Receptor Type Tyrosine Phosphatase Gamma (PTPγ), a new identifier for myeloid dendritic cells and specialized macrophages

Running Title: PTPγ in the hematopoietic system

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ABSTRACT
Protein tyrosine phosphatase (PTP)γ is a receptor-like molecule with a known role in murine hematopoiesis. We analyzed the regulation of PTPγ expression in the human hematopoietic system, where it was detected in human peripheral blood monocyte and dendritic cells (DC) of myeloid and plasmacytoid phenotype. Its expression was maintained during in vitro monocyte differentiation (moDC) and was further increased following maturation with bacterial lipopolysaccharide (LPS), CD40L and TNFα. On the contrary PTPγ was absent when monocytes from the same donor were induced to differentiate in macrophages. B and T lymphocytes did not express PTPγ. PTPγ mRNA was expressed in primary and secondary lymphoid tissues, the highest expression being in the spleen. PTPγ was detected by immunohistochemistry in subsets of myeloid-derived dendritic cells and specialized macrophages (tingible bodies, sinus and alveolar macrophages). Classic macrophages present either in infective or reactive granulomatous reactions did not express PTPγ. Increased PTPγ expression was found associated to decreased capability to induce proliferation and interferon gamma secretion in T cell by moDC from patients affected by advanced pancreatic cancer. Taken together, these results indicate that PTPγ is a finely regulated protein in dendritic cell and macrophage subsets both in vitro and in vivo.
INTRODUCTION

Classical PTPs exist in transmembrane forms (receptor-type PTPs or RPTPs) and non-transmembrane (non-TM) forms and have phosphotyrosine as substrate (described in http://ptp.cshl.edu/). RPTPs can be classified in nine subtypes according to the different combinations of the common motifs that compose their external segments. Receptor-type protein tyrosine phosphatase gamma (PTPγ) forms with PTPζ the subtype V of RPTPs, being characterized by the presence of a carbonic anhydrase-like and a fibronectin type III domains in the N-terminal portion of the extracellular domain. Hematopoietic cells express a number of RPTPs: CD45 is the prototype and is known to play a role in leukocytes, influencing the lymphocyte signaling process after antigen receptor engagement. Experimental evidence is now emerging concerning the involvement of a number of other RPTPs in hematopoiesis and immune response. PTPγ was previously shown to regulate hematopoietic differentiation in a murine model of hemopoietic differentiation but its role in the human system is unknown.

Dendritic cells (DC) represent a specific subset of bone marrow-derived APCs that have a central role in the initiation and regulation of immune responses in both lymphoid and non-lymphoid tissues. They are ubiquitously distributed within the body and share several common features, in particular the expression of high levels of MHC-II molecules in combination with the absence of lineage-specific markers such as CD3, CD14, CD15, CD16, CD19, CD20 and CD56, and are defined as “lineage negative” (lin-) cells. In addition to myeloid DC (MDC), blood DC also include plasmacytoid DC (PDC). PDC have a morphology resembling plasma cells, are devoid of myeloid markers, and can be distinguished from myeloid DC on the basis of membrane markers like CD123, BDCA-2 and BDCA-4. Following virus infection, PDC migrate from the blood to lymph nodes where they produce high concentrations of type I IFN. Several DC types with distinct biological features have been identified in different tissues from mice and humans, including Langerhans cells in the epidermis, interstitial DC in various parenchyma, thymic DC and DC populations found in other lymphoid organs that differ in phenotype, morphology and function. Following activation, DC migrate from peripheral tissues to regional lymph nodes and present the processed antigens to naive T cells. The presence of various maturation stages further complicates the picture: for example five distinct DC subsets within human tonsils have been characterized. Like DC, tissue macrophages are widely distributed in human body, sharing the same microenvironmental niches. Tissue macrophages acquire markedly heterogeneous phenotypes, reflecting their specialized functions in different microenvironments.

Here we demonstrate PTPγ expression in peripheral blood monocytes and in in vitro differentiated DC as well as in subsets of tissue myeloid DC and specialized macrophages. We also demonstrate that increased PTPγ expression associate with a DC phenotype characterized by a decreased capability to induce allogeneic T cell proliferation. This represents a novel finding in the field of myeloid cell biology, and
might find application for the selection and identification of specific DC/specialized macrophages subsets and their activation status both in vitro and in vivo with potentially relevant clinical applications.
MATERIAL and METHODS

Hematopoietic cells and tissues
Circulating human monocytes (>70% pure as assessed by expression of CD14), polymorphonuclear cells (>95% pure as assessed by CD15 expression) and lymphocytes (>95% pure as assessed by morphology and the lack of CD14) were purified by Percoll (Pharmacia Uppsala, Sweden) gradient centrifugation from leukocyte-rich buffy coats obtained from human blood of healthy donors, as described elsewhere. Monocytes were cultured in RPMI 1640 (2x10^6 cells/well/ml) supplemented with 2mM glutamine and 10% heat-inactivated FCS, and maintained in a humidified atmosphere with 5% CO2 at 37°C for various times with or without indicated stimuli. Immature monocytes derived human dendritic cells (moDC) were obtained in vitro as previously described. Briefly purified monocytes from independent donors were cultured in RPMI 1640 (2x10^6 cells/well) containing 10% heat-inactivated FCS, 2mM glutamine and supplemented with 50 ng/ml recombinant human GM-CSF and 20 ng/ml recombinant human IL-4 (Peprotech, Rocky Hill, NJ) for 5-6 days. To induce maturation, immature moDC were treated for 24 h with 100ng/ml LPS (Escherichia coli serotype 026: B6; Sigma, St. Louis MO) or soluble CD40L (1 µg/ml) and enhancer (1 µg/ml) (Alexis Biochemical, Rome, Italy). Part of the cells were subjected to flow cytometry analysis to assess the activation/maturation status.

Circulating human myeloid and plasmacytoid dendritic cells were isolated by magnetic cell sorting using BDCA-1 and BDCA-4 Dendritic Cell Miltenyi Biotec Isolation Kit (Miltenyi Biotec, Bologna, Italy) respectively. In all the experiments >95% of the isolated cells express the myeloid CD11c or the plasmacytoid CD123 DC specific markers respectively.

Mononuclear cells were isolated from non pathologic bone marrow (provided by the Hematology Section from Verona University) by centrifugation on density gradient (Lymphoprep-Nycomed Pharma AS, Oslo, Norway).

We analyzed surgical specimens from human normal skin (obtained from plastic surgery) thymus (obtained from cardiac surgery) and spleen (obtained from post-traumatic splenectomy), reactive palatine tonsils and reactive lymph nodes (foreign body reaction to oil-based contrast media and mycobacterial lymphadenitis). This work was conducted in accordance with a protocol approved by the Spedali Civili di Brescia Institutional Ethical Board and informed consent was obtained from all patients.

From each specimen, a portion was frozen in liquid nitrogen and then stored at -80°C; the remaining tissue block was formalin-fixed and paraffin-embedded. Cryostat sections were air dried overnight at room temperature and fixed in acetone for 10 min before staining.

Twenty-four patients with inoperable or metastatic pancreatic cancer carcinoma (14 males and 10 females; median age of 68 years; range of 54-85 years) and no prior chemotherapy were enrolled in this study, which was conducted under strict observance of the principles of the Declaration of Helsinki. The diagnosis of pancreatic carcinoma was based on typical radiographic findings at ultrasonography, computed
tomography, endoscopic retrograde cholangiopancreatography, and/or endoscopic ultrasonography. After informed consent, peripheral blood samples were obtained from patients and, for comparison, from 15 age and sex matched healthy donors. RNA was extracted with TRIzol® from frozen (-70°C) tissue specimens derived from a minimum of two spleens (trauma), thymus (cardiac surgery), tonsils (hyperplastic), lymph-nodes (hyperplastic) and bone marrows (from healthy donors). RNA was treated as described for QPCR analysis.

**Preparation of opsonized particles**
Heat-killed Saccaromyces cerevisiae (boiled in a water bath for 30 min.) were incubated with anti-yeast IgG as described, washed twice in PBS and stored at 4°C for 2 to 3 days.

**Cytofluorimetry**
The following antibodies were utilized for the phenotyping of peripheral blood samples and DCs: mouse IgG1 anti-human CD1a, CD3, CD14, CD15, CD20, CD56, CD80, CD83, CD86 FITC labelled and anti-CD16, CD11c and CD123 PE labelled (BD Biosciences Pharmingen, San Diego, CA). Briefly, cells were first blocked with 10% vol/vol normal human serum (Invitrogen, Milan, Italy) for 15 minutes at RT, then stained with mAb for 30 minutes at RT. Cells were also incubated with isotype-matched immunoglobulins (BD Pharmingen) as negative controls. Flow cytometry was performed on a Becton Dickinson FACScan flow cytometer. Analysis of flow cytometry data was performed with FCS Express V3 software (De Novo Software).

**Immunostaining of cells and tissues**
Tissue blocks were cut on a cryostat into 5 µm-thick sections, mounted onto poly-L-Lysine slides, air-dried overnight, post-fixed for 10 min in acetone and stained. Primary antibodies used include: PTPγ - goat polyclonal IgG, C-18 (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:20), CD3 (rabbit; DAKO, Glostrup, Denmark; dilution 1:200), CD11c (LeuM5, Becton Dickinson, 1:10), CD21 (IF8, Bio-optica, Milan, 1:20), CD35 (Ber-MAC-DRC, Dako, 1:20), CD123 (7G3, Pharmingen, San Diego, CA, USA; 1:40), DC-SIGN (Pharmingen, San Diego, CA, 1:5), DC-LAMP (104.G4; Immunotech; 1:100), CD68 (clone PG-M1, DAKO, working dilution 1:40). For immunohistochemical staining, PTPγ was incubated for 2 hours, followed by biotinylated anti-goat secondary antibody (Biogenex) and Streptavidin-HRP (Biogenex) For double immunofluorescence staining, primary antibodies were revealed using Texas red- or FITC-conjugated isotype-specific secondary antibodies (Vector Laboratories, Inc. Burlingame, CA, dilution 1:75 and 1:50 respectively). Sections were examined using an Olympus BX60 fluorescence microscope and objectives with numeric apertures of 0.40 (10 x), 0.70 (20 x), 0.85 (40 x), and 0.90 (60 x), equipped with a DP-70 Olympus digital camera (Olympus, Melville, NY). Images were acquired using analySIS Image Processing software (Soft Imaging System, Münster, Germany).
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For paraffin embedded tissue, the staining was developed using NovoLink™ Polymer Detection System (Novocastra Laboratories Ltd., UK) according to the manufacturer's instructions.

For confocal microscopy analysis, cells were washed in PBS and centrifuged on polylysinated-slides, fixed with 4% paraformaldehyde, and permeabilized in PBS containing 0.2% TWEEN-20. The rabbit polyclonal antibody specific for PTPγ extracellular domain\textsuperscript{27} was used at 10 µg/ml concentration in 0.1% TWEEN-20-PBS and incubated for 2 hours at RT. Other monoclonal antibodies FITC conjugated (anti-CD83 Immunotech Marseille, France and anti-HLA DR BD Biosciences, Milan, Italy) were incubated for 1h at 4°C together with secondary Cy3-conjugated goat anti-rabbit antibody (1:1000 dilution; Amersham, Milan, Italy). After final washes in PBS, preparations were mounted on the anti-fading 1,4-diazabicyclo[2,2,2]octane (Sigma) in PBS containing 50% glycerol. All preparations were viewed with a Zeiss LSM 510 confocal microscope equipped with argon (488 nm) and helium/neon (543 nm) excitation beams. Results were analyzed with MetaMorph software (Universal Imaging Corporation, Downingtown, PA).

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

Total RNA from 5x10\(^6\) cells/point was prepared using TRIzol extraction kit (Invitrogen, Life Technologies, Rockville, USA) according to the manufacturer’s instructions. 1 µg of total RNA was reverse transcribed in a volume of 20 µl using oligo (dT)\textsubscript{20} 1µM and 200 U of SuperScript™ II (Invitrogen, Milan, Italy) at 42°C for 1 h as described by the manufacturer. Polymerase chain reaction (PCR) was performed in a GeneAmp PCR System 9700 (PE Applied Biosystems, Milan, Italy) for 35 cycles (30 sec of denaturation at 94°C, 30 sec of annealing at 60°C and 30 sec of elongation at 72°C) in a volume of 25 µl of reaction buffer containing 0,75 U AmpliTaq (PE Applied Biosystems), 0,4 µM of each primer and 0,2 mM dNTPs (Roche, Milan, Italy). The β-actin mRNA amplification was performed for 22 cycles on the cDNA as positive control of reaction efficiency. The same procedure for RNA extraction and PCR was applied to frozen tissue samples. In this case,10 to 20 cryostate tissue sections (20µm each) were resuspended in Trizol and treated as previously described. The primers used were:

- **ACTB 170F** 5'- ATC AAG ATC ATT GCT CCT CCT G -3'; **ACTB 170R** 5' - GCA ACT AAG TCA TAG TCC GCC -3';
- **PTPRG 958F** 5' - CGT CAC CAG TCT CCT CCT G -3'; **PTPRG 1694R** 5' - GTC TGT CAT GTC GTG GTT CC -3';
- **PTPRJ F** 5' - ATG CCA CCG TTT ATT CCC AAG C -3'; **PTPRJ R** 5' - GAC TCG TTA TCG CTG ACT TTC C -3';
- **PTPRC F** 5' - CTG TCG GTG GGT TTG TTT GAG AGG CAT TA -3'; **PTPRC R** 5' - GAC TCG TTA TCG ACT TTC C -3';
- **PTPRE F** 5' - CCG ACA GCA ACG AGA CAA CC -3'; **PTPRE R** 5' - ATT CCG TTG GGC ATC TTC TTG T -3';
- **PTPRU F** 5' - GCT TGC TGT CCT CAT CCT TCT -3'; **PTPRU R** 5' - CAC CAT ACG CCA GAA GTC ATA G -3'.

**Real-Time Quantitative RT-PCR**

cDNA was analysed for the expression of target genes by the SYBR Green I double-stranded DNA binding dye assay, using a SYBR green PCR master mix (PE Applied Biosystems, Milan, Italy) and tested in a
DNA Engine Opticon 2 Continuous Fluorescence Detector (MJ Research Incorporated). Reactions were denatured for 10 min at 95°C, and then subjected to 50 two-steps amplification cycles with denaturation at 95°C for 15 sec followed by annealing/extension at 60°C for 1 min. Values are expressed as number of copies of specific transcript/β-actin. Cloned βACT and PTPγ cDNA were used as reference for cDNA quantification. The primers used were: ACTB 170F; ACTB 170R (170 bp); PTPγ1 F 5’- GCC TTT ACC GTC ACC CTT ATC -3’; PTPγ1 R 5’- AAA GGT ACT ACT TAT GGG GGC -3’ (171 bp).

Data have been obtained with the software Optical monitor version 2.02.24 (Copyright (C) 2002 MJ research, Inc.) and analyzed with the programs GraphPad Prism® version 4.00 and GraphPad InStat® version 3.05.

**Allogeneic T-cell proliferation assay**

MoDC cells were used in primary MLR to stimulate allogeneic responder T cells. T cells were isolated by negative immunomagnetic depletion of non-adherent PBMC from healthy volunteers using a monoclonal antibody mixture containing anti-CD14, CD19, CD20, CD16 and CD36 (BD Pharmingen) and MACS separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Graded numbers of irradiated (30 Gy) moDC from normal subjects or patients were mixed with allogeneic T cells (2 x 10^5/200 µl) in 96-well U bottom culture plates in RPMI 1640 containing 10% heat-inactivated FCS. To determine DNA synthesis, cells were pulsed with 0.5 µCi of [3H]-TdR (GBq/mmol; Du Pont-New England Nuclear, Wilmington, DE) for the last 5 h of a 5-day culture. Cellular DNA was collected on glass fiber filters, and [3H]-TdR incorporation was measured in a β-counter. Results are expressed as mean counts per minute (cpm) ±SD in triplicates. To measure IFN-γ production, 100 µl of supernatants were harvested and frozen at -70°C.

**IFN-γ detection**

IFN-γ was measured using a commercially available ELISA kit (Bender MedSystems, Vienna, Austria) in cell free-supernatants, collected from primary MLR prior to addition of [3H]-TdR. The minimum detectable dose was less than 1.5 pg/ml. All samples were assayed in duplicate.
RESULTS

PTPγ mRNA expression in hematopoietic cells, tissues and differentiated monocytes.

We quantitatively measured the presence of PTPγ transcript in purified peripheral blood cells from normal donors, where it was readily detected in monocytes (Fig. 1A). The low level of expression in lymphocytes is likely due to residual monocyte contamination (an average 5%) present in the preparation, as judged by FACS analysis with anti CD14 antibody (not shown). A similar amount of transcript was detected in MDC and PDC isolated from peripheral blood, while PTPγ transcript was nearly undetectable in Ficoll purified polymorphonuclear cells (PMN). Of the primary and secondary hemopoietic tissues examined (spleen, thymus, lymph nodes, tonsils and Ficoll-purified bone marrow cells), spleen appeared to express the highest mRNA levels (Fig. 1B). When monocytes were driven to differentiate into macrophages (moMΦ) or moDC by cytokines addition (see material and methods), PTPγ was lost in the former case but maintained, or even increased, when monocytes differentiated to immature moDC, as judged by a combination of markers described in Fig. 3. Maturation induced by bacterial lipopolisaccaride (LPS) and CD40L was associated to variable increase of PTPγ expression (Fig. 1C).

PTPγ expressing cells in spleen and other tissues.

To identify the cell type expressing PTPγ in situ, immunohistochemical and double immunofluorescence staining was performed on frozen and formalin-fixed tissue sections from lymphoid organs and normal skin.

In normal spleen, lymph node and tonsil, PTPγ reactivity was dominantly observed in the germinal center. The vast majority of PTPγ expressing cells were tingible body macrophages represented by large cells with abundant cytoplasm (Fig. 2A) containing phagocytosed debris (not shown). The identity of this cell population was confirmed by double immunofluorescence demonstrating coexpression of CD11c (Fig. 2B) and lack of CD21/CD35 (data not shown), the latter excluding a follicular dendritic cells phenotype. Of note, double immunofluorescence analysis revealed a preferential localization of germinal center T-lymphocytes around PTPγ+ cells (insert in Fig. 2B). PTPγ expression was not restricted to the B-cell compartment: in the spleen, it was observed in macrophages and sinus lining cells of the red pulp (Fig. 2A and supplementary figure S1). In addition, PTPγ positive cells with dendritic morphology were found in the interfollicular area of lymph nodes and tonsils (Fig. 2C), as well as in nodal DC-SIGN+ sinus macrophages (Fig. 2D). No expression of PTPγ was detected in mature interdigitating dendritic cells of the nodal T-cell area as well as in plasmacytoid dendritic cells (data not shown).

The expression of PTPγ was evaluated in normal skin by immunohistochemistry. In addition to a diffuse PTPγ reactivity on epidermal keratinocytes, PTPγ positive cells were found in the upper dermis. These cells were characterized by fusate/dendritic morphology and co-expressed DC-SIGN (Fig. 2E), thus corresponding to dermal DC (29,30). In normal thymus, immunohistochemistry confirmed anti-PTPγ reactivity on sparse cells in the medulla and in the cortex as well (Fig. 2F). Positive cells displayed abundant
cytoplasm and irregular morphology (Fig. 2F-H), but did not co-expressed pancytokeratin thus excluding a thymic epithelial cells identity (not shown). Significantly, some PTPγ+ cells co-expressed DC-SIGN (insert in Fig. 2F); in contrast no reactivity was found on the two major thymic DC subset, namely CD123+ plasmacytoid dendritic cells (Fig. 2G) and DC-LAMP+ mature DC (Fig. 2H).

Altogether, these data indicate that PTPγ expression is retained in primary and secondary lymphoid organs by subsets of dendritic cells and some specialized macrophages.

**PTPγ expression is associated with moDC but not moMΦ differentiation: a key role for IL-4.**

Peripheral blood monocytes can differentiate into macrophages or dendritic cells in vitro and regulation of PTPγ expression during monocyte differentiation along these two pathways was investigated. When monocytes were cultured in vitro in the absence of exogenous cytokines, PTPγ was down-regulated within two hours (not shown) and remained absent in moMΦ obtained after five days culture. Under these experimental conditions monocytes differentiate into large adherent cells that represent macrophagic elements, as determined by morphology and by the expression of specific surface markers (Fig. 3). Activation of moMΦ by various cytokines known to regulate macrophage functions, including IL-1β, TNFα, IL-4, GM-CSF, IFNγ, IL-10, LPS and CD40L did not restore PTPγ expression. Furthermore, since the PTPγ-positive “tingible bodies” macrophages of the germinal center exert phagocytic activity, we tested the hypothesis that phagocytosis could trigger PTPγ expression in in vitro cultured macrophages. However, induction of phagocytosis by yeast-IgG did not lead to PTPγ expression (data not shown).

When monocytes from the same donors were differentiated into DC in the presence of IL-4 and GM-CSF, PTPγ expression was maintained throughout the five day culture. Even though PTPγ expression was rapidly lost in cultured monocytes (within two hours, data not shown), its expression could be restored by the addition of GM-CSF and IL-4 in the medium. GM-CSF alone was ineffective, but if IL-4 was added after three days of culture with the sole GM-CSF, PTPγ transcript was re-expressed (Fig. 3). In this case, the resulting phenotype was mixed, as suggested by the expression of the CD64 antigen. When monocytes were cultured in the absence of cytokine for five days, they acquire the macrophagic phenotype here described. In this case, the addition of IL-4 alone or in combination with GM-CSF did not induce PTPγ expression anymore (data not shown). Altogether these data indicate a critical and differentiation-dependent role for IL-4 in the induction and/or maintenance of PTPγ expression.

**PTPγ co-localize with MHC-II on DC plasmamembrane.**

Confocal microscopy analysis of PTPγ expression was performed on moDC using an antibody, anti P4, that can detect PTPγ only in cells fixed and treated with detergents. It is well known that DC maturation induced by stimuli like LPS is associated with the increase of MHC-II and CD83 expression on the cell surface. Analysis of immature and LPS-mature moDC confirmed the membrane expression of PTPγ and its increased expression following LPS stimulation (Fig. 4). The confocal image also suggests a co-
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localization of PTPγ with MHC-II. The specificity of these results was supported by the observation that CD83, another cell surface marker of mature DC, did not appear to co-localize with PTPγ (Fig. 4).

**Specificity of PTPγ expression in moMΦ and moDCs**

In order to determine the specificity of PTPγ modulation within the RPTPs family members, we evaluated the expression profile of four RPTPs known to be expressed by hematopoietic cells. PTPRC (CD45), PTPRJ (CD148) and PTPRE (PTPε) transcripts were found in both moMΦ and moDC and were down-regulated in mature DC. PTPRU (PTPλ) was not expressed by any cell type, while PTPγ was always absent in moMΦ, but expressed and further induced by LPS treatment in moDC (Fig. 5). These data further indicate that PTPγ is a moDC associated phosphatase and is regulated during differentiation and maturation.

**PTPγ expression in specialized and reactive tissue MΦ**

PTPγ expression was also investigated in tissue macrophages present in the normal lung or recruited at the site of inflammation. Alveolar macrophages appear to strongly express PTPγ in their cytoplasm (Fig. 6A); in contrast, PTPγ expression is not observed in multinucleated cells that accumulate at the site of inflammation, in a case of lymphadenitis caused by foreign body reaction (Fig. 6B). In mycobacteria-induced lymphadenitis, granuloma lesions are mostly composed by a collection of specialized monocyte-derived macrophages called epithelioid cells admixed to variable amount of T-lymphocytes. PTPγ expression was completely absent in all the cells that participate to the granuloma reaction including epithelioid cells (Fig. 6C). These data confirm that the lack of PTPγ expression we observed in *in vitro* differentiated macrophages is also occurring *in vivo*, and that PTPγ expression is a feature of selected macrophage subpopulations.

**PTPγ expression correlates with a tolerogenic phenotype**

We recently demonstrated that in pancreatic cancer patients DC acquire a tolerogenic phenotype that is associated to the reduced capability in inducing an allogeneic response and release of INF-γ. We measured the level of PTPγ expression in moDC derived from sex and age matched controls and subjects affected by advanced pancreatic cancer. The latter shows a reduced capability to induce T cell proliferation (Fig. 7A). Immature moDC induced significantly higher IFN-γ production by T cells compared to immature mo-DC generated from patients (median 1784 pg/ml, range 1028-3425 vs median 1045 pg/ml, range 521-1507, p=0.006, Fig. 7B). This feature is associated to an increased expression of PTPγ (p=0.01, Fig. 7C).
DISCUSSION
PTPγ transcript is detected in a variety of human tissues, including lung, stomach, esophagus, colon, liver, spleen and kidney. Aside from the reported expression in the spleen, the pattern of expression and the nature of the cells expressing PTPγ in the human hematopoietic system is unknown. A role for PTPγ in hematopoietic differentiation was described in a murine model of hematopoiesis using embryonic stem cells. In the present study, PTPγ mRNA expression was readily detectable in peripheral blood monocytes and, at lower level, in circulating myeloid and plasmacytoid dendritic cells. In addition, quantitative measurement of mRNA levels in hemopoietic and lymphoid tissues confirmed the spleen as a major PTPγ-expressing organ followed by tonsil, lymph-nodes, thymus and Ficol purified bone marrow cells. Accordingly, immunohistochemical analysis of tissue sections from lymphoid organs revealed PTPγ protein expression in dendritic cells and macrophages. In particular, PTPγ reactivity was observed in tissue-localized DC with an immature phenotype and in specialized macrophages such as alveolar and sinus macrophages as well as tingible bodies macrophages of the germinal center. In the spleen, PTPγ reactivity was also observed in macrophages and sinus lining cells of the red pulp, a major component of the spleen structure, thus providing a possible explanation for the abundance of PTPγ mRNA found in this organ.

In addition, a subset of PTPγ expressing cells that exhibits DC morphology was observed in the interfollicular area of tonsil and lymph nodes. These cells have been recently characterized as immature DC expressing DC-SIGN. We also found PTPγ+/DC-SIGN+ dendritic cells in the thymus and skin, in agreement with the previously reported identification of a distinct DC-SIGN+ dendritic cell subset in these organs. Given the selective expression of PTPγ in these myeloid cell subsets, we investigated the expression of this phosphatase and its regulation in an in vitro model of monocyte-derived DC (moDC) and macrophages (moMΦ). In vitro cultured moDC express PTPγ transcript and protein, and this expression is increased by maturation induced by LPS, CD40L or TNFα. Circulating PDC and MDC express low levels of PTPγ transcript, whereas tissue PDC, as well as lymph nodes interdigitating mature DC, do not express detectable levels of PTPγ protein. Despite the fact that MDC and PDC may differ in their lineage of origin (reviewed in ), they share PTPγ expression. Therefore it is likely that PTPγ is finely regulated in different DC populations, mostly reflecting their degree of differentiation, function and tissue location as well.

Other than DC, we also observed PTPγ expression in lymph node sinus macrophages. Although these cells are considered macrophages, the sinus conduit system in mice is known to contain both CD11b+/CD11c+ as well as CD11b+CD11c+ mononuclear cells. Interestingly, some authors proposed to consider marginal sinus macrophages “sinus DC” based on the selective expression of the Ki-M9 antigen. Lung alveolar MΦ also express PTPγ. It is of note that PTPγ+ DC coexpressed DC-SIGN, a feature shared by both sinus and alveolar MΦ. This suggests that the “PTPγ+/DC-SIGN+” phenotype may identify specific subsets of tissue macrophages which share functional features with DC in terms of high level of antigen presentation.
Tangible bodies macrophages are CD11c+ myeloid cells poorly characterized in term of function. Their main role in the context of the germinal centre reaction is related to their scavenger function of apoptotic germinal center B-cells (reviewed in 41,42). Here we found that they express PTPγ and physically interact with germinal center T-cells. Taken together these data suggest a more complex role of these cells in the generation of the germinal center reaction.

In contrast, in the interfollicular area, PTPγ+ DC cells can interact and instruct a mixture of lymphoid cells recently immigrated from the peripheral blood through the high endothelial venules. In keeping with these hypotheses, the specific co-localization of PTPγ and MHC-II, in association to its tyrosine phosphatase activity, might suggest its involvement in the regulation of the “immunological synapse” 43,44.

Little is known about factors that modulate PTPγ mRNA expression. Human astrocytoma cells express this phosphatase under the influence of IL-1α/IL-1β and/or TNFα. 17β-Estradiol, which down-regulates PTPγ expression in normal and breast cancer cells, is the only other molecule known to modulate its expression so far 45. Based on the data reported above and on the fact that cytokines represent important factors for myeloid cell differentiation/activation, we attempted to identify in vitro conditions that could modulate PTPγ expression. IL-4 is a critical cytokine that prevents PTPγ down-modulation induced by in vitro culture of monocytes. Under these experimental conditions PTPγ expression is up-regulated by pro-inflammatory stimuli like LPS, CD40L and TNFα. Since we observed that certain specialized macrophage populations express PTPγ in vivo, we wondered whether PTPγ could be induced also in moMΦ in vitro. However, moMΦ failed to express PTPγ in vitro in any of the experimental conditions investigated such as IL-1β, TNFα, IL-4, IFNγ, IL-10 and GM-CSF, alone and in combination. The same cells did not express PTPγ following LPS and CD40L stimulation or after the ingestion of IgG coated yeast particles. This results mimic the absence of PTPγ expression by foreign-bodies reactive MΦ and by epithelioid cells from granulomatous lymphadenitis in vivo, which are assumed to derive from circulating monocytes 46-48.

In addition, it must be considered that PTPγ expression in moDC and moMΦ is unique within the members of the transmembrane PTP family expressed in hemopoietic cells. It also appears to be the only one whose expression is increased following maturation. Altogether PTPγ can be considered as an in vitro marker of the DC differentiation pathway, whereas in vivo its expression is present in a subset of immature DC SIGN+ DC and in certain subsets of specialized macrophages, namely the one localized in lung, in the germinal center and sinus conduit in the lymph node. In parallel with the in vitro data, in vivo inflammatory macrophages do not express PTPγ. Interestingly, IL-4, the key cytokine for the expression of PTPγ in vitro, is also known to increase the expression of DC-SIGN 49. Since PTPγ expression in vivo is restricted to certain subsets of DC and macrophages, it is likely that this protein might play a role in determining the functional heterogeneity that characterize these...
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populations. In keeping with this hypothesis, the results of our investigation strongly suggest that pancreatic carcinoma-associated cytokines lead to the development of a predominant tolerogenic DC2 phenotype, characterized by low expression levels of co-stimulatory molecules and poor T cell allostimulatory capacity. These features associate with higher PTPγ expression (this manuscript). DC capability to induce tolerance has been demonstrated in both immature and mature DCs. Therefore the evidence that PTPγ expression i) is observed in immature DC in vitro and in vivo, ii) is induced by LPS and CD40L in moDCs and iii) characterize a subset of tolerogenic mo-DC in cancer patients, are all in keeping with the possibility that PTPγ levels, other that its presence or absence, could represent a crucial event that define or modulate specific DC and other myeloid cells function.

The future identification of PTPγ ligand and the better understanding of the role of this protein in cell function will contribute to improve our understanding about the biological role of DC and macrophage subpopulations.

Aknowledgements: The authors thank Brian Weaver (Department of Pathology and Immunology, Washington University, St. Louis, MO) and Martin Pelletier (Department of Pathology, University of Verona) for the careful review of the manuscript.

Obituary: This article is dedicated to D.L., a serious scientist who, despite troubles, never lost his interest in life science, and a deeply caring person with an irrepressible sense of humor.
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Figure 1
Figure 2
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Figure 3
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Figure 4
Figure 5
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Figure 6

A

B

C

Figure 7
FIGURE LEGEND:

Figure 1: Expression of PTPγ in peripheral blood, hematopoietic tissues and differentiated monocytes.
Number of copies of PTPRG mRNA/10^6 copies of ACTB estimated by QPCR analysis in peripheral blood cells of individual donors (monocytes: n=8; PBL: n=6; PMN: n=5 ; myeloid DC: n=4 ; plasmacytoid DC: n=4) (A) and in primary and secondary hematopoietic tissues (spleen: n=3 subjects; thymus: n=2 subjects; tonsil: n=2 subjects; lymph nodes: n=3 subjects; bone marrow: n=8 subjects) (B). QPCR analysis of PTPγ expression in monocytes (n=8 subjects) induced to differentiate to macrophages (moMΦ: n=5 subjects) or dendritic cells (moDC: n=9 subjects) in the presence or absence of LPS (n=7 subjects) and CD40L (n=9 subjects) (C). Statistically meaningful p values are indicated for A and C (calculated with unpaired t test with Welch correction). The graphs are box-plots in which median, 25th, 75th percentiles, the minimum and the maximum values are presented as vertical boxes and lines.

Figure 2: PTPγ expression in lymphoid organs and skin.
Sections are obtained from spleen (A), human tonsil (B), lymph node (C and D), skin (E) and thymus (F-H).
In the spleen, PTPγ stains tingible body macrophages of a B-cell follicle, as well as macrophages (white arrowhead) and sinus lining cells (black arrowheads) in the red pulp (A; Gc: germinal centre; Mz: marginal zone). Double immunofluorescence staining shows that all PTPγ found in B-cell follicle co-expressed CD11c on the plasma membrane (B) and some of them clusterize with CD3+ germinal centre T-cells (insert in B). PTPγ cells with dendritic morphology are obvious in the interfollicular area (IF), the latter highlighted by the presence of high endothelial venules (black arrow head) (C) and in the large majority of DC-SIGN+ sinus macrophages (D, yellow cells).
In normal skin, PTPγ positivity is diffuse in the epidermis; in the dermis, scattered PTPγ+ cells are observed and the majority of them show an irregular morphology and co-express DC-SIGN (E, yellow cells).
In normal thymus, scattered PTPγ+ cells are observed in the cortex (Tc) and in the medulla (Tm) (F), the latter highlighted by the presence of Hassal’s bodies (arrow head); no reactivity is observed on the majority of thymocytes (F-H). PTPγ cells are irregular in morphology, show abundant cytoplasm (F-H) and some of them coexpress DC-SIGN (insert in F). Absence of PTPγ positivity is observed on CD123+ plasmacytoid dendritic cells (G) and in DC-LAMP+ mature dendritic cells (H) populating the thymic medulla.
Indirect immunoperoxidase technique was applied and nuclei were counterstained with hematoxilin (A, C and F). Double immunofluorescence was performed using FITC and Texas-red conjugated secondary antibodies as labeled in the panels (B, D, E, G and H).
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Figure 3: PTPγ expression is differentially modulated by IL4 and GM-CSF

IL-4 and GM-CSF were added either individually or in combination at the beginning of the incubation. In one case IL-4 has been added after three days of GM-CSF treatment (†). Cytocentrifuged cells were stained with May-Grunwald. Large cells with large, vacuolated cytoplasm are shown in the absence of cytokines. These cells do not express PTPγ and are characterized by a macrophage phenotype (CD14+, CD64+, CD1a-, CD80-). PTPγ starts to be expressed only when IL-4 is added, but in the absence of GM-CSF the number of cells obtained in culture is decreased and the phenotype is macrophagic. When both cytokines are added, PTPγ expression is present and both the morphology and surface markers shift to a typical DC phenotype (CD14-, CD64-, CD1a+, CD80+). In the case IL-4 addition is delayed (†), the phenotype is intermediate, as CD64 is still expressed and the cytoplasmatic processes typical of DCs are much less apparent. ACTB expression is shown to demonstrate the specificity of the signal (one experiment representative of three is shown).

Figure 4: PTPγ expression is increased by LPS and appear to co-localize with MHC II on DCs plasma membrane.

moDC were left untreated (upper panels) or treated with LPS for 24h (lower panels) and then stained with control antibody (Rabbit (Rbt) IgG), anti-PTPγ affinity purified polyclonal antibody P4, followed by anti Rbt IgG-Cy3 (red), FITC conjugated anti MHC-II and anti CD83 antibodies (green). Inserts show the double staining of PTPγ expressing cells (red) with MHC-II (third column) and CD83 (last column) both in green. PTPγ, MHC-II and CD83 signals all increase at the cell surface upon LPS treatment. MHC-II and especially CD83 also appear as bright, green spot in a perinuclear localization. Yellow colour indicates co-localization of the selected proteins that is particularly evident at the surface of the cells (insert, third column from left, lower panel) corresponding to LPS-treated moDC stained with anti MHC II and PTPγ antibodies.

Figure 5: PTPγ regulation is unique among other RPTPs.

Expression of PTPγ mRNA in comparison to a selection of RPTPs in resting and activated moMΦ and moDC. Lane 1: marker; lane 2: resting macrophages; lane 3: macrophages activated with LPS 100 ng/ml for 24 hours; lane 4: resting DC; lane 5: DC activated with LPS 100 ng/ml for 24 hours. The genes analyzed are indicated on the left side of the picture.

Figure 6: Variability of PTPγ expression in tissue macrophages.

Sections were obtained from normal lung (A), a case of foreign-body reaction to oil-based contrast media (B) and a case of mycobacterial lymphadenitis (C).

In normal lung, strong PTPγ reactivity is observed in the cytoplasm of alveolar macrophages, some of them showing obvious anthracosis (arrow heads). In nodal foreign-body reaction, numerous PTPγ sinus
macrophages are present; no PTPγ reactivity is observed in numerous multinucleated giant cells surrounding lipidic drops (B, arrow heads). In mycobacterial lymphadenitis, only rare PTPγ+ cells are found outside the granuloma and no staining is noticeable in epithelioid macrophages (C, star). Indirect immunoperoxidase technique was applied and nuclei were counterstained with hematoxilin.

Figure 7: The tolerogenic phenotype of pancreatic cancer patients-derived moDC is associated with decreased PTPγ expression

A: Allostimulatory function (MLR) of immature moDC generated from advanced or metastatic pancreatic carcinoma patients (n=24, black circles) vs control immature moDC (n=15, empty circles). Results are expressed as means ±SD of [3H-TdR] uptake after 5 days of MLR culture in triplicate and show a reduced capability to stimulate T cell proliferation by the former. CPM for T cells alone are 3963±984.

B: IFN-γ production by allogeneic T cells stimulated by immature moDC from normal donors (n=15) and advanced or metastatic pancreatic carcinoma patients (n=15). Cytokine levels were detected by ELISA in cell free-supernatants collected from primary MLR prior to addition of 3H-TdR.

C: PTPγ expression is increased in metastatic pancreatic carcinoma patients (n=9) in comparison with normal donors (n=11).

For B and C, median, 25th, 75th percentiles, the minimum and the maximum values are presented as vertical boxes and lines.
Receptor type tyrosine phosphatase gamma (PTPγ), a new identifier for myeloid dendritic cells and specialized macrophages

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