Phase-dependent roles of E-selectin during chronic contact hypersensitivity responses.

Phase-Dependent Roles of E-selectin

during Chronic Contact Hypersensitivity Responses

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Numbers of text pages: 21

Numbers of tables: 0

Numbers of figures: 8

Running title: E- AND P-SELECTINS IN CHRONIC CONTACT HYPERSENSITIVITY

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Abstract

Chronic contact hypersensitivity (CH) models induced by repeated hapten exposure exhibit chronic dermatitis and immunologic abnormalities resembling atopic dermatitis. To assess the contribution of endothelial selectins (P- and E-selectins) to cutaneous chronic inflammation, chronic CH responses were assessed in mice lacking P- or E-selectin. Elicitation with oxazolone on the ears of P-selectin$^{-/-}$ mice 7 days after the sensitization induced a typical delayed-type hypersensitivity response similar to that found in wild-type mice. By contrast, a significant increase in ear swelling was observed in E-selectin$^{-/-}$ mice 36 to 48 hours after first elicitation. E-selectin$^{-/-}$ mice showed augmented P-selectin upregulation, and administration of anti-P-selectin monoclonal antibody significantly inhibited the enhanced ear response, suggesting that the enhanced ear-swelling response in E-selectin$^{-/-}$ mice resulted from compensatory increase in P-selectin expression. In the late phase of chronic CH, acceleration of ear swelling was significantly reduced in both E- and P-selectin$^{-/-}$ mice relative to wild-type littermates. Thus, the loss of P- or E-selectin suppressed inflammatory responses during the chronic phase of the chronic models, while early-phase inflammatory responses are exacerbated by E-selectin blockade. Collectively, P- and E-selectins cooperatively regulate CH response, although their roles may be different depending on the phase of the reaction.

Introduction

Leukocyte recruitment from the circulation into a site of inflammation involves adhesive interactions between leukocyte and the vascular endothelium, and has been implicated in the pathology of various inflammatory diseases [1-3]. Leukocytes first tether and roll on vascular cells, before they are activated to adhere firmly and subsequently emigrate into the extravascular space. The selectin family, which mediates tethering and rolling of leukocytes, consists of three cell-surface molecules expressed by leukocytes (L-selectin), vascular endothelium (E- and P-selectin), and platelets (P-selectin) [1]. E-selectin expression is induced within several hours after activation with inflammatory cytokines, while P-selectin is rapidly mobilized to the surface of activated endothelium or platelets [1].

Interaction of adhesion molecules during the process of leukocyte migration into inflammatory sites is complex and highly regulated, where three selectin members have partially overlapping functions [4, 5]. Specifically, E-selectin-deficient (E-selectin$^{-/-}$) mice do not exhibit significant defects of leukocyte rolling and inflammation, which are virtually eliminated by the additional blockade of P-selectin [6-9]. In addition, the relative contribution of each adhesion molecule to the inflammation varies according to the tissue site and the nature of the inflammatory stimuli [10]. For example, in experimental models such as Arthus reaction and Con A- or LPS-induced dermal inflammation, inhibition or loss of E-selectin expression does not induce a significant reduction of leukocyte rolling and recruitment, while inhibition or loss of P-selectin expression results in a significant reduction of leukocyte rolling and recruitment [11, 12]. By contrast, loss of P-selectin exacerbates certain inflammatory responses, such as glomerulonephritis and collagen-induced arthritis [13, 14]. Therefore, it is important to evaluate the differential contribution of each adhesion molecule to the diseases and their experimental models.

Contact hypersensitivity (CH) is a cutaneous immune reaction in sensitized individuals to subsequent contact with the sensitizing hapten, where local endothelial cell activation plays a critical role. Repeated application of a contact-sensitizing agent in mice induces chronic CH responses that is clinically relevant to human skin allergic diseases [15]. Repeated epicutaneous application of antigen (Ag) results in a shift in the time course from a typical delayed-type hypersensitivity (DTH) to an immediate-type hypersensitivity (ITH) response followed by a late phase reaction [15-17]. This ITH response is site-restricted and Ag-specific [15, 16], and is associated with a change from a local Th1-type to Th2-type cytokine pattern with elevated serum IgE levels [15, 16, 18]. Since atopic dermatitis (AD) is often caused by repeated epicutaneous exposure to various environmental Ags, this is considered to serve as a potential animal model for AD. In acute CH models, L-selectin$^{-/-}$ mice and ICAM-1$^{-/-}$ mice exhibit reduced responses, with the combined loss of both molecules resulting in additional reductions [19]. Furthermore, mice lacking both E-selectin and P-selectin expressions have an impaired DTH response, whereas no such impairment was seen in E- or P-selectin$^{-/-}$ mice [20].

We previously reported that the induction of an ITH response following chronic Ag exposure was completely eliminated by deficiency or blockade of L-selectin or ICAM-1, while development of the accompanying late phase reaction was significantly inhibited. These findings suggest that adhesion molecules are potential therapeutic targets for regulating human allergic reactions [21]. However, the relative contribution of E- and P-selectins to this CH model remains unknown. The current study demonstrates that loss of E- or P-selectin inhibits the chronic inflammatory response, while loss of E-selectin alone exacerbates inflammatory response in early phase of this CH model, mainly due to excess expression of P-selectin.

Materials and Methods

Mice

P-selectin$^{-/-}$ [22], E-selectin$^{-/-}$ mice [8], and wild-type C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were healthy, fertile, and did not display evidence of infection or disease. All mice were backcrossed between 10 generations onto the C57BL/6 genetic background. Mice used for experiments were 12 to 16 weeks old. All mice were housed in a pathogen-free barrier facility and screened regularly for pathogens. All studies and procedures were approved by the Committee on Animal Experimentation of Kanazawa University Graduate School of Medical Science.

Sensitization and elicitation procedure

Mice were sensitized with 20 μl of a 1% oxazolone solution (4-ethyoxymethylene-2-phenyloxazolone; Sigma-Aldrich, St. Louis, MO) in acetone/sesame seed oil (4:1) applied to the right ear (10 μl on the dorsal side and 10 μl on the ventral side of the right ear) as described elsewhere [23]. Starting 7 days following sensitization, 20 μl of 1% oxazolone was repeatedly applied to the original sensitized right ear as above at 2-day intervals until day 10. An identical amount of acetone/sesame seed oil (4:1) was administered to the left ear as control.

Ear thickness was measured using a dial thickness gauge (Ozaki Seisakusho Co., Tokyo, Japan) under light ether anesthesia at various time points during the course of the experiment. For detailed time-course analysis of ear-swelling reactions, ear thicknesses were measured before and 0.5, 1, 3, 6, 9, 12, 24, 36, and 48 h after each elicitation on days 0, 4, and 8. The each ear lobe was measured three times at each time point and the mean of those values was used for analysis. Six mice were used per each group in all experiments.

Histological examination and immunohistochemical staining

A central strip of the ear was fixed in 3.5% paraformaldehyde and then paraffin embedded. Sections (6 μm) were stained using hematoxylin and eosin (H&E) for general histological evaluation and toluidine blue for mast cell staining. Dermal leukocyte infiltration was evaluated by averaging the numbers of leukocytes present in 12 high-power fields (0.07 mm^2). Each section was examined independently by two investigators in a blinded manner, and the mean was used for analysis. For immunohistochemistry, frozen tissue sections of skin biopsies were acetone-fixed and then incubated with 10% normal rabbit serum in PBS (10 min, 37°C) to block non-specific staining. Sections were then incubated with rat monoclonal antibodies (mAbs) specific for mouse CD4 (RM4-5, BD PharMingen, San Diego, CA), and CD8 (53-6.7, BD PharMingen). Rat IgG (Southern Biotechnology Associates Inc., Birmingham, AL) was used as a control for non-specific staining. Sections were then incubated sequentially (20 min, 37°C) with a biotinylated rabbit anti-rat IgG (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA), then horseradish peroxidase-conjugated avidin-biotin complexes (Vectastain ABC Kit, Vector Laboratories). Sections were developed with 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide, and then counterstained with methyl green.

Blocking study by mAbs

For a blocking study using mAbs to P-selectin and/or L-selectin and/or ICAM-1, mAbs were injected intravenously into E-selectin$^{-/-}$ mice 24 h after the elicitation on day 0. Abs used in this blocking study included mAbs to murine L-selectin (MEL14, rat IgG2a, 100 μg per mouse; BD PharMingen, San Diego, CA) [24], mAbs to murine P-selectin (RB40.34, rat IgG1, 30 μg per mouse; BD PharMingen) [25] and mAbs to murine ICAM-1 (3E2, Armenian hamster IgG, 100 μg per mouse; BD PharMingen) [26]. These were the mAb concentrations

required to inhibit L-selectin-, P-selectin- and ICAM-1-dependent leukocyte recruitment in vivo as previously described [27, 28]. Irrelevant isotype-matched, purified rat IgG1 mAb (R3-34), rat IgG2a mAb (R35-95) and Armenian hamster IgG mAb (Ha4/8) served as controls (30 μg per mouse; BD PharMingen).

RNA isolation and real-time RT-PCR

Total RNA was isolated from the ear with mutant and wild-type mice using QIAGEN RNeasy spin columns (QIAGEN Ltd., Crawley, UK) and digested by DNase I (QIAGEN Ltd.) to remove chromosomal DNA in accordance with manufacturer's protocol. Total RNA was reverse transcribed to cDNA using Reverse Transcription System with random hexamers (Promega, Madison, WI), and mRNA expression of ICAM-1 and P-selectin was analyzed by real-time quantitative RT-PCR using the TaqMan® system (Applied Biosystems, Foster City, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize mRNA. We obtained all sequence-specific primers and probes from TaqMan® Gene Expression Assays (Applied Biosystems). Real-time PCR was performed on an ABI Prism 7000 Sequence Detector (Applied Biosystems) according to the manufacture's instructions. Relative expression of real-time PCR products was determined using the $\Delta\Delta C_T$ technique as previously described [29]. Briefly, we normalized each set of samples using the difference in threshold cycle (C_T) between the target gene and GAPDH: $\Delta C_T = (C_{T\ \text{target gene}} - C_{T\ \text{GAPDH}})$. Relative mRNA levels were calculated by the expression $2^{-\Delta\Delta CT}$ where $\Delta\Delta C_T = \Delta C_{T\ \text{sample (n)}} - \Delta C_{T\ \text{calibrator (n)}}$. Each reaction was done in, at least, triplicate. One of the control samples was chosen as a calibrator sample.

Statistical Analysis

The Mann-Whitney U-test was used for determining the level of significance of differences in sample means and Bonferroni's test was used for multiple comparisons. All data are shown as mean ± SEM.

Results

Ear-swelling response by repeated Ag elicitation

Mice sensitized on the right ears with oxazolone 7 days before the first elicitation were repeatedly exposed to oxazolone at 2-day intervals on the originally sensitized ear for 8 days. Acetone/sesame seed oil was applied to left ears as control. In addition, no swelling response was observed in the naïve mice treated on the ear with 1% oxazolone solution (data not shown). Total ear thickness was measured immediately before each elicitation. Following repeated oxazolone elicitation, wild-type mice exhibited dramatic increase in the total ear thickness, whereas repeated applications of carrier alone had no detectable effect (Fig. 1). In P-selectin$^{-/-}$ mice, increases in total ear thickness were almost identical to those found in wild-type mice. Initial velocity of ear-swelling response in wild-type mice and P-selectin$^{-/-}$ mice was relatively slow until day 4, when it was accelerated. By contrast, E-selectin$^{-/-}$ mice exhibited a more rapid onset of ear swelling on day 0, followed by an almost linear increase in thickness until day 6, resulting in a significant increase in total ear thickness in E-selectin$^{-/-}$ mice compared with wild-type mice from days 2 to 6 (1.5-5.1-fold, $p < 0.05$). There were no significant differences in total ear thickness between wild-type mice and E-selectin$^{-/-}$ mice on day 8 or 10 (Fig. 1). Thus, in the early-phase response, loss of E-selectin significantly enhanced the increase in total ear thickness by accelerating the velocity of ear swelling, while loss of P-selectin did not affect total ear thickness.

The time course of ear-swelling response in P- and E-selectin$^{-/-}$ mice

A detailed time course of ear-swelling reaction by repeated oxazolone elicitation was assessed on day 0, 4 and 8. After the first challenge (day 0), wild-type mice exhibited typical DTH response that peaked at 12 to 24 h and then gradually decreased (Fig. 2A). Similar results were obtained in P-selectin$^{-/-}$ mice. In contrast, E-selectin$^{-/-}$ mice exhibited

ear-swelling response that was similar with wild-type mice until 24 h, while ear swelling did not decrease 24 to 48 h after the first elicitation (Fig. 2A). By contrast to the day-0 response, the acceleration of ear-swelling reaction was significantly reduced in E-selectin$^{-/-}$ mice compared with wild-type mice at 3 to 48 h after the elicitation on day 4 (>33% decrease, $p < 0.05$), while there were no significant differences between P-selectin$^{-/-}$ mice and wild-type mice (Fig. 2B). On day 8 in both of P-selectin$^{-/-}$ mice and E-selectin$^{-/-}$ mice, the magnitudes of ear-swelling reaction peak were significantly suppressed relative to wild-type mice (33% decrease, $p < 0.05$ in P-selectin$^{-/-}$ mice and 50% decrease, $p < 0.05$ in E-selectin$^{-/-}$ mice; Fig. 2C). Thus, the loss of P- or E-selectin inhibited inflammatory response in the chronic phase, while acute inflammatory response was rather exacerbated by loss of E-selectin.

Histological examination of the ear-swelling reaction by repeated Ag exposure

To assess histological characteristics associated with the ear-swelling reaction, ear biopsies were examined on day 0 and 8. The ear biopsies from wild-type mice taken 24 h after the Ag exposure revealed edema in the subcutaneous tissue (data not shown). Similar results were obtained by observation of tissues from P-selectin$^{-/-}$ and E-selectin$^{-/-}$ mice (data not shown). At 48 h after the Ag challenge, subcutaneous edema was only modestly observed in wild-type mice and P-selectin$^{-/-}$ mice (Fig. 3A). By contrast, subcutaneous edema with leukocyte infiltration was more prominent in E-selectin$^{-/-}$ mice 48 h after the elicitation (Fig. 3A). However, 24 h after elicitation on day 8, decreases in area of edema and in infiltrated leukocytes were observed in the subcutaneous tissue from P-selectin$^{-/-}$ and E-selectin$^{-/-}$ mice relative to wild-type mice (Fig. 3B).

Mast cell numbers were also assessed in the Ag-administrated ears from P- and E-selectin$^{-/-}$ mice and wild-type mice on day 0 and 8. Mast cell numbers 48 h after the first challenge were comparable between wild-type mice and P-selectin$^{-/-}$ mice, but were

significantly higher in E-selectin$^{-/-}$ mice than wild-type mice (1.6-fold, $p < 0.0001$; Figures 4A and 5A). Whereas, following the repeated epicutaneous hapten application, mast cell numbers 24 h after elicitation on day 8 were slightly but significantly reduced in P-selectin$^{-/-}$ mice (12% decrease, $p < 0.05$) and E-selectin$^{-/-}$ mice (17% decrease, $p < 0.05$) compared with wild-type mice (Figs. 4B and 5C). Additionally, numbers of neutrophils, CD4^{+} T cells, CD8^{+} T cells, and macrophages were not significantly different between P- or E-selectin$^{-/-}$ mice and wild-type mice before or at any time points after elicitation (data not shown). Thus, although deficiency of E-selectin enhanced mast cell accumulation in the early phase, loss of E- or P-selectin expression led to significant inhibition of mast cell accumulation in the chronic phase.

Contribution of endothelial selectins to the enhanced ear-swelling response in E-selectin$^{-/-}$ mice

A significant increase in ear swelling was observed in E-selectin$^{-/-}$ mice 24 to 48 hours after the first elicitation on day 0 (Fig. 2A). To assess the mechanisms of this enhanced ear-swelling response in E-selectin$^{-/-}$ mice, a blocking study using mAbs was performed. E-selectin$^{-/-}$ mice were intravenously treated with mAbs to L-selectin and/or P-selectin, or ICAM-1, or all, simultaneously with the Ag administration (Fig. 6A) or after 24 h (Fig. 6B). E-selectin$^{-/-}$ mice which received injection of isotype-matched control mAbs exhibited kinetics and magnitudes of ear swelling similar to those of untreated E-selectin$^{-/-}$ mice (Fig. 2A and data not shown). When E-selectin$^{-/-}$ mice were treated with mAbs to L-selectin and/or P-selectin, or ICAM-1, or all simultaneously with the Ag administration on day 0 (Fig. 6A), ear-swelling responses were significantly inhibited relative to wild-type mice and resulted in a significant further reduction of ear-swelling response compared with E-selectin$^{-/-}$ mice at 36 and 48 h after elicitation (Fig. 6A). Especially, the blockade of P-selectin in E-selectin$^{-/-}$ mice

remarkably resulted in 70-80% inhibition of the ear-swelling response in wild-type mice, and the inhibitory effect of P-selectin loss in E-selectin$^{-/-}$ mice on ear-swelling responses at 48 h after elicitation was greater than both of L-selectin loss and ICAM-1 loss in E-selectin$^{-/-}$ mice.

Injection of mAbs to L-selectin, P-selectin, or ICAM-1 24 hr after the Ag administration also significantly inhibited the enhanced ear-swelling response in E-selectin$^{-/-}$ mice (Fig. 6B). Although the effect was modest compared with the 0-hr treatment, treatment with anti-P-selectin mAb significantly suppressed the exacerbated inflammatory response at 48 h after elicitation even compared with treatment with anti-L-selectin or anti-ICAM-1 mAb (Fig. 6B). Thus, the enhanced ear-swelling response in E-selectin$^{-/-}$ mice may result from compensatory increase in P-selectin expression. Furthermore, the combination of P- and L-selectins with or without ICAM-1 blockade in E-selectin$^{-/-}$ mice significantly inhibited the enhanced ear-swelling response, which was equivalent to that found in wild-type mice (Fig. 6B).

Mast cell recruitment was also reduced in E-selectin$^{-/-}$ mice with cell adhesion molecules blockade. Mast cell numbers of E-selectin$^{-/-}$ mice treated with anti-P-selectin mAb were more reduced than anti-L-selectin or anti-ICAM-1 mAb treatment (Fig. 7 and data not shown). These results were in parallel with the ear-swelling response, suggesting that the increase in mast cell numbers was mediated by adhesion molecules, especially P-selectin.

P-selectin expression was markedly up-regulated in E-selectin$^{-/-}$ mice

Since the results indicated that P-selectin expression may compensate the loss of E-selectin, expression of P-selectin, as well as ICAM-1, was assessed in wild-type mice, P-selectin$^{-/-}$ mice, and E-selectin$^{-/-}$ mice 12, 24, 36, and 48 h after elicitation on day 0. ICAM-1 and P-selectin mRNA expression levels in the dermis were quantified by real-time

RT-PCR. At 48 h after the first elicitation, ICAM-1 mRNA level was decreased in P-selectin$^{-/-}$ mice compared with wild-type mice, although there was no significant difference (Fig. 8A). Furthermore, E-selectin$^{-/-}$ mice showed identical expression level of ICAM-1 as wild-type mice. Moreover, at different time points (12, 24 and 36 h after the first challenge), no significant difference of ICAM-1 mRNA expression levels were observed in E- and P-selectin$^{-/-}$ mice relative to wild-type mice (data not shown). After the challenge, P-selectin expression was upregulated both in wild-type and E-selectin$^{-/-}$ mice (Fig. 8B). P-selectin expression level in E-selectin$^{-/-}$ mice was slightly higher than that in wild-type mice after 12 and 24 h, although there was no significant difference. In contrast to P-selectin mRNA expression in wild-type mice that remained similar levels until 48 h, P-selectin mRNA expression levels in E-selectin$^{-/-}$ mice significantly increased after 36 h (4.5-fold, $p < 0.05$) and 48 h (5.6-fold, $p < 0.05$) compared with wild-type mice. Thus, E-selectin deficiency in early phase of CH resulted in the up-regulation of P-selectin expression.

Discussion

Assessing the contribution of selectins to chronic CH responses following repeated Ag exposure, the current study has demonstrated the critical role of P-selectin and E-selectin in chronic CH responses induced by repeated epicutaneous Ag elicitation, suggesting the cooperative regulation by all selectins. We previously showed that the loss of L-selectin, ICAM-1, or both significantly inhibited chronic skin inflammation [21]. While P-selectin$^{-/-}$ mice exhibited typical early-phase CH response that was similar to that found in wild-type mice, resolution of ear swelling was disturbed in E-selectin$^{-/-}$ mice 36 to 48 h after the first elicitation (Fig. 2A). On the other hand, in the chronic phase of this CH model, acceleration of ear swelling was significantly reduced both in E- and P-selectin$^{-/-}$ mice compared with wild-type mice (Fig. 2C). Thus the loss of E-selection significantly altered the kinetics of ear swelling during the CH response; early-phase inflammatory responses were exacerbated by E-selectin blockade, while the loss of P- or E-selectin resulted in suppressed velocity of inflammatory responses during the chronic phase.

Loss of P- or E-selectin resulted in no inhibition of ear-swelling response 24 h after the first elicitation (Fig. 2A), which is consistent with a previous report [20]. At 36 and 48 h after the first elicitation, ear-swelling responses was not affected by loss of P-selectin, while loss of E-selectin resulted in impaired resolution of ear swelling (Fig. 2A). This enhanced ear-swelling response in E-selectin$^{-/-}$ mice was inhibited by administration of mAbs to cell adhesion molecules 24 h after the first challenge, especially by blocking mAbs to P-selectin (Fig. 6). In addition, augmented P-selectin expression was observed in E-selectin$^{-/-}$ mice 36 and 48 h after the first elicitation (Fig. 8). Taken together, these results suggest that the enhanced ear-swelling responses in E-selectin$^{-/-}$ mice are mainly due to compensatory increase in P-selectin. Furthermore, the concomitant administration of anti-P- and anti-L-selectin mAbs completely abrogated the augmented swelling response in E-selectin$^{-/-}$

mice, suggesting that the expression of L-selectin ligands may be also upregulated by the loss of E-selectin. Thus, while E-selectin-deficiency generally results in minimal phenotypic alteration in most inflammatory models, E-selectin plays a significant role in early-phase CH reaction by influencing on the expression levels of other adhesion molecules.

By contrast, ear-swelling responses were significantly reduced in both of E- and P-selectin$^{-/-}$ mice on day 8 compared with wild-type mice (Fig. 2C). Chronic Ag exposure leads to a shift in the time course of CH from DTH response that peaked at 24 h to an ITH response [15-17]. Relative contribution of adhesion molecules may change during the shift, which may result in increasing the dependency on endothelial selectins Alternatively, recruitment of Th2 cells to the skin may be more dependent on selectins than Th1 cells, although there have been no studies supporting this. Further investigation is required to clarify the mechanism.

Mast cell recruitment into tissues is considered to occur by release of immature mast cell precursors from the bone marrow into the peripheral blood, following by migration of these precursors into tissue and their subsequent differentiation into mature mast cells [30]. Also, increase in mast cell numbers at sites of inflammation have been reported [31]. In the present study, chronic Ag exposure induced mast cell recruitment that was significantly inhibited by loss of P-selectin or E-selectin (Fig. 4B and 5C). These results suggest that chronic inflammatory response is dependent on the recruitment of mast cells, which is regulated by expression of adhesion molecules on the cells. In agreement with these results, mast cell numbers were significantly increased in E-selectin$^{-/-}$ mice 48 h after the first elicitation (Fig. 4A and 5A). Moreover, mast cell recruitment was significantly reduced in E-selectin$^{-/-}$ mice treated with anti-P-selectin mAbs (Fig. 7). Several studies have shown that rolling of immature bone marrow-derived mast cell precursors is mediated by the interaction of P-selectin and P-selectin glycoprotein ligand-1 [32, 33]. Additionally, in the passive Arthus

reaction, cutaneous and peritoneal mast cell recruitment is reduced in mice lacking P-selectin or E-selectin [12]. Therefore, our results suggest that P-selectin and E-selectin regulate mast cell recruitment into inflammatory sites presumably through the peripheral blood, and indicate that mast cells play a critical role in the progression of inflammatory responses.

AD is a chronic inflammatory skin disease with an allergic and genetic background [34, 35]. The prevalence of AD has increased in recent years, and it is now estimated to affect up to 20% of the general population [34]. The present study demonstrates the differential contribution of P-selectin and E-selectin in murine model of chronic CH responses that are clinically relevant to human skin allergic diseases, such as AD [15]. The results indicate that chronic imflammatory responses are inhibited by P-selectin or E-selectin blockade, while the loss of E-selectin exacerbated acute imflammatory response. Thus, while anti-adhesion therapy with mAb to these selectins is potentially beneficial for AD treatment, disrupting E-selectin expression alone may lead to the exacerbation of acute inflammatory response, and simultaneous blockade of E- and P-selectins may be a preferable strategy.

Acknowledgments

We thank Ms. M. Matsubara and Ms. Y. Yamada for technical assistance. This work is supported by the Grant-in-Aid from the Ministry of Education, Science, and Culture of Japan.

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Figure Legends

Figure 1. Total ear thickness following repeated 1% oxazolone solution elicitation in P-selectin$^{-/-}$, E-selectin$^{-/-}$, and wild-type mice. Mice sensitized on the ears with oxazolone 7 days before the first elicitation were repeatedly exposed for 8 days at 2-day intervals. Repeated adminiatration with acetone/sesame seed oil alone served as control. Ear thickness was measured just before each elicitation until 10 days. All values represent the mean ± SEM of results obtained from 6 mice in each group. Statistical analysis results are described in the Results section. Error bars are not shown in case SEM values are smaller than their symbols.

Figure 2. Time course of ear-swelling responses in P-selectin$^{-/-}$, E-selectin$^{-/-}$, and wild-type mice repeatedly elicited with oxazolone on days 0 (A), 4 (B), and 8 (C). Mice sensitized on the ears with oxazolone 7 days before the first elicitation were repeatedly exposed for 8 days at 2-day intervals. Ear thickness was measured at before and 0.5, 1, 3, 6, 9, 12, 24, 36, and 48 hours after each elicitation. The values indicate the difference of the ear thickness between before and these time points. All values represent the mean ± SEM. These results were obtained from 6 mice in each group. Statistical analysis results are described in the Results section. Error bars are not shown in case SEM values are smaller than their symbols.

Figure 3. Histological sections of the ear pinnae from P-selectin$^{-/-}$ mice, E-selectin$^{-/-}$ mice, and wild-type mice 48 hours after oxazolone challenge on day 0 (A) and 24 hours after on day 8 (B). The repeated epicutaneous oxazolone elicitation was performed as described in figure 2. The sections were stained with H&E. Original magnifications, ×100 (A), ×40 (B).

Figure 4. Histological sections showing mast cell accumulation in the ear pinnae from P-selectin$^{-/-}$ mice, E-selectin$^{-/-}$ mice, and wild-type mice 48 hours after oxazolone challenge on day 0 (A) and 24 hours after on day 8 (B). The repeated epicutaneous oxazolone challenge was performed as described in figure 2. Mast cells were detected as cells with metachromatic staining of granules in toluidine blue-stained sections (arrows). Original magnifications, ×100.

Figure 5. Mast cell numbers at 48 hours after oxazolone elicitation on day 0 (A), 6 hours after on day 4 (B), and 24 hours after day 8 (C) in the dermis from P-selectin$^{-/-}$ mice, E-selectin$^{-/-}$ mice, and wild-type mice repeatedly elicited with oxazolone on the ears. The repeated epicutaneous oxazolone challenge was performed as described in figure 2. Mast cell numbers per dermis section high-power microscopic field (0.07 mm^2) were determined by counting in paraffin sections stained with toluidine-blue. All values represent the mean ± SEM of results from 6 mice in each group.

Figure 6. The effect of adhesion molecule blockade by mAbs on ear-swelling responses. E-selectin$^{-/-}$ (E-sel$^{-/-}$) mice were treated i.v. with mAbs to L-selectin (L-sel), P-selectin (P-sel), and ICAM-1, simultaneously with the elicitation on day 0 (A) or after 24 hours (B). Ear thickness was measured at 36 hours (left) and 48 hours (right) after oxazolone challenge on day 0. The repeated epicutaneous oxazolone challenge was performed as described in figure 2. The values indicate the difference of the ear thickness between before and each time point. All values represent the mean ± SEM. These results were obtained from 4 mice in each group. Statistical analysis results are described in the Results section.

Figure 7. Histological sections at 48 hours after oxazolone challenge on day 0 in the ear pinnae from wild-type mice, E-selectin$^{-/-}$ mice and E-selectin$^{-/-}$ mice treat with mAb to P-selectin 24 h after the first elicitation as was done in Fig. 6B. The sections were stained with H&E and toluidine blue. Mast cells were detected as cells with metachromatic staining of granules in toluidine blue-stained sections (arrows). Original magnifications, ×100.

Figure 8. mRNA expression of ICAM-1 (A) and P-selectin (B) in the dermis from P-selectin$^{-/-}$ mice, E-selectin$^{-/-}$ mice, and wild-type mice on day 0. mRNA expression was analyzed by real-time PCR. Relative expression of real-time PCR products was determined by using the Ct method to compare target gene and GAPDH mRNA expression. All values,

which indicate mRNA expressions relative to those in wild-type mice at 48 hours, represent the mean ± SEM. These results were obtained from 5 mice in each group.

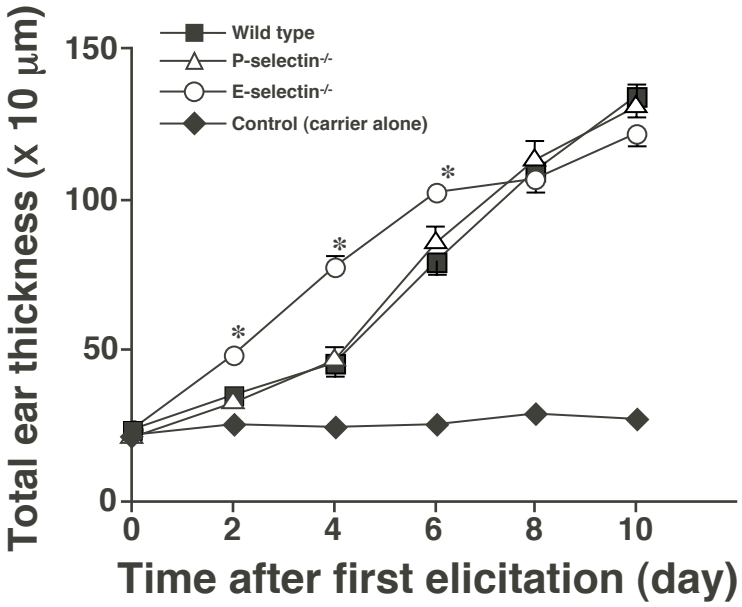

Figure 1
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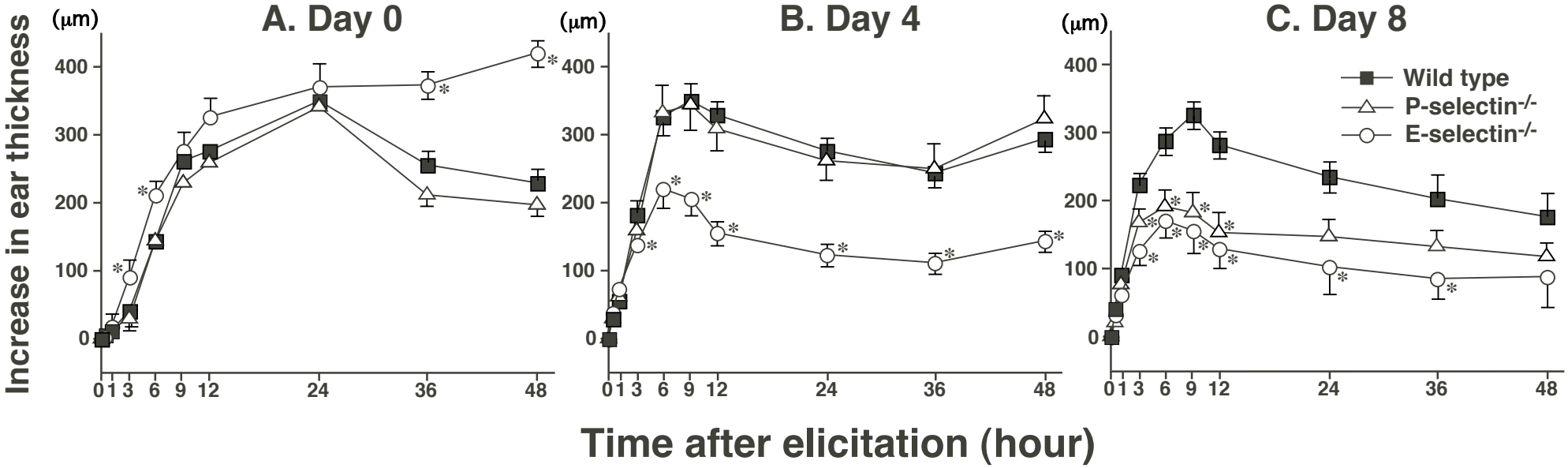

Figure 2
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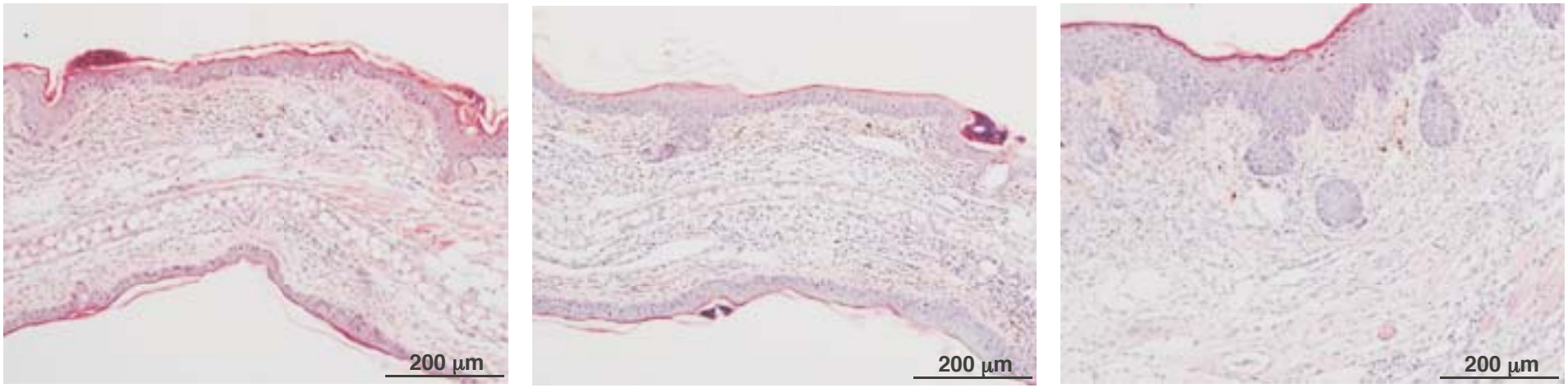
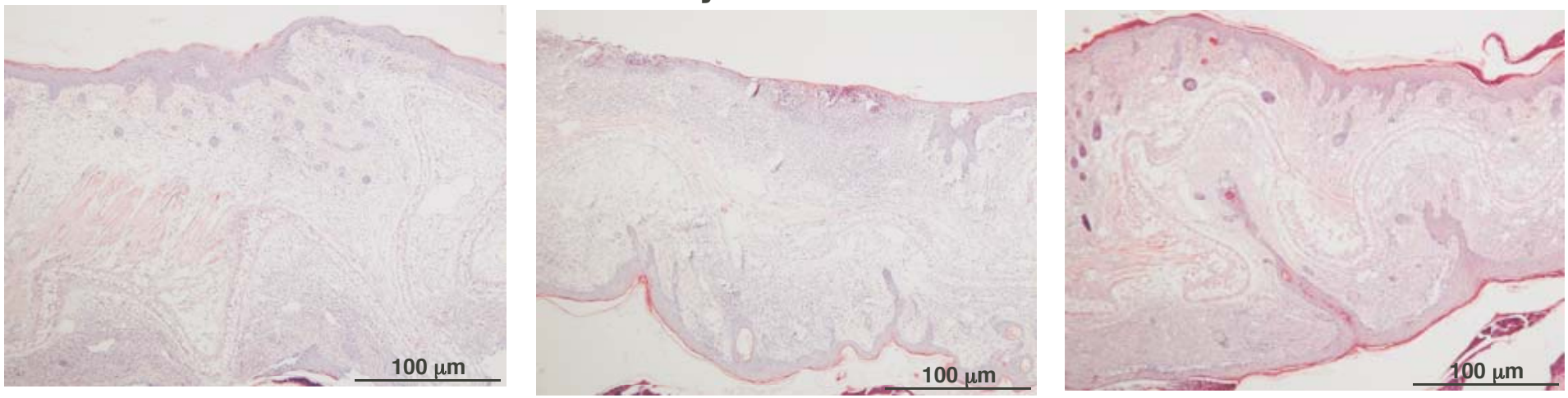
A. 48 hours after the elicitation on day 0



B. 24 hours after the elicitation on day 8



Wild-type **P-selectin$^{-/-}$** **E-selectin$^{-/-}$**

Figure 3
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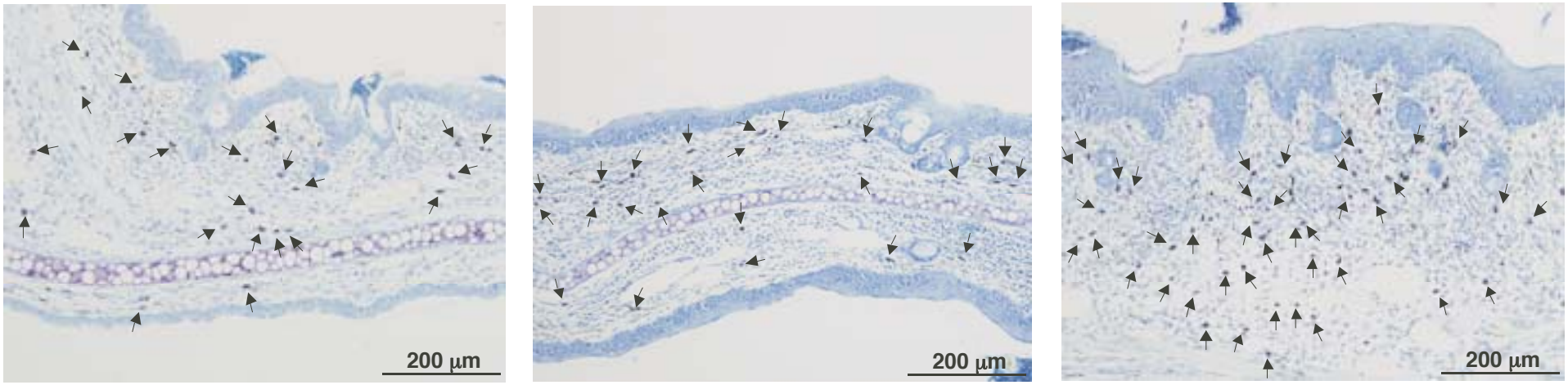
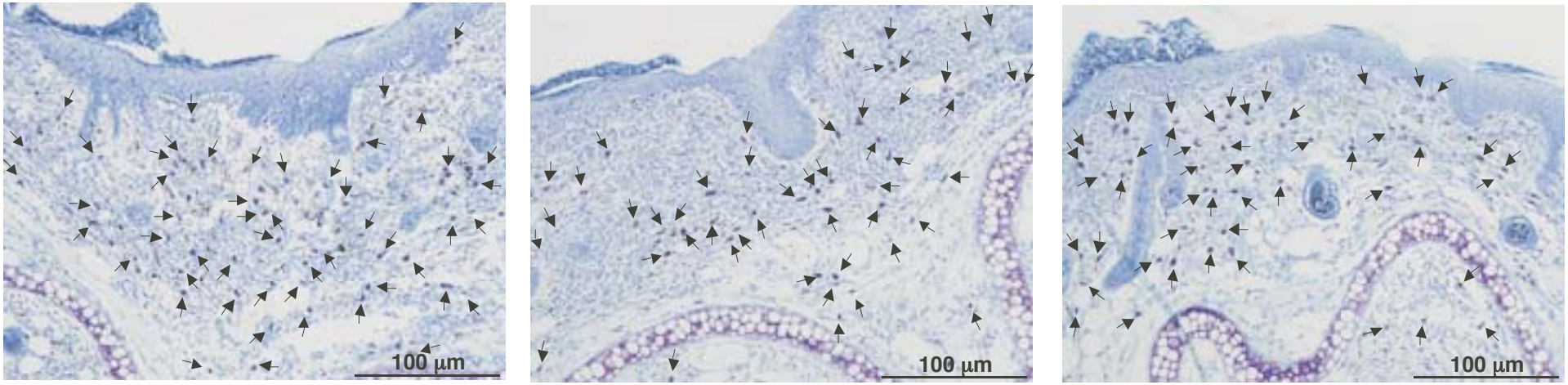
A. 48 hours after the elicitation on day 0



B. 24 hours after the elicitation on day 8



Wild-type **P-selectin$^{-/-}$** **E-selectin$^{-/-}$**

Figure 4
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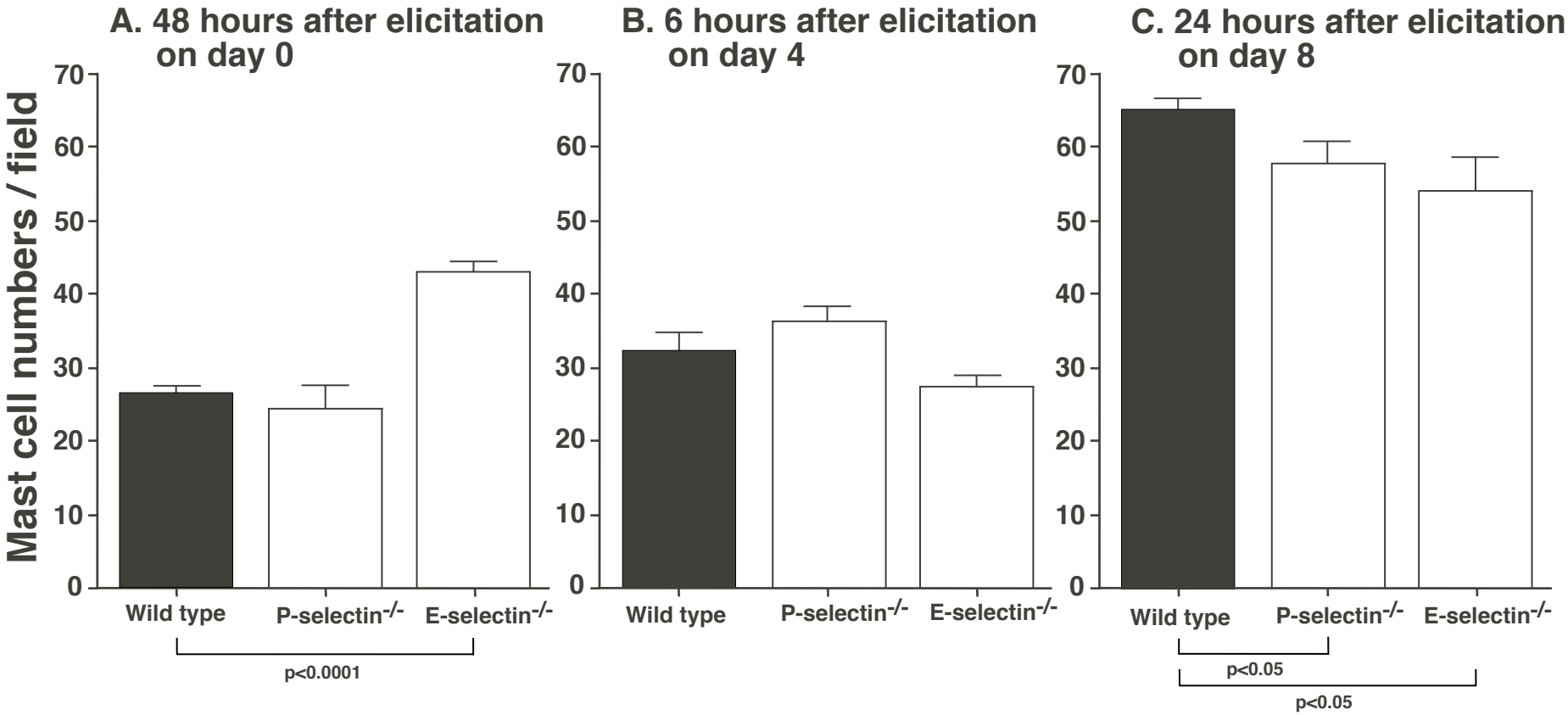

Figure 5
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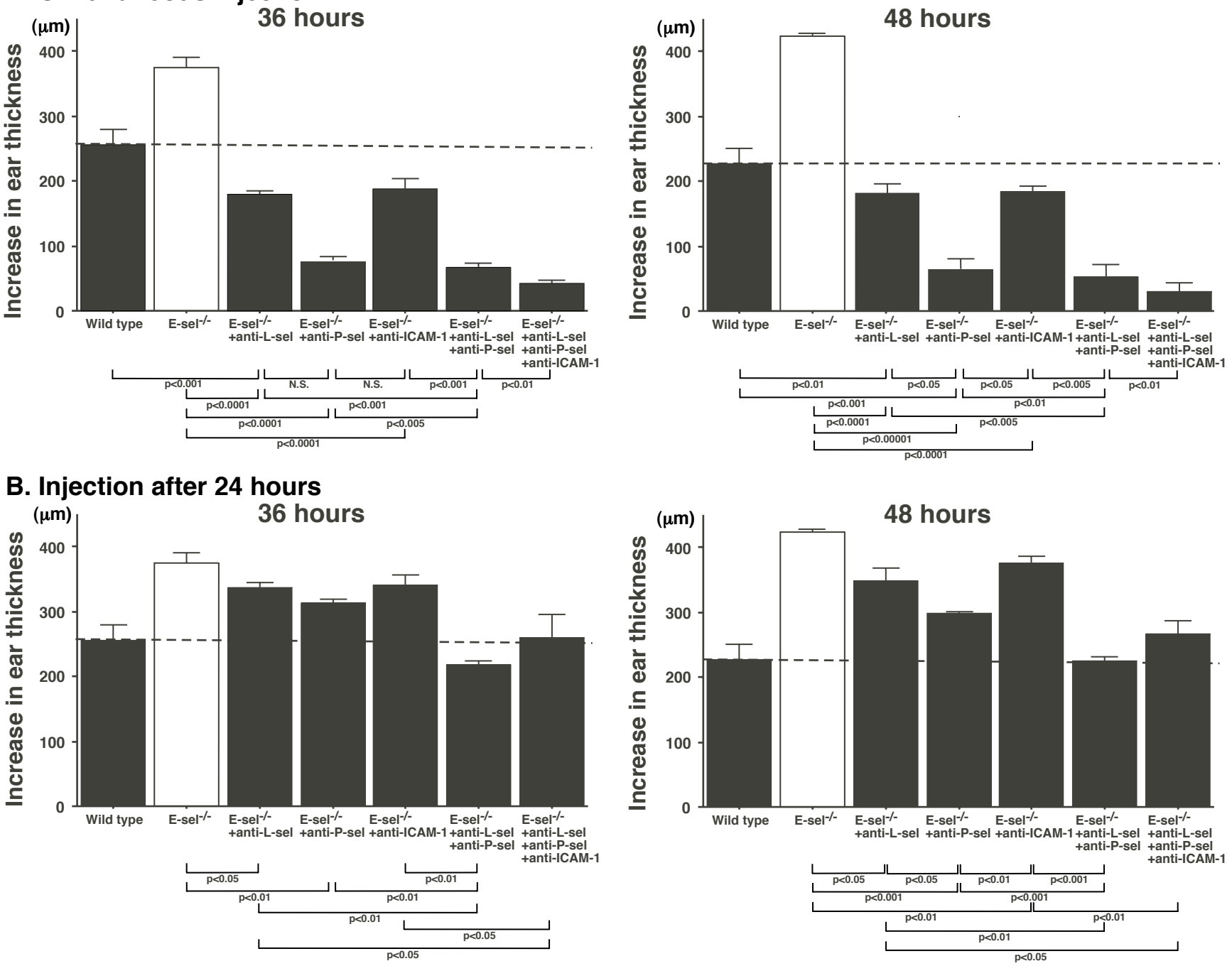

Figure 6
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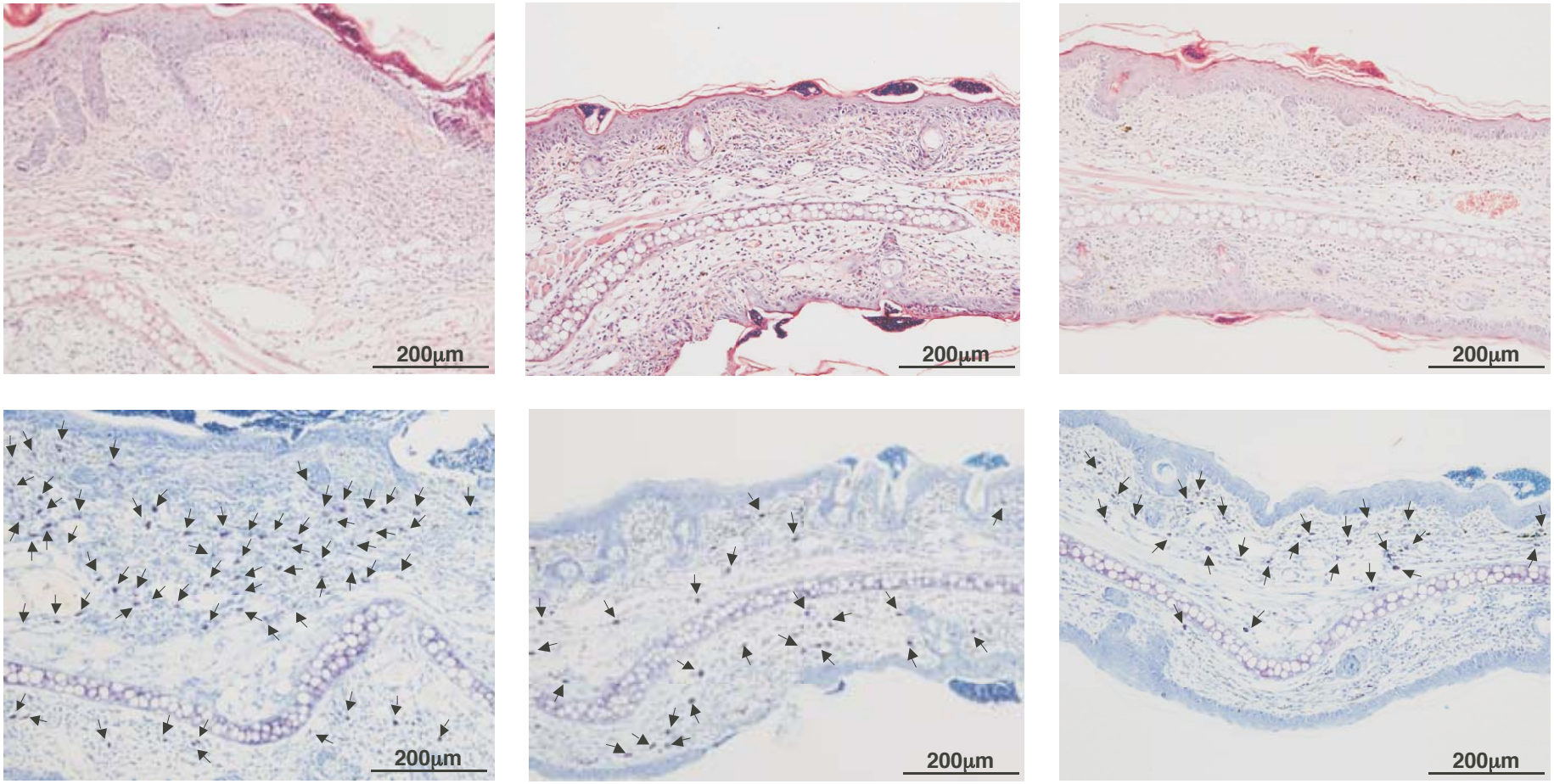
48 hours after the elicitation on day 0



E-selectin$^{-/-}$ **E-selectin$^{-/-}$+P-selectin mAb** **Wild-type**

Figure 7
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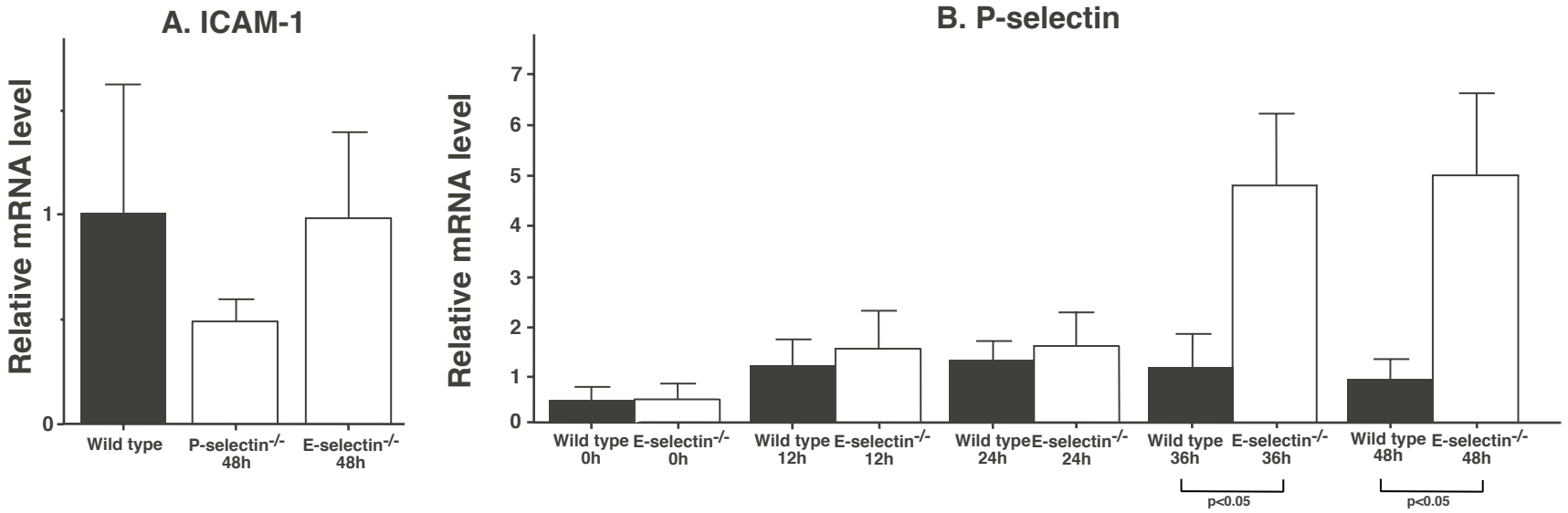

Figure 8
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