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Human Immature Monocyte-Derived Dendritic Cells Express the G Protein-Coupled Receptor GPR105 (KIAA0001, $P2Y_{14}$) and Increase Intracellular Calcium in Response to its Agonist, Uridine Diphosphoglucose

Lisa Skelton,* Mike Cooper,† Marianne Murphy,* and Adam Platt[1†]

Dendritic cells (DC) are essential to the initiation of an immune response due to their unique ability to take-up and process Ag, translocate to lymph nodes, and present processed Ag to naive T cells. Many chemokines, chemokine receptors and other G protein-coupled receptors (GPCRs) are implicated in these various aspects of DC biology. Through microarray analysis, we compared expression levels of chemokines, their cognate receptors, and selected GPCRs in human monocytes and in vitro monocyte-derived immature and mature DC. Hierarchical clustering of gene expression clearly distinguishes the three cell types, most notably highlighting exceptional levels of expression of the GPCR GPR105 within the immature monocyte-derived DC (MDDC) gene cluster. Little or no expression was observed within the monocyte and mature MDDC cluster. Putative functionality of the GPR105 receptor was demonstrated by an observed calcium flux in immature MDDC treated with the potent GPR105 agonist, uridine 5′-diphosphoglucose (UDP-glucose), while no response to the nucleotide sugar was seen in monocytes and mature MDDC. This UDP-glucose-induced calcium response was, at least in part, pertussis toxin-sensitive. Moreover, immature MDDC from some donors treated with UDP-glucose exhibit an increase in expression of the costimulatory molecule CD86, which correlates with the intensity of the UDP-glucose-induced calcium flux. Together, these data demonstrate differential expression of GPR105 on immature and mature MDDC and suggest a role for the receptor and its agonist ligand in DC activation. ***The Journal of Immunology,*** **2003, 171: 1941–1949.**

Dendritic cells (DCs)[2] are extremely potent APC s, often described as sentinels of the immune system (1). They normally reside within peripheral tissue in what is described as an immature form, sampling the microenvironment for foreign Ags. Following uptake of Ag, and in response to inflammatory signals, DC mature and migrate to lymph nodes where they present Ag to naive T cells (1–2). Immature human DC can be derived in vitro from CD14-positive peripheral blood monocytes in the presence of GM-CSF and IL-4 (3). These cells express the chemokine receptors CCR1 and CCR5 (4–5) consistent with their requirement to migrate to areas of inflammation where ligands for these receptors, such as CC chemokine ligand (CCL)3 (macrophage inflammatory protein (MIP)-1α), CCL4 (MIP-1β), CCL5 (RANTES), and CCL15 (MIP-5, HCC2), are expressed (4, 6). Exposure to a variety of inflammatory or bacterial stimuli, including monocyte-conditioned medium (7), leads to maturation of monocyte-derived DC (MDDC), and these in vitro-derived cells are referred to as "mature" DC. Coordinated changes in gene expression occur upon DC maturation, including down-regulated expression of CCR1 and CCR5 and up-regulated expression of CCR7 (8–10). This change in chemokine receptor expression enables the chemotactic recruitment of DC to T cell-rich areas of lymph nodes that express the CCR7 ligands CCL19 (EBI1 ligand chemokine, MIP-3β) and CCL21 (secondary lymphoid-tissue chemokine, 6Ckine) (11). DC maturation also results in increased expression of many proteins other than chemokines, such as the costimulatory molecule CD86 (B7.2), fundamental in the ability of DC to interact with naive T cells (12).

Induction of DC maturation through exposure to necrotic tumor cells (13) has led to the proposal that "danger signals" (14) could have a major role in the initiation of an immune response. Indeed, ATP, through interaction with the G protein-coupled receptor (GPCR) P2Y11, can play a role in DC maturation (15) and ATP has been shown to be nonlytically released from stressed (mechanically agitated) cells (16). It is an attractive hypothesis that other small molecules may be released as danger signals from cells at inflammatory and/or tumor sites, either through passive diffusion following traumatic lysis or through regulated exocytosis. As dendritic cells express many P2Y purinergic receptors (17), there is a possibility that purine/pyrimidine-derived molecules may influence dendritic cell maturation, although UTP does not seem to have a direct effect (18).

The GPCR GPR105 (KIAA0001), a distant relative of the P2Y family of receptors (19), recently renamed as the $P2Y_{14}$ receptor (20), was first cloned from an immature myeloid cell line (21) and uridine 5′-diphosphoglucose (UDP-glucose) was later identified as a potent agonist ligand (19). No function has yet been attributed to this receptor, although the rat orthologue, VTR 15–20, is regulated by in vivo challenge with LPS and has been implicated in neuroimmune function (22). The rat and mouse orthologues for GPR105, exhibiting 81 and 83% amino acid identity, respectively,

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[2] Abbreviations used in this paper: DC, dendritic cell; CCL, CC chemokine ligand; MIP, macrophage inflammatory protein; MDDC, monocyte-derived DC; GPCR, G protein-coupled receptor; UDP-glucose, uridine 5′-diphosphoglucose; MCM, monocyte-conditioned medium; $[Ca^{2+}]_i$, intracellular calcium; FLIPR, fluorometric imaging plate reader; FIU, fluorescence intensity unit; UTR, untranslated region.

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both respond to the putative ligand, UDP-glucose (23); however, existence of other physiological ligands remains a possibility. In this report, we demonstrate high levels of expression of GPR105 by human immature MDDC, but much lower levels by mature MDDC and monocytes. We further report that UDP-glucose induces increased intracellular calcium in immature MDDC, which is in part, pertussis toxin-sensitive, and correlates with an increase in costimulatory molecule expression.

Materials and Methods

Materials and reagents for cell culture

The medium used throughout was RPMI 1640 (Invitrogen, Paisley, U.K.), supplemented with 10% heat inactivated FCS (Sigma-Aldrich, St. Louis, MO), 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen), 2 μM L-glutamine (Invitrogen), and 10 mM HEPES (Invitrogen). Cytokines used for DC differentiation were recombinant human GM-CSF (R&D Systems, Minneapolis, MN) and recombinant human IL-4 (R&D Systems).

Monocyte-conditioned medium (MCM)

MCM was prepared as previously described (7). Briefly, using Ig-coated Petri dishes (100 mm, Falcon; BD Labware, Franklin Lakes, NJ), 4 ml of human γ-globulin (Jackson ImmunoResearch Laboratories, West Grove, PA) at 10 mg/ml in PBS was incubated in the Petri dishes for 2 min. The Petri dish was washed three times in PBS. Monocytes ($1–2 \times 10^7$) (see below) were added in 5–10 ml of medium. The cells were incubated for 1 h at 37°C, and then washed with warm medium to remove nonadherent cells. Five milliliters of medium per 1×10^7 monocytes (starting number) were then added, the dishes were then incubated for 24 h at 37°C, 5% CO_2. MCM was collected and stored at −20°C.

Monocyte and MDDC generation and chemokine assays

PBMCs were separated from peripheral blood by sedimentation in Ficoll-Hypaque (Pharmacia Biotech, Upsala, Sweden) and monocytes were subsequently isolated via negative selection using a monocyte isolation kit and the MACS column system (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes were either used directly to obtain RNA, for the preparation of conditioned medium, in calcium flux assays or for in vitro DC generation. For generation of MDDC, monocytes were cultured with 5 ng/ml GM-CSF + 50 ng/ml IL-4 (R&D Systems) at 1.5×10^6 cells/well in six-well tissue culture plates (Falcon) for 6 days at 37°C, 5% CO_2. Cytokines were replenished on day 3 by adding 0.5 ml of fresh medium with 30 ng/ml GM-CSF and 300 ng/ml IL-4. On day 6, nonadherent cells were collected by aspiration with a Pasteur pipette and either used directly for RNA preparations or transferred to new six-well plates with fresh cytokines as on day 3. To obtain mature and immature day 9 MDDC, day 6 DC were cultured for a further 72 h either with (mature) or without (immature) the addition of 1 ml of MCM. Immature and mature MDDC were characterized by flow cytometry, the ability to take-up FITC-dextran, and their T cell stimulatory capacity in a DC:T cell MLR (see results). For chemokine assays, day 9 immature and mature (MCM-treated) MDDC were washed thoroughly and replated in 24-well plates at 5×10^5 cells/well. Culture supernatants were collected at 48 h and assayed by ELISA for constitutive chemokine production. Duoset ELISA development kits from R&D Systems were used for CCL17 (thymus and activation-regulated chemokine) and CCL15 (leukotactin) and an R&D Systems quantikine ELISA kit was used for CCL23 (myeloid progenitor inhibitory factor-1, Ckβ8) measurement. For UDP-glucose-treated DC experiments, day 6 MDDC were cultured for a further 48 h either with (+UDP-glucose) or without (no treatment) 1 mM UDP-glucose (ICN Biomedicals, Irvine, CA), used at <0.04 endotoxin units/ml as assessed by the *Limulus* amebocyte assay, repeated daily.

Microarray construction, hybridization, and quantitation

Sequences corresponding to 3′-untranslated regions (UTRs) for all known human chemokines and receptors, plus related receptors, GPCRs and their ligands, and suitable housekeeping proteins (see supplementary material for list)[3] were cloned either from IMAGE clones (Research Genetics, Huntsville, AL) or Universal cDNA library (Clontech Laboratories, Palo Alto, CA). 3′-UTRs were amplified by PCR with oligonucleotides designed to produce an amplicon of ~500–900 bp, the specific 3′-UTR sequence chosen for low homology to other genes and repetitive sequences. Microarrays were printed using GMS 417 Microarrayer (Affymetrix, Santa Clara, CA) and ~100 pg of PCR products were printed in duplicate on Hybond N^+ membranes (Pharmacia Biotech). The printed membranes were denatured, neutralized, and cross-linked according to manufacturer's instructions.

Radiolabeled cDNA probes were synthesized in 30-μl reactions containing 0.1–1 μg of total cellular RNA, 2 μg poly(dT) (Research Genetics), 0.5 mM each dATP, dGTP, and dTTP (Amersham, Bucks, U.K.), 50 μCi [α-^{33}P]dCTP (NEN, Boston, MA), 60 U SUPERNaseIn (Ambion, Austin, TX), 10 mM DTT, 6 μl of 5× first-strand buffer, and 300 U SuperScript II (Invitrogen), incubated at 42°C for 2 h. RNA template was hydrolyzed at 60°C for 30 min in 0.05% SDS, 25 mM EDTA, and 0.45 M NaOH, the reaction was neutralized by HCl to 0.45 M, and Tris, pH 7.5, to 250 mM and 15 μg of human Cot-1 DNA was added (Invitrogen). Unincorporated nucleotides were removed with Bio-Spin 6 chromatography columns (Bio-Rad, Hercules, CA). Microarrays were prehybridized at 55°C for 2–3 h in 8 ml of ExpressHyb solution (Clontech Laboratories) with 200 μg of salmon testes DNA (Sigma-Aldrich, Poole, U.K.), and 10 μg of poly(dA) (Research Genetics). Probes were denatured at 95°C for 3 min, then hybridized to the microarray for 16–20 h at 55°C. Microarrays were washed with 0.2× SSC, 0.1% SDS for 1 h, twice at 55°C, and once at 65°C. Hybridized microarrays were exposed to low energy PhosphorImager screens and scanned by Storm860 (Molecular Dynamics, Sunnyvale, CA). Data was extracted using IMAGENE software (BioDiscovery, Los Angeles, CA) and normalized to the average expression level of the housekeeping gene panel, with final expression levels expressed as the percent average of this panel. Data manipulations were performed with Microsoft Excel, and hierarchical clustering was achieved using GeneSpring software (Silicon Genetics, Redwood City, CA).

RNA extraction and Northern hybridization

Total cellular RNA was extracted using an RNeasy mini kit (Qiagen, Crawley, U.K.) following manufacturer's protocol. Northern blots were constructed by transfer of 5 μg of total cellular RNA, separated on a 1.2% agarose formaldehyde gel, onto ζ-Probe GT membrane (Bio-Rad) according to manufacturer's instructions. Double-stranded probes were synthesized with a Strip-EZ DNA kit (Ambion); 905-bp GPR105 probes consisted of nucleotides 1427–2332 of National Center for Biotechnology Information accession number XM_003091. Hybridization was performed overnight at 65°C as per manufacturer's instructions.

Flow cytometry

Cell surface markers were evaluated using FITC-conjugated Abs to CD86 (Serotec) and CD80 (BD PharMingen, San Diego, CA), and PE-conjugated Abs to CD83 (BD PharMingen) and HLA-DR (BD PharMingen) by two-color flow cytometry. Cell surface CCR1 and CCR5 were determined using directly conjugated PE and FITC Abs (R&D Systems), respectively. Cell surface CCR7 was determined using a three-step technique using an unconjugated mouse Ab to CCR7 (Insight Biotechnology), anti-mouse biotin (Insight Biotechnology), and streptavidin PE (Insight Biotechnology, Wembley, Middlesex, U.K.). Briefly, cells were washed with wash buffer, PBS (Invitrogen) containing 5% FCS (Sigma-Aldrich) and 0.1% sodium azide (Sigma-Aldrich), and then were incubated with optimal concentrations of unconjugated mAbs, FITC- and PE-conjugated mAbs, or matched isotype controls for 30 min on ice. Cells were washed in ice-cold wash buffer and cells incubated with directly conjugated Abs were analyzed immediately. Cells incubated with unconjugated Abs were then incubated with anti-mouse biotin for 30 min on ice, washed extensively, and finally were incubated with streptavidin PE for 30 min on ice, washed in ice-cold buffer, and analyzed in a FACSCalibur equipped with CellQuest software (BD Biosciences, Mountain View, CA). Live cells were gated on their forward and side light scatter properties, where indicated the percentage of positive cells was recorded.

Endocytosis

Endocytic ability was measured by the cellular uptake of FITC-dextran (Sigma-Aldrich) analyzed by flow cytometry. The MDDC preparations were incubated in medium containing 0.5 mg/ml FITC-dextran for 30 min at 37°C or washed and incubated in 0.5 mg/ml FITC-dextran for 30 min at 4°C (control). Cells were then washed extensively in ice-cold wash buffer and the uptake of FITC-dextran was determined by flow cytometry. Results were expressed as the percentage of positive cells.

Allogeneic T cell proliferation

T cell stimulatory capacity of the immature and mature MDDC populations was assessed by their ability to drive proliferation of naive allogeneic T

[3] The on-line version of this article contains supplemental material.

cells, in a MLR. T cells were purified from PBMC via negative selection using a pan T cell isolation kit and the MACS column system (Miltenyi Biotec). The DC preparations were washed extensively and irradiated for 45 min (^{137}Cs source). The T cells (1×10^6/ml), used as responder cells, were stimulated with 1×10^4 irradiated DC/ml for 5 days at 37°C, 5% CO_2, in round-bottom, 96-well microtiter plates. Cell proliferation was assessed by adding 1 μCi/well of methyl [^{3}H]thymidine (10 Ci/mmol; Pharmacia Biotech) during the last 16 h of culture. Cultures were then harvested onto glass fiber filter mats using a Skatron plate harvester (Molecular Devices, Sunnyvale, CA), and the radioactivity was counted using liquid scintillation.

Ca^{2+} flux measurement

The response of cells to UDP-glucose (Sigma-Aldrich or ICN Biomedicals, see figure legends 4–6), histamine (Sigma-Aldrich), CCL3 (R&D Systems), and CCL19 (R+D Systems) was investigated by measuring intracellular calcium $[Ca^{2+}]_i$ changes using the Ca^{2+}-sensitive dye Fluo4-AM, in conjunction with a fluorometric imaging plate reader (FLIPR) (Molecular Devices, Berkshire, U.K.). Where indicated, cells were pretreated with 10 ng/ml pertussis toxin for 18 h. Agonists were prepared in HBSS, 0.2% BSA, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM HEPES (all from Sigma-Aldrich). Fluo-4 (Molecular Probes, Eugene, OR) was dissolved in DMSO (HYBRI-MAX; Sigma-Aldrich) and pluronic acid (20% in DMSO). Cells were then washed in the above buffer and incubated for 1 h at 37°C, 5% CO_2 with Fluo-4 (final concentration 4 μM). Cells were then washed twice and plated into 96-well black-wall clear-bottom microplates (Corning-Costar, Acton, MA) at 0.5–1 $\times$ 10^5/well. The plates were centrifuged at $210 \times g$, for 3 min, with no brake and rested at 37°C, 5% CO_2 for 10 min. The cell plate was loaded into the FLIPR and a signal test was taken and laser power adjusted to obtain a basal level of ~10,000 fluorescence intensity units (FIU). The cells were then excited at 488 nm using the FLIPR laser and fluorescence emission was determined using a charge-coupled device camera with a bandpass interference filter (510–560 nm). Fluorescence readings were taken at 1.5-s intervals for 70 s and a further 10 readings were taken at 6-s intervals. Agonists or buffer (negative control) were added after 15 s using the FLIPR. *Max − Min* fluorescence data, with negative control subtraction, was exported for each well. Where required, data was then imported into Excel and EC_{50}s were calculated using XLfit version 2.0.7a, and a one-site dose response model: $Y = A + ((B - A)/(1 + ((C/X)^D)))$.

Results

Immature and mature MDDC were characterized for surface marker expression, the ability to take-up FITC-dextran, and their allogeneic T cell stimulatory capacity

Day 9 immature and mature (MCM-treated) MDDC were characterized for surface marker expression by flow cytometry. Immature MDDC were HLA-DR (MHC class II) medium, CD80 low, CD86 negative, and CD83 negative, mature MDDC were HLA-DR high, CD80 high, CD86 high and CD83 positive (Fig. 1*A*). The two DC populations were also tested in functional assays to assess their capability to take-up Ag (FITC-dextran uptake) and stimulate naive T cell proliferation (allogeneic T cell proliferation). Mature MDDC demonstrate a reduced capacity to take-up FITC-dextran (Fig. 1*B*) and an increased capacity to activate naive T cells (Fig. 1*C*). These mature DC also stimulate greater IFN-γ production from naive T cells than their immature counterparts and demonstrate constitutive production of IL-12p40 (data not shown). Although constitutive expression of IL-12p70 is not seen with these MDDC, the immature MDDC will produce IL-12p70 on activation with *Staphylococcus aureus* and IFN-γ (data not shown), consistent with IL-12p70 production as an event dependent on time and nature of the stimulant (24, 25). The change in expression of cell surface markers described and altered abilities to take-up Ag and stimulate naive T cells represents a process commonly referred to as maturation (1–3). Thus, we have immature and mature DC populations suitable for investigating the changes in gene expression that occur on dendritic cell maturation.

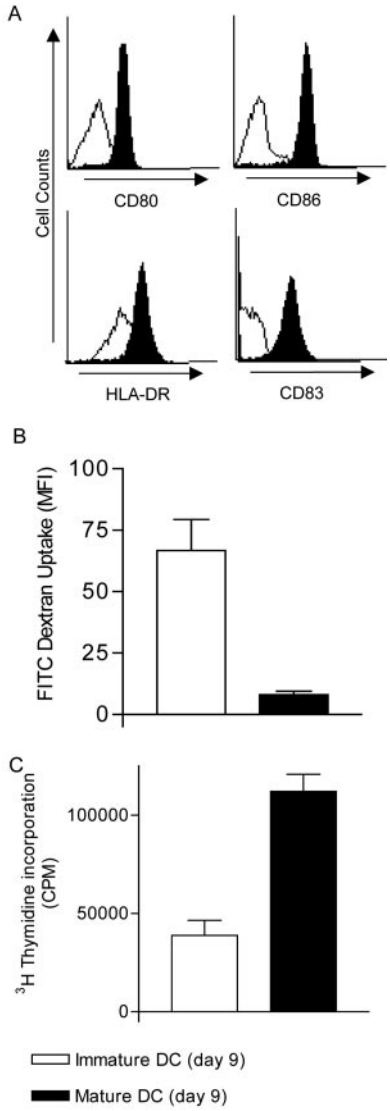
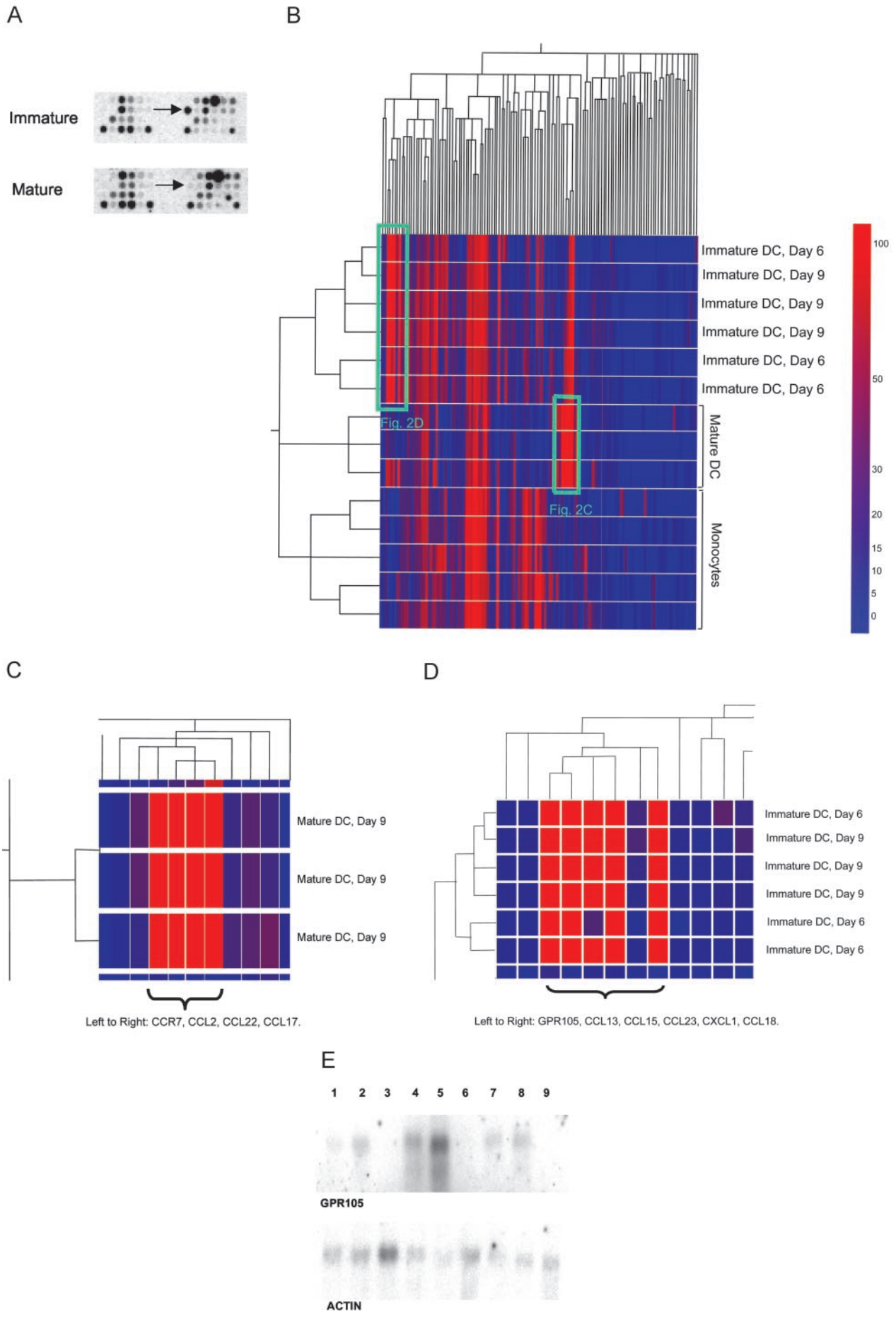


FIGURE 1. Characterization of in vitro monocyte-derived immature and mature DC. Day 6 immature MDDC were incubated with or without MCM (closed or open histograms/bars, respectively) for 72 h and (*A*) analyzed by flow cytometry for cell marker expression; one representative experiment of six is shown. *B*, Assessed for endocytic ability by FITC-dextran uptake, data are means (+SEM) of three experiments. *C*, Tested for ability to drive allogeneic T cell proliferation in a MLR, data are means (+SEM) of three experiments.

Hierarchical clustering of gene expression profiles highlights exceptional levels of GPR105 in immature MDDC

Through use of our custom made microarray, we investigated the expression by monocytes, immature and mature MDDC of the entire superfamily of chemokines and their receptors, as well as other selected GPCRs (Fig. 2*A*). The resulting gene expression data was analyzed by hierarchical clustering performed blind to description of cell type. Data are grouped together solely on the similarity between gene expression profiles. This analysis resulted in the formation of three clearly defined clusters, correlating with the three cell populations (monocytes, immature MDDC, or mature MDDC) (Fig. 2*B*). Within the mature DC cluster (Fig. 2, *B* and *C*), elevated expression levels of CCR7, CCL2 (monocyte chemoattractant protein-1), CCL22 (macrophage-derived chemokine), and CCL17 (thymus and activation-regulated chemokine) are observed, consistent with the mature phenotype of these cells (5, 9,



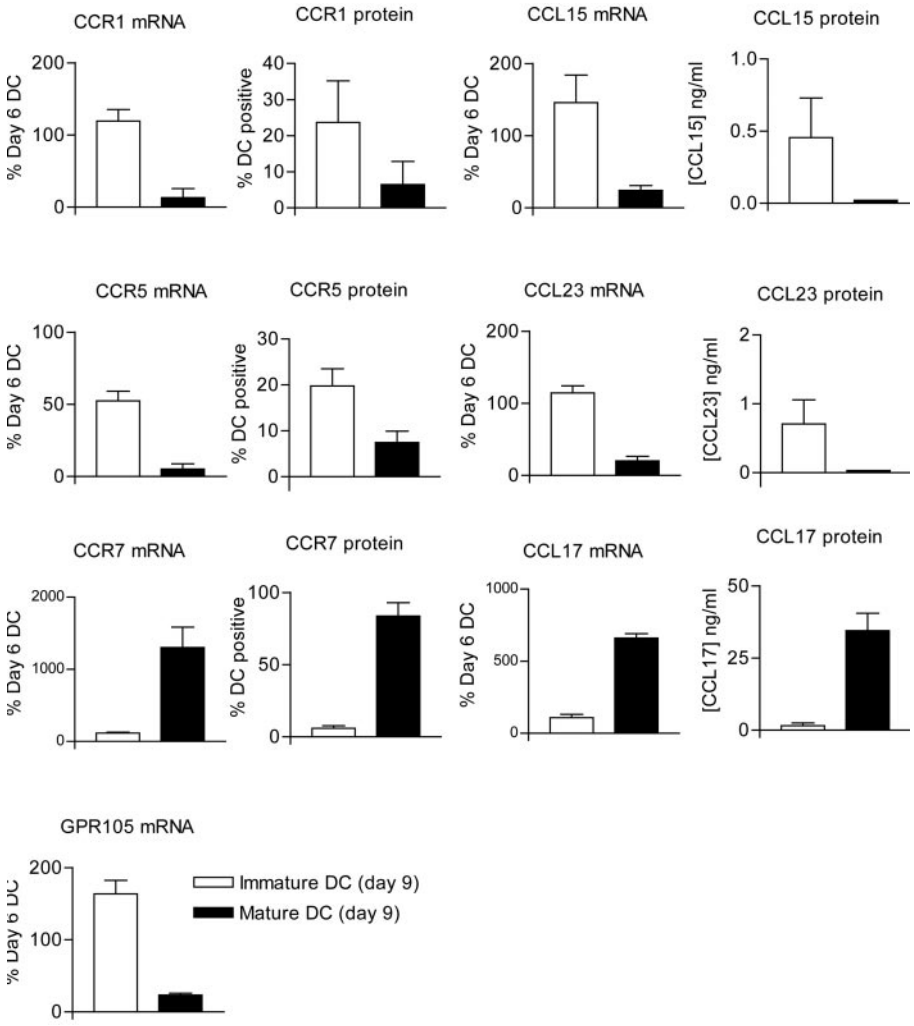


FIGURE 3. Mature MDDC up-regulate CCR7 and CCL17 and down-regulate CCL23, CCL15, CCR1, CCR5, and GPR105. Transcript and protein expression of specific chemokines and receptors by MDDC subsets is shown. Day 6 immature MDDC were incubated with or without MCM (mature (■) and immature (□), respectively) for 72 h. Transcript values as determined by hybridization of RNA to a microarray (as described in *Materials and Methods*) are expressed as the percentage of day 6 immature DC; data are means (+SEM) of three experiments. Cell surface protein levels, as determined by flow cytometry (as described in *Materials and Methods*), are expressed as percentage of positive DC; data are means (+SEM) of three experiments for CCR5 and two experiments for CCR1 and CCR7. Constitutive chemokine production, as determined by ELISA (as described in *Materials and Methods*) are expressed as nanograms per milliliter of culture supernatant, data are means (+SEM) of two experiments.

26–28). An entirely separate cluster grouped together the immature DC samples, these cells distinguished by elevated expression levels of CCL13 (monocyte chemoattractant protein-4), CCL15 (leukotactin-1), CCL23 (myeloid progenitor inhibitory factor-1, CKβ-8) and CCL18 (pulmonary and activation-regulated chemokine, MIP-4) (Fig. 2, *B* and *D*). CCR1 and CCR5 are also elevated in the immature DC samples (Fig. 3), this along with the CCL23 expression profile are consistent with published work (8, 29). However, the most surprising up-regulation of gene expression in the immature DC gene cluster is that of the GPCR GPR105 (Figs. 2*D* and 3). Expression of this gene is notably absent from the monocyte cluster and, with the exception of a low expression level exhibited by one donor, from the mature DC cluster as well. Northern hybridization was used to confirm the expression pattern of GPR105, expression in immature MDDC, but not mature MDDC and monocytes (Fig. 2*E*). Together, this data identifies very distinct gene expression profiles distinguishing immature DC from mature DC (Fig. 3). CCL17 and CCR7 are markedly up-regulated by mature DC, while CCL23, CCL15, CCR1, CCR5, and GPR105 all show a significant down-regulation of expression upon DC maturation. Flow cytometry and ELISA analyses demonstrate a direct correlation between protein and message levels for CCR1, CCR5, CCR7, CCL23, CCL15, and CCL17 (Fig. 3).

Calcium signaling induced by UDP-glucose correlates with GPR105 expression

As we observed a higher level of GPR105 mRNA expression by immature MDDC, in comparison to mature MDDC and monocytes, it was of great interest to determine whether this particular subset of DC would respond to the GPR105 agonist ligand, UDP-glucose. We measured calcium signaling of monocytes, immature and mature MDDC in response to UDP-glucose (Fig. 4) as well as CCL3, a positive control for monocytes and immature DC expressing CCR1 and CCR5, and CCL19, a positive control for mature

FIGURE 2. Expression of GPR105 is increased in immature MDDC. Day 6 immature MDDC were incubated with or without MCM (mature and immature, respectively) for 72 h. RNA was extracted and hybridized to a microarray as described in *Materials and Methods. A,* A comparison of the same area of the microarray hybridized with probes synthesized from either immature or mature MDDC (day 9); the arrow indicates the spot corresponding to GPR105. *B,* Hierarchical clustering of microarray gene expression data from monocytes and DC. Genes represented on the array are clustered along the horizontal axis and samples are clustered on the vertical axis of the dendogram. Expanded views of the mature DC cluster (*C*) and immature DC cluster (*D*) are indicated. The bar on the right side of *B* indicates the level of expression: blue = low, red = high. *E,* Northern hybridization analysis of GPR105 and β-actin expression by day 6 immature MDDC (*lanes 1*, *4*, and *7*), day 9 immature MDDC (*lanes 2*, *5*, and *8*), and day 9 mature MDDC (*lanes 3*, *6*, and *9*).

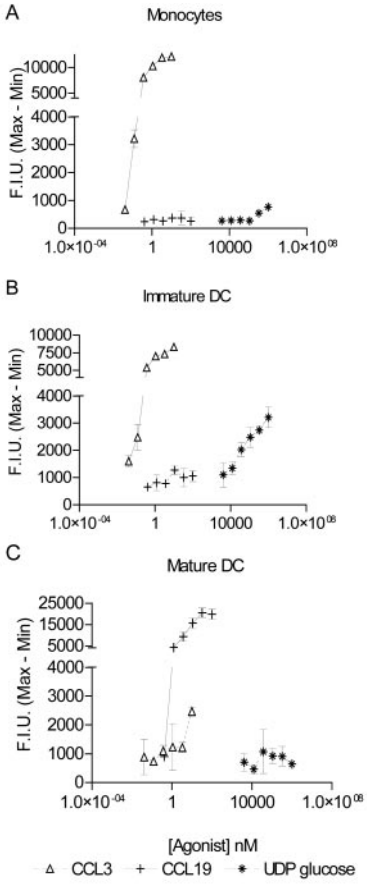


FIGURE 4. Increased $[Ca^{2+}]_i$ is seen in response to UDP-glucose in immature MDDC, but not monocytes or mature MDDC. CCL3-, CCL19-, and UDP-glucose- (Sigma-Aldrich) induced calcium responses were measured using Fluo4-AM in monocytes (*A*), immature DC (*B*), and mature (MCM-treated) DC (*C*). Responses were measured as peak increase in fluorescence units minus basal (FIU *Max* − *Min*), in triplicate, and are given as mean ± SD. One representative experiment of three (monocytes) or four (immature and mature DC) is shown.

DC expressing CCR7. As predicted by the tightly regulated expression of CCR1, CCR5, and CCR7 in these cell types (9), monocytes and immature MDDC both exhibit increased $[Ca^{2+}]_i$ concentrations in response to CCL3 (EC_{50} values: 0.025 nM ± 0.0006 SEM ($n = 3$) monocytes, 0.22 nM ± 0.07 SEM ($n = 4$) immature DC) while mature MDDC show elevated $[Ca^{2+}]_i$ concentrations in response to CCL19 (EC_{50}: 9 nM ± 3.9 SEM ($n = 4$)). No or very little response was observed from immature DC to CCL19 or from mature DC to CCL3. Consistent with the observed expression pattern of GPR105, immature MDDC increase $[Ca^{2+}]_i$ concentrations in response to UDP-glucose (a GPR105 agonist ligand) (EC_{50}: 11.5 μM ± 2.1 SEM ($n = 4$)), while no significant response was observed from mature MDDC or monocytes.

UDP-glucose induces CD86 expression in immature MDDC in some donors; this correlates with the magnitude of a UDP-glucose induced calcium flux.

As GPR105 expression is confined to immature DC and treatment of these cells with UDP-glucose elicited a calcium flux, we sought to determine whether UDP-glucose was capable of inducing a phenotypic change in immature MDDC. Immature (day 6) DC were treated either with or without UDP-glucose, and expression of CD86 (often cited as a classical marker of DC maturation (1, 30), was quantified by flow cytometry. Overall, UDP-glucose-treated DC showed an increase in CD86 levels, although clearly not all donors responded (Fig. 5*A*). In four donors, UDP-glucose-induced calcium flux and UDP-glucose-induced CD86 expression were evaluated in parallel. This revealed a correlation between the magnitude of a UDP-glucose-induced calcium flux and the increase in CD86 expression ($r^2 = 0.87$) (Fig. 5*B*).

UDP-glucose-induced calcium flux is, at least in part, pertussis toxin-sensitive

The calcium responses of immature DC to histamine, CCL3, and UDP-glucose were investigated in immature MDDC with and without pertussis toxin pretreatment. The calcium flux of immature DC to CCL3 was abolished with pertussis toxin pretreatment (Fig. 6*C*) and that of histamine remained intact (Fig. 6*A*). These data are consistent with the pertussis toxin-sensitive nature of the Gi proteins known to couple to the CCL3 receptors, CCR1 and CCR5 (31) and the pertussis toxin-resistant nature of the Gq and Gs proteins that couple to the histamine receptors H1 and H2, respectively (32), present and functional on monocyte-derived immature DC (33). The UDP-glucose initiated calcium flux was down-modulated in response to pertussis toxin pretreatment (Fig. 6*B*). A paired *t* test revealed statistically significant down-regulation of the UDP-glucose-initiated calcium flux by pertussis toxin pretreatment at 10 μM UDP-glucose ($n = 5$, $p = 0.02$) and 100 μM UDP-glucose ($n = 5$, $p = 0.003$). This demonstrates that the increase in intracellular calcium in response to UDP-glucose is at least in part through a pertussis toxin-sensitive G protein.

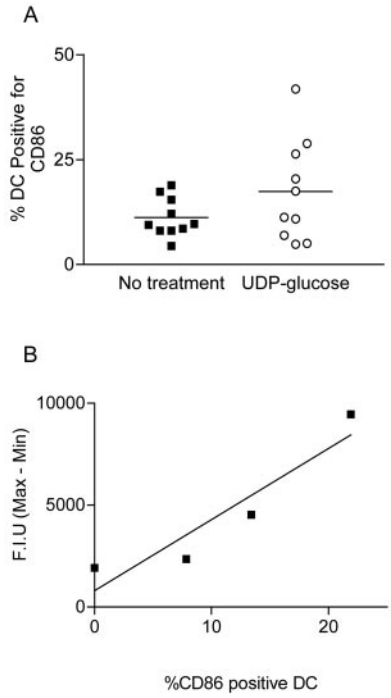


FIGURE 5. UDP-glucose induces CD86 expression in immature MDDC in some donors; this correlates with the magnitude of a UDP-glucose-induced calcium flux. *A*, Day 6 immature MDDC were incubated with (○) or without (●) 1 mM UDP-glucose (ICN Biomedicals), repeated daily, for 48 h. CD86 expression levels were determined by flow cytometry, each symbol represents a single data point, the median of each group is demonstrated by a solid line. *B*, UDP-glucose (ICN Biomedicals) -induced calcium responses were measured using Fluo4-AM in day 6 immature MDDC, the same DC were evaluated for CD86 up-regulation in response to UDP-glucose as in *A*, each symbol represents a single data point, linear regression fit is shown, $r^2 = 0.87$.

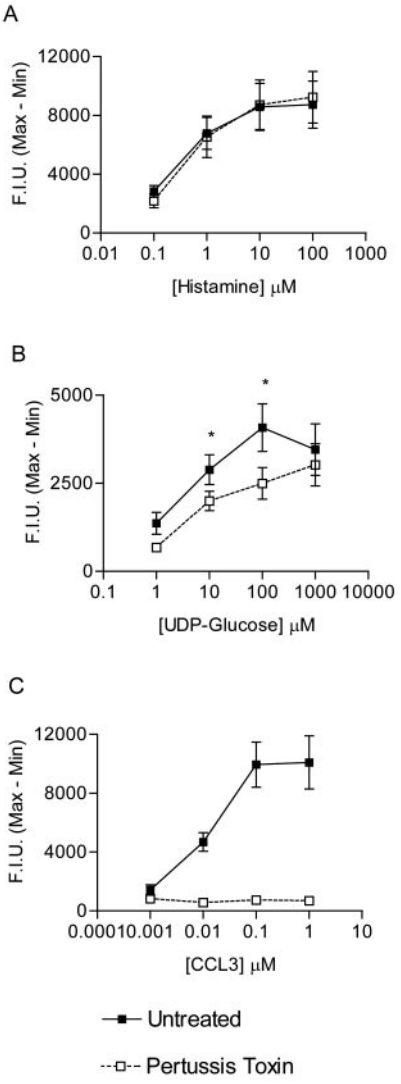


FIGURE 6. UDP-glucose-induced calcium flux is, in part, pertussis toxin-sensitive. $[Ca^{2+}]_i$ responses of day 8 or 9 pertussis toxin-treated (dashed line, □) or untreated (solid line, ■) immature MDDC to (*A*) histamine, (*B*) UDP-glucose (ICN Biomedicals), or (*C*) CCL3; data are means (± SEM) of five experiments. ∗, $p < 0.05$ as compared with untreated (Student's *t* test).

Discussion

DC respond to signals in peripheral tissues which induce a sequential maturation process, conferring a unique ability to both take-up Ag in the periphery and to present processed Ag to naive T cells in lymph nodes. In this study, we demonstrate discrete expression of GPR105 within the immature MDDC subset and suggest a possible role for this receptor in DC activation.

Hierarchical clustering of microarray gene expression profiles of immature and mature MDDC revealed patterns of chemokine expression that distinguish both stages of maturation. Immature MDDC exhibit higher levels of expression of CCL13, 15, 18, and 23 than mature MDDC while mature MDDC exhibit increased expression of CCL2, CCL22, and CCL17. Expression of CCL13 and CCL18 by human in vitro MDDC has been previously noted (29, 34), but the effect of maturation on regulation has not been studied extensively. Our results confirm the expression of these molecules in MDDC and demonstrate a down-regulation on maturation (Fig. 2, *B* and *D*). CCL23 is reported to be constitutively produced by MDDC and its expression down-regulated by inflammatory mediators (35) as is demonstrated in our transcript analysis. Expression of CCL15 by DC has not been previously reported but, intriguingly, this chemokine has high protein homology and functional similarities with CCL23 (36), suggesting some redundancy of function. Our work also demonstrates constitutive production of CCL15 and CCL23 protein from immature but not mature MDDC. Consistent with our data, the proinflammatory chemokine CCL2 is reported to be produced upon maturation of DC in a sustained manner (5) and expression of the constitutive chemokine CCL17 is up-regulated on maturation (5, 26). The high level of CCL22 transcript seen on maturation in this study is consistent with DC as a major source of this chemokine, both in vitro and in vivo (27). Other inflammatory chemokines such as CCL3, CCL4, and CXC chemokine ligand 8 (IL-8) are also reported to be produced at high levels in response to maturation signals, however, the increase is transient, lasting only for a few hours (5, 37). Transcript levels for these chemokines either stayed the same or decreased in mature MDDC (data not shown), probably a reflection of analyzing the cells 72 h after addition of the maturation stimulus.

Expression of particular chemokine receptors has also previously been associated with DC development, including CCR7 with mature DC and CCR1, CCR5, and CCR6 with immature DC (6, 38–41). As expected, CCR7 predominates in the mature MDDC gene expression cluster, and CCR1 and CCR5 decrease significantly on maturation. We were able to demonstrate cell surface levels of these chemokine receptors, as analyzed by flow cytometry, that correlate well with the transcript levels. Significant levels of CCR6 were not observed in our MDDC (data not shown), consistent with several reports that MDDC, unlike $CD34^+$ progenitor-derived DC, do not express CCR6 (38–40).

Our most exciting observation was the strong correlation of the GPR105 expression pattern with other genes demonstrating the immature MDDC cluster. Immature MDDC express GPR105 at substantially higher levels than mature MDDC, with expression by monocytes similar to that of mature MDDC. Interestingly, we have not observed significant levels of expression of the GPR105 transcript by any other cell type that we have analyzed with this microarray, including primary human keratinocytes and bronchial epithelial cells, in vitro-derived osteoclast cultures and primary T cells, as well as the cell lines HUT-78 and Caco-2 all with and without treatment with various proinflammatory stimuli (data not shown). Expression of GPR105 by myeloid DC purified directly from blood awaits investigation.

Calcium signaling via the GPR105 agonist UDP-glucose by immature MDDC (but not mature MDDC or monocytes) adds support for the expression of functional GPR105 by this DC subset. Although definitive confirmation that UDP-glucose is acting through GPR105 on immature MDDC awaits the development of specific anti-GPR105 Abs, antagonizing chemical entities, or other specific gene-targeting approaches, the correlative data is compelling. Indeed, UDP-glucose-initiated calcium fluxes were not affected by preincubation with the endotoxin binder, polymyxin B (data not shown). Specific signaling of UDP-glucose in immature MDDC warranted investigation of the role of this receptor in DC development. To this end, immature MDDC were incubated with UDP-glucose and analyzed by flow cytometry. In some donors, UDP-glucose-treated immature MDDC are induced to express increased levels of the costimulatory molecule CD86, the increase in expression of CD86 correlates with the magnitude of an UDP-glucose-induced calcium flux.

To further substantiate that UDP-glucose is working through GPR105, we investigated the pertussis toxin sensitivity of an UDP-glucose-initiated calcium flux in immature MDDC. The ability to block a calcium flux with pertussis toxin is limited to receptors

coupled with pertussis toxin-sensitive Gi G proteins. A previous report suggests that GPR105 is coupled to such G proteins (19). Our data demonstrate that the calcium signal obtained is at least in part working through pertussis toxin-sensitive G proteins. There is precedence for GPCRs to associate with different G protein classes and therefore demonstrate incomplete sensitivity to pertussis toxin (42, 43).

But what is the physiological consequence of UDP-glucose-mediated DC signaling? Although the downstream effect of this UDP-glucose-initiated calcium flux is not clear, the presence of UDP-glucose and other glycolytic metabolites at high levels in tumor cells (44) is intriguing. This leads us to speculate that UDP-glucose could be acting as a tumor specific danger signal. Indeed, supernatants from necrotic cancer cells, but not other cell types, can induce maturation characteristics in immature DC (13). Furthermore, immunogenic tumors, such as melanoma (45), are present in areas where immature DC, such as Langerhans cells, are highly prevalent. Therefore, we suggest that increased levels of UDP-glucose released from necrotic tumor cells might reach a danger threshold and, through GPR105-mediated signaling, in concert with other signals, initiate an immune response, via induction of DC maturation.

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References

1. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature 392:245.*
2. Rescigno, M., C. Winzler, D. Delia, C. Mutini, M. Lutz, and P. Ricciardi-Castagnoli. 1997. Dendritic cell maturation is required for initiation of the immune response. *J. Leukocyte Biol. 61:415.*
3. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. *J. Exp. Med. 179:1109.*
4. Pashenkov, M., N. Teleshova, M. Kouwenhoven, V. Kostulas, Y. M. Huang, M. Soderstrom, and H. Link. 2002. Elevated expression of CCR5 by myeloid ($CD11c^+$) blood dendritic cells in multiple sclerosis and acute optic neuritis. *Clin. Exp. Immunol. 127:519.*
5. Sallusto, F., B. Palermo, D. Lenig, M. Miettinen, S. Matikainen, I. Julkunen, R. Forster, R. Burgstahler, M. Lipp, and A. Lanzavecchia. 1999. Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *Eur. J. Immunol. 29:1617.*
6. Sozzani, S., W. Luini, A. Borsatti, N. Polentarutti, D. Zhou, L. Piemonti, G. D'Amico, C. A. Power, T. N. Wells, M. Gobbi, et al. 1997. Receptor expression and responsiveness of human dendritic cells to a defined set of CC and CXC chemokines. *J. Immunol. 159:1993.*
7. Reddy, A., M. Sapp, M. Feldman, M. Subklewe, and N. Bhardwaj. 1997. A monocyte conditioned medium is more effective than defined cytokines in mediating the terminal maturation of human dendritic cells. *Blood 90:3640.*
8. Sallusto, F., and A. Lanzavecchia. 2000. Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression. *Immunol. Rev. 177:134.*
9. Sozzani, S., P. Allavena, G. D'Amico, W. Luini, G. Bianchi, M. Kataura, T. Imai, O. Yoshie, R. Bonecchi, and A. Mantovani. 1998. Differential regulation of chemokine receptors during dendritic cell maturation: a model for their trafficking properties. *J. Immunol. 161:1083.*
10. Caux, C., S. Ait-Yahia, K. Chemin, O. de Bouteiller, M. C. Dieu-Nosjean, B. Homey, C. Massacrier, B. Vanbervliet, A. Zlotnik, and A. Vicari. 2000. Dendritic cell biology and regulation of dendritic cell trafficking by chemokines. *Springer Semin. Immunopathol. 22:345.*
11. Baekkevold, E. S., T. Yamanaka, R. T. Palframan, H. S. Carlsen, F. P. Reinholt, U. H. von Andrian, P. Brandtzaeg, and G. Haraldsen. 2001. The CCR7 ligand elc (CCL19) is transcytosed in high endothelial venules and mediates T cell recruitment. *J. Exp. Med. 193:1105.*
12. Ni, K., and H. C. O'Neill. 1997. The role of dendritic cells in T cell activation. *Immunol. Cell Biol. 75:223.*
13. Sauter, B., M. L. Albert, L. Francisco, M. Larsson, S. Somersan, and N. Bhardwaj. 2000. Consequences of cell death: exposure to necrotic tumor cells , but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J. Exp. Med. 191:423.*
14. Gallucci, S., and P. Matzinger. 2001. Danger signals: SOS to the immune system. *Curr. Opin. Immunol. 13:114.*
15. Wilkin, F., X. Duhant, C. Bruyns, N. Suarez-Huerta, J. M. Boeynaems, and B. Robaye. 2001. The P2Y11 receptor mediates the ATP-induced maturation of human monocyte-derived dendritic cells. *J. Immunol. 166:7172.*
16. Burnstock, G. 1997. The past, present and future of purine nucleotides as signalling molecules. *Neuropharmacology 36:1127.*
17. Berchtold, S., A. L. Ogilvie, C. Bogdan, P. Muhl-Zurbes, A. Ogilvie, G. Schuler, and A. Steinkasserer. 1999. Human monocyte derived dendritic cells express functional P2X and P2Y receptors as well as ecto-nucleotidases. *FEBS Lett. 458:424.*
18. la Sala, A., S. Sebastiani, D. Ferrari, F. Di Virgilio, M. Idzko, J. Norgauer, and G. Girolomoni. 2002. Dendritic cells exposed to extracellular adenosine triphosphate acquire the migratory properties of mature cells and show a reduced capacity to attract type 1 T lymphocytes. *Blood 99:1715.*
19. Chambers, J. K., L. E. Macdonald, H. M. Sarau, R. S. Ames, K. Freeman, J. J. Foley, Y. Zhu, M. M. McLaughlin, P. Murdock, L. McMillan, et al. 2000. A G protein-coupled receptor for UDP-glucose. *J. Biol. Chem. 275:10767.*
20. Abbracchio, M. P., J. M. Boeynaems, E. A. Barnard, J. L. Boyer, C. Kennedy, M. T. Miras-Portugal, B. F. King, C. Gachet, K. A. Jacobson, G. A. Weisman, and G. Burnstock. 2003. Characterization of the UDP-glucose receptor (re-named here the P2Y(14) receptor) adds diversity to the P2Y receptor family. *Trends Pharmacol. Sci. 24:52.*
21. Nomura, N., N. Miyajima, T. Sazuka, A. Tanaka, Y. Kawarabayasi, S. Sato, T. Nagase, N. Seki, K. Ishikawa, and S. Tabata. 1994. Prediction of the coding sequences of unidentified human genes. I. The coding sequences of 40 new genes (KIAA0001-KIAA0040) deduced by analysis of randomly sampled cDNA clones from human immature myeloid cell line KG-1 (supplement). *DNA Res. 1:47.*
22. Charlton, M. E., A. S. Williams, M. Fogliano, P. M. Sweetnam, and R. S. Duman. 1997. The isolation and characterization of a novel G protein-coupled receptor regulated by immunologic challenge. *Brain Res. 764:141.*
23. Freeman, K., P. Tsui, D. Moore, P. C. Emson, L. Vawter, S. Naheed, P. Lane, H. Bawagan, N. Herrity, K. Murphy, et al. 2001. Cloning, pharmacology, and tissue distribution of G-protein-coupled receptor GPR105 (KIAA0001) rodent orthologs. *Genomics 78:124.*
24. Ebner, S., G. Ratzinger, B. Krosbacher, M. Schmuth, A. Weiss, D. Reider, R. A. Kroczek, M. Herold, C. Heufler, P. Fritsch, and N. Romani. 2001. Production of IL-12 by human monocyte-derived dendritic cells is optimal when the stimulus is given at the onset of maturation, and is further enhanced by IL-4. *J. Immunol. 166:633.*
25. Kalinski, P., J. H. Schuitemaker, C. M. Hilkens, E. A. Wierenga, and M. L. Kapsenberg. 1999. Final maturation of dendritic cells is associated with impaired responsiveness to IFN-γ and to bacterial IL-12 inducers: decreased ability of mature dendritic cells to produce IL-12 during the interaction with Th cells. *J. Immunol. 162:3231.*
26. Vissers, J. L., F. C. Hartgers, E. Lindhout, M. B. Teunissen, C. G. Figdor, and G. J. Adema. 2001. Quantitative analysis of chemokine expression by dendritic cell subsets in vitro and in vivo. *J. Leukocyte Biol. 69:785.*
27. Vulcano, M., C. Albanesi, A. Stoppacciaro, R. Bagnati, G. D'Amico, S. Struyf, P. Transidico, R. Bonecchi, A. Del Prete, P. Allavena, et al. 2001. Dendritic cells as a major source of macrophage-derived chemokine/CCL22 in vitro and in vivo. *Eur. J. Immunol. 31:812.*
28. Yanagihara, S., E. Komura, J. Nagafune, H. Watarai, and Y. Yamaguchi. 1998. EBI1/CCR7 is a new member of dendritic cell chemokine receptor that is up-regulated upon maturation. *J. Immunol. 161:3096.*
29. Hashimoto, S., T. Suzuki, H. Y. Dong, S. Nagai, N. Yamazaki, and K. Matsushima. 1999. Serial analysis of gene expression in human monocyte-derived dendritic cells. *Blood 94:845.*
30. Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y. J. Liu, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. *Annu. Rev. Immunol. 18:767.*
31. Luther, S. A., and J. G. Cyster. 2001. Chemokines as regulators of T cell differentiation. *Nat. Immunol. 2:102.*
32. Hill, S. J., C. R. Ganellin, H. Timmerman, J. C. Schwartz, N. P. Shankley, J. M. Young, W. Schunack, R. Levi, and H. L. Haas. 1997. International Union of Pharmacology. XIII. Classification of histamine receptors. *Pharmacol. Rev. 49:253.*
33. Mazzoni, A., H. A. Young, J. H. Spitzer, A. Visintin, and D. M. Segal. 2001. Histamine regulates cytokine production in maturing dendritic cells, resulting in altered T cell polarization. *J. Clin. Invest. 108:1865.*
34. Adema, G. J., F. Hartgers, R. Verstraten, E. de Vries, G. Marland, S. Menon, J. Foster, Y. Xu, P. Nooyen, T. McClanahan, et al. 1997. A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells. *Nature 387:713.*
35. Nardelli, B., D. K. Morahan, G. W. Bong, M. A. Semenuk, B. L. Kreider, and G. Garotta. 1999. Dendritic cells and MPIF-1: chemotactic activity and inhibition of endogenous chemokine production by IFN-γ and CD40 ligation. *J. Leukocyte Biol. 65:822.*
36. Youn, B. S., S. M. Zhang, E. K. Lee, D. H. Park, H. E. Broxmeyer, P. M. Murphy, M. Locati, J. E. Pease, K. K. Kim, K. Antol, and B. S. Kwon. 1997. Molecular

cloning of leukotactin-1: a novel human β-chemokine, a chemoattractant for neutrophils, monocytes, and lymphocytes, and a potent agonist at CC chemokine receptors 1 and 3. *J. Immunol. 159:5201.*

37. Fischer, F. R., Y. Luo, M. Luo, L. Santambrogio, and M. E. Dorf. 2001. RANTES-induced chemokine cascade in dendritic cells. *J. Immunol. 167:1637.*
38. Yang, D., O. M. Howard, Q. Chen, and J. J. Oppenheim. 1999. Cutting edge: immature dendritic cells generated from monocytes in the presence of TGF-β 1 express functional C-C chemokine receptor 6. *J. Immunol. 163:1737.*
39. Power, C. A., D. J. Church, A. Meyer, S. Alouani, A. E. Proudfoot, I. Clark-Lewis, S. Sozzani, A. Mantovani, and T. N. Wells. 1997. Cloning and characterization of a specific receptor for the novel CC chemokine MIP-3α from lung dendritic cells. *J. Exp. Med. 186:825.*
40. Greaves, D. R., W. Wang, D. J. Dairaghi, M. C. Dieu, B. Saint-Vis, K. Franz-Bacon, D. Rossi, C. Caux, T. McClanahan, S. Gordon, et al. 1997. CCR6, a CC chemokine receptor that interacts with macrophage inflammatory protein 3α and is highly expressed in human dendritic cells. *J. Exp. Med. 186:837.*
41. Sallusto, F., P. Schaerli, P. Loetscher, C. Schaniel, D. Lenig, C. R. Mackay, S. Qin, and A. Lanzavecchia. 1998. Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur. J. Immunol. 28:2760.*
42. Haribabu, B., D. V. Zhelev, B. C. Pridgen, R. M. Richardson, H. Ali, and R. Snyderman. 1999. Chemoattractant receptors activate distinct pathways for chemotaxis and secretion: role of G-protein usage. *J. Biol. Chem. 274:37087.*
43. Ahamed, J., and H. Ali. 2002. Distinct roles of receptor phosphorylation, G protein usage, and mitogen-activated protein kinase activation on platelet activating factor-induced leukotriene C_4 generation and chemokine production. *J. Biol. Chem. 277:22685.*
44. Eigenbrodt, E., M. Reinacher, U. Scheefers-Borchel, H. Scheefers, and R. Friis. 1992. Double role for pyruvate kinase type M2 in the expansion of phosphometabolite pools found in tumor cells. *Crit. Rev. Oncog. 3:91.*
45. Brinckerhoff, L. H., L. W. Thompson, and C. L. Slingluff Jr. 2000. Melanoma vaccines. *Curr. Opin. Oncol. 12:163.*