Differential regulation of human and murine P-selectin expression and function in vivo

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Leukocytes roll on P-selectin after its mobilization from secretory granules to the surfaces of platelets and endothelial cells. Tumor necrosis factor (TNF), IL-1β, and lipopolysaccharide increase synthesis of P-selectin in murine but not in human endothelial cells. To explore the physiological significance of this difference in gene regulation, we made transgenic mice bearing the human *Selp* gene and crossed them with mice lacking murine P-selectin (*Selp*$^{-/-}$). The transgenic mice constitutively expressed human P-selectin in platelets, endothelial cells, and macrophages. P-selectin mediated comparable neutrophil migration into the inflamed peritoneum of transgenic and wild-type (WT) mice. Leukocytes rolled similarly on human or murine P-selectin on activated murine platelets and in venules of the cremaster muscle subjected to trauma. However, TNF increased murine P-selectin in venules, slowing rolling and increasing adhesion, whereas it decreased human P-selectin, accelerating rolling and decreasing adhesion. Both P- and E-selectin mediated basal rolling in the skin of WT mice, but E-selectin dominated rolling in transgenic mice. During contact hypersensitivity, murine P-selectin messenger (m) RNA was up-regulated and P-selectin was essential for leukocyte recruitment. However, human P-selectin mRNA was down-regulated and P-selectin contributed much less to leukocyte recruitment. These findings reveal functionally significant differences in basal and inducible expression of human and murine P-selectin in vivo.

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Abbreviation used: ATF-2, activating transcription factor 2.

During inflammation, leukocytes tether to and roll on vascular surfaces through interactions of glycosylated ligands with P-selectin and E-selectin (McEver and Zhu, 2010). They then decelerate, arrest, and migrate into extravascular tissues through integrin-dependent mechanisms (Ley et al., 2007). The inducible expression of P- and E-selectin is a key regulator of inflammation (McEver, 1997; Vestweber and Blanks, 1999). Endothelial cells of skin and bone marrow constitutively synthesize E-selectin (Schweitzer et al., 1996; Weninger et al., 2000; Chong et al., 2004). In other tissues, mediators such as TNF, IL-1β, or LPS must stimulate endothelial cells to transcribe mRNA for E-selectin. Activation of the *Sele* gene requires cooperative binding of NF-κB, activating transcription factor 2 (ATF-2), and other transcription factors (Collins et al., 1995). This signaling pathway is conserved in all mammals studied, including mice and humans.

P-selectin is constitutively synthesized in megakaryocytes/platelets and endothelial cells, where it is packaged into storage granules (Vestweber and Blanks, 1999). It is also expressed by resident peritoneal macrophages (Tchernychev et al., 2003). Two distinct mechanisms regulate the inducible expression of P-selectin. In the first mechanism, thrombin, histamine, or other secretagogues rapidly mobilize P-selectin from storage granules to the plasma membrane (Vestweber and Blanks, 1999). This mechanism does not require new protein synthesis and is conserved in mice and humans. In the other mechanism, TNF, IL-1β, or LPS increases murine P-selectin mRNA and protein in endothelial cells in vitro and in vivo (Sanders et al., 1992; Weller et al., 1992; Hahne et al., 1993). As for the murine or human *Sele* gene, activation of the murine *Selp* gene requires

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cooperative binding of NF-κB, ATF-2, and other transcription factors (Pan et al., 1998b). This signaling pathway is conserved in all mammals studied (Auchampach et al., 1994; Bischoff and Brasel, 1995; Doré and Sirois, 1996), with the important exception of humans and other primates. The promoter of the human *Selp* gene lacks canonical binding sites for NF-κB and ATF-2 (Pan and McEver, 1993; Pan et al., 1998a). In vitro, TNF does not increase, or even decreases, P-selectin mRNA in human endothelial cells (Burns et al., 1995; Yao et al., 1996, 1999). In vivo, infusion of *Escherichia coli* into baboons, which releases LPS and stimulates secretion of TNF, increases mRNA for E-selectin but not for P-selectin (Yao et al., 1999).

The biological significance of this species difference in inducible expression of the *Selp* gene is not known. The issue is important given the widespread use of murine models of inflammation to predict physiological or pathological mechanisms in humans. In many of these models, P-selectin plays a central role, and TNF, IL-1β, and LPS are major mediators (Ley, 2003; Ley and Kansas, 2004). It is not known whether the observed functions of P-selectin require that such mediators up-regulate the murine *Selp* gene. To address this issue, we generated transgenic mice that expressed the human *Selp* gene. We used these mice to compare the basal and inducible expression of human and murine P-selectin and their functional consequences in vivo.

RESULTS

Generation of transgenic mice that express the human *Selp* gene

We reasoned that a transgene containing the entire human *Selp* gene plus long flanking sequences might confer tissue-specific, basal, and inducible expression of human P-selectin in mice as the native gene does in humans. We isolated a bacterial artificial chromosome clone comprising all 17 exons and 16 introns of the human gene (Johnston et al., 1990), plus 70 kb of 5′ flanking sequence and 29 kb of 3′ flanking sequence (Fig. 1 A). After confirming its integrity by pulsed field gel electrophoresis (Fig. 1 B), the 149-kb insert was injected into fertilized murine eggs. Founder mice among the offspring were identified by PCR of tail genomic DNA with primer pairs located 30 kb upstream of exon 1, at the exon 9/intron 9 junction, and within exon 17 (Fig. 1, A and C). We amplified all three PCR products from three founders termed *TghSelp1*, *TghSelp2*, and *TghSelp3*, suggesting that the 149-kb transgene was intact. Unless stated otherwise, we bred founder mice with mice lacking murine P-selectin ($Selp^{-/-}$) to generate lines that expressed human but not murine P-selectin. All lines were healthy and fertile. Blood counts and leukocyte differentials were normal, but there was a small but significant increase in platelet counts (Table S1). Quantitative PCR documented a single transgene in each line that was transmitted stably for at least 10 generations. RT-PCR of total lung RNA from each line yielded an mRNA transcript of the expected size (Fig. 1 D). This transcript comigrated with human P-selectin mRNA from transfected Chinese hamster ovary cells expressing human P-selectin cDNA, indicating

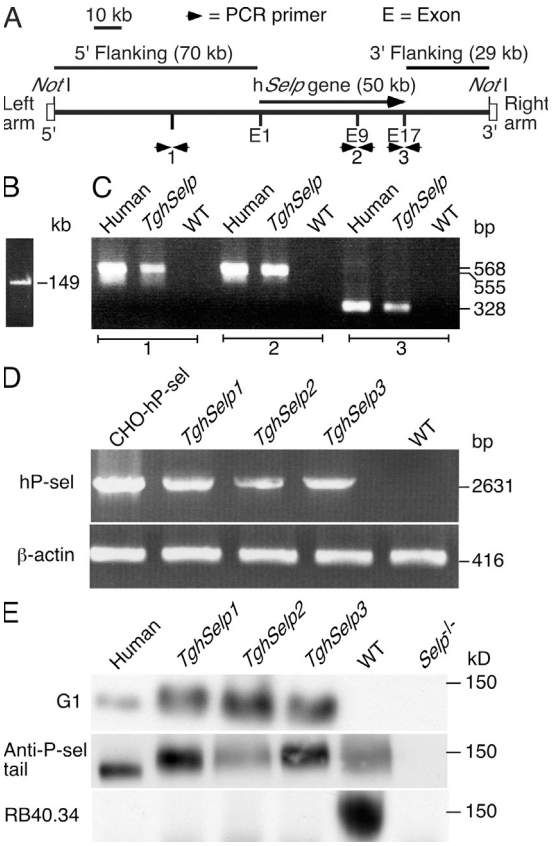

Figure 1. Generation of transgenic mice that express the human *Selp* gene. (A) Schematic of bacterial artificial chromosome clone containing all 17 exons and 16 introns of the human *Selp* gene plus large 5′ and 3′ flanking sequences. The 149-kb insert is shown as a horizontal line between *NotI* sites in the vector. Arrows mark locations of three pairs of PCR primers used to evaluate incorporation of the gene into DNA of transgenic mice. (B) The intact nature of the insert released by *NotI* from the vector was confirmed by pulsed-field gel electrophoresis. (C) PCR analysis of transgenic founders. Tail genomic DNA was amplified using the three pairs of primers depicted in A. Human genomic DNA and tail genomic DNA from WT mice were used as positive and negative controls, respectively. (D) RT-PCR of transcripts for human P-selectin. Total RNA was obtained from lungs of three transgenic lines (*TghSelp1*, *TghSelp2*, and *TghSelp3*, each backcrossed with $Selp^{-/-}$ mice), from lungs of WT mice, and from Chinese hamster ovary (CHO) cells transfected with an expression vector containing a full-length cDNA encoding human P-selectin. The primers for the human P-selectin cDNA were located 10 bp upstream of the ATG initiating codon and 131 bp downstream of the TAA polyadenylation signal, yielding the expected 2,631-bp product. As a positive control, RT-PCR of a 416-bp fragment of murine β-actin mRNA was performed. (E) Western blots of platelet lysates from the indicated genotype. Lysates fractionated by SDS-PAGE under nonreducing conditions were probed with G1, a mAb which binds to the lectin domain of human P-selectin; anti–P-sel tail, a rabbit antiserum which recognizes the cytoplasmic domain of both human and murine P-selectin; or RB40.34, a mAb which binds to the lectin domain of murine P-selectin. The P-selectin bands appear broader in murine than in human platelet lysates because more total protein from murine lysates was loaded in the gel. The data in B–E are representative of at least three experiments.

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that the human transgene was correctly spliced after its transcription. No transcript was amplified from WT mice, confirming specificity. Western blots of platelet lysates probed with G1, a mAb to the lectin domain of human P-selectin, and with a polyclonal antibody that recognizes the cytoplasmic domain of human and murine P-selectin demonstrated that transgenic mice expressed full-length human P-selectin that comigrated with P-selectin from human platelets (Fig. 1 E). Neither antibody bound to platelet lysates from *Selp*$^{-/-}$ mice. RB40.34, a mAb to murine P-selectin, bound to murine P-selectin in WT lysates but not to human P-selectin in *TghSelp* lysates (Fig. 1 E).

Preliminary analysis suggested similar characteristics in all three transgenic lines. In this paper, we describe experiments with the *TghSelp3* line, denoted simply as *TghSelp* mice. To avoid phenotypes from inadvertent disruption of an endogenous gene after integration of the transgene, we initially studied mice with the transgene on only one allele. These are termed *TghSelp*$^{+/-}$ mice. *TghSelp*$^{+/+}$ mice bearing the transgene on both alleles have been maintained for >2 yr with no unexpected abnormalities. Therefore, we used these mice in some experiments as indicated.

Cell-specific expression of human P-selectin in transgenic mice

A mAb to human P-selectin bound to thrombin-activated but not resting platelets from *TghSelp*$^{+/-}$ mice (Fig. 2 A). This suggests that human P-selectin was correctly sorted to α-granules of resting platelets and was mobilized to the plasma membrane after activation. The mAb did not bind to leukocytes or erythrocytes (unpublished data). Activated platelets from mice bearing the human or murine *Selp* gene on both alleles expressed twice as much human or murine P-selectin, respectively, as mice bearing the gene on one allele (Fig. 2 B). To directly quantify the number of P-selectin molecules on activated platelets, we measured saturable binding of ^{125}I-labeled mAb S12 to human P-selectin or mAb RB40.34 to murine P-selectin. *TghSelp*$^{+/-}$ mice expressed 1,770 ± 282 (n = 3) molecules and WT mice expressed 2,727 ± 589 (n = 13) molecules per activated platelet. Given that the *TghSelp*$^{+/-}$ mice carry the transgene on only one allele, the expression levels of human and murine P-selectin per allele are comparable.

Like WT mice, *TghSelp*$^{+/-}$ mice expressed P-selectin on resident peritoneal macrophages (Fig. 2, C and D). Antibody to the platelet-specific marker CD41 did not stain the macrophages, excluding a contribution of adherent platelets to P-selectin expression (unpublished data).

We stained tissue sections from *TghSelp*$^{+/-}$ and WT mice with an antibody that recognizes human but not murine P-selectin. Human P-selectin was found only on the endothelium in heart, lung, and other tissues of *TghSelp*$^{+/-}$ mice (Fig. 3 A and not depicted). Expression was observed primarily on veins and venules, although a few arteries and arterioles were stained. A similar preference for venules has been observed for P-selectin in human tissues (McEver et al., 1989) and for murine P-selectin in WT mice (Gotsch et al., 1994). The antibody did not stain tissues from WT mice, and nonimmune antibody did not stain tissues from *TghSelp*$^{+/-}$ mice (Fig. 3 A). Immunofluorescence of cultured endothelial cells from lungs of WT and *TghSelp*$^{+/-}$ mice revealed that both murine and human P-selectin colocalized with von Willebrand factor in Weibel-Palade bodies (Fig. 3 B). Collectively, these results demonstrate that the transgenic mice constitutively express human P-selectin in platelets, endothelial cells, and resident peritoneal macrophages.

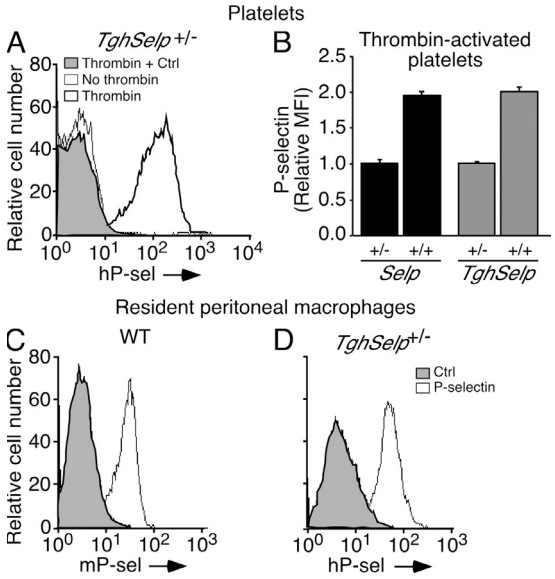

Figure 2. Transgenic mice express human P-selectin on activated platelets and on resident peritoneal macrophages. (A) Flow cytometric analysis of resting (no thrombin) or thrombin-activated platelets from *TghSelp*$^{+/-}$ mice stained with PE-conjugated anti–human P-selectin mAb AK4 or PE-conjugated isotype-control mAb (Ctrl). (B) Flow cytometric analysis of thrombin-activated platelets from mice heterozygous (+/−) or homozygous (+/+) for the murine or human *Selp* gene stained, respectively, with FITC-conjugated RB40.34 or PE-conjugated AK4 or matched conjugated isotype control mAbs. The data represent the relative mean fluorescence intensity (MFI) ± SEM from six mice of each genotype. (C and D) Flow cytometric analysis of resident peritoneal macrophages from WT mice stained with FITC-conjugated anti–murine P-selectin mAb RB40.34 or FITC-conjugated isotype control mAb (C), or from *TghSelp*$^{+/-}$ mice stained with PE-conjugated anti–human P-selectin mAb AK4 or PE-conjugated isotype-control mAb (D). The data in A, C, and D are representative of at least five experiments.

TNF and LPS increase expression of murine P-selectin mRNA but decrease expression of human P-selectin mRNA in transgenic mice

We injected TNF, LPS, or control human serum albumin i.v. into mice that express the human *Selp* transgene and the endogenous murine *Selp* gene (*TghmSelp*). Quantitative RT-PCR was performed to measure the levels of murine and human P-selectin mRNA in different tissues 3 h after TNF injection or 3.5 h after LPS injection. Compared with controls, TNF or LPS markedly increased murine P-selectin mRNA in the heart, lung, and liver (Fig. 4 A). However, TNF or LPS significantly decreased human P-selectin mRNA

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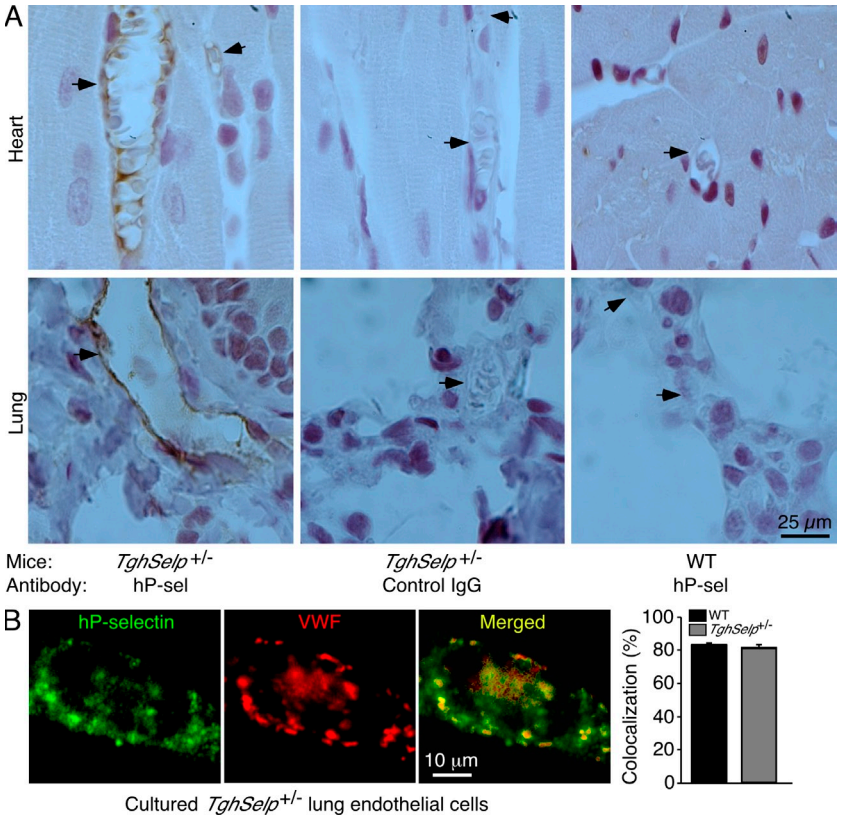

Figure 3. Transgenic mice express human P-selectin in endothelial cells. (A) Sections of heart or lung from *TghSelp*$^{+/-}$ or WT mice were incubated with biotinylated sheep anti–human P-selectin IgG (anti–hP-sel, which does not crossreact with murine P-selectin) or control sheep IgG, followed by a streptavidin–horseradish peroxidase complex. The slides were developed with a peroxidase substrate. Brown reaction product marks sites of antibody binding. Arrows mark endothelial cells lining venules. (B) Cultured lung endothelial cells from WT or *TghSelp*$^{+/-}$ mice were fixed, permeabilized, and stained with antibodies to P-selectin (green) and von Willebrand factor (VWF, red). Fluorescent images were visualized with a confocal microscope. Shown are representative images of cells from *TghSelp*$^{+/-}$ mice. Yellow staining indicates overlapping distribution of human P-selectin and murine VWF in the merged images. Colocalization of VWF and P-selectin fluorescence was quantified with an Imaris colocalization module. The bar graph depicts the mean ± SEM of the percentage of colocalized VWF and P-selectin pixels from three representative images of cells from WT and *TghSelp*$^{+/-}$ mice. The images in A and B are representative of at least three experiments.

in these and other organs (Fig. 4 B and not depicted). The inability of TNF or LPS to up-regulate the human *Selp* gene in transgenic mice corresponds to the inability of these mediators to up-regulate the *Selp* gene in cultured human endothelial cells.

Murine leukocytes roll on human P-selectin expressed on activated platelets from transgenic mice

To examine whether the transgenic mice expressed functional human P-selectin, we perfused murine bone marrow–derived leukocytes over monolayers of activated platelets from WT or *TghSelp*$^{+/-}$ mice. The leukocytes rolled specifically on P-selectin expressed by platelets of both genotypes (Fig. 5, A and B). Rolling on *TghSelp*$^{+/-}$ platelets was reversed by the blocking anti–human P-selectin mAb G1 but not by the nonblocking anti–human P-selectin mAb S12 or the blocking anti–murine P-selectin mAb 5H1. Conversely, rolling on WT platelets was eliminated by mAb 5H1 but not by mAb G1 or S12.

Leukocytes roll with equivalent velocities on human or murine P-selectin expressed in trauma-activated venules but roll faster on human than murine P-selectin expressed in TNF-activated venules

We compared rolling of leukocytes on murine or human P-selectin in postcapillary venules of the cremaster muscle of WT or *TghSelp*$^{+/-}$ mice. Leukocyte rolling was assessed as the

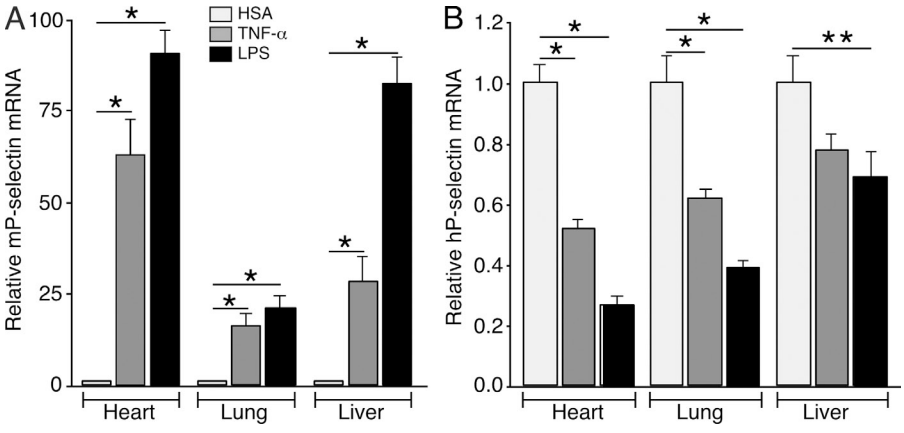

Figure 4. TNF and LPS increase expression of murine P-selectin mRNA but decrease expression of human P-selectin mRNA in transgenic mice. *TghmSelp* mice that express both human and murine *Selp* genes were injected i.v. with 1% human serum albumin (HSA) as control or with 1 μg TNF or 50 μg LPS. After 3 h (TNF) or 3.5 h (LPS), total RNA was isolated from heart, lung, and liver. Real-time quantitative RT-PCR was used to measure mRNA for murine P-selectin (mP-selectin; A) and human P-selectin (hP-selectin; B). Expression was normalized to mRNA for murine β2-microglobulin determined in parallel and expressed as n-fold difference relative to the calibrator mRNA for P-selectin in control mice injected with HSA. The data represent the mean ± SEM for nine mice in each group. *, $P < 0.01$; **, $P < 0.05$.

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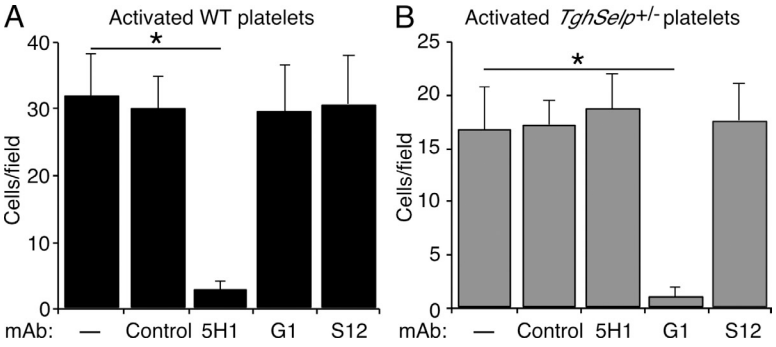

Figure 5. Murine leukocytes roll on human P-selectin expressed on activated platelets from transgenic mice. Murine bone marrow–derived leukocytes were perfused at 1 dyn/cm² over monolayers of activated platelets from WT mice (A) or *TghSelp*$^{+/-}$ mice (B) in the presence or absence of blocking anti–mouse P-selectin mAb 5H1, isotype-matched control mAb (rat IgG1), blocking anti–human P-selectin mAb G1, or nonblocking anti–human P-selectin mAb S12. After 5 min, the number of rolling cells was measured. The data represent the mean ± SEM of three experiments. *, $P < 0.01$.

rolling flux fraction, defined as the number of rolling leukocytes divided by the total number of leukocytes passing through the same vessel. Mild trauma during exposure of the cremaster muscle mobilizes P-selectin from Weibel-Palade bodies to the venular surface (Ley et al., 1995). In this model, we observed similar rolling flux fractions (Fig. 6 A) and mean rolling velocities (Fig. 6 C) in postcapillary venules of WT and *TghSelp*$^{+/-}$ mice. The P-selectin dependency of rolling was confirmed by injecting mAbs i.v. In *TghSelp*$^{+/-}$ mice, rolling was abrogated by G1,

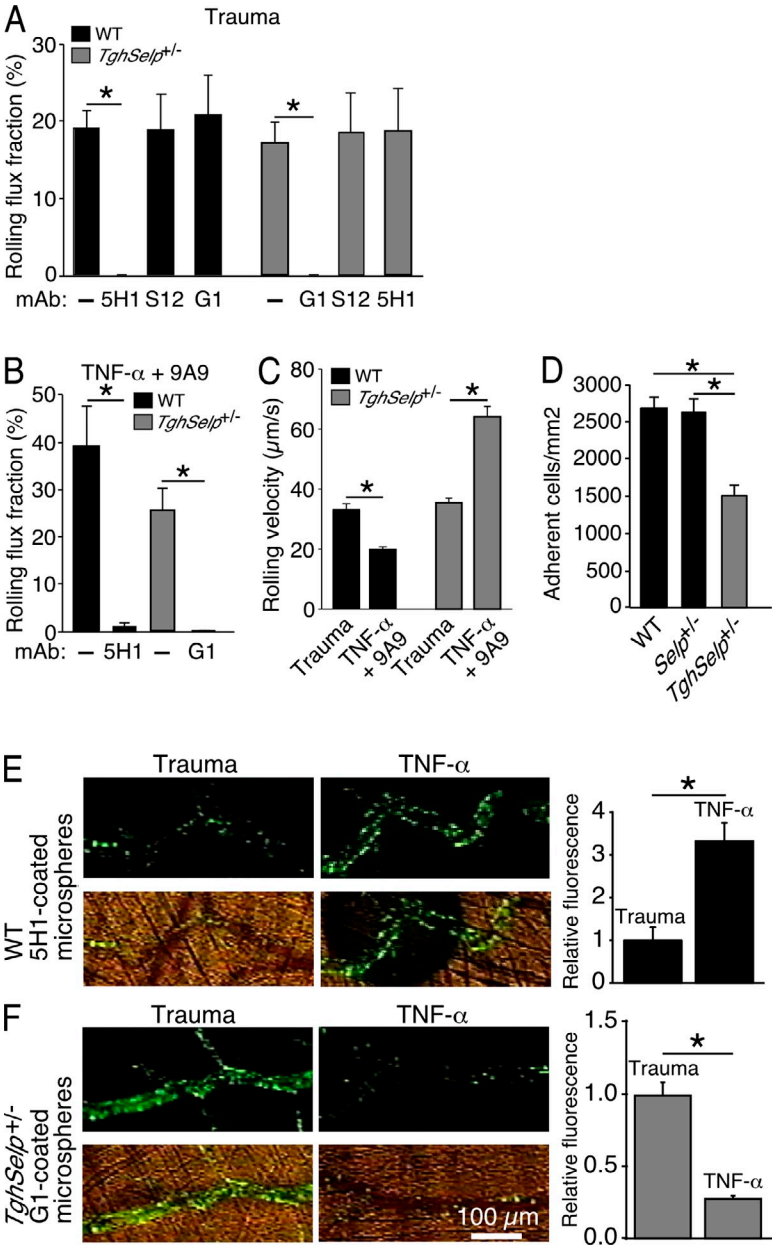

Figure 6. Leukocytes roll with equivalent velocities on human or murine P-selectin expressed in trauma-activated venules but roll faster on human than murine P-selectin expressed in TNF-activated venules. (A) Leukocyte rolling flux fractions were measured in venules of cremaster muscle from WT and *TghSelp*$^{+/-}$ mice subjected to trauma to mobilize P-selectin to the venular surface. Rolling fluxes before or after injection of the indicated mAb were measured within 1 h after tissue exposure. (B) WT and *TghSelp*$^{+/-}$ mice were injected with TNF into the scrotum and with anti–murine E-selectin mAb 9A9 i.v. After 3 h, leukocyte rolling flux fractions were measured in venules of the cremaster muscle before and after injection of the indicated mAb. (C) Leukocyte rolling velocities in trauma- or TNF-stimulated venules from WT and *TghSelp*$^{+/-}$ mice. (D) Number of firmly adherent leukocytes in TNF-stimulated venules from WT, *Selp*$^{+/-}$ (heterozygous for murine *Selp*), and *TghSelp*$^{+/-}$ mice. All mice were injected with mAb 9A9 i.v. to block E-selectin when TNF was administered. At the end of each experiment, anti–P-selectin mAb 5H1 (for murine P-selectin) or mAb G1 (for human P-selectin) was injected i.v. This injection reversed rolling, confirming its dependency on P-selectin. (E and F) Representative images of mAb 5H1- or G1-coated fluorescent microspheres (injected through a carotid artery catheter) adhering to murine or human P-selectin in trauma- or TNF-stimulated venules from WT or *TghSelp*$^{+/-}$ mice. For each genotype, the top row depicts fluorescence images that are overlaid on the corresponding bright field images in the bottom row. The bar graphs quantify the relative fluorescence density of the adherent microspheres. The data in A and B represent the mean ± SEM of 8–54 venules from five to nine mice for each group. The data in C represent the mean ± SEM of 370–730 leukocytes in 37–73 venules from five to eight mice for each group. The data in D represent the mean ± SEM of 25 to 36 venules from four to five mice for each group. The data in E and F represent the mean ± SEM of 13 to 25 venules from three to four mice for each group. *, $P < 0.01$.

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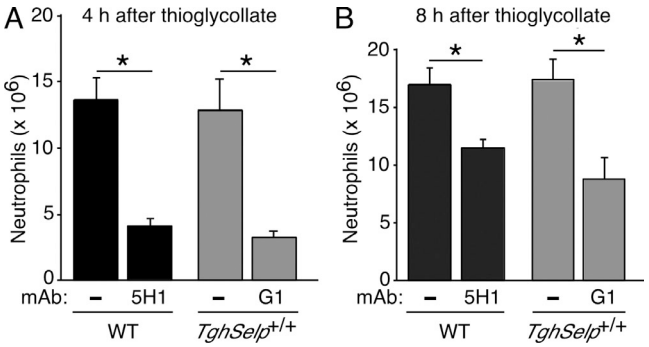

Figure 7. Human and murine P-selectin mediate equivalent neutrophil recruitment during thioglycollate-induced peritonitis. WT or *TghSelp*$^{+/+}$ mice were injected with thioglycollate i.p. Some mice were injected with mAb 5H1 or G1 i.v. at the time of thioglycollate challenge. After 4 h (A) or 8 h (B), peritoneal cells were lavaged and neutrophils were quantified by flow cytometry. The data represent the mean ± SEM of 8–12 mice in each group. *, P < 0.01.

a blocking mAb to human P-selectin, but not by S12, a non-blocking mAb to human P-selectin or by 5H1, a blocking mAb to murine P-selectin. Conversely, rolling in WT mice was eliminated by mAb 5H1 but not by mAb G1 or S12 (Fig. 6 A).

Intrascrotal injection of TNF increases synthesis of murine P-selectin and E-selectin (Jung and Ley, 1997). In this model, leukocytes roll on both selectins and then arrest through integrin–ligand interactions (Kunkel and Ley, 1996). To examine rolling mediated solely by P-selectin, we injected anti–murine E-selectin blocking mAb 9A9 i.v. when TNF was administered. 3 h after TNF injection, the rolling flux fraction increased in both WT and *TghSelp*$^{+/-}$ mice (Fig. 6, compare A and B), although the increase was less in *TghSelp*$^{+/-}$ mice. Injecting mAb 5H1 or G1 at this time point eliminated rolling, confirming its dependency on murine or human P-selectin in WT or *TghSelp*$^{+/-}$ mice, respectively (Fig. 6 B). TNF significantly decreased rolling velocities on murine P-selectin (Fig. 6 C), which is consistent with previous results (Sperandio et al., 2001). In sharp contrast, TNF significantly increased rolling velocities on human P-selectin (Fig. 6 C). To determine the functional significance of the divergent rolling velocities on P-selectin, we measured the number of arrested (firmly adherent) leukocytes in venules from mice injected with TNF and anti–E-selectin mAb 9A9. Significantly fewer leukocytes arrested in venules of *TghSelp*$^{+/-}$ mice than in venules of WT mice (*Selp*$^{+/+}$) or mice heterozygous for the murine *Selp* gene (*Selp*$^{+/-}$; Fig. 6 D).

Because TNF reduced human P-selectin mRNA levels (Fig. 4 B), we asked whether TNF reduced the density of human P-selectin on venular surfaces. We injected fluorescent microspheres bearing mAb 5H1 or mAb G1 through a catheter in the carotid artery. As assessed by fluorescence microscopy, flowing microspheres reached the cremaster muscle within 5 s and disappeared from circulation within 10 min, the time when images were recorded. 5H1-coated microspheres adhered to venules of WT mice (Fig. 6 E), and G1-coated microspheres adhered to venules of *TghSelp*$^{+/-}$ mice

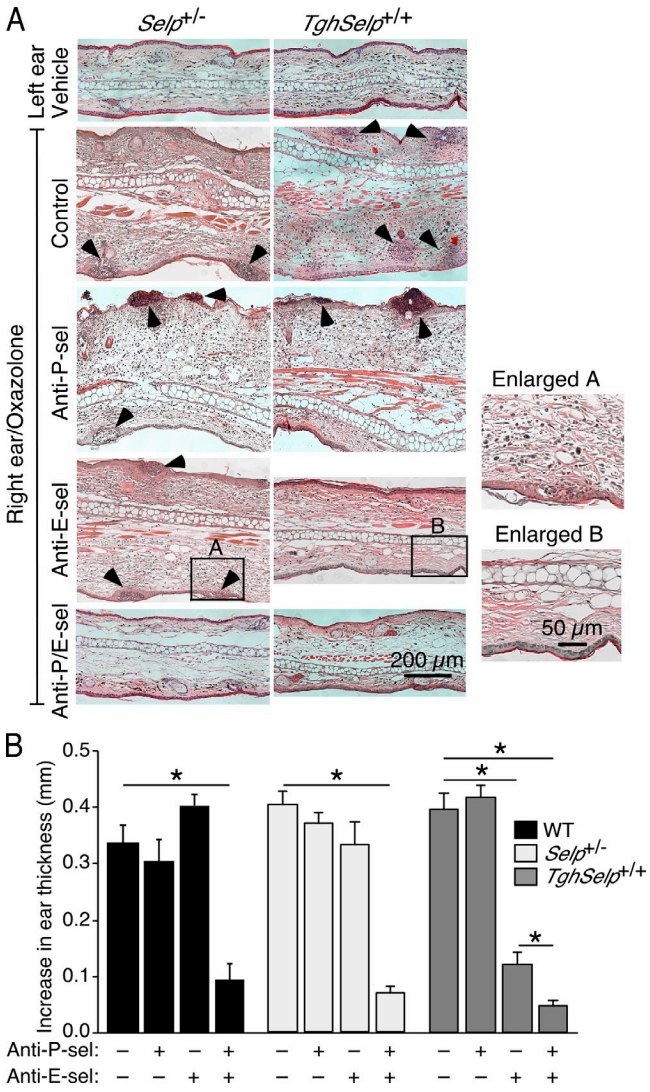

Figure 8. Human P-selectin contributes less than murine P-selectin to inflammation during contact hypersensitivity. WT mice, mice heterozygous for murine *Selp* (*Selp*$^{+/-}$), or transgenic mice homozygous for human *Selp* (*TghSelp*$^{+/+}$) were sensitized with 2% oxazolone on the abdomen and paws. After 7 d, the mice were challenged with 1% oxazolone on the right ear and with vehicle only on the left ear. Immediately before challenge, some mice were injected i.v. with anti–murine E-selectin mAb 9A9 (anti–E-sel), anti–P-selectin mAb (anti–P-sel; 5H1 for *Selp*$^{+/+}$ and *Selp*$^{+/-}$ mice or G1 for *TghSelp*$^{+/+}$ mice), or both anti–E-selectin and anti–P-selectin mAbs. Control mice were injected with isotype control mAb or saline, which yielded identical results. (A) Sections of left and right ears collected 24 h after challenge were stained with hematoxylin and eosin. Arrowheads show microabscesses. Areas within boxes marked A and B are shown at higher magnification on the right. (B) The increase in ear thickness 24 h after challenge was measured. The data represent the mean ± SEM of 5–10 mice in each group. *, P < 0.01.

(Fig. 6 F). Neither 5H1- nor G1-coated microspheres adhered to venules of *Selp*$^{-/-}$ mice (unpublished data). Significantly more 5H1-coated microspheres adhered to TNF-stimulated than to trauma-stimulated venules of WT mice (Fig. 6 E). In marked contrast, significantly fewer G1-coated microspheres adhered to TNF-stimulated than to trauma-stimulated

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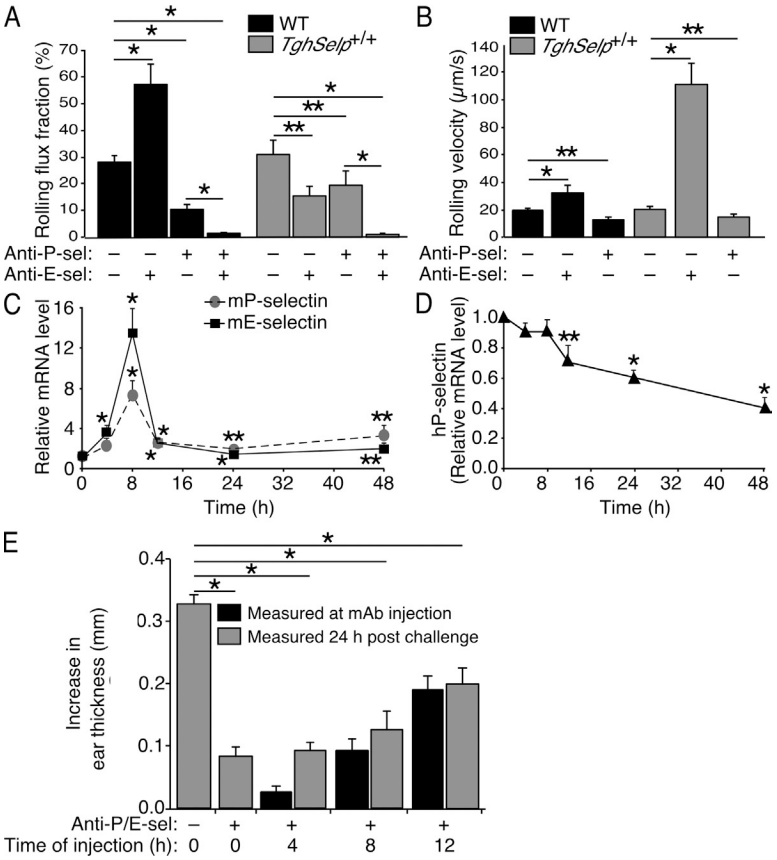

Figure 9. Human P-selectin contributes less than murine P-selectin to basal leukocyte rolling in dermal venules, and human P-selectin mRNA decreases during contact hypersensitivity. (A and B) Rolling flux fractions (A) and rolling velocities (B) of rhodamine 6G–labeled leukocytes were measured in noninflamed ear venules of WT or *TghSelp*$^{+/+}$ mice using fluorescence microscopy. Mice were injected i.v. with anti–murine E-selectin mAb 9A9 (anti–E-sel), anti–P-selectin mAb (anti–P-sel; 5H1 for WT mice or G1 for *TghSelp*$^{+/+}$ mice), or both anti–E-selectin and anti–P-selectin mAbs. Several venules were recorded before and after each mAb injection. (C and D) *TghmSelp* mice expressing both human and murine *Selp* genes were sensitized and challenged as in Fig. 8. At the indicated interval after challenge, total RNA was isolated from the right ear (challenged with oxazolone) and the left ear (challenged with vehicle). Quantitative RT-PCR was used to measure levels of mRNA for murine P-selectin and E-selectin (C) or human P-selectin (D). The results represent the relative expression of mRNA in the right ear divided by that in the left ear. (E) WT mice were injected i.v. with isotype control mAb (−) or combined anti–E-selectin mAb 9A9 and anti–P-selectin mAb 5H1 immediately before antigen challenge (0 h) or 4, 8, or 12 h after antigen challenge. Changes in ear thickness were measured at the time of antibody injection and 24 h after antigen challenge. The data in A and B represent the mean ± SEM of 6–10 mice. The data in C and D represents the mean ± SEM of 5–14 mice. The data in E represent the mean ± SEM of 8–11 mice in each experimental group. *, $P < 0.01$; **, $P < 0.05$. The p-values in panels C and D are versus the baseline measurement at time 0.

venules of *TghSelp*$^{+/-}$ mice (Fig. 6 F). These data suggest that TNF causes faster leukocyte rolling in *TghSelp*$^{+/-}$ mice by decreasing the P-selectin density on the venular surface.

Human and murine P-selectin mediate equivalent neutrophil recruitment during thioglycollate-induced peritonitis

Selectins mediate neutrophil migration into the peritoneum after local administration of thioglycollate (Robinson et al., 1999). We found that comparable numbers of neutrophils migrated into the peritoneum of WT and *TghSelp*$^{+/+}$ mice 4 and 8 h after thioglycollate challenge (Fig. 7, A and B). Neutrophil influx was significantly reduced by i.v. injection of blocking mAb to murine or human P-selectin, respectively. These data demonstrate that human P-selectin, like murine P-selectin, contributes significantly to neutrophil recruitment in this model of acute inflammation.

Human P-selectin contributes less than murine P-selectin to inflammation during contact hypersensitivity

Both P- and E-selectin contribute to inflammation in murine models of antigen-dependent contact hypersensitivity (Labow et al., 1994; Staite et al., 1996; Catalina et al., 1999; Hwang et al., 2004; O'Leary et al., 2006). A common model sensitizes mice with the hapten oxazolone, followed by topical challenge of one ear with oxazolone and the other ear with vehicle as control. We used this model to assess the relative contributions of murine and human P-selectin to inflammation 24 h after challenge. We compared WT mice (*Selp*$^{+/+}$) or heterozygous mice (*Selp*$^{+/-}$) with transgenic mice homozygous for human *Selp* (*TghSelp*$^{+/+}$; Fig. 8, A and B). In all mice, swelling and leukocyte infiltration were greatly increased in the ear challenged with oxazolone (Fig. 8, A and B). In *Selp*$^{+/+}$ or *Selp*$^{+/-}$ mice, individual blockade of murine P- or E-selectin with mAbs did not affect swelling or leukocyte infiltration, whereas injecting mAbs to both selectins markedly decreased both parameters (Fig. 8, A and B). In *TghSelp*$^{+/+}$ mice, anti–human P-selectin mAb had no effect on swelling or infiltration, whereas anti–murine E-selectin alone significantly reduced both parameters (Fig. 8, A and B). Combined blockade with anti–human P-selectin and anti–murine E-selectin further reduced swelling and infiltration to control levels. These data demonstrate that human P-selectin contributes much less than murine P-selectin to inflammation in this model.

Human P-selectin contributes less than murine P-selectin to basal leukocyte rolling in dermal venules

WT mice constitutively express both P- and E-selectin in dermal venules and use both selectins to mediate basal leukocyte rolling in skin (Yamada et al., 1995; Weninger et al., 2000). P-selectin primarily captures flowing leukocytes, whereas E-selectin primarily controls rolling velocities. Blocking both selectins with mAbs is required to eliminate rolling (Weninger et al., 2000). One explanation for the lesser role of human P-selectin in contact hypersensitivity could be lower constitutive

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expression of human P-selectin in dermal venules of transgenic mice. We observed similar basal leukocyte rolling flux fractions and rolling velocities in unchallenged ears of WT and *TghSelp*$^{+/+}$ mice (Fig. 9, A and B). Anti–murine or anti–human P-selectin mAb reduced rolling flux in WT or *TghSelp*$^{+/+}$ mice; however, the reduction was much greater in WT mice. Anti–P-selectin mAb decreased rolling velocities in both WT and *TghSelp*$^{+/+}$ mice, confirming the major function of E-selectin in controlling rolling velocities. Anti–E-selectin mAb increased rolling flux in WT mice but decreased rolling flux in *TghSelp*$^{+/+}$ mice. Anti–E-selectin mAb increased rolling velocities in both WT and *TghSelp*$^{+/+}$ mice, but the increase was much more dramatic in *TghSelp*$^{+/+}$ mice (Fig. 9, A and B). These data demonstrate that human P-selectin contributes much less than E-selectin to basal leukocyte rolling in dermal venules, most likely as the result of a lower cell surface density of P-selectin. As a result, E-selectin has a greater role in capturing leukocytes and an even more dominant function in controlling rolling velocities.

Murine P-selectin mRNA increases, but human P-selectin mRNA decreases, during contact hypersensitivity

Many cytokines, including TNF, are expressed during contact hypersensitivity (McHale et al., 1999; Wang et al., 2003). Another explanation for the lesser role of human P-selectin in contact hypersensitivity could be the failure of TNF and other mediators to increase expression of the human *Selp* gene. Accordingly, we quantified mRNA for murine and human P-selectin in the ears of sensitized *TghmSelp* mice, which express both murine and human P-selectin, at intervals after challenge with oxazolone. The amount of mRNA for murine P-selectin rose dramatically, peaking at approximately sevenfold above baseline at 8 h but remaining elevated at 48 h, closely paralleling the kinetics of murine E-selectin mRNA induction (Fig. 9 C). In contrast, the level of mRNA for human P-selectin did not increase and, indeed, slowly declined after challenge (Fig. 9 D). It was reported that administering mAbs to P- and E-selectin to WT mice 2 h after challenge failed to prevent the ear swelling and leukocyte infiltration observed after 24 h, suggesting that the selectins functioned only in a brief temporal window after challenge (Hwang et al., 2004). If so, the different responses of the murine and human *Selp* genes to cytokines might occur too late to explain the different contributions of murine and human P-selectin to inflammation. However, we observed that injecting anti-selectin mAbs into WT mice 2 h after challenge had the same antiinflammatory effects as administering mAbs before challenge (unpublished data). Furthermore, injecting mAbs to P- and E-selectin into WT mice 4, 8, or 12 h after challenge blocked further swelling at 24 h (Fig. 9 E). These data demonstrate that both murine selectins contribute to inflammation for at least 12 h after challenge in our model. Therefore, one explanation for the lesser role of human P-selectin in contact hypersensitivity could be the failure of cytokines to up-regulate the human *Selp* gene.

DISCUSSION

The mechanisms for inducible expression of the *Selp* gene in humans have diverged sharply from those in mice. The physiological significance of this divergence has received little attention despite the widespread use of mice to probe the functions of P-selectin in vivo. We have developed transgenic mice bearing the entire human *Selp* gene that recapitulate the basal and inducible expression of P-selectin observed in humans. Importantly, the cis elements of the gene are sufficient to confer human-like expression of P-selectin in mice; other human gene products are not required. Our data confirm shared functions of human and murine P-selectin but also reveal key differences that reflect specific tissue environments and/or inflammatory challenges.

Like humans and WT mice, *TghSelp* mice expressed P-selectin in platelets and endothelial cells, where it was stored in secretory granules and mobilized to the plasma membrane by agonists such as thrombin. Like WT mice (Tchernychev et al., 2003), *TghSelp* mice expressed P-selectin in resident peritoneal macrophages, implying that human macrophages also express P-selectin. These data argue that the human *Selp* transgene has all of the components for cell-specific expression of P-selectin in vivo. Activated platelets expressed similar levels of human or murine P-selectin when normalized for the number of alleles of the corresponding gene. Human and murine P-selectin also exhibited functional equivalence. Murine leukocytes rolled similarly on thrombin-activated platelets and in trauma-stimulated cremasteric venules of WT and *TghSelp*$^{+/-}$ mice. These results suggest similar basal synthetic rates for P-selectin in WT and *TghSelp* mice in these tissues. As noted subsequently, this may not be true in all tissues.

P-selectin contributed much less than E-selectin to basal leukocyte rolling in dermal venules of *TghSelp*$^{+/+}$ mice, which is in sharp contrast to the importance of both selectins for rolling in dermal venules of WT mice (Yamada et al., 1995; Weninger et al., 2000). This is most likely a result of the lower basal expression of human P-selectin in dermal venules, which may explain the less frequent detection of P-selectin in human skin (Jung et al., 1997). It will be important to determine whether differential basal expression of human and murine P-selectin is regulated by NF-κB and/or ATF-2 or by other transcription factors. Furthermore, the relevance to humans of P-selectin–mediated basal trafficking of hematopoietic cells in mice should be reevaluated. Hematopoietic stem cells home through interactions with P- and E-selectin in bone marrow sinusoids of mice (Frenette et al., 1998b; Mazo et al., 1998). In contrast, only E-selectin has been detected in bone marrow sinusoids of humans (Schweitzer et al., 1996). T cell progenitors home by interacting with P-selectin on thymic endothelial cells of mice (Rossi et al., 2005; Scimone et al., 2006). The lipid S1P regulates thymic expression of the murine *Selp* gene through postulated NF-κB– and ATF-2–dependent mechanisms (Gossens et al., 2009). However, the human *Selp* promoter lacks binding sites for NF-κB and ATF-2 (Pan and McEver, 1993; Pan et al., 1998a). The *TghSelp* mice provide a model to critically test the contribution of

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human P-selectin to basal trafficking of hematopoietic cells in skin, bone marrow, and thymus.

We found that human and murine P-selectin contributed equivalently to neutrophil migration into the peritoneum 4 and 8 h after injection of thioglycollate. Previous bone marrow transplant experiments demonstrated that leukocyte migration into the peritoneum does not require P-selectin on platelets (Frenette et al., 1998a). Therefore, sufficient human P-selectin must be expressed on endothelial cells of *TghSelp* mice to support the response. Leukocyte migration in this model may be more dependent on local macrophage release of chemokines CXCL1 and CXCL2 instead of cytokines that activate NF-κB–dependent genes in endothelial cells (Cailhier et al., 2005).

Systemic administration of TNF or LPS induced massive increases in mRNA for murine, but not human, P-selectin in mice that expressed both murine and human *Selp* genes. The failure of these mediators to up-regulate the human *Selp* gene in vivo mirrors previous in vitro results in human endothelial cells (Burns et al., 1995; Yao et al., 1996) and in vivo data in baboons (Yao et al., 1999). In vitro, the differential responsiveness to TNF and related mediators is the result of critical differences in binding sites for NF-κB and ATF-2 in the promoters of the murine and human *Selp* genes (Pan et al., 1998a,b). Whether this is the sole explanation for differential responsiveness of these genes in vivo requires further study. TNF and LPS actually decreased mRNA for human P-selectin in transgenic mice, in accordance with experiments with cultured human endothelial cells (Burns et al., 1995; Yao et al., 1996; Yano et al., 2006). How these mediators decrease expression of human P-selectin is unknown. Our data suggest that this decreased expression is physiologically important in some settings.

After intrascrotal injection of TNF in WT mice, neutrophils roll on both P- and E-selectin in venules of the cremaster muscle. Signaling through engagement of selectin ligands and chemokine receptors causes integrin-dependent deceleration, firm adhesion, intraluminal crawling, and emigration into extravascular tissues (Ley et al., 2007). Both selectins must be blocked to reduce the number of firmly adherent and emigrated neutrophils (Kunkel and Ley, 1996). We found that compared with trauma alone, TNF increased the density of murine P-selectin in venules, whereas it decreased the density of human P-selectin. In the presence of blocking mAb to E-selectin, neutrophils rolled much faster in venules expressing human rather than murine P-selectin, and fewer neutrophils arrested in venules of *TghSelp* than WT mice. The consequence of this TNF-mediated decrease in human P-selectin is a dominant role for E-selectin in neutrophil recruitment. Our results are consistent with the observation that anti–human E-selectin mAb blocks neutrophil influx into human skin grafts in SCID mice after intradermal injection of TNF (Yan et al., 1994).

In WT mice, TNF and other mediators contribute to contact hypersensitivity, in part by augmenting selectin expression (Harari et al., 1999; McHale et al., 1999; Kneilling et al., 2009). We found that mRNA for both murine P- and E-selectin increased 7–14-fold in the affected ear 8 h after challenge and remained elevated for at least 48 h. Like many previous studies (Labow et al., 1994; Staite et al., 1996; Catalina et al., 1999; Hwang et al., 2004; O'Leary et al., 2006), we observed that both P- and E-selectin must be blocked to suppress ear swelling and leukocyte infiltration during contact hypersensitivity in WT mice. In marked contrast, human P-selectin contributed much less than murine E-selectin to the inflammatory response in *TghSelp* mice, in accordance with its lower basal expression in dermal venules and the decline in mRNA for human P-selectin in the ear after challenge. Our data are consistent with the increased expression of E-selectin, but not P-selectin, in skin biopsies from humans or nonhuman primates with contact hypersensitivity (Griffiths et al., 1991; Silber et al., 1994; Jung et al., 1997).

Although canonical NF-κB– and ATF-2–dependent activators do not up-regulate the human *Selp* gene, sustained increased expression of P-selectin has been observed on the surfaces of endothelial cells in human tissues with chronic or allergic inflammation (Grober et al., 1993; Johnson-Tidey et al., 1994; Symon et al., 1994). This may be a result of cytokines, such as oncostatin M or IL-4, that increase transcription of the human *Selp* gene through mechanisms that do not require NF-κB and ATF-2 (Yao et al., 1996, 1999; Khew-Goodall et al., 1999; Woltmann et al., 2000; Miyazaki et al., 2006). In vitro, oncostatin M up-regulates expression of human and murine P-selectin with different kinetics (Yao et al., 1996, 1999), suggesting additional species-specific regulatory mechanisms that should be explored in vivo. Other factors may regulate the density of human P-selectin on the plasma membrane, including its basal synthetic rate, its mobilization from secretory granules, its proteolytic shedding from the cell surface (Michelson et al., 1996; Hartwell et al., 1998), and its endocytosis (Setiadi et al., 1995; Setiadi and McEver, 2003), delivery to lysosomes (Green et al., 1994), or recycling to new Weibel-Palade bodies (Subramaniam et al., 1993). The importance of P-selectin for leukocyte recruitment may also depend on the levels of E-selectin, chemokines, and integrin ligands that are regulated by specific tissue environments and inflammatory challenges. In some responses to infection or tissue injury, platelets may be a more important source of P-selectin than endothelial cells (Zarbock et al., 2007). Our results underscore the need for caution in extrapolating the functions of P-selectin obtained in mice to humans, particularly in the many models where mediators are generated that activate NF-κB– and ATF-2–dependent genes. Therefore, the *TghSelp* mice should be valuable tools to probe the functions of human P-selectin in basal leukocyte trafficking and in numerous models of inflammation and thrombosis.

MATERIALS AND METHODS

Generation of transgenic mice expressing the human *Selp* gene. A human genomic bacterial artificial chromosome library (Genome Systems) was screened with PCR primers for exon 9 and intron 9 (Table S2) of the human *Selp* gene (Johnston et al., 1990). A clone containing the entire human *Selp* gene was isolated. The size of the *NotI*-released insert was analyzed using pulsed-field gel electrophoresis with a CHEF DR II apparatus (Bio-Rad Laboratories). The integrity of the human *Selp* gene was confirmed

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by PCR with primers complimentary to its exons, introns, and 5′ and 3′ flanking regions and by partial sequencing. The DNA was purified using a NucleoBond BAC Maxi kit (Takara Bio Inc.) and microinjected into fertilized (C57BL/6 X SJL) F2 murine eggs at the University of Michigan Transgenic Animal Model Core. Founder mice were identified by PCR of tail DNA using primer pairs located, respectively, at 30 kb upstream of the promoter, at the exon 9/intron 9 junction, and in exon 17. The sequences of the primers are listed in Table S2. Mice from three independent founders were backcrossed with C57BL/6J mice (The Jackson Laboratory) to establish *TghmSelp* transgenic lines that coexpressed the human *Selp* transgene and the endogenous murine *Selp* gene. Alternatively, they were backcrossed with $Selp^{-/-}$ mice in the C57BL/6J background (Bullard et al., 1995; The Jackson Laboratory) to generate *TghSelp* transgenic lines that expressed human but not murine P-selectin. This paper reports experiments with mice derived from founder line 3. Mice were typically analyzed at 8–12 wk of age. In earlier studies, littermate controls lacking the transgene were used. Transgenic mice were crossed into the C57BL/6J background for at least 10 generations. All studies of P-selectin function in vitro or in vivo were performed on fully backcrossed mice. Lines that carried the transgene on one allele or on both alleles were maintained. All animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee of the Oklahoma Medical Research Foundation.

Peripheral blood counts. Peripheral blood counts were measured with a Hemavet 950 veterinary hematology analyzer (Drew Scientific, Inc.).

Flow cytometry. Whole blood collected by cardiac puncture from anesthetized mice was transferred directly into a tube containing EDTA (2.7 mM final concentration). To study platelets and RBCs, 2 µl of blood was diluted 1:100 in PBS containing 2.7 mM EDTA (PBS/EDTA). In some experiments, diluted blood was incubated with 0.5 U/ml bovine thrombin for 5 min at room temperature to activate platelets. To study leukocytes, RBCs in 100 µl of blood were lysed by adding 1.4 ml of 150 mM NH_4Cl, 10 mM $NaHCO_3$, and 1 mM EDTA for 3 min at room temperature. Leukocytes were centrifuged at 150 *g* for 5 min, washed once with PBS/EDTA, and resuspended at 10^6/ml in 200 µl PBS/EDTA. Resident peritoneal cells were collected by flushing the peritoneal cavity with 10 ml PBS/EDTA. All antibody incubations were performed on ice. Cells were preincubated with 2.5 µg/ml murine Fc Block (anti–murine CD16/CD32; BD) for 5 min. Cells were then incubated for 30 min with 10 µg/ml of FITC-labeled RB40.34 (rat IgG1, anti–murine P-selectin; BD), PE-labeled AK4 (murine IgG1, anti–human P-selectin; BD), PE-labeled F4/80 (rat IgG2b, macrophage marker; AbD Serotec), or labeled isotype-control mAbs. The cells were then washed, resuspended in PBS containing 0.2% BSA and 2.7 mM EDTA, and analyzed on a FACScan instrument using CellQuest software (BD). Platelets, RBCs, and leukocyte subpopulations were distinguished by their light scatter properties. Peritoneal macrophages were also identified with mAb F4/80.

Quantification of P-selectin expression on platelets. Binding of ^{125}I-labeled murine anti–human P-selectin mAb S12 or rat anti–murine P-selectin mAb RB40.34 to resting or activated platelets was performed as previously described (McEver and Martin, 1984). In brief, prostaglandin E_1 (Sigma-Aldrich) was added to EDTA-anticoagulated blood at 25 nM. To obtain platelet-rich plasma, blood was centrifuged at 150 *g* for 10 min. The platelet-rich plasma was centrifuged at 2,500 *g* for 10 min, and the isolated platelets in the pellet were resuspended at 2×10^8/ml in PBS containing 0.2% BSA and 2.7 mM EDTA. 200 µl of platelets were incubated with a saturating concentration of ^{125}I-labeled mAb (1 µg/ml, pretitrated in a pilot study) for 30 min in the presence or absence of 1.0 U/ml bovine thrombin. After centrifugation over a 9:1 mixture of *n*-butyl phthalate (J.T. Baker) and Apiezon A oil (M&I Materials), the radioactivity of platelet pellets was counted. The number of P-selectin molecules on each platelet was calculated by assuming a 1:1 ratio of bound mAb to protein.

RT-PCR. Total RNA from murine lungs or from transfected Chinese hamster ovary cells expressing human P-selectin (Setiadi et al., 1995) was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. RNA integrity was verified by ethidium bromide staining after electrophoresis and quantified by optical density at 260 nm. 5 µg RNA in a 20-µl reaction volume was reverse-transcribed using oligo (dT) 12–18 primers with the SuperScript II RT System (Invitrogen) as specified by the manufacturer. PCR of human P-selectin cDNA (Johnston et al., 1989) was performed with primers located 10 bp upstream of the initiating ATG and 131 bp downstream of the TAA polyadenylation signal. PCR for murine β-actin cDNA was performed as a positive control. The sequences of the primers are listed in Table S3.

Western blots. Platelets were incubated in 25 mM Tris, pH 7.4, 137 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 mM NaF, 10 mM sodium phosphate, and 10 mM sodium pyrophosphate containing protease inhibitors (Roche). Lysates were fractionated in a 7.5% SDS-PAGE gel under nonreducing conditions and transferred to a polyvinylidene fluoride membrane. The membrane was blocked for 2 h in 5% nonfat dry milk in 10 mM Tris-HCl, pH 7.5, and 100 mM NaCl containing 0.1% Tween-20 (TBST) and then incubated for 2 h with 1 µg/ml of biotinylated murine anti–human P-selectin mAb G1 (Geng et al., 1990), a 1:5,000 dilution of rabbit serum raised to the 25 carboxyl-terminal residues of the human P-selectin cytoplasmic domain (Green et al., 1994), or 1 µg/ml of rat anti–murine P-selectin mAb RB40.34 (Ley et al., 1995). The blot was washed three times in TBST and incubated for 1 h with the appropriate secondary reagent coupled to horseradish peroxidase. After three washes, bound antibodies were detected using enhanced chemiluminescence (GE Healthcare).

Immunohistochemistry. Murine tissues were fixed in 10% formalin, embedded in paraffin, and processed into 5-µm sections. Sections were deparaffinized and microwaved in antigen unmasking solution (Vector Laboratories). Air-cooled sections were treated with 3% hydrogen peroxide in PBS to block endogenous peroxidase activity, then with Avidin-Biotin blocking kit (Vector Laboratories) to block endogenous biotin, and finally with protein block (Dako) to reduce other nonspecific antibody binding. The treated sections were incubated for 1 h with 5 µg/ml biotinylated sheep anti–human P-selectin antibody (does not cross react with murine P-selectin; R&D Systems), biotinylated control sheep IgG (Vector Laboratories), or buffer containing no primary antibody. The sections were then incubated for 1 h with streptavidin-conjugated horseradish peroxidase complex solution (1:300 dilution; Vectastain Elite ABC; Vector Laboratories). The slides were developed with DAB peroxidase substrate kit (Vector Laboratories) and counterstained with hematoxylin. All incubations were performed at room temperature and were separated by three washes with PBS.

Immunofluorescence of cultured murine endothelial cells. Endothelial cells isolated from murine lung were cultured as previously described (Yao et al., 2005). The cells were fixed in 4% paraformaldehyde for 5 min at room temperature, permeabilized with 0.01% saponin, and incubated with rabbit anti–human von Willebrand factor antibody (cross reacts with murine von Willebrand factor; Dako) and goat anti–human P-selectin antibody (cross reacts with murine P-selectin; Ferrell Farms) for 30 min, followed by donkey anti–rabbit IgG conjugated to Alexa Fluor 568 and donkey anti–goat IgG conjugated to Alexa Fluor 488 (Invitrogen). All antibodies were used at a 1:100 dilution. Images were visualized on a confocal microscope (Eclipse C1; Nikon). Colocalization of von Willebrand factor and P-selectin fluorescence was quantified with an Imaris colocalization module (Bitplane) and expressed as a percentage of colocalized pixels.

Flow chamber assay. Murine bone marrow leukocytes (Xia et al., 2002) were resuspended at 10^6 cells/ml in HBSS containing 1.26 mM Ca^{2+}, 0.81 mM Mg^{2+}, and 0.5% human serum albumin. 50 µg/ml of human fibronectin (Sigma-Aldrich) was adsorbed to a demarcated region in a 35-mm polystyrene dish. Human platelets (Yago et al., 2008) or murine platelets (10^8 in 50 µl) were allowed to spread on the fibronectin-coated surface and then incubated with 1 U/ml thrombin for 3 min immediately before insertion into a flow chamber. Leukocytes were perfused over the adherent platelets

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at a wall shear stress of 1.0 dyn/cm^2. In some experiments, dishes were preincubated with 10 µg/ml blocking anti–mouse P-selectin mAb RB40.34, isotype-matched control mAb (rat IgG1), blocking anti–human P-selectin mAb G1, or nonblocking anti–human P-selectin mAb S12 (Geng et al., 1990). After 5 min, the number of rolling cells was analyzed using a video microscopy system (Xia et al., 2002; Miner et al., 2008).

Real-time quantitative PCR. To measure the number of copies of the human *Selp* transgene, tail genomic DNA from *TghSelp* mice was used as template. Intron 1 sequence from the murine *Selp* gene (retained in *Selp*$^{-/-}$ mice; Bullard et al., 1995) was amplified to normalize DNA loading between samples. As calibrator, we used a murine embryonic stem cell clone in which 1.4 kb of the murine *Selp* promoter in one allele was replaced with the corresponding sequence from the human *Selp* promoter by homologous recombination.

To measure expression of the human and murine *Selp* genes, *TghmSelp* mice were injected i.v. with 100 µl saline containing 1% human serum albumin as control, 1 µg murine recombinant TNF (R&D Systems), or 50 µg LPS (Sigma-Aldrich). Mice were sacrificed and organs were collected 3 h after TNF injection or 3.5 h after LPS injection. Total RNA was isolated and reverse transcribed as described in RT-PCR. The cDNAs for both human and murine P-selectin were amplified. Murine β2-microglobulin RNA was amplified as endogenous control, and the RNA level in samples from human serum albumin–treated mice was used as calibrator. Quantitative PCR was performed on an ABI Prism 7000 spectrofluorometric thermal cycler (Applied Biosystems) using SYBR green I as a double-strand DNA–specific binding dye. PCR primers (Table S3) were chosen using Primer Express 1.7 Software. In 96-well plates, a 2.5-µl sample was added to a 22.5-µl PCR amplification mixture containing SYBR Green PCR Core Reagents mixture (Applied Biosystems) and 0.2 µM of each primer. The amplification scheme was used according to the manufacturer's protocol. The starting concentration of target in each reaction was quantified according to the relative standard curve method and normalized to the endogenous control (Applied Biosystems; User Bulletin #2). Quantitative PCR assays were conducted in triplicate for each sample. Data are expressed as an *n*-fold difference relative to the calibrator.

Intravital microscopy. Intravital video microscopy of anesthetized mice was performed as previously described (Xia et al., 2002; Miner et al., 2008). The cremaster muscle was isolated and superfused with thermocontrolled (35°C) bicarbonate-buffered saline. Microscopy was performed immediately after isolation or 3 h after intrascrotal injection of murine TNF (R&D Systems, 0.5 µg per mouse in 0.3 ml of sterile saline). A blocking anti–murine E-selectin mAb 9A9 (Kunkel and Ley, 1996; 30 µg in 100 µl saline) was injected i.v. at the time of TNF administration. Microvessel diameters, lengths, and centerline velocities were comparable in mice from all genotypes. Mean leukocyte rolling velocities and rolling fluxes were analyzed offline. The number of firmly adherent cells was divided by the surface area of the vessel wall seen in the field of view. Surface area was calculated for each vessel, using $S = \pi \times d \times l_v$, where d is the diameter, and l_v is the length of the vessel (Smith et al., 2006).

To study leukocyte rolling in the skin, Nair lotion was applied to both sides of the right ear to remove hair. After 5 min, the ear was gently cleaned with a gauze pad. The ear was mounted against the microscope stage, flattened, and held in place by a coverslip. 0.25 mg/kg rhodamine 6G in 100 µl saline (Sigma-Aldrich) was injected through a carotid artery catheter to visualize fluorescently labeled leukocytes by epi-illumination with a light source emitted from a mercury lamp (HB-10103AF). Some mice were injected i.v. with mAbs (30 µg each in 100 µl saline) to human P-selectin (G1), murine P-selectin (5H1), or murine E-selectin (9A9).

The density of P-selectin on venular surfaces was measured by injecting mAb-coated fluorescent microspheres. Anti–human P-selectin mAb G1 or anti–murine P-selectin mAb 5H1 (Ramos et al., 1997) was coupled to 1.82 × 10^{11} fluoresbrite yellow green microspheres (0.5-µm diameter; excitation/emission, 441 nm/486 nm; Polysciences) according to the manufacturer's protocol. 10^{10} microspheres in 100 µl saline were injected through the cannulated carotid artery, and cremaster vessels were observed 10 min later by fluorescence microscopy. G1-coated microspheres were injected into *TghSelp*$^{+/-}$ mice and 5H1-coated microspheres were injected into WT mice. Nonspecific binding was measured by parallel injections of microspheres into *Selp*$^{-/-}$ mice. After experiments, the still images were converted from the finished video. ImageJ software (1.43n; National Institutes of Health) was used to analyze the relative fluorescence intensity. Images were converted from 8-bit to binary values. A region of interest was then selected by drawing a line around the vessel. The relative fluorescence intensity of the bead-covered area within the drawn region was calculated by the software.

Thioglycollate-induced peritonitis. Mice were injected i.p. with 1.5 ml of 0.9% saline or 4% thioglycollate. Some mice received 30 µg anti–human P-selectin mAb G1 or anti–murine P-selectin mAb 5H1 i.v. immediately before administration of thioglycollate. After 4 or 8 h, mice were sacrificed and the peritoneal cavity was lavaged with 7 ml PBS containing 2.7 mM EDTA. The recovered cells were analyzed by flow cytometry. Neutrophils were counted based on scatter properties and high expression of Gr-1 (Mócsai et al., 2002).

Oxazolone-induced contact hypersensitivity. On day 0, mice were sensitized by topical application of 100 µl of 2% oxazolone (4-ethoxymethylene-2-oxazolin-5-one; Sigma-Aldrich) in acetone/olive oil (4:1) to shaved abdominal skin and of 5 µl of the same mixture to each paw. On day 7, mice were challenged by painting the right ear with 1% oxazolone (10 µl to each side). The left ear was painted with acetone/olive oil as control. In some mice, mAbs (100 µg in 100 µl saline) to human P-selectin (G1), murine P-selectin (5H1), or murine E-selectin (9A9) were injected i.v. immediately before challenge or at the indicated time after challenge. Control mice were injected with isotype-matched murine or rat IgG1 (BD) or with saline, which yielded identical results. Ear thickness 24 h after challenge or at the indicated time of mAb injection was measured with an electronic digital micrometer (Marathon Watch). Nonspecific swelling caused by oxazolone-induced irritation was measured in nonsensitized WT mice. Ear thickness was expressed as the absolute increase in millimeters and calculated as (treated ear thickness − control ear thickness − nonspecific swelling). Measurement was performed by an investigator who was blinded to genotypes and treatment protocols. For histology, ear tissue collected 24 h after challenge was fixed with formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin. To investigate gene expression, treated and control ears were removed at various intervals after challenge, frozen in dry ice, and homogenized in lysis buffer (QIAGEN) without thawing. Total RNA was purified using the RNeasy kit (QIAGEN). Quantitative RT-PCR was performed as described in Real-time quantitative PCR.

Statistics. Statistical analysis was performed using the Student's *t* test for unpaired samples.

Online supplemental material. Table S1 provides peripheral blood counts of WT and *TghSelp*$^{+/-}$ mice. Tables S2 and S3 provide the sequences of primers used for PCR or RT-PCR. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20101545/DC1.

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R.P. McEver has equity interest in Selexys, a company that is developing inhibitors of selectins and selectin ligands. The other authors have no conflicting financial interests.

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