by >90% relative to an equivalent concentration of isotype control mAb or no Ab (Fig. 2B). In a separate study we confirmed that the observed CyPA-mediated chemotaxis of murine neutrophils could also be blocked using CsA, a known inhibitor of cyclophilin function. Specifically, we made use of a nonimmunosuppressive analog of CsA, SDZNM811, which has been reported to induce structural changes in CyPA, thereby inhibiting its activity without affecting calcineurin (14). As shown in Fig. 2C, the extent of inhibition of CyPA-mediated neutrophil migration by a previously optimized dose (2  $\mu$ M) of SDZNM811 was equivalent to that induced by anti-CD147 mAb. Of importance, neither anti-CD147 nor

CsA had any effect on FMLP-induced migration of neutrophils (Fig. 2C), demonstrating that both types of inhibition were specific for CyPA-CD147 interactions.

### Blocking CyPA-CD147 interactions significantly reduces LPS-induced lung inflammation

Our final series of studies were designed to establish whether blocking CyPA-CD147 interactions has an effect on inflammatory responses in vivo. For these studies we used a mouse model of acute lung inflammation that closely resembles human ALI. The LPS mouse model of ALI has been previously described and characterized by other groups (15). In this model, LPS is given by intranasal delivery to C57BL/6 mice, resulting in a rapid induction of several proinflammatory cytokines and chemokines as well as a major accumulation of neutrophils in lung tissues and airways within 12–24 h. The data in Fig. 3 show that this model of LPS-induced lung inflammation is also associated with an increase in CyPA protein. Indeed, Western blot analysis conducted on BAL fluid from groups of mice treated with LPS showed a striking increase in extracellular CyPA compared with that from groups of mice that were given PBS alone (Fig. 3A). Densitometric analysis of the protein bands showed the increases in CyPA to be statistically significant (Fig. 3B) and to correlate closely with the elevated numbers of neutrophils present within BAL samples (Fig. 3C).

Having established the presence of elevated CyPA and elevated numbers of neutrophils in our model of LPS-induced tissue inflammation, we next tested the capacity of anti-CD147 treatment to affect this inflammation. Thus, mice were given an i.p. injection of 100 µg of anti-CD147 mAb (or isotype control mAb) 1 day before intranasal delivery of LPS and a second i.p. injection on the same day as intranasal LPS. All mice were killed 18 h later, and their tissue and BAL leukocytes were enumerated by flow cytometry. Fig. 4A shows the mean number of neutrophils recovered from the lung tissues and airways of each group of mice. Strikingly, anti-CD147 treatment reduced neutrophil infiltration by 40–50% (relative to isotype control) at both tissue sites. Both these reductions were statistically significant ( $p < 0.05$ ), with no significant difference between isotype control and untreated groups. The decrease in neutrophilia by anti-CD147 treatment was also observed at the histological level (Fig. 4B). Specifically, the number and severity of inflammatory foci around lung airways and blood vessels were greatly reduced after anti-CD147 treatment. Finally, we tested whether blocking the CyPA arm of CyPA-CD147 interactions could also reduce neutrophil infiltration in this model of acute tissue inflammation. As with our in vitro studies, we made use of the nonimmunosuppressive drug, SDZ-NIM811. For the in vivo blocking studies, mice received a 20 mg/kg body weight dose of the drug (or diluent alone) i.p.; 10 h later they received a second i.p. dose of the drug along with intranasal delivery of LPS. The data in Fig. 5A show that, as observed with anti-CD147 treatment, blocking CyPA significantly ( $p < 0.05$ ) reduced neutrophil infiltration into tissues with ongoing inflammation. There was no significant difference between diluent control and untreated groups. We also tested whether combining anti-CD147 and SDZ-NIM811 intervention regimens might lead to a greater reduction in neutrophilia during lung inflammation. As shown in Fig. 5B, although the individual treatments led to reductions in the total number of tissue neutrophils ranging from 29% (SDZ-NIM811 alone) to 38% (anti-CD147 alone), combining the two treatments reduced neutrophil numbers by only an additional 6% compared with the effect of anti-CD147 treatment. Although each of the observed reductions in neutrophil numbers was statistically significant relative to the untreated group, there were no significant differences between treatment groups. Taken together, these findings suggest that all three regimens inhibited neutrophil influx into tissues to a similar extent, and that anti-CD147 and SDZ-NIM811 intervention regimens are probably acting on the same cyclophilin-CD147 interactions.

## Discussion

One of the hallmarks of inflammation is the extravasation of neutrophils. Although chemotactic factors, including IL-8/CXCL8, platelet-activating factor, and TNF- $\alpha$ , are known to be involved in the mobilization of neutrophils by generating a chemical gradient within the site of inflammation, indirect evidence provided by several disease models shows that extracellular cyclophilins might also be a contributing factor to the establishment of inflammatory pathology. The goal of the current study was to establish the contribution of cyclophilin-mediated recruitment of leukocytes, specifically neutrophils, into tissues during inflammatory responses. Although cyclophilin-induced recruitment of neutrophils and T lymphocytes has previously been studied *in vitro*, this is the first attempt to understand the physiological and pathological significance of cyclophilin-CD147 interactions *in vivo*.

As part of these studies, we demonstrated CD147 expression on circulating murine leukocytes and observed that granulocytes/neutrophils expressed the highest levels. Interestingly, this expression was slightly decreased on tissue neutrophils compared with circulating neutrophils. One potential explanation is that CD147 expression is down-regulated on leukocytes after their entry into tissues, supporting the idea that CD147 is most important for the recruitment phase of leukocytes during an inflammatory response. However, other studies have reported elevated levels of CD147 on leukocytes present within inflammatory sites, including fibroblastlike cells and granulocytes in the synovial joints of rheumatoid arthritis patients (16,17). Whether these differences in expression contribute in any way to the recruitment and/or entry of leukocytes into tissues has not been established; however, using *in vitro* chemotaxis assays, we have observed no difference in the capacity of neutrophils from the circulation vs different tissues to migrate in response to CyPA (K. Arora, unpublished observations).

Our *in vitro* studies clearly demonstrate the capacity of CyPA to induce neutrophil migration. Furthermore, the importance of CD147 to this CyPA-specific chemotaxis was confirmed by the >90% inhibition of the response using anti-CD147 Ab. Most importantly, our studies also demonstrate a role for cyclophilin-CD147 interactions during an inflammatory response. The LPS mouse model of ALI has been previously described and characterized by other groups, and several proinflammatory cytokines and chemokines have been reported to be present within the pulmonary tract of these mice (15). In the current study we now show that this model of lung inflammation is also associated with elevated levels of CyPA. Such findings agree well with other studies of inflammation-mediated diseases in which elevated levels of extracellular cyclophilins were detected in the synovial fluid of rheumatoid arthritis patients (5) and in the serum of patients with ongoing sepsis (4). Strikingly, treatment of the LPS-sensitized mice with anti-CD147 Ab led to a 40–50% inhibition of the development of neutrophilia within lung tissues and airways. This decrease in neutrophil accumulation within tissues was also observed at the histological level, with a greatly reduced number of inflammatory foci present in mice treated with anti-CD147. Additional confirmation of the contribution of cyclophilin-CD147 interactions to neutrophilia during inflammatory responses was provided by treating mice with an inhibitor to the cyclophilin arm of the interaction. Treatment with SDZ-NIM811, a nonimmunosuppressive CsA analog, inhibited neutrophil influx into lung tissues to a similar extent as observed with anti-CD147. Interestingly, treating mice with a combination of anti-CD147 and CsA led to an inhibition of neutrophil infiltration only slightly greater than that induced by the individual treatments. In the absence of a major additive effect from combining the two intervention regimens, such findings suggest that anti-CD147 and CsA are probably acting on the same cyclophilin-CD147 interactions. This is an important issue, because CD147 has also been reported to act as an adhesion molecule *in vivo* (18). Specifically, the capacity of erythrocytes to circulate out of the spleen into the general circulation was significantly impaired after treatment with anti-CD147. However, erythrocyte entry into tissues was not affected, and there was no evidence of a selective retention of erythrocytes or leukocytes within

other organs, suggesting that the adhesion properties of CD147 may be limited to specific subsets of cells and tissues. In unrelated *in vitro* studies, recombinant cyclophilins have been shown to increase leukocyte adhesion to an extracellular matrix (8). However, the mechanism for this increased adhesion was dependent on interaction with glycosaminoglycans, rather than CD147, and was restricted to CyPB. Thus, we think it unlikely that changes in the adhesive properties of neutrophils, due to either cyclophilin-CD147 interactions or our intervention regimens, are responsible for the observed changes in neutrophil accumulation within inflamed tissues. Nevertheless, we acknowledge that changes in CD147-dependent adhesion cannot be formally ruled out in our current *in vivo* studies and remain a possible contributing factor.

To date, there have been many reports of immune-mediated approaches to decrease neutrophil extravasation and/or accumulation in tissues during inflammatory responses. Indeed, studies have reported significant reductions in neutrophilia after the inhibition of different chemokines or their receptors (19–22). Interestingly, regardless of the chemokine or receptor inhibited, the influx of neutrophils into tissues was always reduced by at least 25%, and in many cases by >50%. Given that the total contribution of all reported chemokines cannot be >100%, such findings imply the existence of a chemokine network that is either sequentially or synergistically regulated during acute inflammation. Alternatively, the contributions of some of these chemokines might not be in the initiation of leukocyte recruitment, but, rather, in the amplification of recruitment, the retention of leukocytes in inflamed tissues, and/or providing survival signals for recruited cells. Where extracellular cyclophilins fit within this scheme is unknown, but is currently under investigation. One preliminary observation is that intranasal delivery of CyPA alone is not sufficient to initiate leukocyte influx into tissues (our unpublished observations). Such a finding suggests that the contribution of extracellular cyclophilins during inflammatory responses is dependent on an initial proinflammatory signal, as might be provided by LPS, or on the presence of another factor released during the response.

Taken together, our findings strongly implicate a role for extracellular cyclophilins, via interaction with CD147, on the accumulation of circulating leukocytes within sites of inflammation and suggest that cyclophilin-CD147 interactions contribute directly to the pathogenesis of inflammatory responses. Studies are underway to establish whether such interactions also play a role in other inflammatory diseases, providing potentially novel therapeutic targets for many inflammation-mediated diseases.

## Acknowledgments

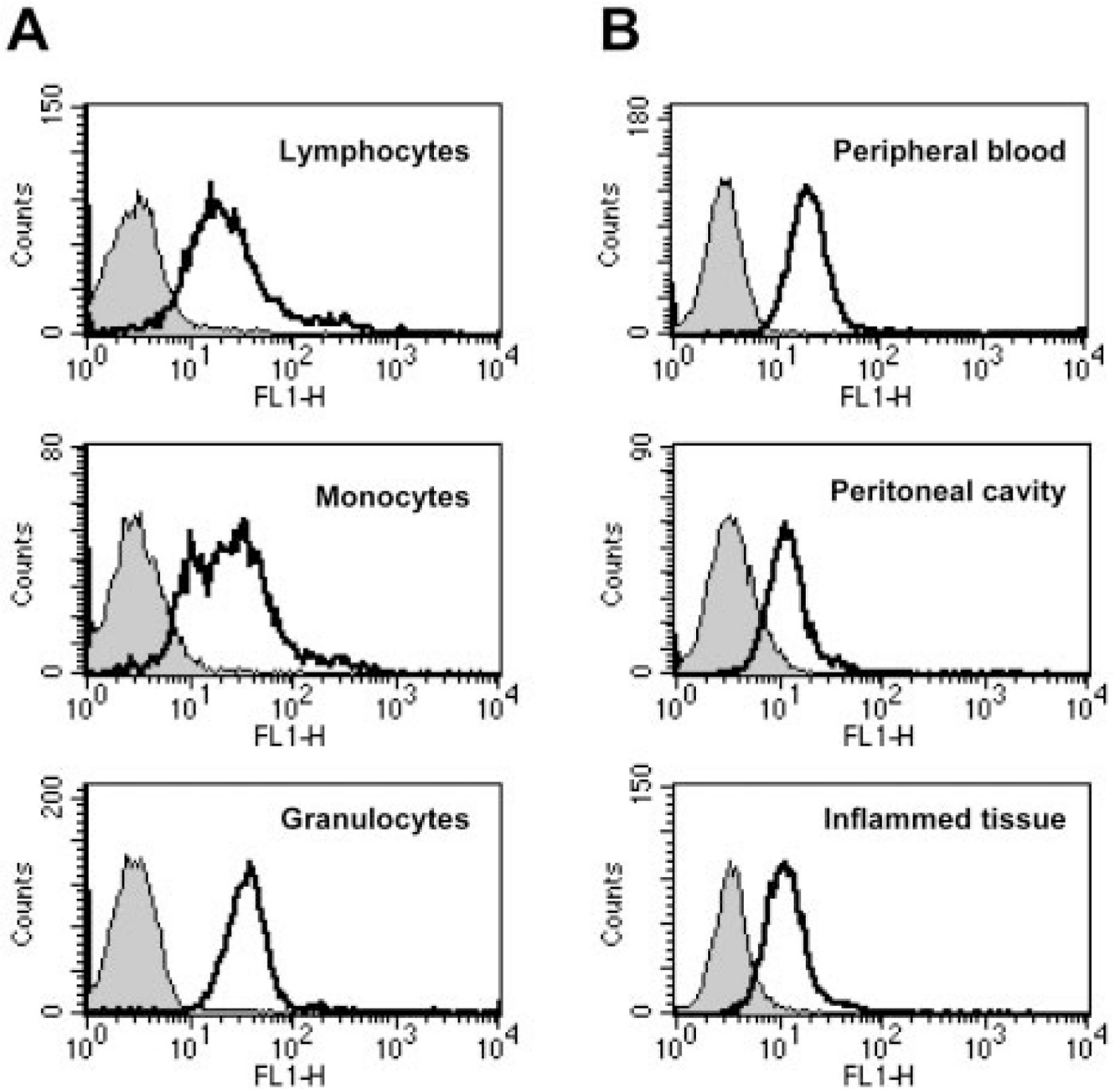
We gratefully acknowledge the National Cell Culture Center for the purification of Abs from RL73.2 and HB-189 hybridomas. We also thank Rustom Falahati and Robin Ruffner for technical help.

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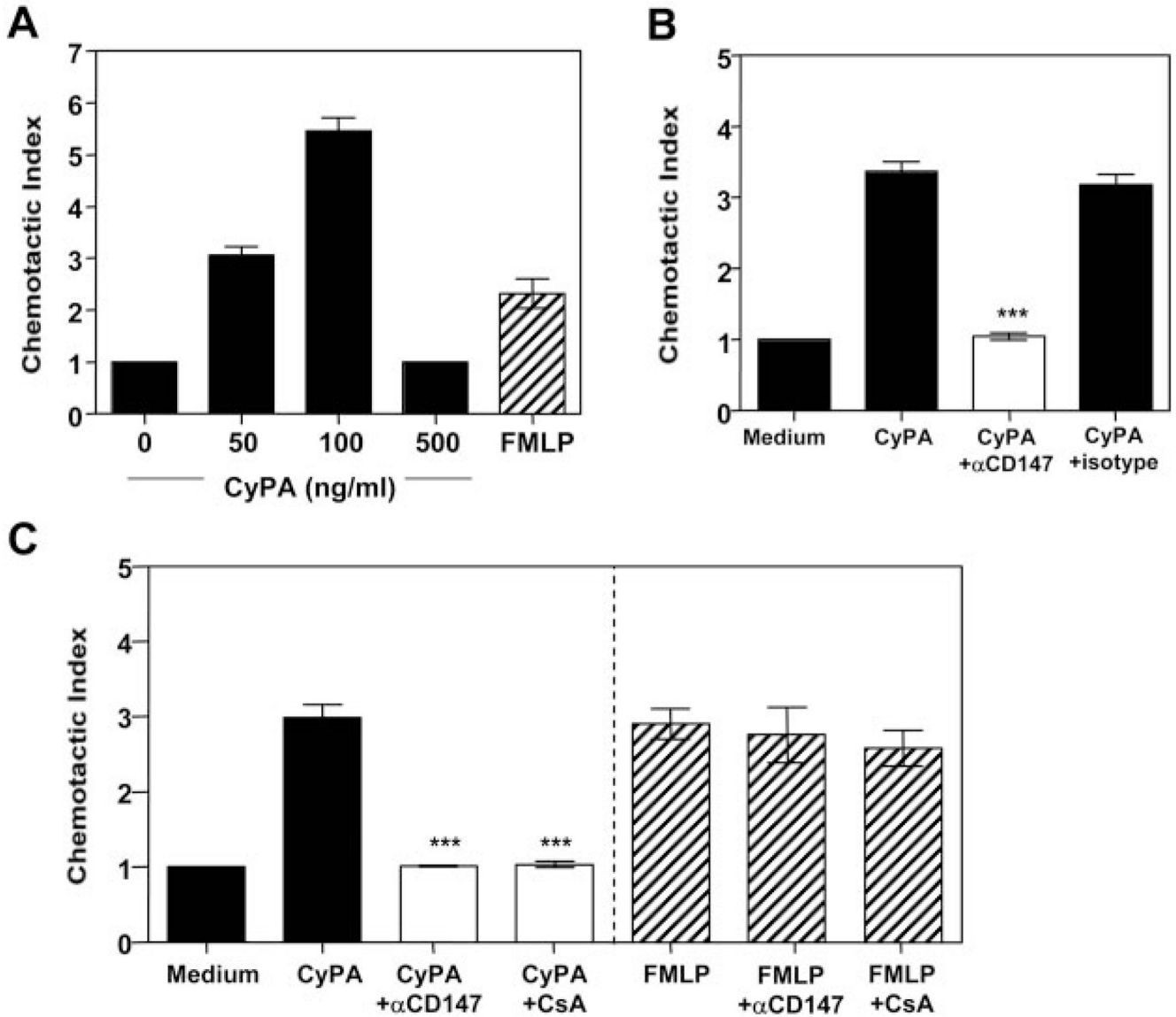


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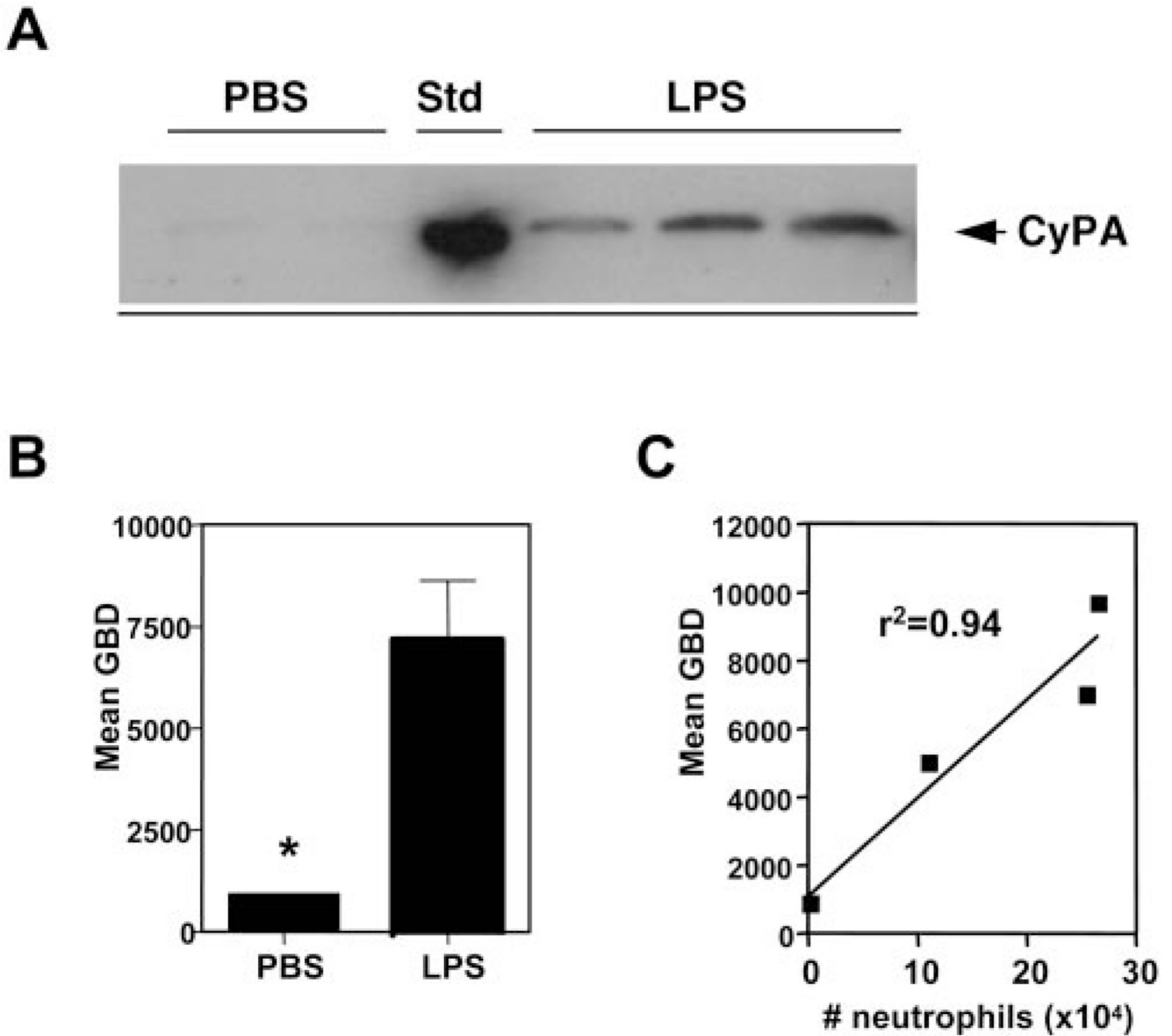


**FIGURE 1.**

CD147 expression on murine leukocytes. *A*, Leukocytes obtained from peripheral blood of C57BL/6 mice were stained with anti-CD147 mAb, or isotype control mAb, followed by FITC-conjugated anti-rat IgG. Analysis gates were set on the lymphoid, monocytic, or granulocytic population using forward scatter/side scatter distributions. ■, Isotype control staining; □, CD147 expression. *B*, Neutrophils from different sites were compared for CD147 expression. Each population was stained with anti-CD147 or isotype control mAb, followed by FITC-anti-rat IgG. Neutrophils were identified by additional staining with PE-conjugated anti-GR-1 mAb. Data show the expression of CD147 (□) relative to isotype control staining (■) after gating on GR-1<sup>+</sup> leukocytes.

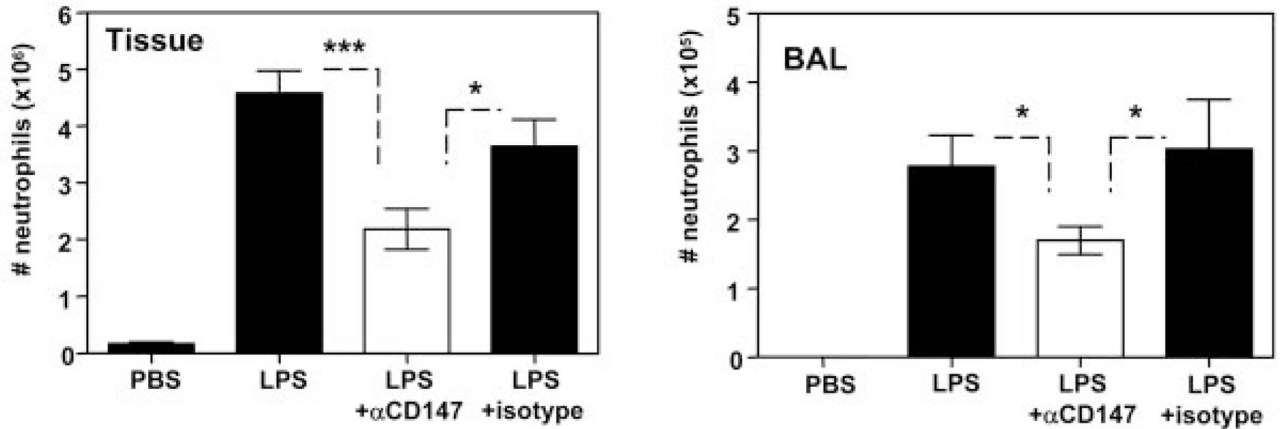
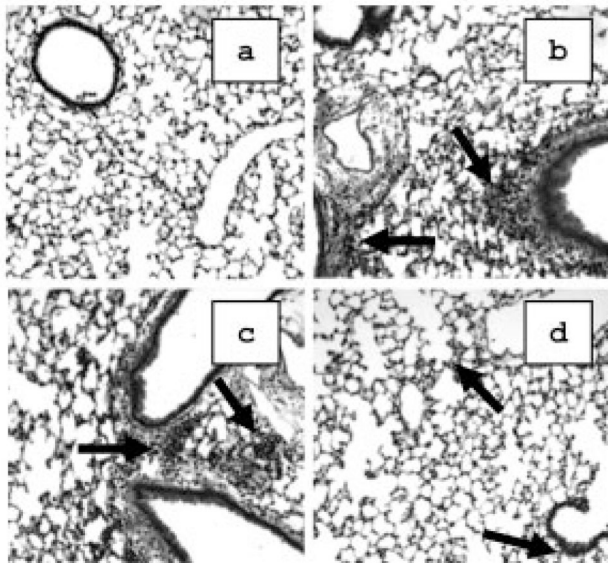
**FIGURE 2.**

Murine neutrophils migrate in response to CyPA in vitro. Neutrophils were enriched from the peritoneal cavity of C57BL/6 mice and set up in 48-well Boyden chambers in the presence of recombinant CyPA, FMLP (as a positive control), or medium alone. *A*, Different concentrations of CyPA were compared, relative to  $10^{-7}$  M FMLP. *B*, A dose of 100 ng/ml CyPA was used with or without the addition of 25  $\mu$ g/ml anti-CD147 or isotype control mAb. *C*, A dose of 100 ng/ml CyPA or  $10^{-7}$  M FMLP was used with or without the addition of 25  $\mu$ g/ml anti-CD147 or 2  $\mu$ M nonimmunosuppressive CsA (SDZ-NIM811). Graphs show the mean ( $\pm$ SE) chemotactic index (number of cells migrating in response to chemotactic agent divided by number of cells migrating to medium alone) for each group ( $n = 4-6$  wells/group). Student's *t* test was used to establish statistical significance between groups of wells treated with anti-CD147 or CsA compared with CyPA alone: \*\*\*,  $p < 0.001$ .

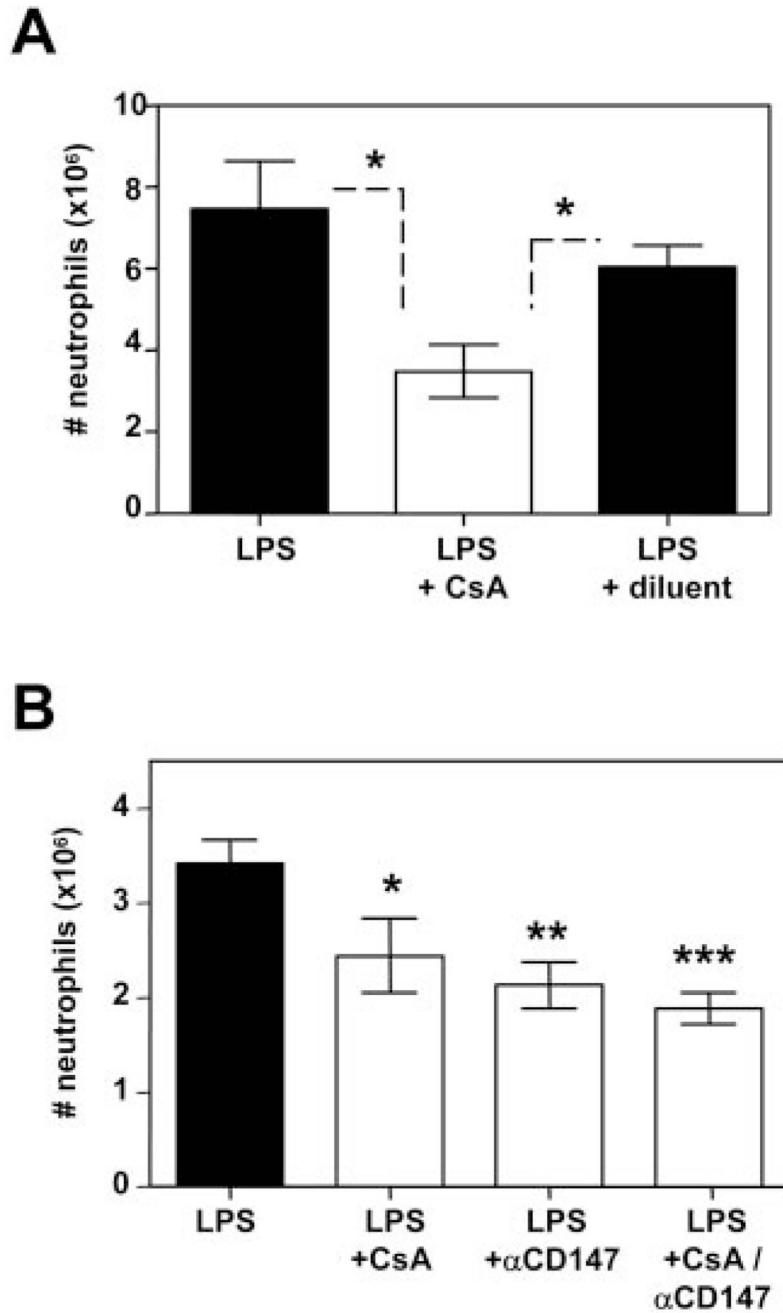
**FIGURE 3.**

Inflammation induced by intranasal LPS is associated with elevated cyclophilins in airways. Groups ( $n = 5$ ) of C57BL/6 mice were given PBS or 2  $\mu\text{g}$  of LPS by intranasal delivery; 18 h later, the animals were killed, and their BAL fluid was collected. **A**, For Western blot analysis, BAL fluid was cleared of leukocytes by centrifugation and pooled within groups of mice. Equivalent volumes of each pool were run under denaturing conditions by SDS-PAGE, followed by transfer onto nylon membranes and then probing with rabbit-anti-CyPA. The Western blot shows detection of CyPA in groups of mice from two or three individual experiments. The standard was a lysate of HEK293T cells. **B**, Densitometry was conducted on the individual lanes from the blot. Data show the mean gel band density (GBD) for the lanes from PBS vs LPS groups. Student's  $t$  test was used to establish statistical significance:\*,  $p < 0.05$ . **C**, Neutrophil numbers were determined in each of the pooled BAL samples by staining cell suspensions with PE-conjugated anti-GR-1, followed by FACS analysis. Data show a correlation between the number of neutrophils recovered and the CyPA content (based on

GBD) for each BAL sample. A correlation coefficient ( $r^2$ ) was calculated between the two parameters.

**A****B****FIGURE 4.**

Inhibition of cyclophilin-CD147 interactions by in vivo anti-CD147 treatment significantly reduces tissue inflammation. **A**, Groups of C57BL/6 mice were given an i.p. injection of 100  $\mu$ g of anti-CD147 or isotype control mAb 1 day before treatment with LPS and then a second injection at the same time as intranasal delivery of LPS. At 18 h after LPS delivery, the mice were killed, and their lung tissue and BAL fluid were collected. Tissue and BAL leukocytes from individual mice were stained with PE-anti-GR-1 and analyzed by FACS. Data show the mean number ( $\pm$ SE) of neutrophils detected in each group of mice ( $n = 7-9$ /group). Student's *t* test was used to establish statistical significance:\*\*\*,  $p < 0.001$ ; \*,  $p < 0.05$ . **B**, In some studies lung tissue was fixed in 10% formalin, sectioned, and stained with H&E for histological analysis. Histology ( $\times 10$  magnification) shows representative sections from the following groups: *a*, PBS alone; *b*, LPS alone; *c*, LPS plus isotype treatment; *d*, LPS plus anti-CD147 treatment. Arrows denote foci of inflammation.

**FIGURE 5.**

Inhibition of cyclophilins by in vivo CsA treatment significantly reduces tissue inflammation. *A*, Groups of C75BL/6 mice were given an i.p. injection of 20 mg/kg body weight SDZNM811 (or diluent alone), and 10 h later a second dose was given i.p. at the same time as intranasal delivery of LPS. All groups were killed 18 h later, and lung tissue was isolated and stained with PE-anti-GR-1 for FACS analysis. Data show the mean number ( $\pm$ SE) of neutrophils detected in lung tissues of each group of mice ( $n = 5$ /group). *B*, Groups of mice were given either two doses of 20 mg/kg SDZNM811 (10 h before intranasal LPS and concurrently with LPS), two doses of 100  $\mu$ g of anti-CD147 mAb (1 day before intranasal LPS and concurrently with LPS), or both treatments. All groups were killed 18 h later, and lung tissue was isolated

and stained with PE-anti-GR-1 for FACS analysis. Data show the mean number ( $\pm$ SE) of neutrophils detected in each group of mice ( $n = 8-10$ /group). For all studies, Student's  $t$  test was used to establish statistical significance: \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ .