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CD312, the human adhesion-GPCR EMR2, is differentially expressed during differentiation, maturation, and activation of myeloid cells

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Abstract

EMR2/CD312 is a member of the adhesion-GPCR family that contains extracellular EGF-like domains. Previously it has been shown to interact with chondroitin sulphate glycosaminoglycans in an isoform-specific manner. Although EMR2 expression has been found to be restricted to human myeloid cells, its expression profile has not yet been systemically characterized. In this report, we show that EMR2 receptor expression is up-regulated during differentiation and maturation of macrophages, and is conversely down-regulated during dendritic cell maturation. We also demonstrate that EMR2 receptor alternative splicing and glycosylation is regulated during myeloid differentiation. In monocytes and macrophages, EMR2 can be specifically up-regulated by LPS and IL-10 via an IL-10-mediated pathway. In inflamed tissues, EMR2 is detected in subpopulations of myeloid cells including macrophages and neutrophils. The results presented here further support the idea that EMR2 plays a role in the migration and adhesion of myeloid cells during cell differentiation, maturation, and activation.

Keywords: Adhesion-GPCR; EGF-TM7; EMR2; Myeloid cell

Blood cells of myeloid lineage, including monocytes (Mo), macrophages (Mϕ), granulocytes (PMN), and dendritic cells (DC) play an essential role not only in the innate immunity but also in the adaptive immunity. Many of the important immunological functions of myeloid cells are critically dependent upon their differentiation and maturation stages and are manifested to a large extent by the diverse array of cell surface receptors [1,2]. In addition to performing such critical functions as pattern recognition, phagocytosis, and adhesion/migration, many of the cell surface proteins can often also be used as specific markers to "phenotype" myeloid cells with regard to their differentiation, maturation, and activation state. Using these markers, it has been well-established that the tissue distribution and the effector functions of myeloid cells are highly heterogeneous *in vivo* [1,2].

Among the numerous myeloid cell surface markers, of special interest is a small group of EGF-TM7 receptors of which the F4/80 (Emr1) glycoprotein is the prototypic member [2–4]. Over the last 25 years, the F4/80 antigen

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(Ag) has been widely used as one of the most specific markers for mouse tissue Mϕ [1]. Most resident tissue Mϕ populations such as the red pulp Mϕ in the spleen, Langerhans cells in the skin and Kupffer cells in the liver express high levels of F4/80 Ag constitutively [5]. However, the expression of F4/80 is tightly regulated based upon the physiological status of cells. For example, F4/80 expression on Langerhans cells decreases after they take up antigens and become migrating DCs. F4/80 is expressed at lower levels on activated Mϕ isolated from Bacille Calmette-Guérin infected animals in comparison to the resting tissue Mϕ [6]. Recently, the F4/80 receptor itself was shown to be involved in the generation of $CD8^+$ T regulatory cells in peripheral immune tolerance, demonstrating a unique immunological function for the EGF-TM7 receptors [7].

The EGF-TM7 receptors belong to a much larger receptor family, the adhesion-GPCR, which is characterized by a hybrid structure consisting of an extended extracellular domain (ECD) and a class B GPCR-like 7TM moiety [8,9]. Within the EGF-TM7 subfamily, tandem repeats of EGF-like motifs are present in the ECD, whereas other adhesion-GPCR members contain different protein modules such as the Ig-, cadherin-, lectin- or thrombospondin-like repeats [8,9]. Hence, the adhesion-GPCR is believed to have a potential dual function of adhesion and signaling. Indeed, endogenous cellular ligands have been identified for several adhesion-GPCRs in recent years [10–12]. Apart from F4/80, 5 additional EGF-TM7 receptors including EMR2, EMR3, EMR4, CD97, and ETL are present in the human genome [13]. Among them, CD97 and EMR2 (recently designated as CD312) are the most studied members. Both receptors contain 5 highly homologous EGF-like motifs in the ECD, and express extensive alternatively spliced isoforms possessing different numbers of the EGF-like motifs [14–16]. This has generated striking functional diversities between the two receptors. Thus, the longest isoforms of both CD97 and EMR2 were found to interact with the same chondroitin sulphate (CS) glycosaminoglycan (GAG) ligand, while only the shorter CD97 isoform was known to bind to its other cellular ligand, CD55 [11,17].

In addition to functional diversity, the expression patterns of the two receptors are also dissimilar. CD97, originally identified as a T cell activation marker, is now known to be expressed ubiquitously in both lymphoid and myeloid cells as well as in certain muscle cells and tumor cells [14,18,19]. EMR2 expression, on the other hand, is restricted to human myeloid cells, suggesting a specific role in myeloid cell biology [16,20]. In order to delineate its functional significance in myeloid cells, we aim to characterize the expression of EMR2 molecules during myeloid cell differentiation/maturation, focusing on its regulation and biochemical properties.

Materials and methods

Reagents. General chemicals were obtained from Sigma (Dorset, UK). Culture media were from Invitrogen. Buffy coats were purchased from the National Blood Service (Bristol, UK). Cell lines were obtained from the Sir William Dunn School of Pathology. The EMR2 stalk-specific 2A1 mAb (CD312, Serotec, Oxford) has been described previously [20]. Rabbit anti-human myeloperoxidase, cathepsin D, and cathepsin G were from Daco A/S (Glostrup, Denmark). Goat anti-rabbit IgG-HRP was from Jackson Immuno Research Laboratories. Cytokines (IL-10, GM-CSF, TNF-α, etc.) and stimulants (LPS, f-MLP) were from R&D and Sigma, respectively. Enzymes for de-glycosylation experiments were from Roche Applied Science.

Cell culture. HEK-293 cells were cultured in DMEM. Myeloid cell lines (THP-1 and HL-60) and primary myeloid cells were incubated in RPMI 1640 and X-Vivo, respectively. EMR2 expression constructs were described previously [17]. *In vitro* differentiation of macrophage-like and neutrophil-like myeloid cell lines was carried out according to the published procedures. In brief, cells at $\sim 1–2 \times 10^5$ cells/ml were cultured in the presence of 10 nM phorbol 12-myristate 13-acetate (PMA) and at $\sim 5 \times 10^4$ cells/ml with 1.3% DMSO + 1 μM all-trans retinoic acid (ATRA) for the differentiation of macrophages and neutrophils, respectively [21]. Human monocyte-derived Mϕ (MDMϕ) and DC were generated as described previously [16]. For cytokine treatment, monocytes and MDMϕ were cultured in media containing appropriate concentrations of various cytokines and stimulants for 24 or 48 h prior to the analysis.

RNA and protein analysis. Total RNA and cell lysate were collected at indicated time points using standard procedures. Northern blot and Western blot analysis were performed as described previously using ^{32}P-labelled EMR2 cDNA probes and 2A1 as primary Ab and goat anti-mouse IgG-HRP as secondary Ab [16]. For the de-glycosylation experiment, equal amounts of protein were incubated with 1 U PNGase F (Roche) and 0.5 mU *O*-glycosidase (Roche) plus 1.0 mU neuraminidase in 20 mM sodium phosphate buffer, pH 7.0 at 37 °C for 20 h prior to Western blot analysis. For FACS analysis, cells were fixed with 4% paraformaldehyde in PBS, blocked with 5% normal goat serum and 0.5% BSA in PBS (blocking buffer) for 1 h at 4°C, and stained for 1 h at 4°C with appropriate mAbs (5 μg/ml) diluted in blocking buffer. Afterwards, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) at 4 °C for 1 h. Cells were analyzed on FACScan. Data were collected and analyzed using CellQuest software.

Immunohistochemistry. Paraffin-fixed tissue sections were cut at 4 μm in thickness and processed for immunohistochemical staining using the Ventana NEXES automated staining system (Ventana Medical Systems, Tucson, AZ, USA) and iView DAB detection kit (Ventana Medical Systems). 2A1 mAb was used at 5 μg/ml without antigen retrieval. An isotype-matched irrelevant mAb was used as a negative control, which consistently resulted in no staining. Tissues were subsequently counterstained with hematoxylin.

Results and discussion

EMR2 is up-regulated during differentiation/maturation of myeloid cells

To investigate the characteristics of EMR2 expression during myeloid cell differentiation/maturation, a well-established *in vitro* myeloid cell differentiation model using human monocytic cell lines, HL-60 and THP-1 was used. Upon treatment of PMA, both cell lines can be induced to display mature macrophage phenotypes while ATRA plus DMSO caused HL-60 to differentiate into neutrophil-like cells [21]. The maturation and differentiation of cells were monitored and confirmed by cell morphology (data not shown) and the down-regulation of cathepsin G (~22 kDa) (Fig. 1A and Supplementary Fig.1). As shown

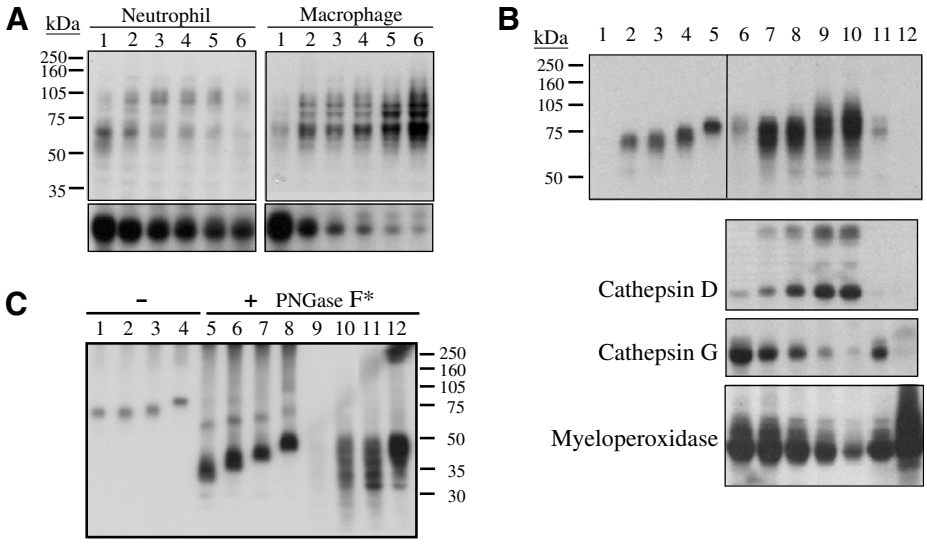

Fig. 1. Analysis of EMR2 expression during differentiation of myeloid cells. (A) Western blot analysis of HL-60 without treatment (lane 1) or treated with ATRA and PMA up to 5 days (lanes 2–6) for neutrophil- and macrophage-like differentiation, respectively. The same blot was probed sequentially with mAbs for EMR2 (top panel) and cathepsin G (bottom panel). (B) Western blot analysis of EMR2 expression in HEK293T cells transfected with individual EMR2 isoforms (mock control, lane 1; EMR2(12), lane 2; EMR2(125), lane 3; EMR2(1235), lane 4; EMR2(12345), lane 5), and primary myeloid cells (Mo, lane 6; day 1 MDMϕ, lane 7; day 2 MDMϕ, lane 8; day 4 MDMϕ, lane 9; day 6 MDMϕ, lane 10; PBMC, lane 11; PMN, lane 12). Differentiation markers (cathepsin D, cathepsin G, and myeloperoxidase) were used to confirm the differentiation status of the cells (lower panels). (C) Western blot analysis of EMR2 glycoproteins either untreated (−) or treated (+PNGase F*) with 1 U PNGase F and 0.5 mU *O*-glycosidase plus 1.0 mU neuraminidase for 20 h before analysis. Samples are from HEK293T cells transfected with EMR2(12) (lanes 1 and 5), EMR2(125) (lanes 2 and 6), EMR2(1235) (lanes 3 and 7), EMR2(12345) (lanes 4 and 8) and Mo (lane 9), day 1 MDMϕ (lane 10), day 4 MDMϕ (lane 11), day 6 MDMϕ (lane 12).

in Fig. 1, EMR2 protein expression was weak in untreated cells but increased in a time-dependent manner in PMA- and ATRA-treated HL-60 and THP-1 cells. As detailed below, multiple EMR2 protein bands (~70 to ~95 kDa) as a result of alternative splicing and differential glycosylation were readily detected in treated cells. Furthermore, we also found the EMR2 RNA transcripts were up-regulated in a similar time-dependent fashion (Supplementary Fig. 1). This indicates that the EMR2 protein expression level is closely related to the differentiation/maturation stages of myeloid cells and that this is mostly regulated at the transcriptional level.

We next examined the EMR2 expression characteristics in primary myeloid cells. Peripheral blood mononuclear cells (PBMC), monocytes, monocyte-derived macrophages (MDMϕ), and polymorphonuclear cells (PMN) were obtained from healthy volunteers and analyzed. The differentiation status of the cells was monitored and confirmed by the expression of various markers such as cathepsin D (~30 and ~40 kDa bands), cathepsin G (~22 kDa), and myeloperoxidase (~55 kDa) (Fig. 1B). The EMR2 proteins (~70 to ~95 kDa) were expressed at low levels in PBMC and monocytes, but increased to a higher level in MDMϕ, consistent with the data obtained from the *in vitro* differentiation of myeloid cell lines. Most interestingly, different species of EMR2 proteins were expressed by MDMϕ at different time points of differentiation. As such, the day 4 and day 6 Mϕ expressed more EMR2 proteins of higher molecular weights than did the day 1 and day 2 Mϕ (Fig. 1B).

Several potential mechanisms can be accounted for such diversities in EMR2 proteins. EMR2 has been found to undergo extensive RNA alternative splicing, producing distinct protein isoforms containing different numbers of EGF-like motifs [16]. We therefore compared the sizes of four individual EMR2 isoforms transiently expressed in HEK293T cells and found that they range from ~70 to ~95 kDa in mass. This size spectrum matched those of EMR2 protein species expressed by MDMϕ, indicating that Mϕ indeed expressed multiple EMR2 isoforms. In addition, EMR2 was expected to be a heavily glycosylated molecule due to the presence of multiple potential *N*- and *O*-glycosylation sites in its extracellular region [16]. Indeed, de-glycosylation experiments showed that carbohydrate moieties represented as much as half of the mass of EMR2 proteins expressed in transfected HEK293T cells and primary myeloid cells (Fig. 1C). Most importantly, multiple EMR2 protein bands were evident after extensive de-glycosylation of Mϕ cell lysate, confirming the expression of distinct EMR2 protein isoforms in these cells. Thus, we concluded that Mϕ expressed diverse EMR2 glycoprotein isoforms whose expression levels were regulated in a differentiation stage-dependent fashion.

In addition, we have also analyzed other primary myeloid cells for EMR2 expression. In neutrophils (PMN), little if any EMR2 expression was detected by Western blotting despite several attempts using different samples (Fig. 1B). This result was unexpected as cell surface expression of EMR2 on PMN was clearly detected by FACS analysis (Fig. 2) and immunohistochemistry (Fig. 4).

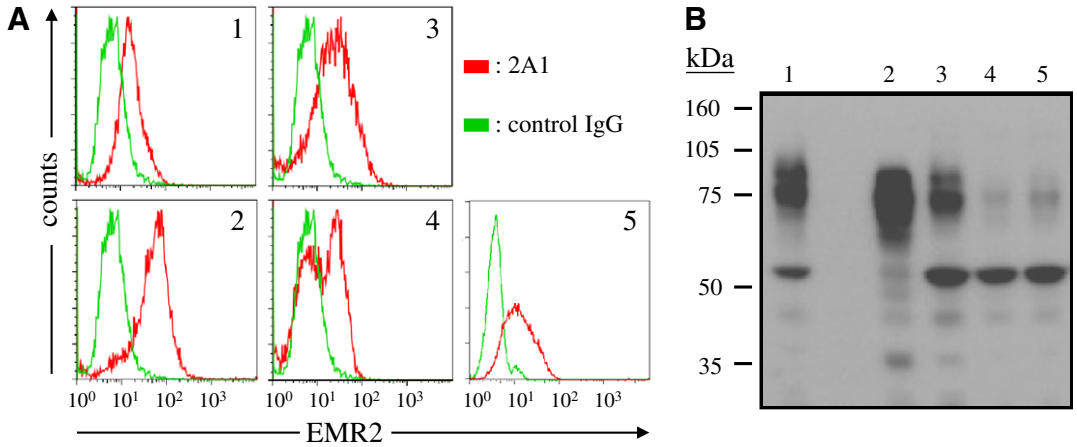

Fig. 2. Analysis of EMR2 expression in primary myeloid cells. (A) Flow cytometric analysis of EMR2 expression in primary monocytes (1), day 6 MDMϕ (2), immature DC (3), mature DC (4), and neutrophils (5). (B) Western blot analysis of EMR2 expression in day 7 MDMϕ (lane 1), day 3 iDC (lane 2), day 6 iDC (lane 3), mDC induced by 20 ng/ml LPS (lane 4), or 20 ng/ml TNF-α (lane 5).

Furthermore, our previous Northern blotting data has shown that PMN is the major cell type expressing EMR2 RNA transcripts [16]. We suspected that the EMR2 antigenic epitope might somehow be destroyed during cell lysate preparation from PMN, probably due to the powerful proteases present within the various vesicles of cells. However, it is equally possible that other unknown mechanisms are responsible for this discrepancy, which requires further study in the future. As for monocyte-derived DC, both FACS and Western blotting analyses showed that EMR2 is expressed at a higher level in immature DC (iDC) than in mature DC (mDC) (Fig. 2), which is in stark contrast to the EMR2 expression patterns found during macrophage differentiation. Taken together, this suggests that the regulation of EMR2 expression in different myeloid cell lineages is uniquely regulated and critically dependent on the differentiation/maturation status of cells.

Specific up-regulation of EMR2 expression in myeloid cells by IL-10 and LPS

In light of the previous finding, we next examined the effect of cytokines on EMR2 expression in monocytes and MDMϕ. Among the numerous cytokines and inflammatory stimuli tested, we found that only IL-10 and LPS significantly up-regulated EMR2 expression (Fig. 3A). Again, the up-regulation of protein expression was correlated to a higher RNA expression level in LPS- and IL-10-treated cells (Supplementary Fig. 2A), indicating that EMR2 expression indeed is mostly controlled at the transcriptional level. Furthermore, we found that the IL-10- and LPS-induced EMR2 up-regulation increased with the duration of the treatment and were both saturable with the optimal concentration of IL-10 and LPS at 20 ng/ml and 1 μg/ml, respectively (Supplementary Fig. 2B–D). Although the effect of IL-10 was generally thought to be anti-inflammatory and LPS pro-inflammatory, it is well-established that LPS can induce IL-10 secretion by monocytes and Mϕ [22]. We therefore tested whether the up-regulation of EMR2 by LPS is mediated by IL-10. Indeed, the addition of a neutralizing anti-IL-10 mAb was found to inhibit the LPS-induced EMR2 up-regulation in a dose-dependent manner (Fig. 3B). No such inhibiting effect was observed with the isotype-control mAb, further confirming the specific involvement of IL-10 in mediating the up-regulation of EMR2 by LPS in monocytes and MDMϕ.

EMR2 expression in selected inflamed tissues

We have previously shown that EMR2 expression *in situ* is generally weak and is limited to certain tissue Mϕ subpopulations [20]. However, not much is known regarding its expression in diseased tissues. To further study the EMR2 expression in pathological conditions, we examined several heavily inflamed tissues including liver abscess, lung abscess and severe acute suppurative appendicitis by immunohistochemistry. Strong EMR2 expression can be readily detected in restricted subpopulations of tissue Mϕ as well as in some but not all infiltrated neutrophils (Fig. 4). This result is further confirmed by double immunohistochemical staining (Supplementary Fig. 3). Although it is generally believed that myeloid cells in inflamed tissues are somehow activated, it is not known at present whether the $EMR2^+$ Mϕ were the resident tissue Mϕ or were derived from recruited blood borne monocytes. These results do however confirm that EMR2 is expressed on infiltrated neutrophils. The differential and restricted staining patterns also confirmed that its expression in myeloid cells is highly regulated.

Our previous finding of CS GAG as a cellular ligand for the largest EMR2 isoform has implicated a role for EMR2 in cell adhesion and migration [11,23]. The results shown here further suggested a dynamic and highly regulated function for EMR2 in myeloid cell biology. As its expression was shown to be up-regulated in differentiated macrophages, EMR2 might be important for the steady-state migration of circulating blood monocytes into tissues and

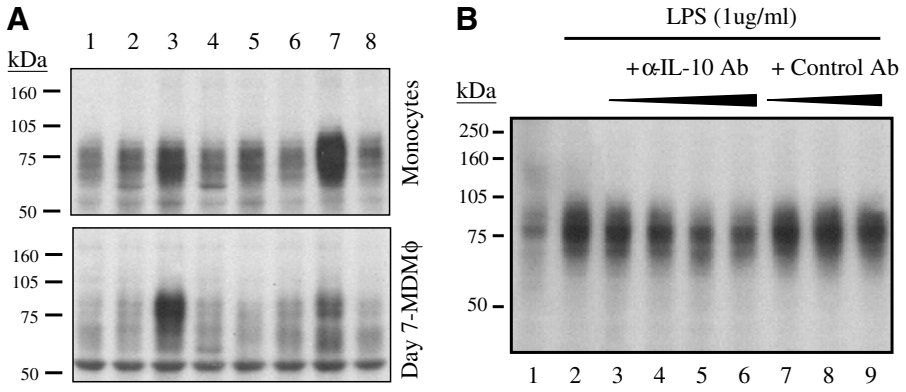

Fig. 3. Regulation of EMR2 expression in primary myeloid cells. (A) Western blot analysis of EMR2 expression in monocytes (top panel) and day 7 MDMϕ (bottom panel) treated with indicated cytokines. Cells were cultured in medium only (lane 1) or medium containing IFN-γ (100 U/ml) (lane 2), LPS (1 μg/ml) (lane 3), IFN-γ (100 U/ml) + LPS (1 μg/ml) (lane 4), TNF-α (20 ng/ml) (lane 5), IL-4 (20 ng/ml) (lane 6), IL-10 (20 ng/ml) (lane 7), or IL-13 (20 ng/ml) (lane 8) for 20 h. (B) Western blot analysis of EMR2 expression in day 7 MDMϕ treated without (lane 1) or with 1.0 μg/ml LPS (lane 2), 1.0 μg/ml LPS plus anti-IL10 (0.5 μg/ml, lane 3, 1.0 μg/ml, lane 4, 5.0 μg/ml, lane 5, 10.0 μg/ml, lane 6), or 1.0 μg/ml LPS plus a control IgG (1.0 μg/ml, lane 7, 5.0 μg/ml, lane 8, 10.0 μg/ml, lane 9) for 20 h.

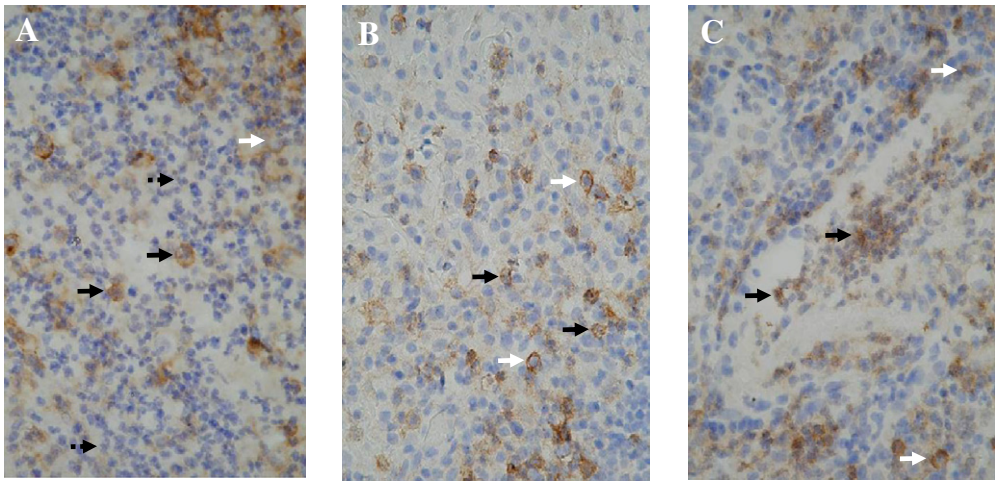

Fig. 4. Immunohistochemical detection of EMR2 in selected inflamed human tissues. Tissue biopsies from liver abscess (A), lung abscess (B), and acute suppurative appendicitis (C) were stained with 2A1. EMR2-expressing neutrophils (black arrow) and macrophages (white arrow) were evident in all inflamed tissues examined. However, EMR2-negative neutrophils (broken black arrow) were also observed. An isotype control Ab did not produce any staining (data not shown). Images are shown at 400× magnification.

their subsequent maturation into tissue macrophages. The detection of EMR2 expression in neutrophils from blood and in inflamed tissues suggested a role in the recruitment of neutrophils into inflamed tissues. Alternatively, EMR2 might be involved in the retention of macrophages and neutrophils within tissues. Likewise, the down-regulation of EMR2 following the activation and maturation of DC also suggested a potential role in the retention or migration of maturing DC. It is interesting to note that the differential expression of EMR2 glycoprotein isoforms seems to be dependent upon the differentiation/maturation status of cells. As only the largest EMR2 isoform is known to interact with CS GAG ligand, the differential expression of different protein isoforms could be another level of control to modulate its cellular adhesion and migration functions. Finally, the specific up-regulation of EMR2 by IL-10 and LPS (via IL-10) in monocytes and macrophages suggests that the EMR2 transcriptional program is different to that of other EFG-TM7 receptors. In the future, it will be desirable to dissect the signaling mechanisms governing the EMR2 transcriptional activity during macrophage differentiation and its up-regulation by IL-10.

Our present study has shown that unlike F4/80, human EGF-TM7 receptor EMR2 is expressed in a wider range of myeloid cells including monocytes, macrophages, neutrophils and DC. Similar to F4/80, however, the expression of EMR2 is highly regulated, depending on the differentiation/maturation stages of the cells and specific cytokine signaling pathways.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.11.148.

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