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Hemopoietic Cell Expression of the Chemokine Decoy Receptor D6 Is Dynamic and Regulated by GATA1

Clive S. McKimmie,* Alasdair R. Fraser,* Chris Hansell,* Laura Gutiérrez,† Sjaak Philipsen,‡ Laura Connell,* Antal Rot,‡ Mariola Kurowska-Stolarska,* Paz Carreno,* Monika Pruenster,‡ Chung-Ching Chu,§ Giovanna Lombardi,§ Christina Halsey,* Iain B. McInnes,* Foo Y. Liew,* Robert J. Nibbs,* and Gerard J. Graham‡*

D6 scavenges inflammatory chemokines and is essential for the regulation of inflammatory and immune responses. Mechanisms explaining the cellular basis for D6 function have been based on D6 expression by lymphatic endothelial cells. In this study, we demonstrate that functional D6 is also expressed by murine and human hemopoietic cells and that this expression can be regulated by pro- and anti-inflammatory agents. D6 expression was highest in B cells and dendritic cells (DCs). In myeloid cells, LPS down-regulated expression, while TGF-β up-regulated expression. Activation of T cells with anti-CD3 and soluble CD28 up-regulated mRNA expression 20-fold, while maturation of human macrophage and megakaryocyte precursors also up-regulated D6 expression. Competition assays demonstrated that chemokine uptake was D6 dependent in human leukocytes, whereas mouse D6-null cells failed to uptake and clear inflammatory chemokines. Furthermore, we present evidence indicating that D6 expression is GATA1 dependent, thus explaining D6 expression in myeloid progenitor cells, mast cells, megakaryocytes, and DCs. We propose a model for D6 function in which leukocytes, within inflamed sites, activate D6 expression and thus trigger resolution of inflammatory responses. Our data on D6 expression by circulating DCs and B cells also suggest alternative roles for D6, perhaps in the coordination of innate and adaptive immune responses. These data therefore alter our models of in vivo D6 function and suggest possible discrete, and novel, roles for D6 on lymphatic endothelial cells and leukocytes. The Journal of Immunology, 2008, 181: 3353–3363.

The inflammatory response is characterized by the accumulation of a variety of innate immune cells which serve to remove pathogens, repair damaged tissues, and scavenge dying and apoptotic cells. The migration of leukocytes into an inflamed site is orchestrated by peptides belonging to the chemokine family of leukocyte chemoattractants which are characterized by the possession of a characteristic cysteine motif which defines the family (1, 2). The family is subdivided into CC, CXC, XC, and CX3C subfamilies on the basis of the specific nature of this motif. Functionally, the chemokines can be classified as inflammatory or constitutive according to the contexts in which they function (2, 3). Specifically, members of the inflammatory subset of chemokines are involved in the initiation and maintenance of the inflammatory response. In regulating leukocyte migration into tissues, chemokines interact with receptors that belong to the seven-transmembrane-spanning family of G protein-coupled receptors (4). There are 11 receptors for CC chemokines, 7 for CXC chemokines, and single receptors for the XC and CX3C chemokines.

In addition to the classical signaling chemokine receptors, a small family of atypical receptors, characterized by promiscuous ligand binding and an apparent inability to signal, has been identified (5). We have been studying one member of this family, D6, which we have previously demonstrated to be a promiscuous receptor with specificity for inflammatory CC chemokines (6, 7). D6 binds at least 12 inflammatory CC chemokines but does not bind constitutive chemokines or those belonging to any of the other three subfamilies (6–8). In vitro analyses indicate that although D6 does not appear to signal, D6 is capable of internalizing its ligands in a β-arrestin-dependent manner and of targeting them for intracellular degradation (9–11). This has led us, and others, to propose a role for D6 as a decay or scavenging receptor for inflammatory CC chemokines (5, 12, 13). Analysis of the inflammatory response in D6-null mice has further indicated that D6 plays an indispensable role in the resolution phase of the inflammatory response in a range of tissue and pathological contexts (14–18). Thus chemokine scavenging by D6 within an inflamed environment appears to be essential for the effective resolution of the inflammatory response.

A key question therefore relates to which D6-expressing cells are the primary vehicles for the inflammation-resolving properties of D6. We have previously reported D6 expression by human lymphatic endothelial cells (LECs) (19) and have postulated that these cells are major vehicles for the inflammation-resolving functions of D6. However, aspects of lymphatic vascular physiology suggest that LECs may not be ideally suited to this purpose (20). For example, lymphatic vessels are separated by relatively large...
distances (100–500 μm), thus chemokines, within intervening tissue spaces, are unlikely to come in contact with, and be degraded by, D6 on LECs. In addition, recent data on the reduced response of D6-null mice in experimental autoimmune encephalomyelitis models (21) suggests additional roles for D6 in the regulation of the immune response that may not be dependent on LEC-expressed D6. This led us to examine other possible cells for D6 expression in the hope of identifying alternative vehicles for in vivo D6 functions. In this study, we show that hemopoietic cells express functional D6 in a dynamic manner. We propose that D6 on motile cells such as leukocytes may be able to migrate to the epicenter of an inflammatory response and provide a more realistic model to explain D6 scavenging of chemokines. In addition, the robust expression of D6 by B cells and dendritic cells (DCs) suggests additional roles for D6 in the coordination of the immune response.

Materials and Methods

Primary cell isolation and analysis

All animal studies were reviewed and approved by local ethical review committees and are licensed by the U.K. Government Home Office. Mouse CD4+ T cells, B cells, and neutrophils were isolated using MACS beads (Miltenyi Biotec) and CD4+ T cells were polarized as previously described (22, 23). Cell purities were verified by staining for CD4 (T cells at >95%), CD19 (B cells at >95%), and hematoxylin of eosinophils (neutrophils at >98%). Mouse bone marrow-derived lineage-negative cells were isolated using bead-depletion strategies. Mouse dendritic cells were derived from GM-CSF or Flt3 ligand-treated bone marrow cultures (24) and were 80–90% pure. CD14 (monocyte, respectively; all from BD Biosciences). Cells were incubated with lineage-specific markers, labeled with goat anti-mouse IgG (Fc-specific) FITC (Sigma-Aldrich). The nochemistry study but has been extensively validated (26) and shown to be able to migrate to the epicenter of an inflammatory response and provide a more realistic model to explain D6 scavenging of chemokines.

Transfection of embryonic stem cells with GATA constructs

Murine embryonic stem cells were maintained as previously described (28) and were transfected with either empty vector or a full-length GATA1 cDNA (in pcDNA3) using the FuGene 6 transfection reagent. Transfected cells were grown in the presence of G418 to select for stable transfectants from which individual clones were isolated and propagated, again as previously described (29).

Chemokine uptake and scavenging assays

Uptake assays were performed on human cell lines and primary cells isolated from PB in complete RPMI 1640 supplemented with 20 mM HEPES to buffer chemokine binding. Similar assays were also performed on splenocytes from wild-type (WT) and D6-null mice. Cells were cultured for 30 min to re-establish cell metabolism at 37°C. Cells were exposed to different concentrations (μg/ml) of chemokine (CCL2-Alexa 488 [Almac Sciences] or a similar concentration of labeled CCL2 plus a 20-fold excess of unlabeled CCL3 (which has previously documented D6-binding capability (6, 7, 30)). Cells were incubated with chemokine for 80 min, washed in PBS, fixed in 2% paraformaldehyde, and analyzed for fluorescence in the FL4 channel of a FACSCalibur flow cytometer.

Alternatively, to assay the level of chemokine scavenging of cells from mixed populations (10^6 splenocytes/ml) were incubated with 1 μM biotinylated CCL3 for 6 h. Supernatant was sampled at 0 and 6 h, cells were removed by centrifugation, and the remaining supernatant was mixed with 2× sample buffer (Sigma-Aldrich). Intact biotin-CCL3 content was assayed by Western blotting (NuPAGE and iBlot; Invitrogen) using streptavidin-HRP and an anti-human CCL3 antibody (Abcam). Samples were run on a MiniPROTEAN Tetra gel in 1× TBE buffer (Invitrogen) and bands were detected using the ECL system (Amersham Biosciences) with an exposure of 1 min. Bands were quantified using the Quantity One software (Bio-Rad). The band density was normalized to the corresponding 18S density.

RT-PCR

RNA was extracted and genomic DNA was removed using RNase-free DNase (Qiagen) followed by RNA concentration was determined using a NanoDrop spectrophotometer. cDNA was synthesized using SuperScript II (Invitrogen) and D6 levels were normalized to GAPDH (one of 10 genes used). Primer sequences were designed using Primer Express software (BioRad). Primer sequences are shown in Table 1.

Immunohistochemistry

Tissues were collected from patients with rheumatoid arthritis/psoriasis with informed ethical consent from North Glasgow National Health Service University Trust. Paraffin-embedded sections were dewaxed and hydrated through decreasing concentrations of ethanol and washed in tap water before treatment in 3% H2O2, for 10 min. Slides were boiled in 1 mM EDTA for 10 min, then blocked at room temperature with horse serum followed by separate blocks with avidin, then biotin. Slides were then probed with anti-human D6 or matched isotype control in 1.5% horse serum. Following washes with PBS/0.025% Tween 20, slides were stained with biotinylated anti-mouse IgG, washed again, and treated with Elite ABC reagent (Vectashield) and Novo red to visualize Ab staining. Tissue sections were imaged to record D6 staining then emerged in xylene to remove coverslips and stained with toluidine blue to reveal mast cell staining. Imaging was conducted using a Axiosstar Plus microscope and Axiovision software.

In silico analysis of the D6 promoter

The intron/exon structure of mouse, rat, humans, and chicken D6 was determined by multiple alignments of the expressed sequence tag database to determine the transcriptional start site, which was further confirmed, in human transcripts, by RACE (data not shown). The 3000-bp region upstream of the most 5′ exon was analyzed online by MatInspector (www.genomatix.de). All transcription factor binding sites were sorted and ranked so that only those scoring above 0.9 for core similarity and 0.8 for matrix similarity were considered.

Cell culture

Human Jurkat, THP-1, K562, Meg01, and Molt-4 cells were grown in complete RPMI 1640. Human HMC-1 cells were grown in IMDM supplemented with 10% FCS, 25 mM HEPES, and glutamine. All primary cultures were maintained in culture in complete RPMI 1640 (supplemented with penicillin/streptomycin, glutamine, and 10% FCS) as necessary.

Human peripheral blood (PB) monocytes were isolated using CD14 beads (Miltenyi Biotec) from freshly drawn venous blood. Monocytes were either used immediately or cultured for 6 days with complete RPMI 1640 to generate mature macrophages (25) or with GM-CSF plus IL-4 to generate DCs. Natural circulating dendritic cell subsets were isolated from human PB using specific kits as per manufacturer’s instructions (untouched plasmacytoid DC (pDC) and standard monocytoid DC (mDC) isolation kits; Miltenyi Biotec). Surface expression of D6 on human leukocytes was quantified using lineage-specific immunostaining. The Ab used in the present study is from an alternative clone to that used in our initial immunochemistry study but has been extensively validated (26) and shown to be specific for D6 on heterologous transfecteds (data not shown). Briefly, PBMCs were initially probed with murine monoclonal anti-human D6 (IgG2a subclass) (19) or matched isotype control Ab. The cells were then washed and labeled with anti-IgG (Fc-specific) FITC (Sigma-Aldrich). The cells were then washed and counterstained with lineage-specific markers, including fluorescence-labeled CD3, CD19 and CD14 (T cell, B cell, and monocyte, respectively; all from BD Biosciences). Cells were incubated with dead cell exclusion reagent (Via-Probe; BD Biosciences), then live cells analyzed using a FACS Calibur flow cytometer with CellQuest software. Circulating natural dendritic cell subsets were assessed for D6 as before, but DC subpopulations were visualized using CD14/CD19 exclusion, followed by gating on CD1c or CD304 (mDC or pDC, respectively). Levels of lineage-specific D6 were expressed as corrected mean fluorescence intensity (corrected MFI; D6 MFI minus MFI of isotype).

DNA microarray analysis

D6 expression in 35-cycle PCR on a 2% agarose-ethidium bromide gel, which was analyzed using a two-tailed, unpaired Student’s t test.

Tissues were collected from patients with rheumatoid arthritis/psoriasis with informed ethical consent from North Glasgow National Health Service University Trust. Paraffin-embedded sections were dewaxed and hydrated through
Results

D6 is expressed by leukocytes in inflamed tissues

Our previous immunostaining for human D6 (19) revealed predominant expression of D6 on LECs in all tissues examined. However, these studies were conducted using a single clone of mAb and non-inflamed tissues typically from the uninvolved margins of tumor biopsies. More recently, when using alternative clones of previously verified mAbs (see Materials and Methods) to examine D6 staining patterns in classically inflamed tissues, we have observed D6 immunoreactivity on isolated leukocyte-like cells that are distinct from LECs and that were not seen in sections stained with either the original clone of Ab or with isotype control Abs (data not shown). Specifically, we have seen such staining patterns in biopsies from atopic dermatitis and rheumatoid arthritis patients (Figs. 1, A and B). Initial phenotypic characterization of these cells in the rheumatoid synovium indicated that they were, in the main, toluidine blue-positive mast cells (Fig. 1B).

These new staining patterns led us to carry out an in silico analysis of the putative D6 promoter in species for which the genomic sequences were available. Note this analysis has been conducted simply to provide tentative insights into transcriptional regulation of D6 and therefore does not represent a functional promoter analysis. For the purposes of this analysis, the putative promoter was defined as being included within a 3-kb sequence immediately 5’ of the transcriptional start site as defined by RACE and expressed sequence tag database searching. Notably, the promoters (as shown for the mouse and human promoters in Fig. 1C) were seen to contain binding sites for a number of hemopoietic transcription factors.
GATA1 (myeloid progenitor, erythroid cell, mast cell, eosinophil, dendritic cell, and megakaryocyte expression) for which there were multiple binding sites. In addition, numerous Smad3/4 transcription factor binding sites (suggestive of TGF-β responsiveness (34)) and NF-κB binding sites (suggestive of responsiveness to inflammatory mediators (35)) were identified, suggesting dynamic regulation of D6 expression. Thus, immunostaining of inflamed tissues and in silico analysis of the putative D6 promoter suggest expression of D6 by a variety of leukocytes and other hemopoietic cell types and potential regulation by inflammatory mediators.

Analysis of D6 expression in mouse hemopoietic tissues and leukocyte subtypes

To further examine the expression of D6 in hemopoietic cells, we first used QPCR to document expression levels in murine hemopoietic tissues compared with levels in tissues in which we had previously detected D6 expression. As shown in Fig. 2A, QPCR analysis indicated D6 expression levels in the spleen that were higher than those seen in the gut but similar to those detected in the skin and lower than those in the lung. Furthermore, moderate expression was also seen in inguinal lymph nodes (LN) with lower levels of expression being detected in the bone marrow.

Next, we examined D6 expression in specific murine cell lineages isolated using positive selection methodology. As shown, we detected expression, albeit at varied levels, in all lineages studied (Fig. 2B), with the highest levels being detected in B cells and DCs and moderate expression being detected in primitive (Sca1+) hemopoietic cells. Mast cells and neutrophils also showed moderate levels of D6 expression. In contrast, expression of D6 was low in LN-derived T cells and in vitro-derived macrophages. Notably, consistently higher expression was seen in splenic compared with LN B cells, a pattern that was also seen with T cells from these two sources. To place D6 transcript levels in context with other CC chemokine receptors that bind D6 ligands, we assayed the level of CCRs 1, 2, 3, and 5 in spleen-derived B cells, DCs, and macrophages (Fig. 2C). In B cells, copies of D6 were higher than any other chemokine receptor, whereas in DCs D6 transcript levels were similar to those of CCRs 1, 2, and 5 but considerably higher than CCR3. In keeping with its low level of expression in murine macrophages, D6 expression levels in these cells were markedly lower than those for the other receptors.

T cells are a pivotal component of all adaptive immune responses and are also important for the regulation of the ongoing immune response and for its ultimate resolution. They are also involved in generating the exaggerated inflammatory responses in D6-deficient mice (14). On the basis of their importance to immune responses and their documented ability to selectively express chemokine receptors depending on the cytokine environment (1), we examined the effects of
in vitro T cell polarization on D6 expression. Our results (Fig. 2D) showed marked increases (20-fold) in D6 expression following Th1 or Th2 polarization of murine CD4^+ T cells. Culture of CD4^+ cells with plate-bound anti-CD3 alone did not increase D6 levels particularly, although addition of soluble CD28 strongly induced D6 up-regulation. This provides strong evidence that activation and polarization of T cells up-regulates D6 expression.

Thus, together, these transcriptional data indicate expression of D6 by a range of murine hemopoietic tissues and cells at levels equivalent to other signaling chemokine receptors that bind D6 ligands. Currently, there are no useable Abs against murine D6; therefore, to further investigate D6 expression, at the protein level, we have examined expression patterns in human leukocytes.

Analysis of D6 expression in human leukocyte subtypes

In agreement with the murine data, a range of human hematopoietic cell lines also expressed D6 (Fig. 3A), with the highest levels being seen in K562 (erythroleukemic cell line), HMC1 (mast cell), Meg01 (megakaryocyte precursor), and THP-1 (monocytic) cells. Importantly, with the exception of THP-1 cells, all of these lineages express and are dependent on GATA1 for differentiation and survival. In further agreement with the murine data, the human T cell lines (Jurkat and Molt4 cells) and primary T cells only expressed low levels of D6 transcripts, whereas primary B cells expressed markedly higher levels of D6. Thus, together these QPCR data indicate that assorted hemopoietic tissues and lineages express D6 at a range of levels, although they do not approach the very high level of expression exhibited by the placental-derived choriocarcinoma BeWo cell line (15, 36).

Comparison of leukocyte D6 expression levels with those of LECs has been hampered by the tendency of these cells to switch off D6 expression following isolation and in vitro culture. Indeed, microarray-based comparisons of in vitro-cultured lymphatic and vascular endothelial cells does not detect LEC-specific expression of D6, despite there being LEC specificity for D6 immunostaining in a variety of tissues (37–39).

To determine whether the transcript levels are reflected in protein expression, we purified a number of human leukocyte subsets from PB and analyzed D6 expression by flow cytometry using an anti-human D6 mAb. As shown in Fig. 3B, we initially gated on monocytes, lymphoid cells, and DCs and then used specific markers to determine lineage-specific D6 expression patterns. In agreement with the murine and human QPCR data, monocytes express D6 protein but only at relatively low levels which are highly variable between different individuals. Examination of cells within the lymphocyte gate indicated that lymphocytes also express D6 protein and that this expression is restricted predominantly to CD20^+ B cells, with only low levels of expression being seen in CD3^+ T cells. Of particular note was that circulating PB DCs of both the monocyteid and plasmacytoid subtypes express relatively high levels of D6 (Fig. 3B) and, importantly, this expression, in monocyte-derived DCs is unaffected by classical stimulators of DC maturation (Fig. 3C). The flow cytometric data are summarized as a plot of MFI vs cell type at the bottom section of Fig. 3B. Thus, flow cytometric analysis confirms that D6 protein is expressed by the leukocyte subtypes that express D6 transcripts.
D6 expression is increased upon differentiation of human monocytes and megakaryocytes

Although murine macrophages display only low levels of D6 expression (Fig. 2B), detection of D6 expression in human monocytes prompted us to further examine expression in macrophages differentiated from these cells as they represent a major component of the intra-tissue inflammatory response. Intriguingly, although D6 transcript levels were low in primary human monocytes, they were increased (100-fold) following differentiation to macrophages, suggesting preferential expression in mature cells (Fig. 4A). However, not all forms of macrophage differentiation induce D6 up-regulation since TPA-matured THP-1 monocytic cells down-regulate expression as they gain an inflammatory macrophage phenotype (data not shown). This may suggest that other responses to the protein kinase C signaling effects of TPA may dominantly suppress D6 transcription in this cell line. In addition, as shown in Fig. 2B, in vitro-derived murine macrophages do not show high levels of D6 expression, suggesting some species specificity to the regulation of expression in macrophages.

To investigate the impact of differentiation on D6 expression in other D6-expressing lineages, we differentiated the megakaryocyte cell line Meg01 by treating it with TPA according to previously published protocols (see Materials and Methods). QPCR analysis (Fig. 4B) of the levels of the mature megakaryocyte marker COX-1 confirmed the induction of differentiation by TPA within 24 h, and analysis of D6 transcript levels (Fig. 4C) indicated considerable elevation of D6 expression in association with Meg01 cell differentiation which, importantly, was also reflected in increased protein levels as detected using flow cytometric analysis (Fig. 4D).

D6 expression can be regulated by the prototypic hemopoietic transcription factor GATA1

As shown in Fig. 1C, the putative D6 promoter has numerous GATA response sites. Given the importance of GATA1 for the differentiation of a number of the cell types identified here as being D6 positive (megakaryocytes, mast cells, myeloid progenitor cells (40, 41), and DCs (32)), we have now specifically investigated the effects of this transcriptional regulator of hemopoietic cell differentiation on the expression of D6. Our initial attempts at expressing GATA1 in hemopoietic progenitor cells were hampered by the tendency of GATA1 to induce terminal differentiation in transfected cells. Thus, to get around this, we initially stably transfected GATA1 into murine embryonic stem cells and examined expression of D6 in these transfectants compared with vector control transfectants. As shown in the PCR analysis

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Maturation of specific hemopoietic precursors is associated with up-regulated D6 expression. A, Monocytes derived from freshly drawn venous blood were cultured on plastic for 6 days to generate macrophage cultures, and D6 transcript levels were determined by QPCR and compared with those seen in freshly isolated monocytes. B–D, Meg01 megakaryocyte precursors were differentiated with TPA for 4 days and expression of megakaryocyte-specific COX-1 transcripts (B), D6 transcripts (C), and D6 protein (D) was determined. The FACS plot (D) shows isotype staining (black), D6 staining at day 0 and at day 4 of differentiation of Meg01 cells. * p < 0.001.

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Expression of D6 is regulated by GATA1. A, Full-length GATA1 constructs were transfected into mouse embryonic stem cells and stable, GATA1-overexpressing clones (six separate clones), obtained. RNA was extracted from these clones as well as from vector control embryonic stem cell clones (four separate clones), and D6 expression was determined by RT-PCR (cDNA was normalized to levels of rRNA before the reverse transcription reaction). Also shown is a densitometric evaluation of the levels of D6 in the individual bands. B, DCs bearing a floxed GATA1 allele and a Tx-inducible Cre recombinase were grown for 5 days with GM-CSF and treated with Tx or a control for 36 h, and D6 gene expression was determined by QPCR. C, Floxed GATA1 mice were treated with Tx to induce GATA1 knockdown in vivo, and harvested bone marrow precursors were treated with GM-CSF for 7 days to induce DC maturation. D6 gene expression was determined by QPCR. All bars represent the mean ± SD of three replicate QPCRs. KO, Knockout.
A putative function for D6 on some leukocytes may be the removal of inflammatory CC chemokines from inflamed sites. To function in such a context, we hypothesized that its expression is likely to be open to regulation by pro- and anti-inflammatory regulators. This suggestion is supported by the tentative identification within the putative D6 promoter of NF-$\kappa$B and SMAD response sites (Fig. 1C), which predict regulation by inducers and suppressors of inflammation. To test this hypothesis, we determined whether inflammatory leukocyte D6 expression could be modulated by agents involved in initiating or suppressing inflammatory responses (42). First, we looked at the effects of inducers of the inflammatory response on D6 expression. LPS

D6 expression is dynamically regulated in mature hemopoietic cells

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A putative function for D6 on some leukocytes may be the removal of inflammatory CC chemokines from inflamed sites. To function in such a context, we hypothesized that its expression is likely to be open to regulation by pro- and anti-inflammatory regulators. This suggestion is supported by the tentative identification within the putative D6 promoter of NF-$\kappa$B and SMAD response sites (Fig. 1C), which predict regulation by inducers and suppressors of inflammation. To test this hypothesis, we determined whether inflammatory leukocyte D6 expression could be modulated by agents involved in initiating or suppressing inflammatory responses (42). First, we looked at the effects of inducers of the inflammatory response on D6 expression. LPS
treatment of the human THP-1 monocytic cell line induced TNF expression, confirming TLR4 signaling in these cells (Fig. 6A). In addition, this induction of inflammatory mediator production was paralleled by a decline in D6 expression levels (Fig. 6B). A dose of 100 ng/ml LPS simultaneously induced a 3-fold increase in TNF expression and a 3- to 4-fold decrease in D6 expression levels. To confirm these observations in primary cells, we treated human monocyte-derived macrophages with LPS and also saw a rapid and significant down-regulation (3- to 4-fold) of D6 expression in these cells following LPS treatment (Fig. 6C). Thus, treatment of D6-expressing monocytes and macrophages with classical proinflammatory mediators reduced D6 expression levels.

In contrast to inducers of inflammation, TGF-β treatment of THP-1 cells consistently resulted in an up-regulation of D6 transcript levels with a doubling being seen following treatment of THP-1 cells with 200 pg/ml TGF-β (Fig. 6D). Flow cytometric analysis confirmed that this induction by TGF-β was reflected in increased D6 protein levels on the THP-1 cells (Fig. 6E). Interestingly, TGF-β was able to reverse the suppression of D6 transcription by LPS in THP-1 cells, suggesting that inflammatory suppressors can be dominant over proinflammatory agents in defining D6 expression levels in vitro (Fig. 6F). The effect of TGF-β on D6-expressing leukocytes was not solely restricted to myeloid cells since the mast cell lines HMC-1 (Fig. 6G) and LAD1 (data not shown) also up-regulated D6 in response to TGF-β, although K562 and Meg01 cells were nonresponsive to TGF-β. Importantly, not all suppressors of inflammation were able to increase D6 expression levels and D6 expression was unaffected by IL-10 or dexamethasone (data not shown).

D6 on leukocytes is functional

Given the ligand-binding profiles of D6 and the other receptors for its ligands, it is possible to identify the combination of CCL2 and CCL3 as ligands that only compete for binding on D6 and thus to identify the presence of functional D6 on cells. As shown in Fig. 7, A and B, flow cytometric analysis of THP-1 cells confirms the presence of functional D6 with substantial levels of CCL3-competable CCL2 accumulation being detected. In contrast, HMC-1 cells could bind and internalize substantially less CCL3-competable CCL2 (Fig. 7C), despite the higher expression of D6 mRNA in these cells compared with THP-1 cells. In addition, examination of specific populations of human leukocytes confirms the presence of functional D6 on both pDCs (Fig. 7D) and mDCs (Fig. 7E).
Interestingly, in contrast to the expression levels shown in Fig. 3B, markedly higher levels of D6 binding and accumulation were detected on the mDCs compared with the pDCs. These data therefore further confirm the presence of D6 on human leukocytes and demonstrate its ligand binding and internalization competence.

To formally demonstrate D6 function on mouse leukocytes, WT and D6-null cells were assayed for their ability to bind and internalize CCL2 (Fig. 8). Spleen-derived B cells express high levels of D6 (Fig. 2) and so were chosen to demonstrate D6-specific chemokine uptake. A small proportion of isolated WT CD19<sup>+</sup> B cells were able to bind and internalize CCL2 in a time-dependent manner (Fig. 8, A and B). Incubation of cells and chemokine at 4°C demonstrated that the level of CCL2 binding to the cell surface was minimal, suggesting that the staining of cells incubated at 37°C predominantly represented active uptake. Significantly, this uptake was CCL3 competitive and was completely absent in D6-null cells, proving that this was by a D6-specific mechanism.

D6-mediated uptake of inflammatory chemokine by leukocytes may have multiple functional consequences, one of which may be the removal of chemokines from sites of inflammation by sequestration and degradation. To formally determine whether D6-expressing cells can effectively remove inflammatory chemokines from their surroundings in a D6-dependent manner, cells were assayed for their ability to degrade biotinylated CCL3 from cell culture medium (Fig. 8C). To this end, splenocytes from either WT or D6-null mice were

**FIGURE 8.** B cell CCL2 uptake and splenocyte CCL3 scavenging is dependent on D6. Spleen-derived B-cells from WT and D6-null mice were allowed to bind and internalize Alexa Fluor 647-labeled CCL2 (at 25 ng/ml) in the absence or presence of a 20-fold molar excess of unlabeled CCL3 which competes with CCL2 for D6 binding. A, FACS plots of Alexa Fluor 647 fluorescence against CD19 staining. B, Percentage cells positive for both CD19 and CCL2. Cells were incubated with chemokine at 37°C for either 40 or 80 min or at 4°C for 80 min to show the level of CCL2 binding in the absence of active uptake. Only WT cells in the absence of CCL3 were positive for CCL2. C, Ten x 10<sup>6</sup> splenocytes/ml were cultured from either WT or D6-null mice in the presence of 1 nM biotinylated CCL3 for 6 h. Culture supernatants were assayed by Western blot for the presence of biotinylated CCL3. Blots are representative of three separate experiments.
incubated with biotinylated CCL3 for 6 h and supernatants were sampled for chemokine levels. Remarkably, WT cells were highly effective at removing chemokines from the medium, such that much of them were gone within 6 h. Conversely, D6-null cells were defective in their ability to sequester chemokines, with a substantial amount of chemokine still being present within the medium after 6 h of culture. Together these data categorically demonstrate that leukocytes can internalize inflammatory chemokines in a D6-specific manner, leading to eventual intracellular degradation of the chemokine.

**Discussion**

It is now clear from many studies that the atypical chemokine receptor D6 is essential for the proper regulation of the in vivo inflammatory response and, accordingly, D6-null mice display enhanced cutaneous and lung inflammation and are more susceptible to tumor development in inflammation-dependent cancer models (14, 16–18). In addition, placental D6 appears to be important for protecting the fetus from inflammation-associated miscarriage (15). We, and others, have previously described D6 immunostaining on LECs (19) and syncytial trophoblasts (15) and had postulated that D6 on these cells is central to its ability to scavenge inflammatory CC chemokines and thus contribute to the resolution of local inflammatory responses. However, there are aspects of lymphatic vascular physiology that do not fit comfortably with this model (20) and do not easily account for possible roles for D6 in the regulation of immune responses (21). Thus, we set out to identify other sources of D6-expressing cells that may be more suited to the scavenging of chemokines at inflamed sites or to the D6-dependent regulation of the immune response. In this study, we show that D6 is expressed by a range of human and murine leukocytes at varied levels. Low level expression is seen on T cells and monocytes but high levels are seen on the APCs, including B cells and DCs. Medium to high levels were also observed on certain hemopoietic progenitor cells, such as the Sca+/lineage-negative cells from mouse bone marrow and the CML-derived human cell lines KS62 and Meg01. Furthermore, D6 expression appears to be regulated during hemopoietic differentiation and is up-regulated as leukocyte precursors terminally differentiate along a range of lineages, including the megakaryocyte and human macrophage lineages. Interestingly, where transcript levels and protein levels were analyzed within the same cell types, there was generally a correlation (see Fig. 3). However, there was not always a correlation between D6 protein levels and activity in functional assays (see the data for DC subtypes in Figs. 3 and 7). This suggests that factors such as other components of the cellular internalization machinery or relative levels of surface and internalized D6 may contribute to cell-specific potency of D6 function.

Importantly, we have also specifically shown the ability of the prototypic hemopoietic transcription factor GATA1 to regulate D6 expression and this is in keeping with the known importance of GATA1 for differentiation along many of the lineages we show here to be positive for D6 expression (32, 33, 41). Both primary human DCs and mouse bone marrow-derived DC cultures express high levels of D6. The recent finding that mouse DC maturation and survival, unlike macrophages, is dependent on GATA1 expression (32) prompted us to explore whether GATA1 knockdown could affect D6 expression. The decrease in D6 transcript levels suggests that function of this transcription factor alone is a prerequisite for expression in mature DCs. In addition, the absence of GATA1 in mouse M-CSF-derived macrophages (32) may explain the low expression of D6 transcripts in this lineage that is closely related to DC. Interestingly, a number of other chemokine receptors have been shown to have GATA response elements in their promoter regions (43–45) and, in the case of CCR5, GATA1 acts to repress expression (43). Thus, it may be that GATA1 differentially regulates proinflammatory (e.g., CCR5) and anti-inflammatory (e.g., D6) chemokine receptors during the orchestration of the inflammatory response.

In addition to differentiation stage-specific regulation of D6 expression, it is clear from the present study that D6 expression can also be dynamically regulated in individual cell types, and these data fit with a model of D6 function in which D6 expression is suppressed during ongoing inflammation but increased during the resolution phase by cytokines such as TGF-β, which appears to be dominant over inflammatory mediators in regulating D6 expression levels. Clearly, therefore, the leukocyte differentiation status, and the context in which leukocytes are functioning, is critical in determining D6 expression levels.

It is of note that our previous immunostaining of human histological sections did not reveal a prominent leukocyte D6 expression profile (19). The present study uses a new clone of mAb which has been previously validated as being specific for D6 (26). In addition, we have complemented the Ab-based analyses with QPCR and functional analyses which together consolidate the data on leukocyte D6 expression. Our previous clone of Ab did detect some limited leukocyte expression (19) and we had also previously reported, but not fully characterized, expression of D6 transcripts in a variety of leukocytes (6, 7). Thus, the present report of expression of D6 by leukocytes is supported by our more limited earlier data. However it appears that the current Ab has a slightly altered specificity and it is our assumption that this reflects its specificity for a posttranslationally added epitope with which the previous Ab has a lower affinity interaction. Thus, our observations may suggest some variability in the epitopes revealed by D6 on LECs and leukocytes reflecting previous precedents within the chemokine receptor field which indicate that different cell staining results for a single receptor can be obtained using different clones of mAbs (46). Similar differential Ab specificities have also been demonstrated for another atypical chemokine receptor, DARC, which has a number of antigenic epitopes detected by a range of different Abs in alternative cellular contexts (47–49).

It is important to note that, although our data support a role for leukocyte D6 in scavenging chemokines at inflamed sites, there may be other roles for D6 on these cells that we have not yet determined. For example, the high levels of expression on B cells and DCs suggest that D6, in addition to regulating inflammation, may also be involved in aspects of initiation of the adaptive immune response. We have recently published results from a collaborative study indicating that D6-null mice have a lessened response in the experimental autoimmune encephalomyelitis model of multiple sclerosis (21). Although we have argued that there may be explanations for this that are consistent with D6 regulation of inflammatory chemokine levels at inflamed sites (20), it is possible that D6 expression on B cells and DCs is indicating a more direct role for D6, perhaps in the process of Ag presentation. In addition, the 20-fold up-regulation of D6 transcripts in T cells following 12 days of culture with anti-CD3/soluble CD28 further suggests as yet undefined roles for D6 in the adaptive immune response. We are currently in the process of examining roles for D6 in the process of Ag presentation and regulation of adaptive immune responses.

As well as being indicative of roles in innate and adaptive immune responses, the expression of D6 by maturing megakaryocytes suggests even broader in vivo functions for D6. Preliminary data suggest expression of D6 protein in human platelets and we are investigating potential roles for platelet D6 in the regulation of systemic chemokine levels and in vascular remodeling (30, 51).

In summary, therefore, we provide evidence for expression of functional D6 on a variety of leukocytes and demonstrate that this expression is dynamically and developmentally regulated. This, therefore, enhances our understanding of the in vivo vehicles for D6 function and, in addition, suggests novel potential functions for D6 in the coordination of innate and adaptive immune responses.
Disclosures

Two of the authors (A.R. and M.P.) are employees of the Novartis Institute for Biomedical Research (Vienna).

References


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