JPET Fast Forward. Published on March 2, 2004 as DOI: 10.1124/jpet.103.063776

063776

Critical role of L-selectin and histamine H4 receptor in zymosan-induced neutrophil recruitment from the bone marrow: Comparison with carrageenan

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JPET Fast Forward. Published on March 2, 2004 as DOI: 10.1124/jpet.103.063776
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063776

a) Running title: Neutrophilia induced by zymosan and carrageenan profoundly differs

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c)

Text pages: 31

Tables: none

Figures: 7

References: 34

Abstract: 238 words

Introduction: 603 words

Discussion: 1788 words

d) Non-standard abbreviations:

Ab: antibody; LT: leukotriene(s); PAF: platelet activating factor; PTX: pertussis toxin;

e) recommended section: Inflammation & Immunopharmacology

JPET Fast Forward. Published on March 2, 2004 as DOI: 10.1124/jpet.103.063776
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Abstract

Zymosan and carrageenan represent two inflammatory stimuli leading to significant neutrophilia when injected into mice. Despite several similarities between the two pro-inflammatory agents, the mechanisms leading to neutrophil influx into the site of stimulus injection are unclear. As demonstrated by antibody (Ab) studies directed against adhesion molecules, L-selectin was pivotal for zymosan-induced but not carrageenan-induced pleurisy. Zymosan but not carrageenan injection into the pleural cavity caused blood neutrophilia and significant release of neutrophils from the bone marrow, events that were inhibited by anti-L-selectin but not anti-MAC-1 Ab pretreatment. PTX, known to regulate cell efflux, abrogated both zymosan- and carrageenan-induced pleurisy, but only zymosan-induced neutrophil release from the bone marrow. Dexamethasone, known to inhibit pleurisy induced by either stimulus, had no effect on bone marrow neutrophil numbers. The Gi/o G protein-coupled H_4 histamine receptor is highly expressed in the bone marrow and on leukocytes and plays an important role in zymosan-induced pleurisy in vivo. Zymosan-triggered neutrophil release from bone marrow was abrogated by pretreatment of mice with thioperamide, a known $H_{3/4}$ receptor antagonist, while H_1 and H_2 receptor antagonists had no effect. Moreover, histamine itself, when injected intravenously, led to a similar time- and dose-dependent decrease of neutrophil numbers in the bone marrow that was inhibited by thioperamide. Since the H_3 receptor is not expressed on neutrophils these findings indicate that both H_4 and L-selectin regulate zymosan-induced neutrophil release from bone marrow and subsequent infiltration in the pleurisy model.

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Acute inflammation can be experimentally induced by numerous stimuli (for example, lipopolysaccharides, phorbol esters, zymosan and carrageenan) and is orchestrated by a complex cellular and biochemical network that involves a multitude of cell types and mediators. Due to the involvement of a high number of molecular players these models are widely used to screen for the efficacy of novel anti-inflammatory drug candidates.

To experimentally induce leukocyte trafficking, carrageenan (sulfated polyanionic polysaccharide) and zymosan have become two commonly used inflammatory agents (Oh-ishi, 1997). Carrageenan-induced pleurisy is an experimental model of acute inflammation characterized by the migration of phagocytic cells. Polymorphonuclear leukocytes are the predominant cell type infiltrating the pleural cavity within the first 12 h following carrageenan injection. Later, polymorphonuclear cells disappear and are replaced by migrating mononuclear cells, which differentiate into macrophages and dominate the reaction up to its resolution at 48 h (Tomlinson et al., 1994) (Willis et al., 1996). These inflammatory cells synthesize and release various mediators of inflammation, among which the kallirein-kinin system and prostaglandins play a pivotal role. Inhibitors of cyclooxygenase, kallikrein-kinin, and bradykinin are capable of blocking carrageenan-induced pleurisy in vivo (Katori et al., 1978) (Dozen et al., 1989). Previous experiments have shown that neutrophil migration is reduced by agents capable of blocking the release of a specific neutrophil attractant from macrophages (Moraes et al., 1993). Likewise, macrophage depletion experiments reducing the resident macrophage population by about 80% significantly blocked neutrophil migration induced by carrageenan, however, such a treatment did not affect neutrophil migration induced by

chemokines (Souza et al., 1988). Thus, these results support the suggestion that macrophages participate in the control of neutrophil migration induced by carrageenan which is mediated by the kallikrein-kinin system and prostaglandins.

Zymosan, the insoluble polysaccharide component of the cell walls of *Saccharomyces cerevisiae*, is also commonly used for pleurisy induction in vivo. Like carrageenan, zymosan injection leads to the migration of phagocytic cells. Neutrophils are the predominant cells found in the pleural cavity up to 12 h after zymosan challenge, before they are replaced by macrophages that dominate the reaction at later stages (Oh-ishi, 1997). Despite these striking similarities in the leukocyte constitutions of the inflammatory exudates, the mechanisms leading to neutrophilia obviously differ between zymosan and carrageenan (Vannier et al., 1989) (Damas and Remacle-Volon, 1986). Histamine, platelet activating factor (PAF), the complement system and leukotrienes (LT), but not prostaglandins, are the main mediators responsible for the cell influx into the pleural cavity in response to zymosan (Imai et al., 1991) (Tarayre et al., 1989). Extensive in vitro studies indicate multiple inflammatory mechanisms of zymosan action including the activation of the alternative complement activation pathway and the generation of the anaphylatoxins C3a and C5a, agents well-known for their capacity to induce the release of histamine, PAF and LT from mast cells. The latter play a critical role in initiating cell migration into the pleural cavity after zymosan-injection. We recently demonstrated that mast cells and the H_4 receptor play an important role in zymosan-induced neutrophil migration into the pleural cavity. Based on the current understanding of the zymosan pathobiology, a cascade is triggered by zymosan binding to toll-like receptor 2, which is

expressed on mast cells, can induce mast cell activation (McCurdy et al., 2003), and mediates the zymosan signal in vivo via MyD88 (Takeshita et al., 2003).

We have undertaken experiments to determine mechanistic similarities and differences responsible for initiating the inflammatory infiltrates induced by zymosan and carrageenan and have shown that although both agents can release bone marrow neutrophils that are likely the source of the pleural infiltrate, the signaling and trafficking mechanisms responsible for this infiltration markedly differ.

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Methods

Chemicals

Dexamethasone, zymosan, histamine, thioperamide, pyrilamine and cimetidine were purchased from Sigma-Aldrich, (Gaithersburg, MD). Carrageenan, wortmannin and all bulk chemicals not further specified were purchased from Wako Chemicals (Osaka, Japan). Anti-L-, E- and P-selectin, anti-MAC-1 and anti-LFA-1 Ab and Ig-matched control Ab were purchased from BD (Biosciences Pharmingen, San Diego, US). Pertussis toxin (PTX) was purchased from Calbiochem (San Diego, CA).

Mice

Balb/c mice were purchased from Charles River Inc. (Yokohama, Japan). Animals were kept under standard conditions in a 12 h day/night rhythm with free access to food and water *ad libitum*. All animals received humane care and the studies have been approved by the internal ethic committee in accordance with the guidelines recommended by the Japanese Association of Laboratory Animal Science.

Zymosan- and carrageenan-induced pleurisy, blood neutrophilia and bone marrow neutrophil release

Mice (Balb/c, ♀, 7-8 weeks old, Charles River Inc.) received a single intrapleural injection of 0.2 ml of PBS containing κ-carrageenan (500 μg/mouse) or zymosan (100 μg/mouse) under anesthetization with ether. Test compounds (PTX, Ab, Y-27632, wortmannin, dexamethasone, and vehicle (V, (PBS)) were administered i.v. (0.2 ml/head) 3 min prior to carrageenan or zymosan. Four hours after injection, mice were euthanized

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and pleural fluid was collected by washing the pleural cavity twice with 2 ml PBS. The cell suspensions were diluted to one tenth in Turk's stain solution (Nacalai Tesque). The number of total cells in the sample was counted under the microscope using a hemocytometer. Cytospin specimens were stained with May-Gruenwald's (Merck, Darmstadt, Germany) and Giemsa's solution (Merck) for leukocyte typing. The distribution of each cell population (neutrophils, eosinophils, macrophages, lymphocytes and others) was counted under microscopy by counting 200-300 cells.

For assessment of neutrophilia and neutrophil release from bone marrow, similarly-treated separate groups of mice were euthanized to obtain and peripheral blood from the abdominal vein at the indicated time points after injection and then mice were completely bled. Immediately after the bleeding, the left femur was isolated and the femoral head and condyles were removed. The displaceable cells were recovered by flushing the lumen of the femur shaft with 1 ml PBS. The cell suspension was diluted to one tenth in Turk's stain solution and cell differentials and counting performed as described above.

Statistics

Data are expressed as means ± SEM. Statistical significance was determined using the unpaired Student's *t*-test if applicable or results were analyzed by using one-way ANOVA and if variances were non-homogeneous differences between groups were assessed using Dunnett's method using commercially available statistic software (GraphPad Software, San Diego, CA). *P* values <0.05 were considered as statistically significant (**P*<0.05, ***P*<0.01).

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Results

The coupling specificity of the $G_{\beta\gamma}$ and G_{α} subunits of $G_{i/o}$ proteins initiates signaling pathways triggered by chemokines and chemoattractants upon binding to their G-protein coupled receptors. Typically, $G_{i/o}$ proteins are sensitive to inhibition by PTX which inhibits cell migration induced by numerous stimuli. In order to compare the PTX-sensitivity of leukocyte recruitment induced by zymosan and by carrageenan, respectively, we evaluated the effect of PTX on neutrophil influx into the pleural cavity of mice. PTX inhibited neutrophil migration triggered by zymosan and by carrageenan however the carrageenan model turned out to be more sensitive (90% versus 60% inhibition of the zymosan response) as shown in Figure 1A and B. Thus, both the chemotaxis inhibitor, PTX, and dexamethasone, the gold standard anti-inflammatory drug, qualitatively showed similar effects on zymosan- and carrageenan-induced inflammation in the mouse (c.f. Figure 1 and (Takeshita et al., 2003)).

To identify potential mechanistic differences between the two models of neutrophilia we next evaluated the contribution of adhesion molecules to neutrophil influx into the pleural cavity induced by zymosan or carrageenan. In neutrophil trafficking, L-, P-, and E-selectins are crucial for the initial adhesion contact and tight adhesion interactions involve members of both Ig-like and the integrin families of adhesion proteins. In particular, the β2 integrins, among which LFA-1 and Mac-1 are most abundantly expressed on neutrophils, are critical for firm adhesion of rolling neutrophils to the endothlial cell surface and transendothelial migration. The central role of these selectins

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and β2 integrins is documented in various models of inflammation however a complete picture of their individual contributions to neutrophilia induced by zymosan or carrageenan, respectively, is not yet available. Mice pretreated with 100 μg of anti-E-selectin Ab showed a significant reduction of neutrophilia in both models, while pretreatment of mice with an anti-P-selectin Ab had no effect. In contrast, pretreatment of mice with an Ab directed against L-selectin reduced the inflammatory response to zymosan only without showing any effect on neutrophil numbers in the pleural cavity exudate of mice challenged by carrageenan (Figure 1C, D, E). To further elucidate the contribution of adhesion molecules we also investigated the role of the β2 integrins. Clearly, their functionality is also a prerequisite to allow neutrophils to invade into tissue upon zymosan or carrageenan injection, as significant reduction (anti-LFA-1 Ab) or even complete abrogation (anti-Mac-1 Ab) was seen under antibody treatment (Figure 2).

In general, zymosan-induced pleurisy was more severe than carrageenan- or chemoattractant (fMLP or LTB_4)-induced pleurisy in mice (c. f. differences in cell numbers in Figures 1and 2 and data not shown). In both cases, however, the relative neutrophil populations and the target body compartment neutrophils infiltrating, i.e. the pleural cavity, were identical. We thus wondered whether potential sources neutrophils were recruited from would differ and measured neutrophil numbers in the peripheral blood and in the bone marrow following intrapleural injection of zymosan or carrageenan. As illustrated in Figure 3A, zymosan injection led to a twofold increase in blood neutrophil counts 2 h after challenge. In parallel, neutrophil numbers in the bone marrow declined identifying the bone marrow as, potentially, a major source of

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neutrophils in the zymosan model. Surprisingly, neutrophil numbers did neither change significantly in the circulation nor in the bone marrow following carrageenan injection (Figure 3B). To investigate the potential link between adhesive function and the mechanism of neutrophil recruitment, we next evaluated the effect of anti-L-selectin and anti-Mac-1 Ab on zymosan-induced neutrophil release from the bone marrow. Neutralization of L-selectin but not of Mac-1 resulted in a highly significant retention of neutrophils in the bone marrow (Figure 3C and D). Pretreatment of mice with PTX also conferred protection against zymosan-induced neutrophil release from bone marrow (Figure 3E). In contrast, no effect at all on bone marrow neutrophils was seen by dexamethasone at doses known to clearly inhibit zymosan-induced pleurisy (Figure 3F) (Takeshita et al., 2003).

We recently demonstrated that zymosan induced pleurisy in a mast cell- and LTB_4-dependent manner (Takeshita et al., 2003) likely through the activation of the H_4 histamine receptor. Thus, we evaluated the contribution of $H_{1\text{-}4}$ receptors to the neutrophil release from bone marrow induced by zymosan. Mice pretreated with maximally tolerable doses of the histamine H_1 receptor antagonist, pyrilamine (10 mg/kg, i.v.), or with the H_2 receptor antagonist, cimetidine (30 mg/kg, i.v.), showed no change in neutrophil release. In contrast, pretreatment of mice with thioperamide, an $H_{3/4}$ antagonist, significantly reduced the release of neutrophils from the bone marrow in a dose-dependent manner (Figure 4).

We next wondered whether histamine itself could mimic zymosan-induced neutrophil recruitment and applied various doses of histamine i.v. and monitored the changes in

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leukocyte numbers in the circulation (not shown) and in the bone marrow. Substantial neutrophil release from the bone marrow was observed within 1 h after challenge, and the dose-response relationship reached a maximum at 60-600 μg histamine per mouse. Neutrophil release from bone marrow induced by a histamine dose of 300 μg/mouse peaked at 2 h after injection (Figure 5B). Pretreatment of mice with thioperamide significantly reduced the histamine-induced release from bone marrow at all doses tested (Figure 5E). Interestingly, and perhaps not surprisingly, the potency of thioperamide in counteracting the release of neutrophils from bone marrow was stronger against histamine compared to zymosan. Again, the H_1 receptor antagonist, pyrilamine (10 mg/kg, i.v.), and the H_2 receptor antagonist, cimetidine (30 mg/kg, i.v.), had no effect (5C and D).

To further substantiate the evidence for the $G_{i/o}$-coupled H_4 receptor being the mediator of histamine-induced neutrophil recruitment, we utilized inhibitors of signal transduction cascades known to be stimulated by histamine/H4 receptor ligation and neutrophil migration. As expected, pretreatment of mice with PTX, the PI3 kinase inhibitor wortmannin, or the Rho kinase inhibitor Y-27632, decreased the number of neutrophils in the release from the bone marrow following histamine injection. Neutrophil release from the bone marrow, however, remained unchanged upon dexamethasone pretreatment (Figure 6).

Finally, we wanted to further characterize and compare the molecular effectors involved in histamine-induced leukocyte trafficking with the zymosan-induced pleurisy and examined the effect of Ab directed against selectins. As shown in Figure 7A, mice

pretreated with L-selectin Ab did not exhibit neutrophilia, and the numbers of neutrophils in the bone marrow remained unchanged compared to non histamine-challenged controls. This resistance was highly specific for L-selectin, as Ab against E- and P- selectin failed to prevent the decrease of neutrophil numbers in the bone marrow following histamine injection (Figure 7B, C). In stark contrast to the zymosan-induced bone-marrow efflux however, anti-Mac-1 was effective in further promoting cell efflux (Figure 7D).

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DISCUSSION

An important finding of these studies highlighting a fundamental difference between zymosan- and carrageenan-induced pleurisy was the observation that zymosan but not carrageenan led to neutrophil recruitment from the bone marrow. This might explain why the number of neutrophils in the pleural cavity induced by zymosan is 2-3 fold higher compared to carrageenan. Although BrdU pulse labeling experiments have not been performed to specifically determine compartmental contributions for either stimulus, carrageenan most likely triggers neutrophil recruitment from the circulating pool in the blood stream or other tissue compartments. It has also been demonstrated that bone marrow derived neutrophils preferentially sequester to the microvasculature of the lung (van Eeden, et al., 1997a; Lawrence et al., 1996). The mechanistic uniqueness of zymosan appears to be mediated by L-selectin, mast cells, (Takeshita et al,. 2003) and probably other mast cell derived products such as leukotriene B_4, and PAF. Further, our Ab and pharmacological experiments show that neutrophil release from the bone marrow is dependent on functional Gi/o signaling, most likely triggered by histamine-H_4 receptor interaction, while it is insensitive to Mac-1 blockade and to steroids.

Physiologically, L-selectin expression is low in the mitotic pool of neutrophils, increases as cells mature in the postmitotic pool of the bone marrow and is constitutively expressed on circulating neutrophils (Van Eeden et al., 1995) (van Eeden et al., 1997b). Importantly, neutrophils that are released from the bone marrow by inflammatory stimuli, express higher levels of L-selectin compared to their circulating counterparts, and they

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progressively lose those L-selectin molecules as they age in the circulation (Van Eeden et al., 1997c). It was reported that glucocorticoids decrease L-selectin expression on circulating neutrophils by downregulating L-selectin expression in the maturation pool of the bone marrow (Nakagawa et al., 1999). Nevertheless, dexamethasone did not affect zymosan-induced neutrophil release from the bone marrow but dose-dependently inhibited their extravasation into the pleural cavity (Figure 3 and (Takeshita et al., 2003)). Given the plurality of anti-inflammatory effects for which dexamethasone may be responsible it is difficult to simplistically conclude a role in any one compartment using this inhibitor, however. Zymosan therefore probably increases the adhesion signal of L-selectin rather than L-selectin expression. However two important caveats must be taken into account. In mice, there is at least one report showing that carrageenan injected into the pleural cavity results not only in acute pleurisy, but also increased neutrophil infiltration into the lung parenchyma (Cuzzocrea et al., 2003). There is also a suggestion in rats (Doherty et al., 1995) that zymosan injection causes plasma leakage and leukocyte extravasation from "milky spots" on the parietal pleural surface. Hence, the source of emigrating neutrophils may be profoundly different in the two models. This could account in part for the differential requirement for L-selectin between the two stimuli, according to the neutrophil source and the necessity for neutrophil rolling as a mechanism for efflux.

Emigrated neutrophils must move through connective tissue and then through the mesothelial lining before gaining entry into the pleural cavity. The profound inability of anti-Mac-1 antibody to affect neutrophil emigration from the bone marrow in response to zymosan only is therefore most intriguing given that the requirement for Mac-1 has been

shown for neutrophil migration (basal to apical direction) across gut epithelium (Parkos et al., 1991). Nonetheless, the lack of, or lower Mac-1 expression in bone marrow compared with inflammatory exudates may account for the lack of effect by Mac-1 antibody on bone marrow extravasation as opposed to the infiltration to the pleural cavity. In the case of histamine-induced bone marrow efflux, it would appear that inhibition of Mac-1 promoted the efflux. It has been shown that histamine prevents complement fragment (C3bi)-induced Mac-1 clustering and chemoattractant-induced upregulation and cell spreading in vitro – the suggestion being that histamine is negatively regulating cell adhesion (Francis, et al., 1991). If a similar scenario occurs in vivo in the bone marrow whereby histamine inhibits Mac-1 function, explaining the result using anti-Mac-1 antibody becomes difficult. However, in the current experiments, no other factor has been characterized as promoting the migratory response of the bone marrow neutrophils thus a characterization of the direct effect of histamine on bone marrow neutrophil Mac-1 expression and functionality may provide clues to the mechanism involved. Even if histamine was negatively regulating bone marrow neutrophil adhesion through Mac-1, such a phenotype does not rule out the promotion of cell efflux through utilization of other adhesion receptors, including L-selectin. Additionally, while complement or chemoattractant-induced Mac-1 clustering and upregulation are relevant to the firm adhesive state and migration capacity, antibody inhibition of the basal adherence may release sufficient numbers of cells to the pool available for efflux. Histamine may then stimulate efflux through activation of L-selectin or other adhesion receptor pairs. A more thorough analysis of Mac-1 expression on bone

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marrow neutrophils and the effect of cell activation is therefore warranted to understand the effect of the anti-Mac-1 antibody.

That the zymosan model turned out to be less sensitive (60% versus 90% inhibition of the response to carageenan) to PTX might be due to PAF, which is released by zymosan- but not by carrageenan-stimulated mast cells. In contrast to other mast-cell-derived chemotactic factors such as C5a, LTB_4, or PGD_2, which all signal through PTX-sensitive G proteins, PAF activation of eosinophils can activate a protein kinase C-driven pathway independent of Gi (Teixeira et al., 1997). It can be speculated that a similar signaling pathway is dominant in mast cells. Indeed, it has been demonstrated that pleurisy induced by zymosan, but not by carrageenan, was significantly reduced in PAF-desensitized or PAF receptor antagonist-treated animals (Martins et al 1989).

Clearly, the results obtained with thioperamide, an $H_{3/4}$ antagonist, strongly argue for H_4 receptor control of neutrophil release from the bone marrow. The fact that histamine injection closely mimicked the effects of zymosan, and that thioperamide was the only histamine receptor antagonist able to block neutrophil recruitment, shows that histamine is an endogenous mediator of zymosan-induced pleurisy and, secondly, H_4 receptor activation is pivotal for this to happen. Interestingly, however, the inhibitory actions of thioperamide or, for instance, PTX, to histamine-induced decreases in neutrophil numbers in the bone marrow was not complete. The absolute numbers of neutrophils harvested from the bone marrow of non-treated mice averaged 16-20 million as illustrated in Figure 2, and dropped to 2.5-5 million after zymosan or histamine application. Thioperamide or PTX pretreatment resulted in a ‘rescue’ of 60-70% of

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neutrophils, i.e. the numbers of neutrophils in the bone marrow were brought back to 10-12 million under drug treatment. The question of why the rescue was not complete is difficult to answer. The most likely explanations, however, are that i) thioperamide and/or PTX did not effectively reach all target receptor molecules activated by histamine, ii) pharmakokinetic properties of the test compounds would not allow for full efficacy, or iii) an additional, non-$G_{i/o}$-coupled, thioperamide-insensitive histamine receptor or histamine-induced biological response plays a role. There is, however, no room for a contribution of H_1 or H_2 receptors, as even at very high doses pyrilamine and cimetidine were without any effect. By using thioperamide as a tool to study H_4 biology, a possible role of H_3 can not be completely ruled out however is rather unlikely given the completely different expression pattern of H_3 compared to H_4, the former being absent on most if not all leukocytes (Gantner et al., 2002). Further, a contribution of H_3 would not explain the lack of full efficacy by thioperamide and PTX in this model. However, in vitro studies performed with histamine-stimulated neutrophils from man, mouse or rats were all negative with regard to chemotaxis induction (data not shown) therefore it is still unclear whether or not histamine induces neutrophil migration directly via H_4 activation in vivo. Histamine can lead to the up-regulation of P-selectin, an endothelial cell adhesion glycoprotein expressed early on during an inflammatory process on the cell surface where it binds to blood leukocytes (Burns et al., 1999). This histamine effect, however, is mediated by the H_1 receptor (Weber et al., 1997). Since pyrilamine failed to influence histamine-induced neutrophil recruitment in our model (c.f. Figure 4), and P-selectin antibody did not appear to inhibit the response, it is unlikely that this is a plausible explanation. In neutrophils, activation of L-selectin induces a variety of

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responses, including calcium flux, activation of the respiratory burst, and, importantly, potentiation of $G_{i/o}$ signaling (Hornquist et al., 1997), which ultimately could explain how H_4 receptor- and L-selectin-derived signals cooperate and then impact the more 'global' migration-inducing signaling events such as activation of PI-3K and Rho (Figure 6). L-selectin cross-linking induces tyrosine phosphorylation as well as activation of MAP kinases, initiating in turn a signaling cascade involving L-selectin phosphorylation, recruitment of the signaling molecules Grb2/Sos, and activation of Ras and Rac2 (Patel et al., 2002) (Ebnet and Vestweber, 1999). Thereby, L-selectin and H_4 signals might synergistically trigger migration in neutrophils in vivo. Putting our findings and the published observations together the remaining unanswered question is how histamine influences the L-selectin system. It can be hypothetized that the sensitivity of the L-selectin system towards activation increases. Possibly, histamine influences the expression levels of L-selectin and/or its ligands in the bone marrow, affects the avidity of L-selectin to their ligand(s), or a combination of both.

Collectively, the similarities and differences between neutrophilia induced by zymosan and carragenan, respectively, can be summarized as follows: Both models depend on E-selectin, Mac-1, LFA-1, Gi/o signaling, are sensitive to dexamethasone (Takeshita et al., 2003), partly depend on TNF-α (unpublished observation), and do not depend on P-selectin. The models clearly differ with regard to the necessity of mast cells, L-selectin, histamine and its action on H_4 receptor, and, the number of cells recruited to the site of injection and the body compartment of neutrophil origin ((Takeshita et al., 2003), this study), and, finally, their sensitivity to inhibitors of Gi/o proteins (carrageenan more

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sensitive, c. f. Figure 1), lipoxygenase (zymosan more sensitive; (Rao et al., 1994)), cyclooxygenase (carrageenan more sensitive; (Calhoun et al., 1987)), and PAF antagonists (zymosan more sensitive; (Martins et al., 1989)).

In utilizing these models to evaluate the efficacy of anti-inflammatory compounds, it is important to bear in mind that, at face value, these two models simply facilitate a general assessment of in vivo trafficking of leukocytes. However, it will be critical to carefully assess their comparability and relevance to the mechanistic intricacies of the physiologic process that one wishes to antagonize, as well as the specific compartments at which one is looking. Given the complexity of leukocyte immune surveillance, trafficking and recruitment, as well as the highly variable kinetics of mediator release and duration of action, a more detailed assessment of the source of recruited leukocytes may have significant impact on the evaluation of the efficacy of a particular therapeutic.

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Footnotes

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JPET Fast Forward. Published on March 2, 2004 as DOI: 10.1124/jpet.103.063776
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Legends for figures

Figure 1: Inhibition by PTX, and effects of anti-selectin Ab on neutrophil migration into the pleural cavity induced by zymosan and carrageenan

Mice received 3 μg/kg of the PTX or the corresponding volume of PBS i.v. (0.2 ml/head) 3 minutes prior to zymosan (A) or carrageenan (B) injection into the pleural cavity. Four hours later, mice were euthanized, pleural fluid collected, and the number of neutrophils microscopically determined. Data are expressed as mean values ± SEM of 5 animals per group. Note the differences in the scale of the Y-axes. Results were analyzed using Student's *t*-test. ***$P<0.001$ versus the respective control group.

Mice received 100 μg/kg of anti-L (C), -E (D), -P (E) or the corresponding control Ab i.v. (0.2 ml/head) 3 minutes prior to zymosan or carrageenan injection into the pleural cavity. Four hours later, mice were euthanized, pleural fluid collected, and the number of neutrophils microscopically determined. Data are expressed as mean values ± SEM of 5 animals per group. Results were analyzed using Student's *t*-test. **$P<0.01$ ***$P<0.001$ versus the respective control group.

Figure 2: Anti-β2 integrin Ab abrogate neutrophils migration into the pleural cavity induced by zymosan or carrageenan

Mice received 100 μg per mouse of anti-LFA-1 (A), anti-MAC-1 (B) or the corresponding control Ab i.v. (0.2 ml/head) 3 minutes prior to zymosan or carrageenan injection into the pleural cavity. Four hours later, mice were euthanized, pleural fluid collected, and the number of neutrophils microscopically determined. Data are expressed

as mean values ± SEM of 5 animals per group. Statistical differences were analyzed using Sudent's *t*-test. ***P*<0.01 ****P*<0.001 versus the respective control group.

Figure 3: Time course of zymosan- or carageenan-induced changes in neutrophil numbers in blood and bone marrow and effect of anti-L-selectin Ab, anti-MAC-1 Ab, PTX and dexmethasone on the zymosan-induced release of neutrophils from the bone marrow

Male Balb/c mice received zymosan (A, 100 μg/mouse) or carrageenan (B, 500 μg/mouse) by intrapleural injection (0.2 ml/cavity). At the time point indicated, mice were euthanized, blood or bone marrow collected, and neutrophil numbers microscopically determined (blood: open circles; bone marrow: full circles). Data are expressed as mean values ± SEM of 5 animals per group.

Mice received equal doses (100 μg/mouse) of anti-L-selectin (C), anti-MAC-1 (D), or control Ab. PTX (E, 3 μg/mouse), dexamethasone (3, 30 mg/kg; F), or the corresponding volume of PBS (0.2 ml/head) were administered i.v. 3 minutes prior to zymosan injection into the pleural cavity. Two hours later, mice were euthanized, cells in bone marrow collected, and the number of neutrophils microscopically determined. Data are expressed as mean values ± SEM of 5 animals per group. Statistical differences were analyzed using Student's *t*-test. ****P*<0.001 versus respective control group.

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Figure 4: Prevention of zymosan-induced release of neutrophils from the bone marrow by the H_4 antagonist thioperamide

Mice received various doses of the histamine receptor antagonists indicated or the corresponding volume of vehicle (V; 0.2 ml/head, i.v.) 3 minutes prior to zymosan injection (100 μg/mouse) into the pleural cavity. Two hours later, mice were euthanized, bone marrow cells collected, and the number of neutrophils microscopically determined. Data are expressed as mean values ± SEM of 5 animals per group. Statistical differences were analyzed by using one-way ANOVA and differences between groups were assessed using Dunnett's method (*P<0.05, **P<0.01). Statistical differences were analyzed using student's *t*-test where appropriate (H_1, H_2).

Figure 5: Dose-response and time course of histamine induced release of neutrophils from the bone marrow of the mouse and effects of histamine receptor antagonists

Male Balb/c mice received histamine by intravenous injection (0.2 ml/mouse) at the doses indicated (A) or at a dose of 300 μg (B). Two hours later (A) or at the time points indicated (B), mice were euthanized, bone marrow cells collected, and the number of neutrophils microscopically determined. Data are expressed as mean values ± SEM of 5 animals per group. Results were analyzed using one-way ANOVA and differences between groups were assessed using Dunnett's method (*P<0.05, **P<0.01).

Mice received various doses of the histamine receptor antagonists as indicated (C, D and E) or the corresponding volume of vehicle (V; 0.2 ml/head, i.v.) 3 min prior to histamine injection into the abdominal vein. Two h later, mice were euthanized, bone marrow cells collected, and the number of neutrophils microscopically determined. Data are expressed

as mean values ± SEM of 5 animals per group. Statistical differences were analyzed by using one-way ANOVA and differences between groups were assessed using Dunnett's method (*P<0.05, **P<0.01). Statistical differences were analyzed using student's *t*-test where appropriate (H_1, H_2).

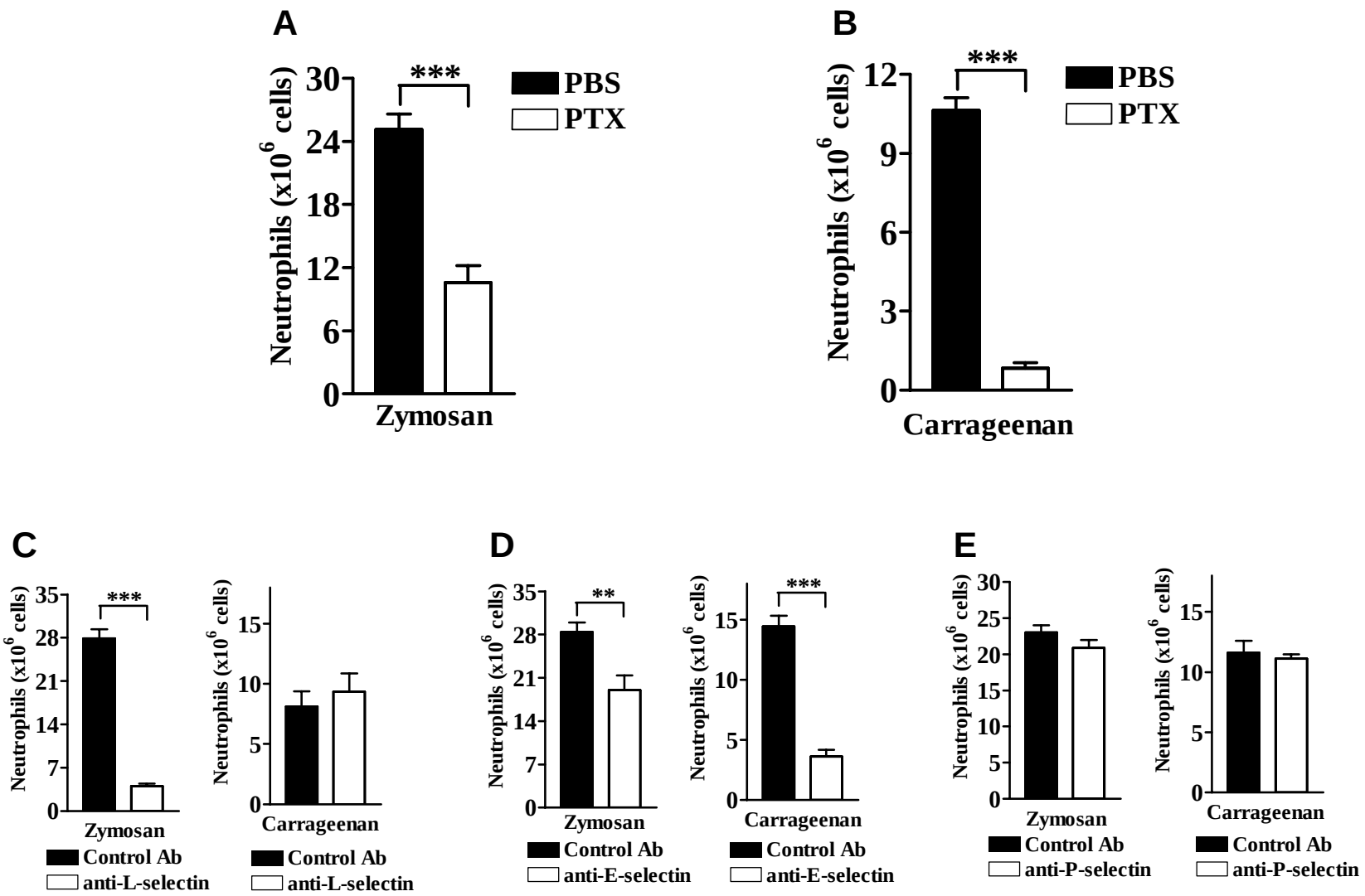
Figure 6: Inhibition of histamine-induced neutrophil recruitment by PTX, wortmannin or Y-27632

Mice received an i.v. injection of PTX (3 μg per mouse, A), wortmannin (WM, 3 mg/kg, B), Y-27632 (5 mg/kg, C), dexamethasone (30 mg/kg, D), or the corresponding volume of vehicle (PBS, 0.2 ml/head, V) 3 min prior to histamine application (300 μg/mouse, i.v.). Two h later, mice were euthanized, bone marrow cells collected, and the number of neutrophils microscopically determined. Data are expressed as mean values ± SEM of 5 animals per group. Results were analyzed using Statistical differences were analyzed using student's *t*-test. **P<0.01 ***P<0.001 versus respective vehicle-treated control group.

Figure 7: Effect of anti-selectin and anti-β2 integrin Ab on histamine-induced release of neutrophils from the bone marrow

Mice received 100 μg/kg of the anti-L- (A), -E (B), or -P (C) selectin Ab or anti-Mac-1 (D), or the corresponding volume of the respective isotype-matched control Ab in PBS i.v. (0.2 ml/head) 3 minutes prior to histamine injection into the abdominal vein. Two h later, mice were euthanized, bone marrow cells collected, and the number of neutrophils

microscopically determined. Data are expressed as mean values ± SEM of 5 animals per group. Results were analyzed using Statistical differences were analyzed using student's *t*-test. ***P*<0.01 ****P*<0.001 versus respective control group.



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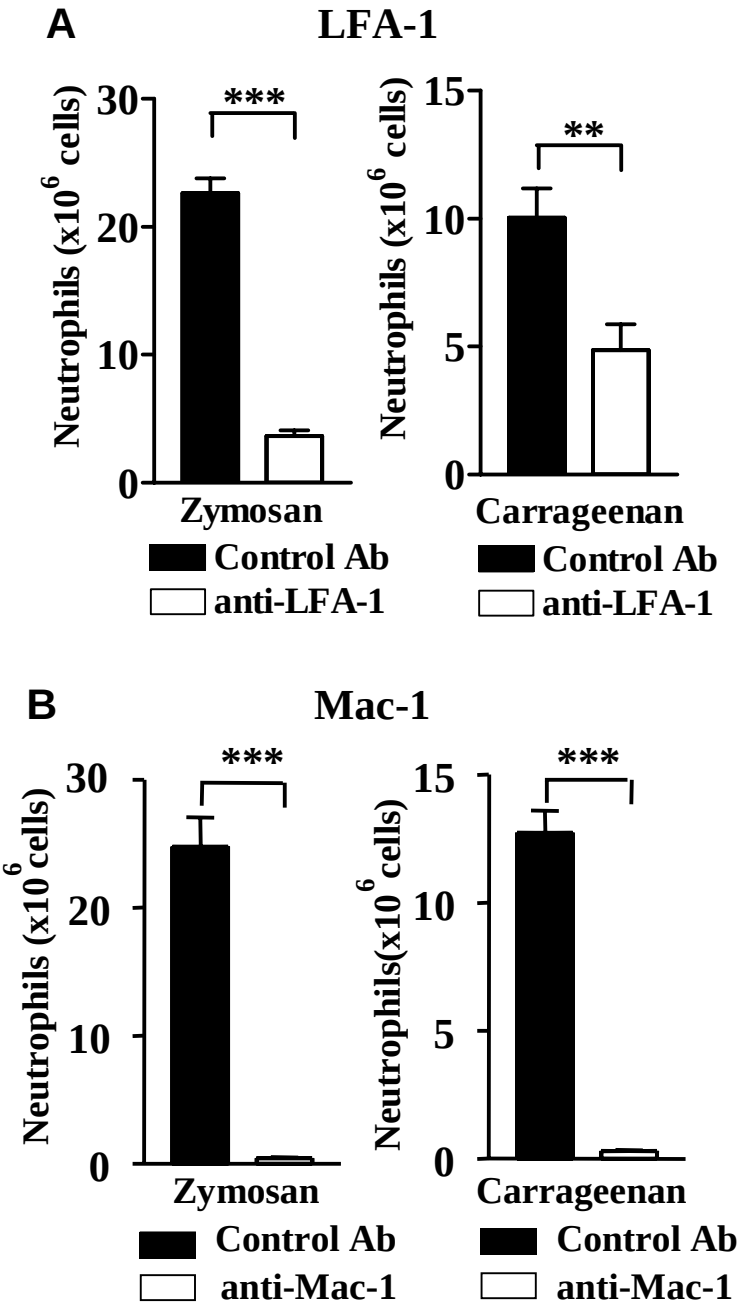

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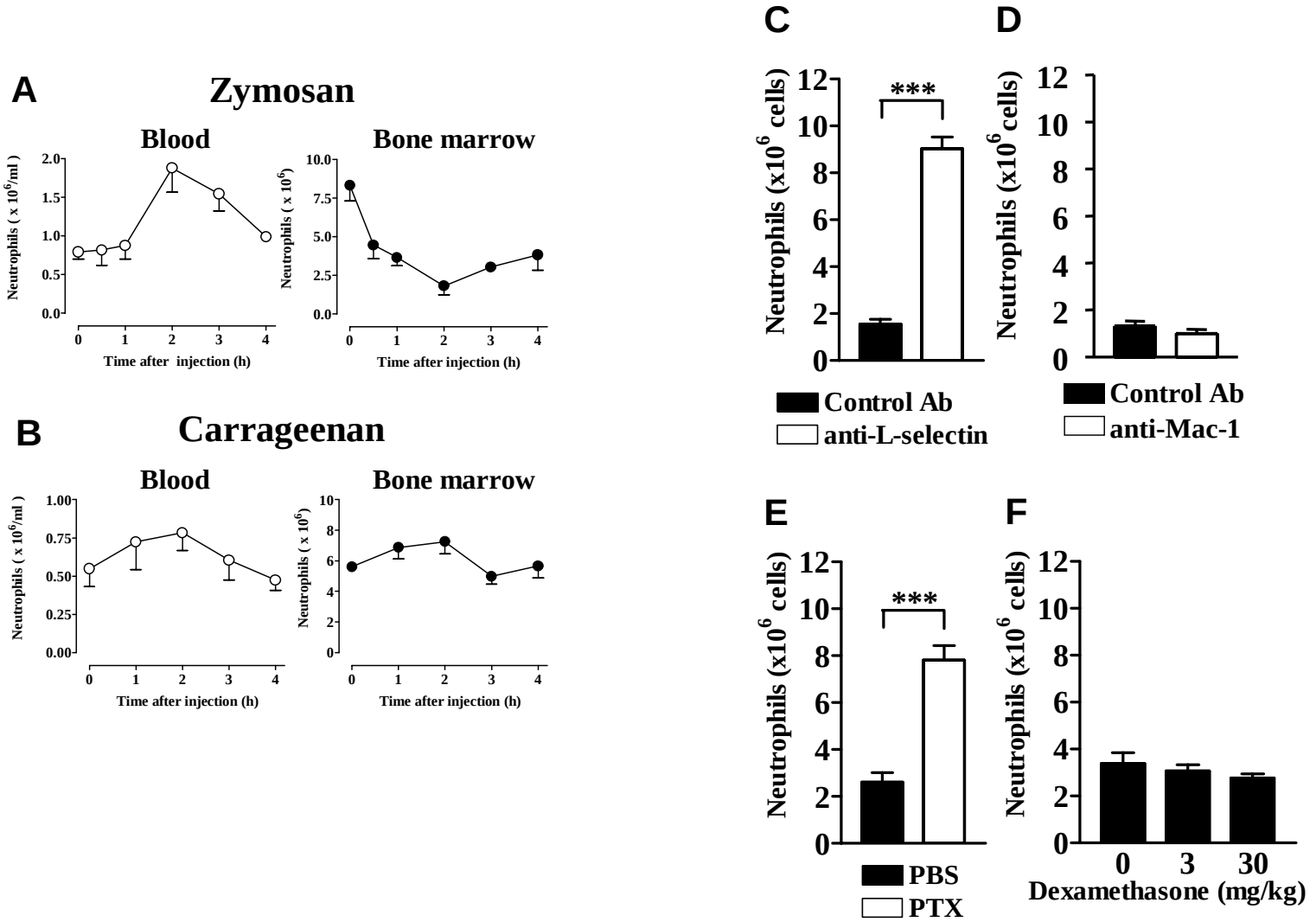

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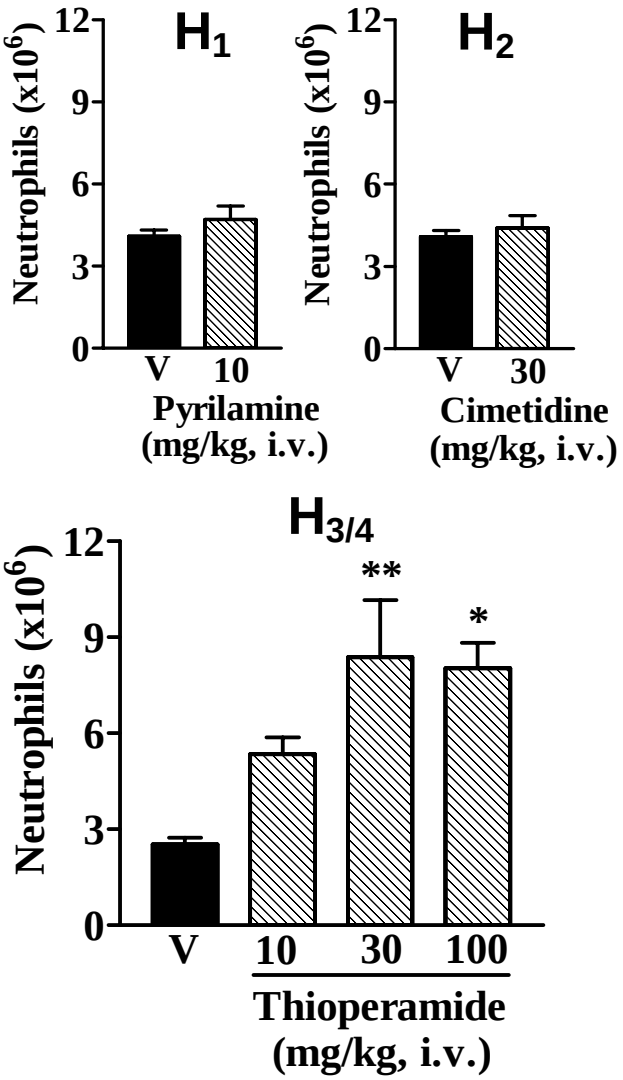

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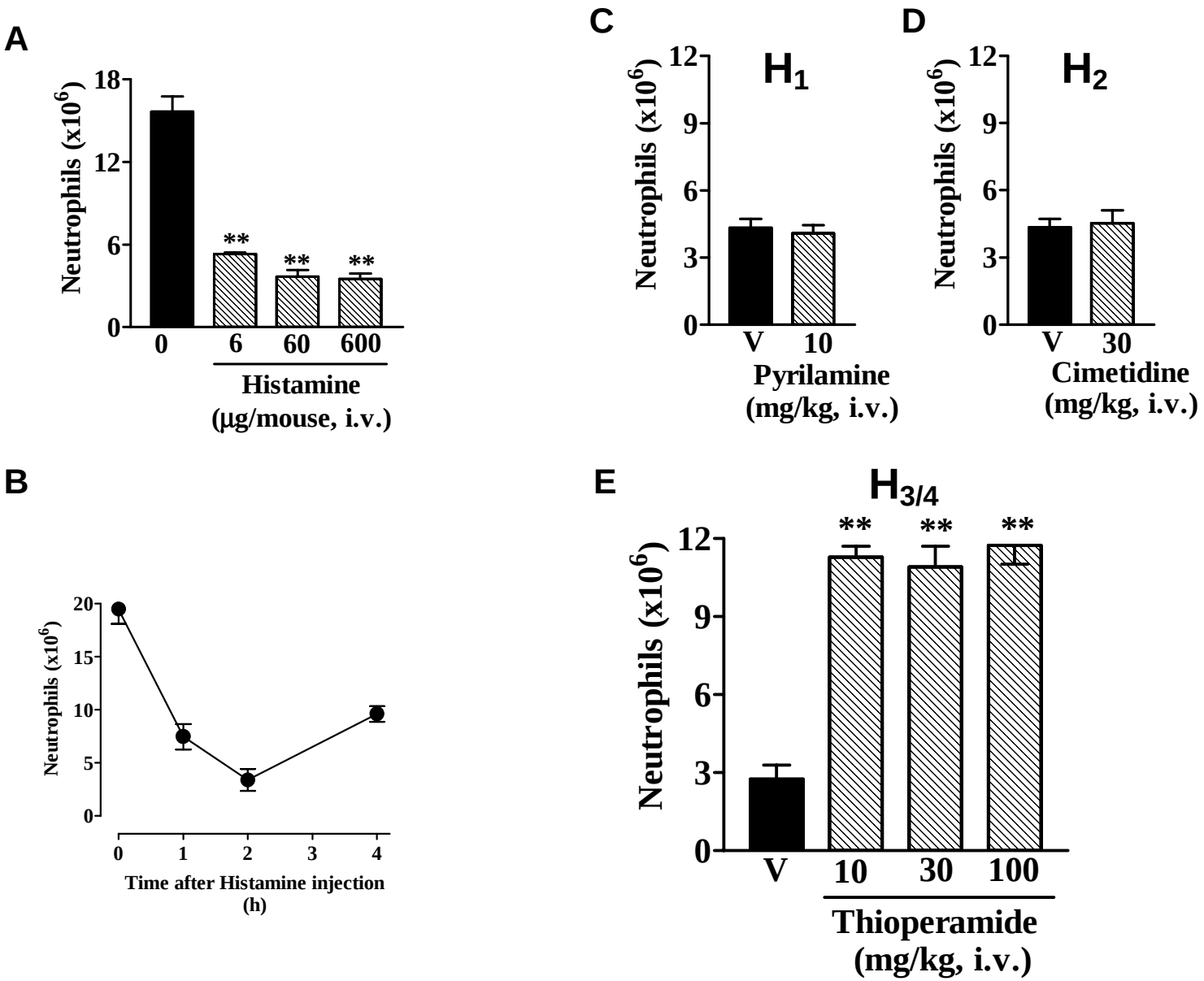

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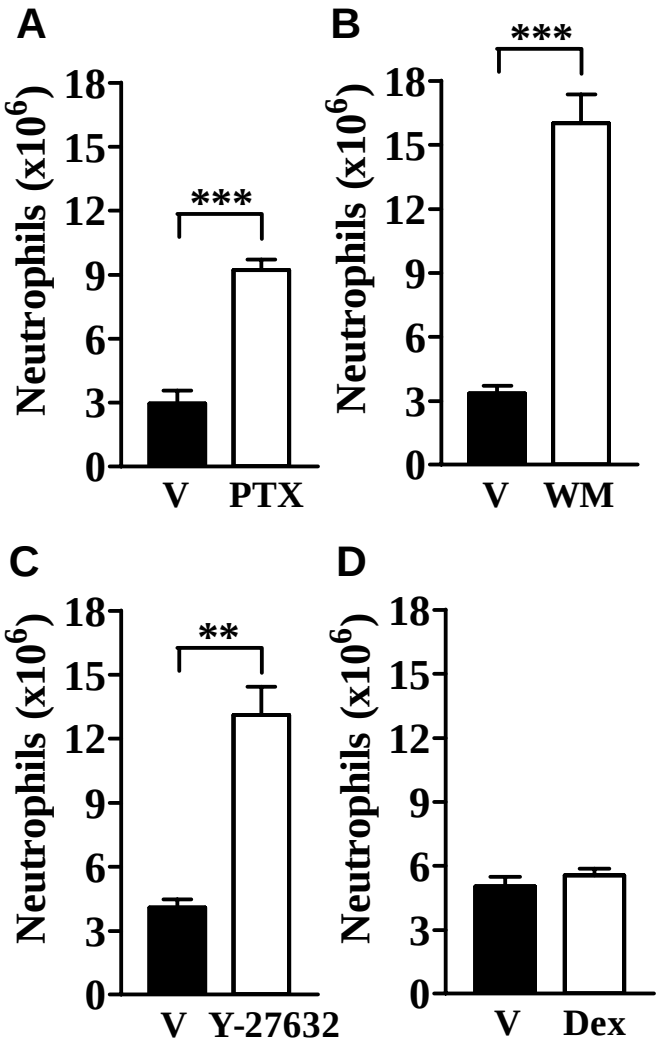

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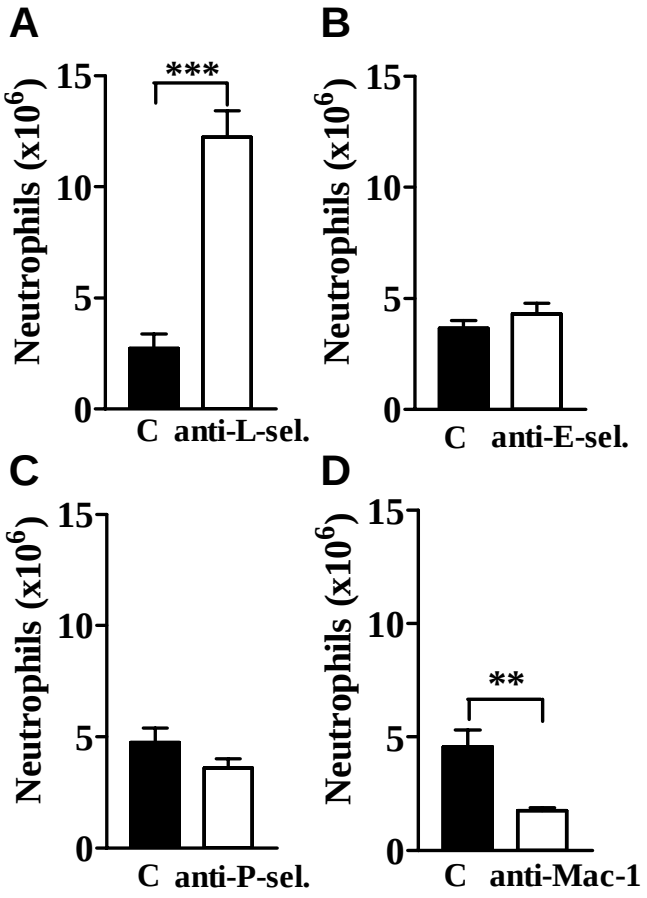

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