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Duffy Antigen Facilitates Movement of Chemokine Across the Endothelium In Vitro and Promotes Neutrophil Transmigration In Vitro and In Vivo¹

Janet S. Lee^{*}, Charles W. Frevert^{*}, Mark M. Wurfel^{*}, Stephen C. Peiper[†], Venus A. Wong^{*}, Kimberley K. Ballman^{*}, John T. Ruzinski^{*}, Johng S. Rhim[‡], Thomas R. Martin^{*}, and Richard B. Goodman^{2,*}

^{*}Medical Research Service, Veterans Affairs Puget Sound Health Care System and Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Washington School of Medicine, Seattle, WA 98108

[†]Department of Pathology, Medical College of Georgia, Augusta, GA 30912

[‡]Department of Surgery, Center for Prostate Disease Research, Uniformed Services University of the Health Sciences, Bethesda, MD 20814

Abstract

The Duffy Ag expressed on RBCs, capillaries, and postcapillary venular endothelial cells binds selective CXC and CC chemokines with high affinity. Cells transfected with the Duffy Ag internalize but do not degrade chemokine ligand. It has been proposed that Duffy Ag transports chemokines across the endothelium. We hypothesized that Duffy Ag participates in the movement of chemokines across the endothelium and, by doing so, modifies neutrophil transmigration. We found that the Duffy Ag transfected into human endothelial cells facilitates movement of the radiolabeled CXC chemokine, growth related oncogene- α /CXC chemokine ligand 1 (GRO- α /CXCL1), across an endothelial monolayer. In addition, neutrophil migration toward GRO- α /CXCL1 and IL-8 (IL-8/CXCL8) was enhanced across an endothelial monolayer expressing the Duffy Ag. Furthermore, GRO- α /CXCL1 stimulation of endothelial cells expressing the Duffy Ag did not affect gene expression by oligonucleotide microarray analysis. These in vitro observations are supported by the finding that IL-8/CXCL8-driven neutrophil recruitment into the lungs was markedly attenuated in transgenic mice lacking the Duffy Ag. We conclude that Duffy Ag has a role in enhancing leukocyte recruitment to sites of inflammation by facilitating movement of chemokines across the endothelium.

Duffy Ag is a minor blood group Ag and the erythrocyte receptor for the malarial parasite *Plasmodium vivax* (1). The Duffy Ag on erythrocytes binds selective CXC and CC chemokines, and is proposed to function as a chemokine sink in the circulation (2–5).

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² Address correspondence and reprint requests to Dr. Richard B. Goodman, Seattle Veterans Affairs Medical Center, Pulmonary, S-111-Pulm, 1660 South Columbian Way, Seattle, WA 98108. goodmanr@u.washington.edu.

Postcapillary venular endothelial cells, the site of leukocyte emigration in most organs, also express the Duffy Ag, even in individuals who do not express Duffy Ag on their erythrocytes (6, 7). The expression of Duffy Ag is up-regulated on endothelial cells during inflammatory states in the kidney (8, 9). Its high affinity binding to several CXC and CC chemokines, focal expression at the site of leukocyte emigration, up-regulation during tissue inflammation, and conserved endothelial expression even in individuals whose erythrocytes lack Duffy Ag all suggest a biological role for Duffy Ag in regulating inflammatory cell recruitment to sites of inflammation. Some have postulated that endothelial Duffy Ag could have a proinflammatory role by facilitating the access of chemokines to circulating leukocytes and enhancing leukocyte migration (7, 9). Alternatively, endothelial Duffy Ag could reduce the intensity of inflammation by sequestering chemokines at sites of inflammation (10).

We have recently shown that there is enhanced Duffy Ag expression on parenchymal vascular beds and the alveolar septa of human lung tissue during suppurative pneumonia, a process defined by neutrophilic infiltration of the airspaces (11). The goals of this study were to determine whether Duffy Ag enhances movement of chemokines across the endothelium in vitro and whether Duffy Ag alters neutrophil migration toward chemokines in vitro and in vivo. Herein, we report that Duffy Ag, stably transfected into an immortalized human endothelial cell line and displaying high affinity binding to growth-related oncogene (GRO)- α /CXC chemokine ligand (CXCL) 1 (GRO- α /CXCL1), facilitates the movement of radiolabeled ^{125}I -labeled (^{125}I) GRO- α /CXCL1 across an endothelial monolayer. We show that the presence of Duffy Ag enhances neutrophil transendothelial migration toward GRO- α /CXCL1 and IL-8/CXCL8 in vitro. Stimulation of Duffy transfectants with GRO- α /CXCL1 does not result in adhesion molecule gene up-regulation or other alterations in the endothelial cell gene profile. Finally, we show that the absence of Duffy Ag in vivo markedly attenuates IL-8/CXCL8-mediated neutrophil recruitment into the airspaces of mice lacking the Duffy Ag. These findings suggest that endothelial Duffy Ag has an active role in chemokine-mediated neutrophil recruitment by promoting the transendothelial movement of chemokines.

Materials and Methods

Endothelial cell culture

HUVECs were immortalized by transformation with human papilloma virus-16 E6-E7 genes (12). The immortalized HUVEC line (IVEC), clone 4-5-2G, has an indefinite lifespan in culture but is nontumorigenic. The cell line demonstrates cobblestone morphology, expresses Factor VIII-related Ag, takes up Dil-Ac-LDL, and expresses the integrin subunits $\alpha_v\beta_3$, $\alpha_v\beta_5$, β_1 , α_2 , α_3 , β_4 , and α_6 , consistent with its endothelial origin. IVEC were maintained in endothelial growth medium-2 containing 2% FCS (BioWhittaker, Walkersville, MD).

³Abbreviations used in this paper: GRO, growth-related oncogene; CXCL, CXC chemokine ligand; ^{125}I , ^{125}I -labeled; IVEC, immortalized HUVEC; CCL, CC chemokine ligand; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; HSA, human serum albumin; PMN, polymorphonuclear neutrophil; fl., calcein fluorescence; Duffy⁺ cells, endothelial Duffy transfectants.

Cloning of human Duffy cDNA

The human Duffy cDNA was cloned by the PCR using primers designed from GenBank AF055992, flanking the open reading frame and containing the native start site (13). A human pancreas cDNA library served as the PCR template. PCR was conducted for 30 cycles according to the manufacturer's protocol (PerkinElmer/Cetus, Norwalk, CT) using *Taq* polymerase. PCR products were blunt-end ligated into a mammalian cell expression plasmid vector, pcDNA3.1/hygromycin B⁺ (Invitrogen, Carlsbad, CA) containing a hygromycin resistance gene and sequenced. PCR clones were selected for the correct sequence and proper orientation in the expression vector.

Transfection and stable expression of Duffy Ag

IVEC were transfected with the pcDNA3.1/hygromycin B⁺ vector containing the Duffy PCR insert. The transfectants were selected by resistance to hygromycin B (Boehringer Mannheim, Indianapolis, IN). Individual clones were isolated using cloning cylinders and subcloned by the limiting dilution technique. Individual clones were then tested for Duffy Ag surface expression by flow cytometry (FACScan; BD Biosciences, San Jose, CA) using the mAb α Fy6, a generous gift from Dr. P. Rubinstein of the New York Blood Center (New York, NY). Clones that were used for subsequent studies showed greater than one log₁₀ increase in mean fluorescence intensity as compared with mock-transfected cells incubated with α Fy6. This shift in mean fluorescence intensity displayed by Duffy-transfected cells labeled with α Fy6 was specific, because cells incubated with an isotype mouse IgG1 control Ab did not show a significant shift.

Receptor binding assay

¹²⁵I-GRO- α /CXCL1 (specific activity 2200 Ci/mmol) was obtained from Amersham Life Science (Piscataway, NJ) and New England Nuclear (Boston, MA). Unlabeled IL-8/CXCL8 and GRO- α /CXCL1 were purchased from PeproTech (Rocky Hill, NJ). Unlabeled RANTES/CC chemokine ligand (CCL) 5, monocyte chemotactic protein-1 (MCP-1/CCL2), and macrophage inflammatory protein-1 α (MIP-1 α /CCL3) were obtained from R&D Systems (Minneapolis, MN). Stable transfectants were seeded into 96-well break-apart plates (Dynatech Laboratories, Chantilly, VA) at 60,000 cells/well.

To determine the K_d of GRO- α /CXCL1 to the Duffy Ag, each well was incubated with 0.2 nM ¹²⁵I-GRO- α /CXCL1 and increasing concentrations of unlabeled GRO- α /CXCL1 for 90 min. Nonspecific binding was defined as the amount of radioactivity measured in the well containing 1 μ M unlabeled ligand. The total number of cells was counted in parallel wells. All samples were performed in triplicates or quadruplicates. Data were analyzed using the LIGAND program (Biosoft, Cambridge, U.K.). The K_d was obtained by Scatchard analysis.

To determine the chemokine binding properties of the expressed Duffy Ag, each well was incubated with 0.2 nM ¹²⁵I-GRO- α /CXCL1 and varying concentrations of unlabeled ligand at 37°C for 90 min. Wells were washed twice with binding buffer (RPMI 1640, 0.2% BSA), and dried. The radioactivity in each well was measured by scintillation counting (ICN Biomedicals, Costa Mesa, CA). Nonspecific binding was defined as the amount of radioactivity measured in the well containing 1 μ M unlabeled ligand.

Endothelial gene expression profile

Duffy transfected endothelial cells were incubated at 1×10^7 in the presence or absence of 20 nM GRO- α /CXCL1 for 4 h at 37°C. Duffy transfectants stimulated with TNF- α at 10 ng/ml served as the positive control. At the end of the incubation, cells were harvested and RNA was isolated using the Qiagen RNeasy Midi kit (Valencia, CA). Biotin-labeled cRNA was generated using the Enzo BioArray HighYield RNA Transcript Labeling kit (Enzo Biochem, Farmingdale, NY) and hybridized with the Affymetrix U95Av2 oligonucleotide array (Affymetrix, Santa Clara, CA) then washed and stained per the manufacturer's protocol. Data from each array was analyzed using the software package Microarray Suite 5.0 (Affymetrix, Santa Clara, CA). After normalization by average intensity, the expression level of each gene was compared in the presence or absence of GRO- α /CXCL1 or TNF- α . The data were then filtered to remove all genes with a <2-fold increase or decrease in expression at a significance level of $p < 0.001$ (Wilcoxon rank-sum test).

¹²⁵I-GRO- α transit assay

A peptide sequence from the N terminus of human Duffy Ag (STENSSQLDFEDVWNSS) was used to generate goat anti-human Duffy polyclonal Ab in collaboration with Invitrogen (Huntsville, AL). Serum from the immunized goat was affinity purified and the goat anti-Duffy Ig was characterized by its ability to displace ¹²⁵I-GRO- α /CXCL1 from Duffy transfectants. We determined that the inhibitory concentration of the anti-Duffy Ab required to displace 50% of ¹²⁵I-GRO- α /CXCL1 from Duffy binding sites (IC₅₀) was 16 nM (see Fig. 3). Nonimmune goat IgG was used as the control Ab (Zymed Laboratories, San Francisco, CA).

Costar 24-well plates containing polycarbonate transwell filters with a pore size of 3 μ m were used for the transit experiments (Corning Costar, Cambridge, MA). The transwell filters were precoated with human placental type IV collagen at 10 μ g/cm² (Sigma-Aldrich, St. Louis, MO). Duffy expressing endothelial cells were seeded at 2×10^5 /well onto the collagen-coated membrane, allowed to adhere, and grown to confluence at least 7 days before each experiment.

On the day of the experiment, cells were pretreated with the respective Ab (0.49 μ M) for 30 min. Following pretreatment with Ab, medium was changed to contain 0.1% human serum albumin (HSA), 2.5 μ M 10,000 m.w. Texas Red dextran, 20 nM GRO- α /CXCL1, 0.2 nM ¹²⁵I-GRO- α /CXCL1, 0.49 μ M of either anti-Duffy Ab or control Ig in the bottom well. Cells were incubated for 4 h at 37°C in 5% CO₂. At the end of the incubation, the amounts of ¹²⁵I-GRO- α /CXCL1 and Texas Red dextran were measured from a 50- μ l aliquot obtained from the top well using a gamma counter (1470 Wizard; Wallac Oy, Turku, Finland) and cytofluorometer set at excitation wavelength 590 nm and emission wavelength 645 nm (Cyto-Fluor II; PerSeptive Biosystems, Foster City, CA), respectively. The percent of ¹²⁵I-GRO- α /CXCL1 crossing the monolayer was calculated using the following equation: ¹²⁵I-GRO- α /CXCL1 crossing = 100% \times (cpm of sample wells – cpm of medium alone)/equilibrium cpm, where equilibrium cpm was defined as the radioactivity in a 50- μ l aliquot after mixing the contents of the top and bottom wells.

In all wells of each experiment, Texas Red dextran, a fluorophore-conjugated dextran of 10,000 m.w. (Molecular Probe, Eugene, OR), was placed on the same side as the chemokine and served as a marker to determine the diffusion of a low m.w. compound similar in size to chemokines. The percent dextran crossing was used to control for any differences in the integrity of the monolayer from well to well and was calculated using the following equation: % dextran crossing = $100\% \times (\text{fluorescence of sample wells} - \text{fluorescence of medium alone/equilibrium fluorescence})$, where equilibrium fluorescence was defined as radioactivity in a 50- μl aliquot after manually mixing the contents of a top and bottom well.

Neutrophil transendothelial migration assay

Polymorphonuclear neutrophils (PMN) were isolated from a healthy volunteer, and loaded with calcein-AM as previously reported (14). PMN at 1×10^6 in a 200- μl volume of RPMI 1640 without phenol red (BioWhittaker, Walkersville, MD) containing 0.1% HSA (Centeon, Kankakee, IL) were placed in the top well of the transwell. IL-8/CXCL8 at 1 nM or GRO- α /CXCL1 at 20 nM were placed in the bottom chamber in a total volume of 1.0 ml of RPMI 1640 containing 0.1% HSA. The transwell chambers were incubated for 3 h at 37°C in 5% CO₂. The transwell insert was removed from each well, and 1.0 ml of 2% Triton-X was added to the bottom chamber contents, mixed, and the fluorescence of each well was measured using emission and excitation wavelengths of 485 and 530 nm, respectively. To calculate the percentage of neutrophils migrating from the top to the bottom chamber, a maximal fluorescence for neutrophils was derived. At time 0, 200 μl of 1×10^6 PMN were added to 800 μl of medium. The mixture was added to 1.0 ml of 2% Triton-X, and calcein fluorescence (fl.) was measured. The percent of PMN migration was calculated using the following formula:

$$\% \text{ PMN migration} = \frac{\text{fl. of sample}}{\text{maximal fl.}} \times 100$$

mDuffy knockout and wild-type mice

Mice with targeted deletion of the Duffy gene have been previously described (10). Briefly, a 1.1-kb *neo* gene eliminating a segment of the intron and 90 bp of exon II was inserted into the genomic Duffy locus by homologous recombination. Embryonic stem cells containing the targeted mutation were injected into C57BL/6 blastocysts. Duffy^{-/-} mice and their wild-type littermates were bred and housed in specific pathogen-free conditions at the Seattle Veteran's Affairs vivarium until the day of the experiment. Equal numbers of male and female mice, age- and weight-matched, in the N2F₂ generation were included in each group. Homozygous Duffy knockout and wild-type mice were generated by breeding Duffy heterozygote breeding pairs. Mice were weaned at 3 wk of age, at which time they were genotyped using tail snip DNA.

Intratracheal instillation of IL-8

Mice were weighed and anesthetized using a ketamine/xylazine solution (70 mg/kg ketamine, 10 mg/kg xylazine). The mice were placed on a 45° incline board, a fiber optic light source was placed just above the thoracic inlet (IntraLux 6000-1; Volpi Manufacturing, Auburn, NY), and the vocal cords were directly visualized using a small catheter introducer

(BD Biosciences, Rutherford, NJ). The trachea was cannulated with a 22-gauge gavage feeding needle (Kent Scientific, Litchfield, CT) connected to a tuberculin syringe. The plunger was removed from the tuberculin syringe and 100 μ l of saline was placed at the distal end before its use. The correct position of the gavage needle in the trachea was verified by movement of the saline column in the tuberculin syringe. Recombinant human IL-8/CXCL8 (1×10^{-6} M) (Peprotech, Piscataway, NJ) was diluted in PBS containing 0.1% HSA or PBS + 0.1% HSA alone (Centeon) and a volume of 0.15 ml/100 g was instilled directly into the trachea through the gavage needle.

At 6 h, mice were euthanized with 120 mg/kg pentobarbital. Whole blood was obtained by direct cardiac puncture and the plasma was collected, aliquoted, and stored in -70°C until use. The thoracic cavity was opened by midline incision. The trachea was exposed and cannulated with a 24-gauge catheter, which was secured with a 2-0 silk suture. Whole lung lavage was performed using 0.9% NaCl containing 0.6 mM EDTA instilled in one aliquot of 1.2 ml, followed by three aliquots of 1.0 ml.

BAL processing

Total cell counts in the BAL fluid were performed using a Neubauer chamber. Cell differentials were performed by counting 100 consecutive cells on cytospin preparations stained with DiffQuik.

Results

Cloning of human Duffy cDNA

Sequence analysis confirmed that the Duffy cDNA isolated was the spliced isoform derived from two exons (GenBank AY167991). This isoform is the predominant transcript in vivo (15, 16) (Fig. 1). The Duffy cDNA encoded the Fy^b epitope (A125). It also contained a silent mutation (G15C), and a polymorphism (G298A: Ala100Thr) that has no observable effect on surface protein expression in vivo or ^{125}I -IL-8/CXCL8-specific binding in vitro (4, 17). The cloned Duffy cDNA also contained either a new polymorphism (A649G:Ile217Val) or a PCR-induced mutation. This Ile217Val substitution occurs within the transmembrane region of the protein and is a conservative mutation.

Duffy expressing endothelial cells bind selective CXC and CC chemokines

Binding analysis was undertaken to calculate the K_d of GRO- α /CXCL1 using 0.2 nM ^{125}I -GRO- α /CXCL1 and increasing concentrations of unlabeled GRO- α /CXCL1 (Fig. 2). Nonspecific binding was defined as the amount of bound ^{125}I -GRO- α /CXCL1 in the presence of 1 μM unlabeled GRO- α /CXCL1. The binding of ^{125}I -GRO- α /CXCL1 was saturable as the amount of total GRO- α /CXCL1 increased. Scatchard analysis indicated a K_d value of 5.6 nM, similar to what has been observed on erythrocytes and endothelial cells (3, 6, 7).

The ability of various chemokines to compete with ^{125}I -GRO- α /CXCL1 for binding to Duffy expressing cells was tested using 0.2 nM ^{125}I -GRO- α /CXCL1 incubated in the presence of increasing concentrations of unlabeled chemokines (Fig. 3). In addition, we

tested the ability of a goat anti-human Duffy polyclonal Ab (α hDuffy Ab) to block binding of ^{125}I -GRO- α /CXCL1 on the Duffy transfectants. The inhibitory concentration of unlabeled ligand required to displace 50% of radiolabeled GRO- α /CXCL1 (IC_{50}) was measured for GRO- α /CXCL1, MCP-1/CCL2, RAN- TES /CCL5, α hDuffy Ab, IL-8/CXCL8, and MIP1- α /CCL3 using Equilibrium Binding Data Analysis software (Biosoft, Cambridge, U.K.). The relative IC_{50} values observed were: GRO α < MCP-1 < α hDuffy Ab < RANTES < IL-8 <<< MIP-1 α . Thus, the binding profile of the endothelial Duffy transfectants (Duffy $^+$ cells) was similar to the findings observed in mouse erythrocytes, and K562 cells stably transfected with the Duffy cDNA (18, 19).

Interestingly, the endothelial Duffy transfectants showed a higher than anticipated IC_{50} of IL-8. IL-8/CXCL8 was a less effective antagonist of ^{125}I -GRO- α /CXCL1 in the endothelial Duffy transfectants. This may be due to differences in the membrane environment on transfected cells vs erythrocytes, as previously suggested for the slightly higher K_d values observed in K562 Duffy transfectants than native erythrocytes (18). Alternatively, the lower affinity of IL-8/CXCL8 for the Duffy transfectants may be the result of a previously unreported polymorphism (A649G:Ile217Val).

Endothelial gene expression following binding of ligand to the Duffy Ag

The Duffy Ag is a member of the family of 7-transmembrane domain receptors, but it lacks the DRY amino acid motif that allows coupling to G proteins (1). The Duffy Ag lacks the ability to transduce a signal given the lack of the DRY motif. Direct evidence for this is based upon the inability to show increases in intracellular calcium ion concentrations following Duffy Ag-ligand binding in transfected 293 cells (4) and the inability of Duffy Ag to stimulate GTPase activity (20). To determine whether the binding of GRO- α /CXCL1 to the Duffy Ag produces a signal that alters gene transcription in endothelial cells, the gene expression pattern of Duffy transfectants was examined using oligonucleotide microarrays (Table I). A 2-fold or higher change in gene expression was chosen as significant.

Of the 12,000 genes examined, there were no significant changes in the gene expression profile following stimulation of Duffy $^+$ cells with GRO- α /CXCL1 as compared with unstimulated Duffy $^+$ cells (Table I). In addition, neither CXCR1 nor CXCR2 mRNA expression were noted in Duffy $^+$ cells with or without stimulation. To show that the Duffy transfected cells could be stimulated to produce changes in their gene expression profile compared with baseline, transfected cells were also challenged with TNF- α . TNF- α served as the positive control stimulus after preliminary studies showed that incubation with TNF- α at 10 ng/ml increased the expression of ICAM-1 on the surface of Duffy transfectants (Duffy $^+$ cells) by flow cytometry (data not shown). The gene expression profile in TNF- α -stimulated Duffy $^+$ cells showed that ICAM-1 mRNA expression was 7.5-fold higher than in un-stimulated Duffy $^+$ cells. Moreover, TNF- α stimulation resulted in up-regulation of 42 genes including VCAM-1, endothelial-selectin (E-selectin), ICAM-1, MCP-1/CCL2, IL-8/CXCL8, GRO- β /CXCL2, GRO- γ /CXCL3, and down-regulation of ~142 genes, similar to published findings (Table I) (21). Thus, GRO- α /CXCL1 stimulation of Duffy transfected endothelial cells does not alter gene transcription. Moreover, genes up-regulated following

TNF- α stimulation of Duffy transfectants help to characterize and validate the transfected cells as possessing an endothelial phenotype.

^{125}I -GRO- α movement across an endothelial monolayer expressing Duffy Ag

A transwell system was used to test the hypothesis that Duffy Ag participates in chemokine movement across the endothelium (Fig. 4). GRO- α /CXCL1 and tracer amounts of ^{125}I -GRO- α /CXCL1 were placed in the bottom well below either a Duffy⁺ and Duffy⁻ endothelial monolayer, in the presence or absence of blocking Ab against the Duffy Ag. The concentration of Ab used was 30-fold greater than its IC₅₀ required to displace ^{125}I -GRO- α /CXCL1 bound on Duffy transfected cells. Chemokine movement from the bottom to top well was determined by measuring the amount of ^{125}I -GRO- α /CXCL1 recovered from the top well following a 4-h incubation at 37°C.

The data showed that inhibition of Duffy Ag with blocking Ab results in a significant reduction in the translocation of ^{125}I -GRO- α /CXCL1 across a Duffy⁺ endothelial monolayer (Fig. 4). To demonstrate that the Duffy-specific Ab did not alter the integrity of Duffy⁺ endothelial monolayers, we also measured the movement of 10,000 m.w. dextran (an inert molecule the size of GRO- α /CXCL1) across each well tested and showed that the translocation of dextran was not significantly different across Duffy⁺ endothelial monolayers in the presence of either anti-Duffy or control Ab. As expected, anti-Duffy Ab had no effect on either GRO- α /CXCL1 and dextran translocation across Duffy⁻ endothelial monolayers. Notably, translocation of GRO- α /CXCL1 across Duffy⁺ monolayers cannot be directly compared with Duffy⁻ monolayers given the relative “leakiness” of Duffy⁻ monolayers, as evidenced by the greater translocation of dextran across Duffy⁻ monolayers. Nevertheless, the blocking Ab studies support the conclusion that Duffy Ag facilitates the movement of GRO- α /CXCL1 across an endothelial monolayer.

In vitro neutrophil migration assay

To test whether the presence of Duffy on endothelial cells modifies neutrophil migration toward either GRO- α /CXCL1 or IL-8/CXCL8, Duffy expressing cells (Duffy⁺) and untransfected cells (Duffy⁻) were seeded onto transwells as previously described (Figs. 5 and 6) (22). We determined that 1 nM IL-8/CXCL8 and 20 nM of GRO- α /CXCL1 produced equivalent chemotactic activity for neutrophils and, thus, these concentrations were selected for study (23). In four separate experiments performed either in triplicate or quadruplicate, we showed that GRO- α /CXCL1-mediated neutrophil migration is enhanced across a Duffy⁺ monolayer following a 2-h incubation (*, $p < 0.05$; Fig. 5). There were no significant differences in random migration or dextran crossing between Duffy⁺ and Duffy⁻ monolayers. Although, IL-8-mediated neutrophil migration appeared greater across Duffy⁺ monolayers, it was not statistically significant at 2 h. However, following a 3-h incubation, the differences in IL-8-mediated neutrophil migration across Duffy⁺ monolayer compared with Duffy⁻ monolayers reached statistical significance (*, $p < 0.05$; Fig. 6). Although Duffy Ag is not essential for neutrophil migration, the in vitro data showed that Duffy Ag can facilitate both GRO- α /CXCL1 and IL-8/CXCL8-mediated neutrophil transendothelial migration.

Neutrophil recruitment into the airspaces of mice lacking the Duffy Ag

We tested whether Duffy Ag modifies neutrophil migration *in vivo* by examining neutrophil recruitment into the pulmonary airspaces following intratracheal instillation of human IL-8/CXCL8 in mice lacking the Duffy Ag (Fig. 7). Direct instillation of chemokine into the distal airways provides a direct test of whether Duffy Ag has an effect on chemokine-mediated inflammatory cell recruitment into local tissue beds. IL-8/CXCL8 was chosen as the stimulus because others have shown that murine Duffy Ag also binds angiogenic CXC chemokines, including human IL-8/CXCL8 (19). Initial dose response studies using 0.01, 0.1, and 1 μM IL-8/CXCL8 yielded peak neutrophil recruitment into the airspaces in mice with 1 μM IL-8/CXCL8 (data not shown); thus, 1 μM IL-8/CXCL8 was used for the *in vivo* studies. Compared with wild-type littermates, there was significantly less neutrophil recruitment into the airspaces following intratracheal instillation of IL-8/CXCL8 in Duffy^{-/-} mice, suggesting that Duffy Ag contributes to chemokine-mediated neutrophil migration (Fig. 7). We also examined plasma levels of IL-8/CXCL8 to determine whether there were measurable differences in plasma IL-8/CXCL8 levels in Duffy^{-/-} vs Duffy^{+/+} litter-mates. Plasma IL-8/CXCL8 levels were undetectable in all mice studied using a standard ELISA with a detection limit of 31 pg/ml. Lung homogenates showed no significant differences in IL-8/CXCL8 concentrations between Duffy^{-/-} and Duffy^{+/+} animals, indicating that the amounts instilled were similar in both groups of animals. To address the possibility that the deletion of the Duffy Ag in the knockout mice resulted in reduced peripheral neutrophil counts or functional defects in neutrophil chemotaxis, we measured total peripheral neutrophil counts and *in vitro* neutrophil chemotaxis in Duffy^{-/-} animals and found no differences as compared with Duffy^{+/+} animals.

Discussion

We have previously shown that Duffy Ag expression is enhanced in human lungs with a histologic diagnosis of suppurative pneumonia, a process defined by neutrophilic infiltration of the airspaces (11). The enhanced Duffy Ag expression occurs along microvessels and alveolar septa, particularly in regions of neutrophil accumulation (11). This is in contrast to normal lungs and lungs with acute lung injury, which showed only low level Duffy Ag expression. These observations suggest that expression of Duffy Ag is regulated in the lung microvasculature by the inflammatory process, and that Duffy Ag may have a functional role in the lung parenchyma during inflammation. Because of the up-regulation of Duffy Ag at tissue sites of inflammation, its colocalization to areas of inflammatory cell accumulation, and its multiple chemokine binding properties (9, 11), the main focus of this study was to test whether Duffy Ag plays a role in modifying chemokine-mediated neutrophil recruitment *in vitro* and *in vivo*. We showed enhanced neutrophil transmigration across a Duffy⁺ monolayer toward two different chemokines, GRO- α /CXCL1 and IL-8/CXCL8. In addition, we found that Duffy knockout mice have markedly reduced neutrophil counts in the bronchoalveolar lavage following intratracheal instillation of IL-8/CXCL8 as compared with wild-type littermates. These findings support the interpretation that Duffy Ag is important in facilitating chemokine-mediated neutrophil migration. The *in vitro* and *in vivo* approaches are complementary and support the conclusion that Duffy Ag has an important biological function in tissue sites of inflammation.

To gain a better understanding of how Duffy Ag contributes to neutrophil migration, we examined whether GRO- α /CXCL1-Duffy Ag interactions change the endothelial gene expression profile. We showed that stimulation of Duffy transfectants with GRO- α /CXCL1 had no observable effect in altering endothelial gene transcription. This is the first direct evidence that GRO- α /CXCL1 binding to the Duffy Ag does not produce intracellular signaling events resulting in changes in gene transcription. Despite the ability of the Duffy endothelial transfectants to express molecules that might affect neutrophil transmigration when stimulated with TNF- α (e.g., VCAM-1, E-selectin, ICAM-1), this is not a mechanism that would explain the differences we observed in the neutrophil transmigration experiments with GRO- α /CXCL1, because GRO- α /CXCL1 binding in these cells does not up-regulate the expression of adhesion molecules or other genes that might affect neutrophil migration.

It has been previously postulated that the Duffy Ag can participate in chemokine transport across endothelial cells (7, 24). We determined whether Duffy Ag contributes to chemokine movement across the endothelium in vitro, and showed that the presence of Duffy Ag facilitates movement of ^{125}I -GRO- α /CXCL1 across a Duffy⁺ monolayer. Thus, our in vitro data are in keeping with the hypothesis that Duffy Ag on endothelial cells can function as a shuttling molecule for chemokines.

Others also have shown lines of evidence to suggest that the function of the Duffy Ag is different in endothelial cells than in erythrocytes (7, 24, 25). In situ binding studies performed in rabbit skin show that ^{125}I -IL-8 originating from the abluminal side is internalized by venular endothelial cells, localizes to caveolae, and is subsequently presented on the luminal surface (24). The molecule participating in the transport and presentation of IL-8 in endothelial cells is unknown. However, it binds IL-8 and RANTES but not MIP-1 α (24), similar to the binding profile of the Duffy Ag (24, 26). In addition, K562 erythroleukemic cells stably transfected with Duffy cDNA rapidly internalize radiolabeled GRO- α /CXCL1, but this GRO- α /CXCR1 is not degraded following internalization (7). Furthermore, immunoelectron microscopy and immunohistochemistry studies have localized Duffy Ag to both apical and basal membrane domains of endothelial cells as well as within caveolae (25). These independent findings support the concept that the potential biological role of Duffy includes internalization and transport of chemokines across endothelial cells (7).

Animal studies have produced discrepant results about the function of Duffy Ag in vivo (10, 27); however, differences in dose and time intervals make direct comparisons of the studies difficult. Luo et al. (27) found that Duffy^{-/-} mice had reduced neutrophil accumulation in the lungs and intestines 24 h following i.p. injection of 10 mg/kg LPS or 3% thioglycolate. These findings suggest that Duffy Ag may facilitate neutrophil accumulation in tissue following specific inflammatory stimuli (27). Our in vitro and in vivo data are in keeping with these findings and provide direct support for the role of the Duffy Ag in chemokine-mediated neutrophil recruitment into the lungs.

In contrast, Dawson et al. (10) found increased neutrophil accumulation in the lungs and livers of Duffy^{-/-} mice 2 h following i.p. injection of 30 mg/kg LPS, and they concluded that Duffy Ag functions as a chemokine sink. However, with an overwhelming dose of LPS

in circulation at the early time, the exaggerated systemic inflammatory response observed in the Duffy^{-/-} mice could be explained by the absence of Duffy Ag on erythrocytes that normally bind excess chemokines in circulation. This interpretation is supported by the findings of Olszyna et al. (28), who showed that in humans with experimental endotoxemia, erythrocyte-associated IL-8 peaked 2 h following LPS injection, then fell as neutrophil-associated IL-8/CXCL8 began to rise at 4 and 6 h. The findings of Dawson et al. (10) and those of Luo et al. (27) highlight the complex roles of the Duffy Ag in two different locations: one expressed on erythrocytes in the circulation and the other expressed predominantly on endothelial cells in tissue.

Endothelial Duffy Ag is genetically conserved, even in individuals who do not express Duffy on their RBCs (6, 7). It is expressed on endothelial cells of the postcapillary venules where leukocytes emigrate (6). Duffy Ag is also up-regulated during inflammation and binds CXC and CC chemokines (4, 6, 8, 9). The data presented herein are the first direct evidence that Duffy Ag can promote neutrophil recruitment into tissue such as the lungs, when IL-8/CXCL8 is the stimulus. The in vitro data suggest a potential mechanism whereby Duffy Ag on endothelial cells contributes to transendothelial movement of chemokine. We conclude that endothelial Duffy Ag is a biologically relevant molecule that participates in regulating inflammatory cell recruitment to tissue sites of inflammation.

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                    1
5'-TGAACCAAACGGTGGC ATG GGG AAC TGT CTC CAC AGG GCG GAG CTC 30
                    M G N C L H R A E L 10
TCC CCC TCA ACT GAG AAC TCA AGT CAG CTG GAC TTC GAA GAT GTA 75
S P S T E N S S Q L D F E D V 25
TGG AAT TCT TCC TAT GGT GTG AAT GAT TCC TTC CCA GAT GGA GAC 120
W N S S Y G V N D S F P D G D 40
#
TAT GAT GCC AAC CTG GAA GCA GCT GCC CCC TGC CAC TCC TGT AAC 165
Y D A N L E A A A P C H S C N 55
CTG CTG GAT GAC TCT GCA CTG CCC TTC TTC ATC CTC ACC AGT GTC 210
L L D D S A L P F F I L T S V 70
CTG GGT ATC CTA GCT AGC AGC ACT GTC CTC TTC ATG CTT TTC AGA 255
L G I L A S S T V L F M L F R 85
*
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P L F R W Q L C P G W P V L T 100
CAG CTG GCT GTG GGC AGT GCC CTC TTC AGC ATT GTG GTG CCC GTC 345
Q L A V G S A L F S I V V P V 115
TTG GCC CCA GGG CTA GGT AGC ACT CGC AGC TCT GCC CTG TGT AGC 390
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L G Y C V W Y G S A F A Q A L 145
CTG CTA GGG TGC CAT GCC TCC CTG GGC CAC AGA CTG GGT GCA GGC 480
L L G C H A S L G H R L G A G 160
CAG GTC CCA GGC CTC ACC CTG GGC CTC ACT GTG GGA ATT TGG GGA 525
Q V P G L T L G L T V G I W G 175
GTG GCT GCC CTA CTG ACA CTG CCT GTC ACC CTG GCC AGT GGT GCT 570
V A A L L T L P V T L A S G A 190
TCT GGT GGA CTC TGC ACC CTG ATA TAC AGC ACG GAG CTG AAG GCT 615
S G G L C T L I Y S T E L K A 205
*
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L Q A T H T V A C L A V F V L 220
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L P L G L F G A K G L K K A L 235
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G M G P G P W M N I L W A W F 250
ATT TTC TGG TGG CCT CAT GGG GTG GTT CTA GGA CTG GAT TTC CTG 795
I F W W P H G V V L G L D F L 265
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V R S K L L L L S T C L A Q Q 280
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A L D L L L N L A E A L A I L 295
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H C V A T P L L L A L F C H Q 310
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A T R T L L P S L P L P E G W 325
TCT TCT CAT CTG GAC ACC CTT GGA AGC AAA TCC TAG TTCTC 1008
S S H L D T L G S K S - 336

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FIGURE 1.

Human Duffy Ag sequence cloned by PCR (GenBank AY167991). Base sequence numbers reference the start codon. GenBank AF055992 sequence was used to design primers (primer targets are underlined). Predicted transmembrane amino acid sequences are highlighted.

Three sequence variances from GenBank AF055992, which encodes the Fy^x mutation, are indicated with an asterisk. The Duffy Ag cDNA possesses the following: 1) G15C, a silent mutation; 2) A125 which codes the Fy^b epitope; 3) C265 that renders full surface expression of Fy^b, in contrast to C265T: Arg89Cys which codes the Fy^x mutation; 4) G298A:Ala100Thr, a previously reported polymorphism that does not alter surface

expression of Fy^b; 5) A649G:Ile217Val, a chemically conserved new polymorphism or PCR mutation.

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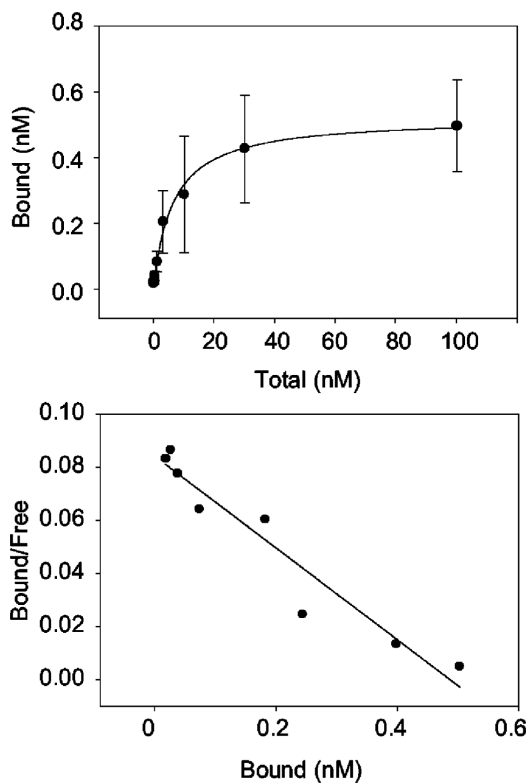


FIGURE 2. Saturation curve and Scatchard plot of GRO- α /CXCL1 binding. Duffy transfected endothelial cells show saturable binding of ^{125}I GRO- α /CXCL1 (*top panel*) and a calculated K_d for GRO- α /CXCL1 of 5.6 nM (*bottom panel*). Duffy transfectants were incubated with 0.2 nM ^{125}I GRO- α /CXCL1 and increasing concentrations of unlabeled GRO- α /CXCL1. Each condition was performed in triplicate or quadruplicate; $n = 2$ independent experiments.

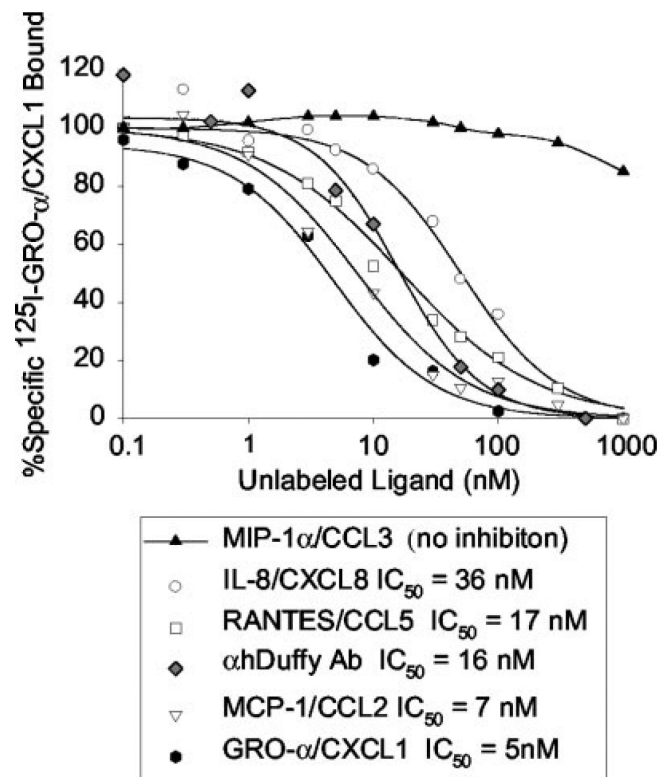


FIGURE 3.

Competition binding studies using ^{125}I -GRO- α /CXCL1 and various unlabeled ligands. Data are presented as the percentage of total bound ^{125}I -GRO- α /CXCL1 as a function of increasing concentrations of unlabeled ligand. A curve fitting program was used to determine the IC_{50} total binding of ^{125}I -GRO- α /CXCL1 to unlabeled GRO- α /CXCL1, IL-8/CXCL8, MCP-1/CCL2, α hDuffy Ab, RANTES/CCL5, MIP-1 α /CCL3, and are shown in the symbol legend. Each condition was performed in triplicate or quadruplicate.

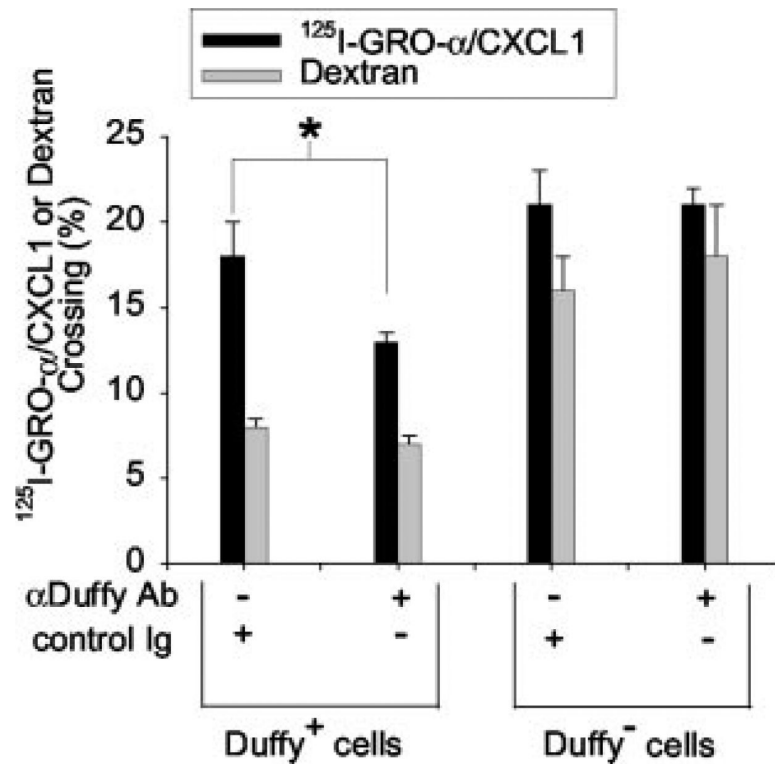


FIGURE 4.

Recovery of ¹²⁵I-GRO-α across Duffy⁺ and Duffy⁻ endothelial cell monolayer in the presence of either anti-Duffy or control Ab. Duffy⁺ and Duffy⁻ endothelial cell monolayers were preincubated in the presence of anti-Duffy Ab or control IgG. Twenty nanomolar concentrations of unlabeled GRO-α/CXCL1, 0.2 nM ¹²⁵I-GRO-α/CXCL1, and 2.5 μM dextran (10,000 m.w. Texas Red dextran) were placed in the bottom wells with 0.5 μM Ab. The amount of ¹²⁵I-GRO-α/CXCL1 and dextran recovered from the top well were measured following a 4-h incubation at 37°C. Recovery of ¹²⁵I-GRO-α/CXCL1 is significantly reduced across a Duffy expressing endothelial monolayer in the presence of anti-Duffy Ab (*, $p = 0.002$ by Mann-Whitney U test, $p = 0.007$ by ANOVA). Data are mean ± SEM of three independent experiments performed in triplicate or quadruplicate. Wells with [H11022]10% dextran crossing were excluded from statistical analysis.

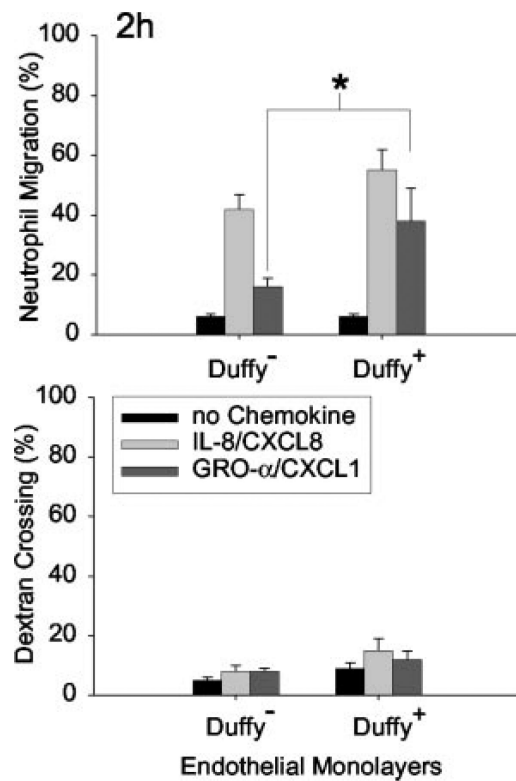


FIGURE 5.

Effect of endothelial Duffy Ag expression on GRO- α /CXCL1-mediated neutrophil migration in vitro. GRO- α /CXCL1-mediated neutrophil migration is enhanced across Duffy⁺ monolayers in vitro following a 2-h incubation (*, $p < 0.05$ by Mann-Whitney U test). IL-8-mediated neutrophil migration is slightly greater across Duffy⁺ monolayers, but this is not statistically significant. No significant differences in the percent dextran crossing were noted between Duffy⁺ and Duffy⁻ mono-layers to account for differences in neutrophil migration. Data are mean \pm SEM of four independent experiments performed in either triplicate or quadruplicate.

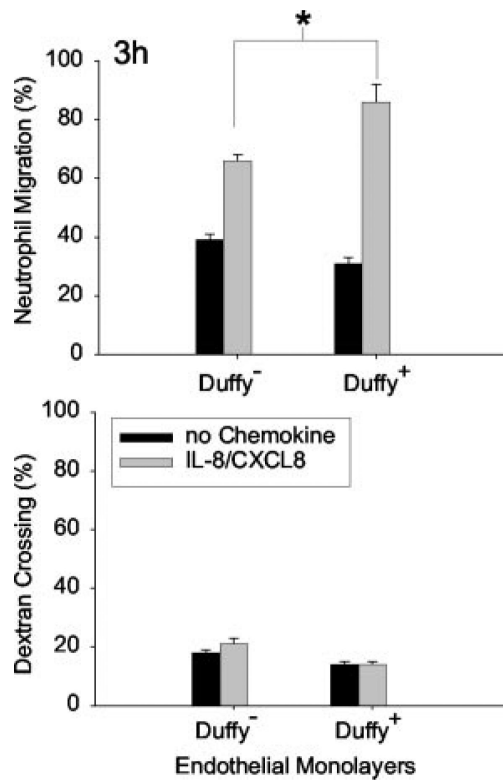


FIGURE 6.

Effect of endothelial Duffy Ag expression on IL-8/CXCL8-mediated neutrophil migration in vitro. IL-8/CXCL8-mediated neutrophil migration is enhanced across Duffy⁺ monolayers in vitro following a 3-h incubation (*, $p < 0.05$ by Mann-Whitney U test). No significant differences in the percent dextran crossing were noted between Duffy⁺ and Duffy⁻ monolayers to account for differences in neutrophil migration. Data are mean \pm SEM of six independent experiments performed in either trip-licate or quadruplicate.

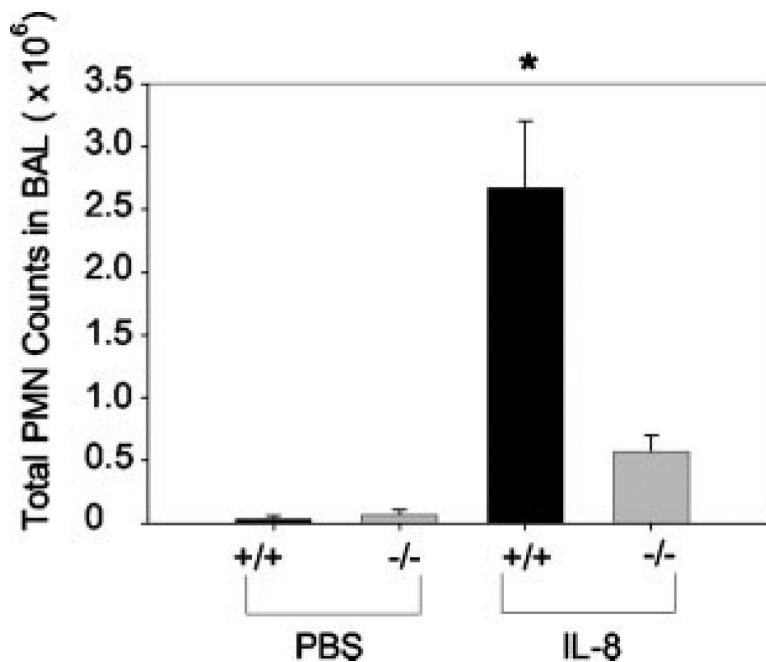


FIGURE 7.

Total neutrophil counts in the BAL of Duffy^{+/+} and Duffy^{-/-} mice 6 h following intratracheal instillation of 1 μ M IL-8/CXCL8. There was minimal neutrophil recruitment into the pulmonary airspaces following instillation of PBS. Alveolar neutrophil recruitment to IL-8/CXCL8 was impaired in Duffy^{-/-} mice. Data are mean \pm SEM combined from two independent experiments performed with $n = 8$ or 9 in each of the PBS groups and $n = 16$ in each of the IL-8/CXCL8 groups. (*, $p < 0.0001$ by Mann-Whitney U test).

Table I

Oligonucleotide microarray analysis^a**Endothelial gene expression profile of Duffy transfectants**

Following GRO- α /CXCL1 stimulation; no significant changes

Following TNF- α stimulation

Up-regulated genes: first 10 of 42 up-regulated genes

1. VCAM-1
2. Human IFN- γ treatment inducible mRNA
3. Human JE gene encoding a monocyte secretory protein (MCP-1)
4. Diubiquitin
5. Human endothelial leukocyte adhesion molecule-1 (E-selectin)
6. ICAM-1
7. Human TNF-inducible early response gene
8. *Homo sapiens* mRNA for KIAA106 protein
9. Human adhesion molecule ninjurin mRNA
10. Human B94 protein mRNA

Down-regulated genes: first 10 of 142 down-regulated genes

1. Human signal transducing adaptor molecule 2A (STAM2) mRNA
2. Human SNF-1-like protein kinase mRNA^b
3. Human homolog of yeast mutL (hPMS1) gene
4. *H. sapiens* mRNA for vascular Rab-GAP/TBC-containing protein^b
5. *H. sapiens* RP58 gene
6. AI961743:wt66f12.x1 *H. sapiens* cDNA
7. Human mRNA for brain-derived neurotrophic factor
8. Human TRF-1-interacting ankyrin-related ADP-ribose polymerase mRNA^b
9. Human myosin H chain 12 (MYO5A) mRNA
10. Human *msg1*-related gene mRNA

^aThe gene expression profile was examined in endothelial Duffy transfectants stimulated with 20 nM GRO- α /CXCL1 or 10 ng/mL TNF- α for 4 h using oligonucleotide microarrays. No genes were found to be significantly up-regulated or down-regulated in the Duffy⁺ cells following GRO- α stimulation. In Duffy⁺ cells stimulated with TNF- α , 42 genes were up-regulated and 142 genes were down-regulated compared with unstimulated cells (the first 10 in each group are shown for simplicity). The results were similar in two independent experiments.

^bSNF, sucrose nonfermenting protein; GAP/TBC, GTPase-activating protein/Tre/Bub2/Cdc16 domain; TRF, telomeric repeat binding factor; msg, missing.