Del-1, an Endogenous Leukocyte-Endothelial Adhesion Inhibitor, Limits Inflammatory Cell Recruitment

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Leukocyte recruitment to sites of infection or inflammation requires multiple adhesive events. Although numerous players promoting leukocyte-endothelial interactions have been characterized, functionally important endogenous inhibitors of leukocyte adhesion have not been identified. Here we describe the endothelially derived secreted molecule Del-1 (developmental endothelial locus–1) as an anti-adhesive factor that interferes with the integrin LFA-1–dependent leukocyte-endothelial adhesion. Endothelial Del-1 deficiency increased LFA-1–dependent leukocyte adhesion in vitro and in vivo. Del-1$^{-/-}$ mice displayed significantly higher neutrophil accumulation in lipopolysaccharide-induced lung inflammation in vivo, which was reversed in Del-1/LFA-1 double-deficient mice. Thus, Del-1 is an endogenous inhibitor of inflammatory cell recruitment and could provide a basis for targeting leukocyte-endothelial interactions in disease.

Leukocyte extravasation is integral to the response to infection or injury and to inflammation and autoimmunity. Leukocyte recruitment comprises a well-coordinated cascade of adhesive events, including selectin-mediated rolling, firm adhesion of leukocytes to endothelial cells, and their subsequent transendothelial migration. The interaction between LFA-1 (also known as αLβ2 and CD11a/CD18) and endothelial intercellular adhesion molecule–1 (ICAM-1) is crucial during firm endothelial adhesion of leukocytes (*1*–*5*). Whereas numerous adhesion receptors promoting inflammatory cell recruitment have been identified, very little information exists about endogenous inhibitors of the leukocyte adhesion cascade (*1*–*7*).

Developmental endothelial locus–1 (Del-1) is a glycoprotein that is secreted by endothelial cells and can associate with the endothelial cell surface and the extracellular matrix (*8*–*10*). Del-1 is regulated upon hypoxia or vascular injury and has been implicated in vascular remodeling during angiogenesis (*10*–*12*). We sought to determine whether endothelially derived Del-1 participates in leukocyte-endothelial interactions.

Del-1 mRNA was observed predominantly in the brain and lung, with no expression in the liver, spleen, or whole blood (Fig. 1A and fig. S1A). Del-1 was expressed in wild-type (WT) but not in Del-1$^{-/-}$ murine lung endothelial cells (Fig. 1B) (*9*). In lung tissues, Del-1 was present in blood vessels (fig. S1B).

To determine whether Del-1 participates in leukocyte recruitment interactions, we studied the adhesion of primary neutrophils to immobi-

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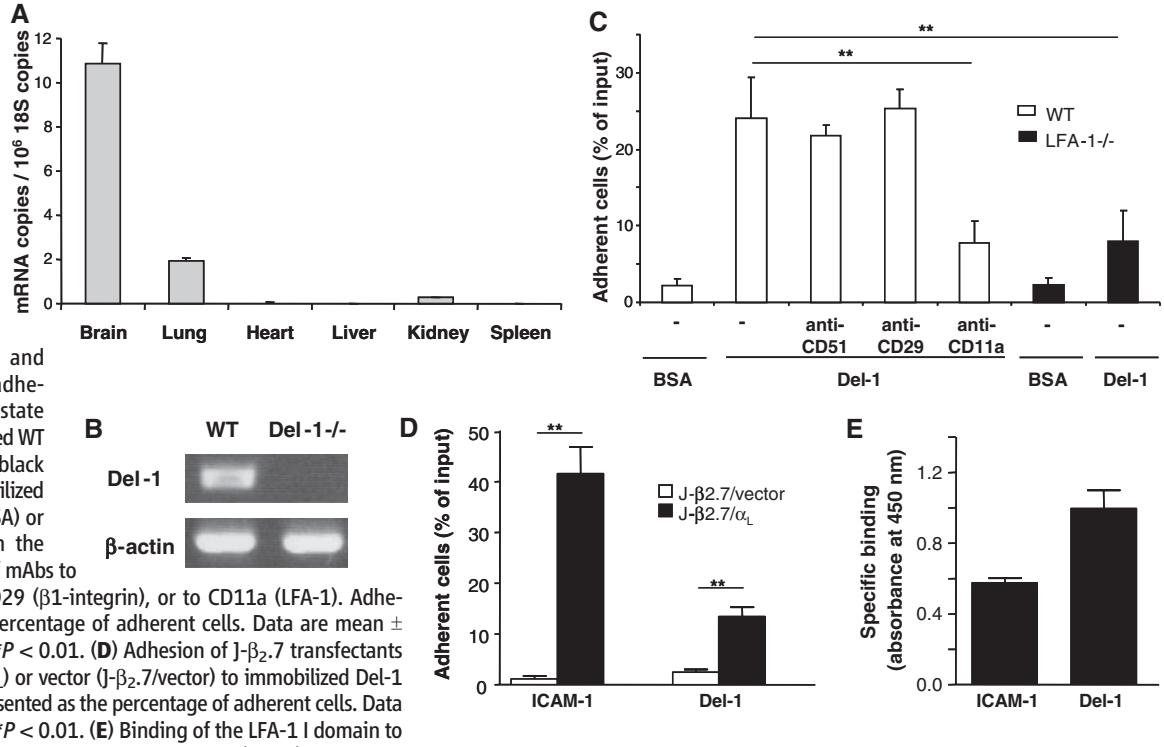

Fig. 1. Del-1 is expressed in endothelial cells and interacts with leukocyte LFA-1. (**A**) Real-time reverse transcription polymerase chain reaction (RT-PCR) demonstrating the expression of Del-1 mRNA in adult mouse tissues. Del-1 mRNA was normalized against 18*S* ribosomal RNA. (**B**) RT-PCR in primary lung endothelial cells from WT and Del-1$^{-/-}$ mice. (**C**) Static adhesion of phorbol 12-myristate 13-acetate (PMA)–stimulated WT (white bars) or LFA-1$^{-/-}$ (black bars) neutrophils to immobilized bovine serum albumin (BSA) or mouse Del-1 is shown in the absence (-) or presence of mAbs to CD51 (αv-integrin), to CD29 (β1-integrin), or to CD11a (LFA-1). Adhesion is presented as the percentage of adherent cells. Data are mean ± SD (n = 3 experiments). **P < 0.01. (**D**) Adhesion of J-β_2.7 transfectants expressing LFA-1 (J-β_2.7/α_L) or vector (J-β_2.7/vector) to immobilized Del-1 or ICAM-1. Adhesion is presented as the percentage of adherent cells. Data are mean ± SEM (n = 3). **P < 0.01. (**E**) Binding of the LFA-1 I domain to immobilized Del-1 or ICAM-1. Data are mean ± SEM (n = 3).

lized Del-1. Mouse neutrophils specifically bound to Del-1 under static conditions. Adhesion was inhibited by a blocking monoclonal antibody (mAb) to CD11a (the αL-integrin subunit) but not by antibodies to αv-integrin or β1-integrin (Fig. 1C), suggesting that LFA-1 mediates the interaction of neutrophils with Del-1. Consistently, LFA-$1^{-/-}$ neutrophils displayed reduced adhesion to Del-1 (Fig. 1C). The residual LFA-1–independent binding of neutrophils to Del-1 was blocked by mAb to Mac-1 (fig. S2A), which is consistent with the fact that LFA-1 and Mac-1 are closely related and share several ligands (*13*). In addition, αL-transfected but not vector-transfected J-β_2.7 cells specifically bound to immobilized Del-1 (Fig. 1D), whereas a direct interaction between Del-1 and the ligand-binding I domain of LFA-1, locked in the open high-affinity conformation, was observed (Fig. 1E and fig. S2B). Thus, Del-1 is a ligand of LFA-1 integrin.

To address whether Del-1 participates in leukocyte-endothelial interactions, we studied neutrophil and monocyte adhesion to WT and Del-$1^{-/-}$ endothelial cells (*14*, *15*). Contrary to our prediction, Del-$1^{-/-}$ endothelial cells promoted significantly higher neutrophil and monocyte adhesion. LFA-1–deficiency on leukocytes and mAb to LFA-1 abolished the enhanced adhesion to Del-$1^{-/-}$ endothelium (Fig. 2A and fig. S3). Thus, enhanced inflammatory cell adhesion to Del-$1^{-/-}$ endothelium is specifically mediated by LFA-1 on leukocytes.

To understand the unexpected inhibitory role of Del-1 in leukocyte-endothelial adhesion, we investigated whether soluble Del-1 interfered with the interaction of LFA-1 with its major ligand, ICAM-1. Mn^{2+}-induced binding of ICAM-1–Fc to murine leukocytes in solution was significantly inhibited by soluble Del-1 (Fig. 2B). Moreover, soluble Del-1 inhibited the LFA-1–dependent adhesion of WT neutrophils to immobilized ICAM-1 under physiologic flow conditions, whereas soluble Del-1 did not affect the weaker adhesion of LFA-$1^{-/-}$ neutrophils to ICAM-1 (Fig. 2C).

The finding that endothelial Del-1 antagonizes LFA-1–dependent adhesion (Fig. 2A) appeared to be discordant with the finding that immobilized Del-1 promoted leukocyte adhesion under static conditions (Fig. 1C). We thus assessed the ability of Del-1 and ICAM-1 to promote adhesion when co-immobilized with P-selectin and the chemokine MIP-2 under physiologic flow conditions at low and high shear rates (0.8 and 2 dynes/cm^2). In this system, leukocytes first roll on selectin and then arrest on the integrin ligand. Whereas ICAM-1 promoted robust firm adhesion of neutrophils at both shear rates, Del-1 promoted only weak adhesion at the lower shear rate and almost none at the higher shear rate (Fig. 2D). We then analyzed how the presence of plate-bound Del-1 would affect the adhesion of neutrophils to ICAM-1 under flow. Increasing concentrations of Del-1 co-immobilized with ICAM-1, P-selectin, and MIP-2 significantly inhibited neutrophil adhesion to ICAM-1 (Fig. 2E). Thus, although it is

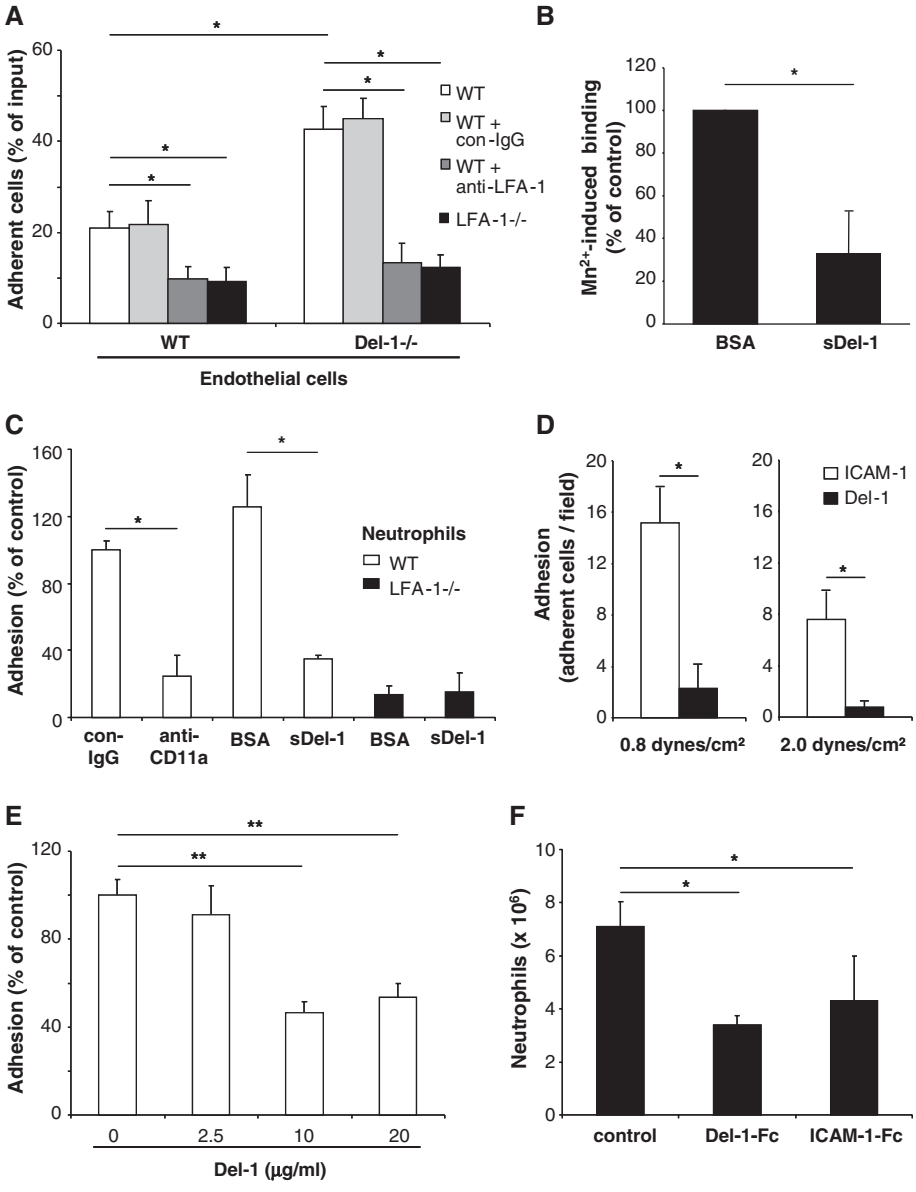

Fig. 2. Del-1 interferes with LFA-1–dependent leukocyte adhesion. (**A**) PMA-induced adhesion of WT neutrophils in the absence (white bars) or presence of isotype control antibody (light gray bars) or mAb to LFA-1 (dark gray bars), or of LFA-$1^{-/-}$ neutrophils (black bars) to WT or Del-$1^{-/-}$ lung endothelial cells, is shown. IgG, immunogolbulin G. Adhesion is presented as the percentage of adherent cells. Data are mean ± SD (n = 4 experiments). *P < 0.05. (**B**) Binding of soluble ICAM-1–Fc to mouse bone marrow mononuclear cells in the presence of $MnCl_2$. Cells were preincubated with BSA or soluble Del-1. Data are mean ± SEM (n = 3). *P < 0.05. (**C**) Adhesion of WT (white bars) or LFA-$1^{-/-}$ (black bars) neutrophils to immobilized P-selectin, MIP-2, and ICAM-1 under flow (0.8 dynes/cm^2) was studied in the presence of mAb to CD11a or isotype control antibody (each mAb at 10 μg/ml) or in the presence of BSA or mouse soluble Del-1 (each at 20 μg/ml). Adhesion is shown as the percentage of control; that is, adhesion of WT neutrophils in the presence of control antibody. Data are mean ± SEM (n = 3 perfusion experiments). *P < 0.05. (**D**) Adhesion of WT neutrophils to immobilized P-selectin, MIP-2, and ICAM-1 (white bars) or Del-1 (black bars) was studied at indicated shear rates. Adhesion is shown as the number of adherent cells per field. Data are mean ± SEM (n = 4 perfusion experiments). *P < 0.05. (**E**) Adhesion of WT neutrophils to immobilized P-selectin, MIP-2, and ICAM-1 was studied in the presence of increasing concentrations of Del-1 that was coimmobilized. Adhesion is shown as the percentage of control; that is, adhesion of WT neutrophils in the absence of immobilized Del-1. Data are mean ± SEM (n = 6 perfusion experiments). **P < 0.01. (**F**) The numbers of neutrophils at 4 hours after intraperitoneal injection of thioglycollate in WT mice are shown. Mice were treated 30 min before thioglycollate injection with intravenous injection of control Fc protein (control), Del-1–Fc, or ICAM-1–Fc. Data are expressed as absolute numbers of emigrated neutrophils. Data are mean ± SD (n = 4 mice per group). *P < 0.05.

a ligand of LFA-1, Del-1 does not promote firm leukocyte adhesion under flow but interferes with leukocyte adhesion to endothelial ICAM-1.

We then assessed the ability of soluble Del-1 expressed as an Fc fusion protein to inhibit neutrophil recruitment in vivo in acute thioglycollate-induced peritonitis (*14*). Intravenous administration of Del-1–Fc 30 min before thioglycollate injection significantly reduced neutrophil accumulation,

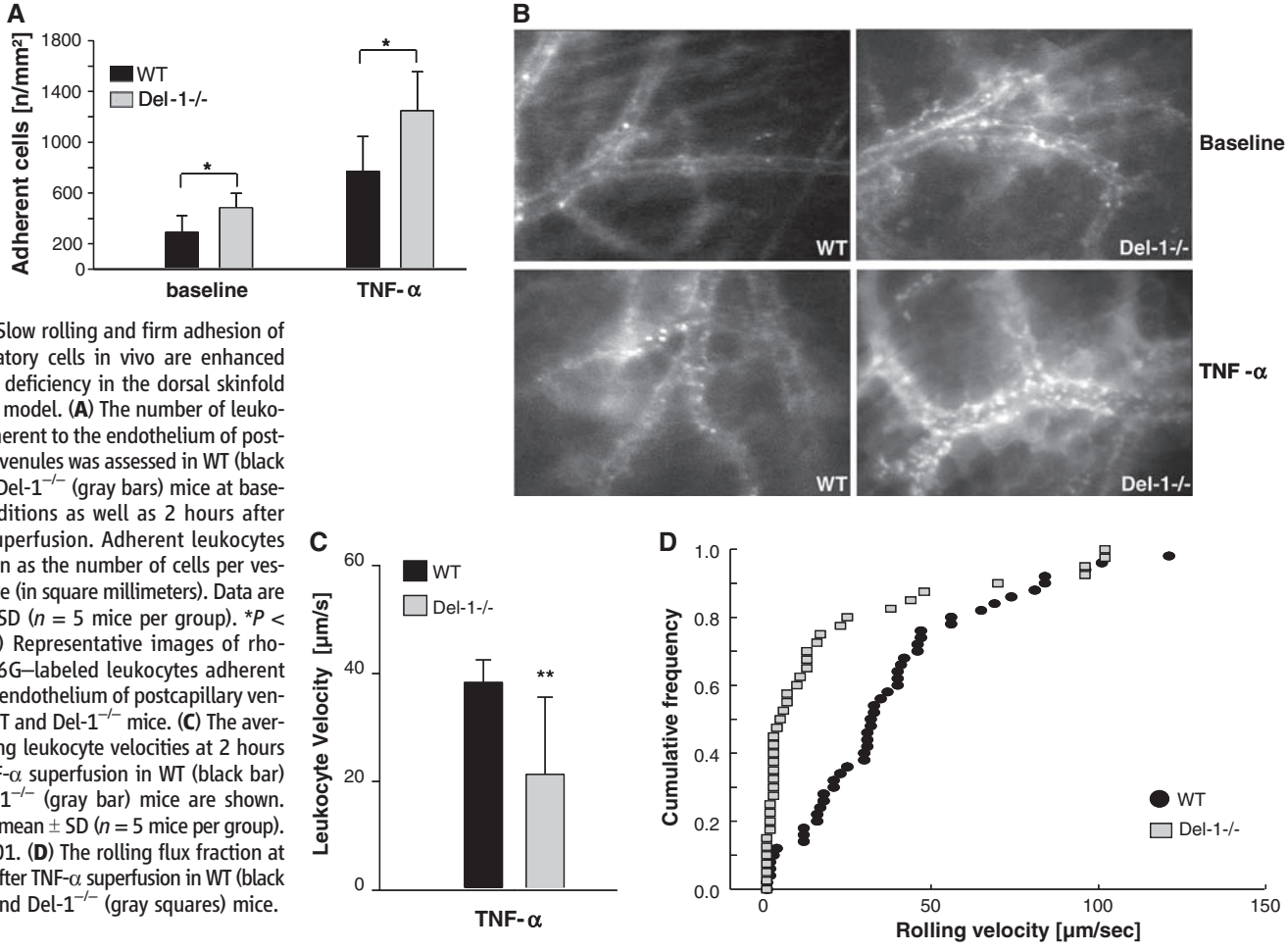

Fig. 3. Slow rolling and firm adhesion of inflammatory cells in vivo are enhanced by Del-1 deficiency in the dorsal skinfold chamber model. (**A**) The number of leukocytes adherent to the endothelium of postcapillary venules was assessed in WT (black bars) or Del-1$^{-/-}$ (gray bars) mice at baseline conditions as well as 2 hours after TNF-α superfusion. Adherent leukocytes are shown as the number of cells per vessel surface (in square millimeters). Data are mean ± SD (n = 5 mice per group). *P < 0.05. (**B**) Representative images of rhodamine 6G–labeled leukocytes adherent onto the endothelium of postcapillary venules of WT and Del-1$^{-/-}$ mice. (**C**) The average rolling leukocyte velocities at 2 hours after TNF-α superfusion in WT (black bar) and Del-1$^{-/-}$ (gray bar) mice are shown. Data are mean ± SD (n = 5 mice per group). **P < 0.01. (**D**) The rolling flux fraction at 2 hours after TNF-α superfusion in WT (black circles) and Del-1$^{-/-}$ (gray squares) mice.

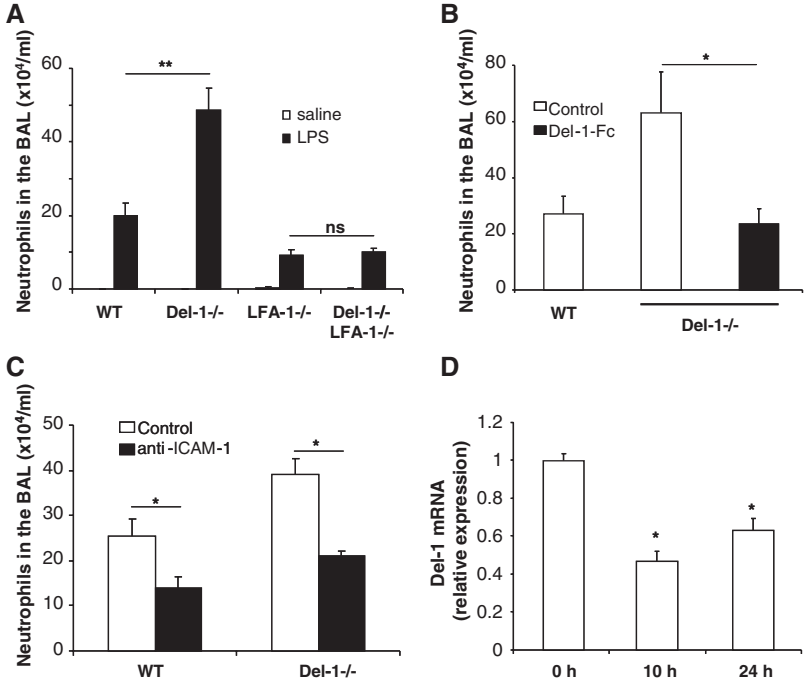

Fig. 4. Increased inflammatory cell recruitment in vivo due to Del-1 deficiency. (**A**) The numbers of neutrophils in the BAL fluid in WT, Del-1$^{-/-}$, LFA-1$^{-/-}$, or Del-1$^{-/-}$LFA-1$^{-/-}$ mice are shown at 24 hours after nasal administration of saline (white bars) or LPS (black bars). Neutrophil recruitment upon saline inhalation was negligible. Data are expressed as absolute numbers and are mean ± SEM (n = 11 to 16 mice per group). **P < 0.01; ns, not significant. (**B**) Thirty minutes before LPS administration, WT or Del-1$^{-/-}$– mice received intravenous injections of BSA (control, white bars) or Del-1-Fc (black bar) (each at 90 μg per mouse). Data are expressed as absolute numbers and are mean ± SEM (n = 4 to 11 mice per group). *P < 0.05. (**C**) Thirty minutes before LPS administration, WT or Del-1$^{-/-}$ mice received intravenous injections of isotype control IgG (white bars) or anti-ICAM-1 (black bars) (each at 85 μg per mouse). Data are expressed as absolute numbers and are mean ± SEM (n = 8 or 9 mice per group). *P < 0.05. (**D**) The expression of Del-1 mRNA in mouse lungs at 0 hours, 10 hours, or 24 hours after intranasal LPS administration was analyzed by semiquantitative RT-PCR. The data are shown as relative expression. The ratio of Del-1 mRNA to actin mRNA at 0 hours was set as 1. Data are mean ± SEM (n = 4 mice per group). *P < 0.05 as compared to 0 hours.

as compared to Fc control protein (Fig. 2F). Similarly, ICAM-1–Fc reduced neutrophil recruitment into the peritoneum (Fig. 2F).

To provide further evidence for the role of Del-1 in inflammatory cell recruitment in vivo, we performed intravital microscopy using the dorsal skinfold chamber model (*16*). Del-1$^{-/-}$ mice displayed increased numbers of leukocytes adherent to postcapillary venules both in the baseline condition and upon tumor necrosis factor–α (TNF-α) stimulation (Fig. 3, A and B). Besides firm arrest, the interaction between LFA-1 and ICAM-1 contributes to slow rolling processes (*17*). A significant decrease in rolling velocity accompanied by an increase in the fraction of slow-rolling leukocytes was observed in Del-1$^{-/-}$ mice (Fig. 3, C and D).

We further studied whether Del-1 could regulate inflammatory cell recruitment in vivo, by performing LPS-induced lung inflammation. Del-1$^{-/-}$ mice displayed significantly higher accumulation of neutrophils in the bronchoalveolar lavage (BAL) fluid than did WT mice (Fig. 4A). LFA-1$^{-/-}$ mice displayed reduced neutrophil accumulation in the BAL upon LPS-induced lung inflammation (Fig. 4A) (*18*, *19*). The increased neutrophil recruitment in vivo due to Del-1 deficiency required the presence of LFA-1, because neutrophil accumulation in the BAL in Del-1$^{-/-}$LFA-1$^{-/-}$ mice equaled the accumulation of these cells in LFA-1$^{-/-}$ mice (Fig. 4A). The increased leukocyte recruitment due to Del-1 deficiency could not be attributed to an alteration in peripheral blood counts, because constitutive leukocyte numbers were comparable in WT and Del-1$^{-/-}$ mice (fig. S4). In addition, intravenous administration of soluble Del-1 efficiently reversed the increased neutrophil recruitment in Del-1$^{-/-}$ mice (Fig. 4B).

Furthermore, Del-1 deficiency resulted in an up-regulation of baseline ICAM-1 protein expression by lung endothelial cells, which was overridden upon TNF-α stimulation, whereas vascular cell adhesion molecule–1 (VCAM-1) expression was unaffected (fig. S5). No significant increase in ICAM-1 expression, under baseline or inflammatory conditions, was found in Del-1$^{-/-}$ lungs (fig. S6), suggesting that altered ICAM-1 expression is not involved in the increased leukocyte recruitment to Del-1$^{-/-}$ lungs. Moreover, whereas the increased neutrophil recruitment to the lung upon Del-1 deficiency was completely reversed by leukocyte LFA-1 deficiency (Fig. 4A), the inhibition of ICAM-1 by a blocking mAb (*18*, *20*) decreased neutrophil recruitment by the same extent in both WT and Del-1$^{-/-}$ mice (Fig. 4C), suggesting an involvement of other LFA-1 ligands. Thus, Del-1 deficiency enhances LFA-1–dependent leukocyte recruitment in vivo.

We found that Del-1 acted in an anti-inflammatory fashion; however, the expression of Del-1 in inflammation has not been demonstrated. Thus, we analyzed Del-1 mRNA expression in the lung and in endothelial cells upon inflammatory stimulation. Upon LPS administration, lung Del-1 mRNA was significantly reduced (Fig. 4D). Likewise, TNF-α stimulation of endothelial cells induced a significant decrease in Del-1 expression (fig. S7).

Endogenous inhibitors exist in many aspects of inflammation and immunity (*21*, *22*), attenuating exuberant inflammatory and immune activation. The existence of endogenous inhibitors in the leukocyte adhesion cascade, a central paradigm of inflammation and immunity, has been unclear. In this study, endothelially derived Del-1 was shown to intercept LFA-1–dependent leukocyte-endothelial interactions. Given the importance of LFA-1–dependent leukocyte recruitment in several inflammatory and autoimmune disorders (*13*, *23*–*25*), Del-1 may provide a platform for designing novel attractive therapeutic modalities to target leukocyte-endothelial interactions in disease.

References and Notes

1. T. A. Springer, *Cell* **76**, 301 (1994).
2. N. Hogg, M. Laschinger, K. Giles, A. McDowall, *J. Cell Sci.* **116**, 4695 (2003).
3. D. Vestweber, *Immunol. Rev.* **218**, 178 (2007).
4. B. A. Imhof, M. Aurrand-Lions, *Nat. Rev. Immunol.* **4**, 432 (2004).
5. K. Ley, C. Laudanna, M. I. Cybulsky, S. Nourshargh, *Nat. Rev. Immunol.* **7**, 678 (2007).
6. C. Weber, L. Fraemohs, E. Dejana, *Nat. Rev. Immunol.* **7**, 467 (2007).
7. T. Chavakis, K. T. Preissner, M. Herrmann, *Trends Immunol.* **28**, 408 (2007).
8. C. Hidai, M. Kawana, H. Kitano, S. Kokubun, *Cell Tissue Res.* **330**, 83 (2007).
9. C. Hidai *et al.*, *Genes Dev.* **12**, 21 (1998).
10. H. K. Ho *et al.*, *Circulation* **109**, 1314 (2004).
11. K. Penta *et al.*, *J. Biol. Chem.* **274**, 11101 (1999).
12. J. Zhong *et al.*, *J. Clin. Invest.* **112**, 30 (2003).
13. C. G. Gahmberg *et al.*, *Cell. Mol. Life Sci.* **54**, 549 (1998).
14. Materials and methods are available as supporting material on *Science* Online.
15. E. Y. Choi *et al.*, *Blood* **111**, 3607 (2008).
16. U. Fiedler *et al.*, *Nat. Med.* **12**, 235 (2006).
17. A. Zarbock, C. A. Lowell, K. Ley, *Immunity* **26**, 773 (2007).
18. A. Basit *et al.*, *Am. J. Physiol. Lung Cell. Mol. Physiol.* **291**, L200 (2006).
19. Z. M. Ding *et al.*, *J. Immunol.* **163**, 5029 (1999).
20. T. Kumasaka *et al.*, *J. Clin. Invest.* **97**, 2362 (1996).
21. R. V. Parry, J. L. Riley, S. G. Ward, *Trends Immunol.* **28**, 161 (2007).
22. R. J. Greenwald, G. J. Freeman, A. H. Sharpe, *Annu. Rev. Immunol.* **23**, 515 (2005).
23. M. Lebwohl *et al.*, *New Engl. J. Med.* **349**, 2004 (2003).
24. A. D. Luster, R. Alon, U. H. von Andrian, *Nat. Immunol.* **6**, 1182 (2005).
25. K. Yonekawa, J. M. Harlan, *J. Leukoc. Biol.* **77**, 129 (2005).

26. We thank X. Feng and M. Sardy for generating the Del-1–Fc protein, N. Hogg for the antibody mAb24, Valentis Inc. for recombinant Del-1 and the antibody to mouse Del-1, T. Veenstra for help with mass spectrometry, D. Winkler for help with genotyping, I. Okwumabua for technical assistance, and D. Singer for critically reading the manuscript. This research was supported by the Intramural Research Program of the NIH, NCI (T.C. and M.C.U.); by NIH grants AI067254 (S.L.C.) and RO1 HL082927 (W.C.A.); and by the Deutsche Forschungsgemeinschaft (grants FOR809 and TP6 to C.W.; TR-SFB23 and Exc 147/1 to S.D. and E.C.). A patent application on the anti-inflammatory actions of Del-1 has been filed.

Supporting Online Material

www.sciencemag.org/cgi/content/full/322/5904/1101/DC1
Materials and Methods
Figs. S1 to S8
References

28 August 2008; accepted 9 October 2008
10.1126/science.1165218

Ubiquitin-Like Protein Involved in the Proteasome Pathway of *Mycobacterium tuberculosis*

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The protein modifier ubiquitin is a signal for proteasome-mediated degradation in eukaryotes. Proteasome-bearing prokaryotes have been thought to degrade proteins via a ubiquitin-independent pathway. We have identified a prokaryotic ubiquitin-like protein, Pup (Rv2111c), which was specifically conjugated to proteasome substrates in the pathogen *Mycobacterium tuberculosis*. Pupylation occurred on lysines and required proteasome accessory factor A (PafA). In a *pafA* mutant, pupylated proteins were absent and substrates accumulated, thereby connecting pupylation with degradation. Although analogous to ubiquitylation, pupylation appears to proceed by a different chemistry. Thus, like eukaryotes, bacteria may use a small-protein modifier to control protein stability.

Similar to the eukaryotic 20*S* proteasome, the *Mycobacterium tuberculosis* (*Mtb*) proteasome is a multisubunit barrel-shaped protease composed of two rings of catalytic β subunits sandwiched by rings of α subunits (*1*–*5*). The eukaryotic 26*S* proteasome is composed of a 20*S* core particle and one or two 19*S* regulatory caps, which include adenosine triphosphatases (ATPases) that recognize, unfold, and translocate substrates into the core for degradation [reviewed in (*6*)]. In *Mtb*, Mpa (*Mycobacterium* proteasome ATPase) shares homology with regulatory cap ATPases. Substrates of the *Mtb* proteasome have been identified (*7*), but it remains unclear how they were targeted for degradation. Proteins delivered to the eukaryotic proteasome are usually conjugated with

ERRATUM

Post date 21 August 2009

Reports: "Del-1, an endogenous leukocyte-endothelial adhesion inhibitor, limits inflammatory cell recruitment" by E. Y. Choi *et al.* (14 November 2008, p. 1101). The following sentence should be added to the acknowledgments in reference 26: H.F.L. was supported by the German Academy of Sciences (Leopoldina).