Effects of Prostaglandin E_2 on Dendritic Cell functions

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Table of contents

CHAPTER 1

Introduction

Dendritic cells and Immunity

Dendritic cells (DCs) represent a unique but diverse population of cells that are characterized as professional antigen-presenting cells (APC) by their unparalleled ability to sense and capture antigen, and to transfer this information to the adaptive immune system. Therefore, DCs possess the inimitable potential to induce primary immune responses. The induction and maintenance of tolerance is also controlled by DCs as well as the type and magnitude of an adaptive immune response (Banchereau 2000).

Dendritic cells and the Induction of an Immune response

DCs are a heterogeneous population of leukocytes distributed in tissues, which are in contact with the external environment, such as skin, gut and lung (Nestle 1993, Nelson 1994, Sertl 1986). DCs can be found in three developmental stages: precursors, immature and mature DCs (Shortman 2002). Production of DC precursors takes place in the bone marrow in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) and *fms*-like tyrosine kinase-3 ligand (Flt3L) (Karsunky 2003, D'Amico 2003). After leaving the bone marrow, DC precursors can be identified in human blood as HLA-DR^+ mononuclear cells that are negative for other lineage markers like CD3, CD14, CD19, or CD56 (Banchereau 2000). Immature DCs and DC precursors can be divided into a myeloid and a plasmacytoid lineage regarding to their expression of CD11c and interleukin (IL)-3 receptor α chain (CD123). Whereas myeloid DCs express CD11c but only low levels of CD123, plasmacytoid DCs do not express CD11c but high levels of CD123. Plasmacytoid DCs are located primarily in the blood and in T cell areas of lymphoid tissues, where they mediate anti-viral immune responses (Kadowaki 2002, Fonteneau 2003). Myeloid DCs, however, are found in many tissues, where they are classified according to their distribution (Ardavin 2003, Shortman 2002). Thus, Langerhans cells, expressing the C-type lectin Langerin, are located in the epidermis and oral, respiratory, and genital mucosa. Other myeloid DCs are named according to their anatomic localization interstitial, dermal, or submucosal DCs (O'Neill 2004).

DCs are strategically positioned to perform a sentinel function for incoming pathogens. After encounter of antigen DCs can not only activate the antigen-non-specific innate immune system (Fernandez 1999, Foti 1999, Zitvogel 2002), but represent the link to the antigen-specific adaptive immune system (Banchereau 2000). apture of antigen by immature DCs

results in the transition to a mature phenotype, which includes migratory responsiveness to lymph node-derived chemokines. After migration into T cell areas of lymph nodes antigen-presenting mature DCs can prime antigen-specific T cells.

Stable interactions between DCs and T cells lead to the establishment of an immunological synapse (Grakoui 1999), which is essential for T cell priming (Hugues 2004). The immunological synapse is formed by clustering of the T cell receptor (TCR) and CD28 interacting with peptide-bound MHC complexes and the co-stimulatory molecules CD86 or CD80 expressed on mature DCs (Bromley 2001, Lenschow 1996). Although CD28 engagement provides a strong and sufficient signal leading to enhanced expression of transcription factors, cytokines and cytokine receptors, which promote differentiation, effector T cell functions and survival, various receptors of the TNF superfamily exert additional co-stimulatory functions. The expression of those receptors, such as OX40, CD27 and 4-1BB, and the availability of their respective ligands on APCs can reinforce and modulate the outcome of an immune response by promotion of survival and differentiation signals (Watts 2005). Effective priming of T cells results in clonal expansion and differentiation into cytokine-secreting effector T cells and long-lasting memory T cells. The kind and strength of the T cell response is dependent on the affinity of the TCR for the presented peptide, the concentration of presented antigen, and on the state of DC maturation and the type of maturation stimulus (Gett 2003)

Dendritic cell Maturation

Immature DCs are specialized for the capture and processing of antigen, a procedure that results in terminal differentiation into mature DCs specialized for antigen-presentation and T cell stimulation. During the process of maturation, DCs up-regulate co-stimulatory molecules; change their cytokine- and chemokine-secretion profile as well as their own responsiveness to chemokines by alteration of their surface receptor repertoire, enabling migration to secondary lymphoid organs.

Immature DCs can take up components of pathogens, dead or dying cells, proteins or immune complexes via phagocytosis, endocytosis, pinocytosis, and specific receptors, while the type of stimulus determines the maturation program and the subsequent immune response (Guermonprez 2002). Conserved molecular patterns from bacteria and viruses as well as components of damaged cells can bind to Toll-like receptors (TLRs) on DCs, which are highly conserved transmembrane receptors transmitting maturation signals (Medzhitov 1997, Kirk 2005). Other receptors expressed on the surface of DCs also facilitate the internalization and presentation of antigens. Heat shock proteins (hsp) like hsp70 and gp96 can bind to specific hsp receptors (Kuppner 2001, Singh-Jasuja 2000), whereas receptors for the Fc domain of immunoglobulins can bind antibody-opsonized particles. Furthermore, C-

type lectin receptors such as DEC205 (CD205) or the mannose receptor mediate up-take of antigen and subsequently induce the process of maturation (Jiang 1995, Sallusto 1995).
In addition, maturation can be induced by signals provided by T cells, such as binding of the trimeric TNF-like molecule CD40L (CD154) mainly expressed on activated $CD4^+$ T lymphocytes (van Kooten 2000, Roy 1993) to its receptor CD40 on DCs (Caux 1994, Cella 1996). For *in vitro* studies and in clinical DC-based vaccination trials, DCs are most commonly matured using a combination of inflammatory cytokines including TNF-α, IL-1ß, IL-6, and PGE_2 (Jonuleit 1997). Overall, the balance between pro-inflammatory and anti-inflammatory signals in the local microenvironment can influence *in vivo* the outcome of maturation (Banchereau 2000, Kalinski 1998).
Captured antigens can be processed and loaded onto major histocompatibility complex (MHC) class I or II according to their origin. Antigens derived from the cytosolic compartment are presented by MHC I molecules, whereas internalized antigens derived from the extracellular environment are typically presented on MHC II molecules. However, exogenously acquired antigens can be processed onto MHC I molecules by a process termed cross-presentation, a unique feature of DCs (Rossi 2005). Maturation results in increased levels and stability of MHC I and MHC II molecules presenting peptides on the surface of DCs, which allows enhanced presentation of peptides derived from pathogenic sources (Cella 1997, Rescigno 1998, Trombetta 2005).
Interactions between peptide-bound MHC molecules and antigen-specific TCRs of T lymphocytes are not sufficient to induce T cell activation. Additional co-stimulatory signals are necessary to lower the threshold for activation of T cells and provide stimulatory signals for proliferation and survival. Initial activation is dependent on binding of T cell expressed CD28 to its ligands CD80 and CD86. Maturation endows DCs with high surface levels of CD80 and CD86, thus enabling the provision of a strong co-stimulatory signal after engagement of T cells. Additional co-stimulatory molecules, which belong to the TNF superfamily, provide regulatory signals especially for T cell survival, shaping the outcome of the immune response (Watts 2005).
During maturation the chemokine receptor repertoire expressed on the surface of DCs is dramatically altered, thereby regulating the trafficking of DC inside inflamed tissues and the migration to lymphoid organs. At sites of infection DCs produce inflammatory chemokines, such as CCL3 and CCL4, early after antigen up-take to recruit immature DCs as well as other cells of the innate immune system, while simultaneously down-regulating the respective receptors on their own surfaces. In the course of proceeding maturation, expression of the chemokine receptor CCR7 is induced, and DCs become responsive to its ligands CCL21 and CCL19 (Sallusto 1998, Sallusto 1999, Sozzani 1997), thus enabling directional migration of mature, antigen-bearing DCs to secondary lymphoid organs.

Migration of mature Dendritic cells

Migration of DCs is largely directed by chemokine gradients between the periphery and secondary lymphoid organs such as lymph nodes, spleen and mucosal lymphoid tissues. For the induction of an adaptive immune response mature DCs must migrated from peripheral tissues, where they encountered antigen, into specialized compartments of lymphoid structures. Because of the immense repertoire of antigen-specific T lymphocytes, the probability that one T cell clone specific for a particular antigen would meet its antigen presented on DCs is infinitely small, if both cell types were circulating undirected through the blood-lymph system. The directed migration of DCs into T cell areas of lymphoid organs ensures a high contact rate with circulating naïve T cells and enables the screening of the whole T cell repertoire for a specific TCR.

The chemokines receptor CCR7 controls migration of DCs into afferent lymphatic vessels as well as the positioning of DCs within lymphoid organs (Weninger 2003). Adoptively transferred CCR7-deficient DCs failed to migrate to the draining lymph node of wild-type mice, emphasizing the significance of CCR7 expression on DCs (MartIn-Fontecha 2003). Maturation of DCs induces CCR7 surface expression and enables DCs to transport captured antigen to secondary lymphoid organs to induce adaptive immunity (Weninger 2003). Two ligands have been described for CCR7, CCL19 and CCL21, which are secreted by lymphatic endothelium, and by stromal cells within lymph nodes (Gunn 2003, Randolph 2005).

The chemokine receptor CXCR4 is expressed on DCs as well as on T and B lymphocytes (Bleul 1998). Only one ligands has been described for CXCR4, termed CXCL12 (Oberlin 1996), which is among others expressed constitutively by lymphatic tissues. Engagement of CXCL12 to CXCR4 plays a crucial role for migration of cutaneous DCs to lymphoid organs, as CXCR4 antagonists impair the initiation of skin immune responses (Kabashima 2007a). Ligation of CCR7 and CXCR4 by their respective ligands may not only function to provide chemoattractive signals, since they have been recently also implied in modulation of DC functions like maturation, cytoarchitecture and survival (Sanchez-Sanchez 2006, Kabashima 2007b).

Chemokine receptors are seven-transmembrane receptors with seven helical membrane-spanning regions connected by extramembranous loops in such a way that the C-terminus is in the cytoplasm. Chemokine receptors transmit signal through heterotrimeric Gαßγ proteins, which are bound to the intracellular loops of the receptor. In the inactive state, the Gα subunit is bound to guanosine 5'-diphosphate (GDP). Ligation of the chemokine induces a conformational change of the receptor resulting in dissociation of GDP from Gα, which is replaced by guanosine 5'-triphosphate (GTP). The activated GTP-bound Gα subunit dissociates from the receptor and the Gßγ complex. Both, Gα and Gßγ activate a variety of

downstream effectors, which ultimately lead to migration and other physiological responses (Allen 2007).

Dendritic cells in the Modulation of Immune responses and Maintenance of Peripheral Tolerance

DC-mediated priming of T cells requires a combination of specific surface and secreted molecules, all of which acquired by DCs during efficient maturation. The maturation stimulus, however, determines the kind of T cell response that is promoted. By secretion of certain cytokines, mature DCs can induce different types of $CD4^+$ T helper (Th) cells: Th1, Th2, or Th17. Th1 differentiation is promoted by type 1 cytokines like IL-2, IL-12, IL-15 and IFN-γ and facilitates cell-mediated immunity to clear intracellular pathogens. Type 2 cytokines, on the other hand, comprising IL-4, IL-10 and IL-13, induce Th2 responses facilitating humoral immunity involved in clearance of parasites. Secretion of IL-23 favours differentiation into Th17 cells, which produce IL-17 and are associated with inflammation (Weaver 2007). The cytokine production profile of mature DCs promotes differentiation of $CD4^+$ T cells and thereby shapes the resulting immune response.

DCs have been implied in the functional control of regulatory T cells (Tregs). T regs are specialized T cells involved in maintenance of peripheral tolerance to self-antigens by inhibition of $CD4^+$ and $CD8^+$ T cell responses (Sakaguchi 2000). Under steady state conditions, immature DCs can promote tolerance by induction of Tregs both *in vitro* (Roncarolo 2001) and *in vivo* (Bonifaz 2002). Also, IL-10 producing immature MoDCs have been described to be able to induce Tregs (Jonuleit 2000).

CTLA-4 mediated signals provided by Tregs can condition DCs to produce active indoleamine 2,3-dioxygenase (IDO) (Fallarino 2003). IDO is an immunosuppressive enzyme that degrades the essential amino acid tryptophan generating toxic metabolites. By depletion of tryptophan in the microenvironment, production of IDO by DCs can control and limit T cell proliferation (Munn 1998, Terness 2002).

Dendritic cell-based Cancer Vaccination

The unique features to orchestrate innate and adaptive immune responses, to regulate tolerance, and to yield long-lived protective T cell memory make DCs attractive candidates for cancer vaccination. Immunotherapeutic strategies against cancer have the potential to specifically target and reject tumor tissue without damaging the rest of the body, a side effect chemotherapeutic approaches can not avoid.

Strategies of Immunotherapies

Immunological approaches to cancer treatment are to date mainly available in form of monoclonal antibodies. Antibodies can not only block critical functions of tumor cells by specific binding, but can also mediate binding of Fc receptor-bearing cells like phagocytes and NK cells, which attack antibody-bound cells and particles. The improvement of technologies to produce pharmaceutical-grade monoclonal antibodies and the identification of a steadily growing number of tumor-specific antigens has led to the development of therapeutic antibodies (Blattman 2004). Breast cancer and lymphoma patients are currently treated with therapeutic antibodies against Her2/neu or CD20, respectively (von Mehren 2003). As an alternative immunological approach that is based on cell-mediated immunity, adoptive T cell therapy has shown great potential to promote regression of established tumors (Morgan 2006, June 2007). However, neither antibody nor adoptive T cell therapy are likely to induce long-lived protective T cell memory (Palucka 2007).

Due to the potential of DCs to induce and modulate immune responses, DC-based vaccines on the contrary have the potential to induce both tumor-specific effector and memory T cell responses. Two approaches were designed to use DCs as vaccines, either *in vivo* targeting or *ex vivo* generation of DCs (Ueno 2007). *In vivo* targeting can be achieved by application of anti-DC antibodies fused to antigens, whereas the administration of an antigen mix, like MHC I peptides, together with adjuvant is more random and might not be very efficient. By using *in vivo* targeting of DCs it is impossible to control what kind of DC subset acquires the antigen, which could have negative impact on the desired outcome, as for example certain DC populations might preferentially induce tolerance (Palucka 2007). In contrast, *ex vivo* generated DC from bone marrow progenitors or blood precursors can be characterized in detail and manipulated to possess certain characteristics and induce the desired effects.

Tumor Therapy using Dendritic cells

A promising field has emerged in the setting of cancer immunotherapy by generation of human DCs from progenitors *ex vivo*. These DCs can be loaded with selected tumor-specific antigens, and be re-injected into the patient to boost immunity in an antigen-specific manner

(Fong 2000, Nestle 2001, Steinman 2001, Schuler 2002). Generation of DCs *ex vivo* provides the opportunity to optimize antigen-loading and manipulate the maturation status to achieve optimal immune responses (Steinman 2002). Among the desired characteristics of DCs for therapeutic usage is the ability to resist tumor-derived factors like IL-10, TGF-ß or VEGF, which are produced in advanced cancer to suppress DC functions (Rabinovich 2007). Currently, multiple choices of DC subsets, the type of antigen and the way to deliver it, as well as the routes and frequencies of injection are under investigation to optimize DC immunotherapy.

The first trials, in which healthy volunteers were vaccinated with *ex vivo* generated DCs pulsed with tetanus toxoid, influenza matrix peptide, or keyhole limpet hemocyanin, proved to induce antigen-specific immune responses with minimal side effects (Dhodapkar 1999, Davis 2003). Vaccination studies against a variety of advanced cancers followed, using tumor antigen-loaded DCs that were either isolated from blood or generated *ex vivo* from blood precursors, and showed induction of specific immune responses, which in some cases correlated with clinical responses (Schuler 2003). However, early trials focused mainly on the safety and immunogenicity of DC vaccines as a proof of principle (O'Neill 2004), whereas current trials monitor clinical responses and evaluate optimization.

Several procedures have been established to *ex vivo* generate the vast numbers of DCs that are needed for vaccination. $CD34^+$ haematopoietic progenitor cells can be differentiated under addition of GM-CSF and TNF-α into a mixture of interstitial DCs and Langerhans cells (Banchereau 2001). The frequency of DC progenitors can be increased by administration of Flt3L. In another approach, plasmacytoid and myeloid DCs can be isolated directly from leukapheresis products, which does not yield huge numbers, but can be improved again by stimulating patients with Flt3L (Fong 2001a). The most common approach, however, to generate large amounts of human DCs is the generation from peripheral blood monocytes (MoDCs) (Thurner 1999). By culturing monocytes in the presence of IL-4 and GM-CSF, they differentiate to immature, non-proliferation DCs, which express low levels of CD86 and MHCII (Sallusto 1994). The use of IL-4 can thereby be substituted by IL-13. The transition of monocytes to DCs can also occur *in vivo* (Ginhoux 2006, Zhang 2007). A mature phenotype can be induced in MoDCs by stimulation with inflammatory products resulting in high expression levels of MHCII and co-stimulatory molecules (Sallusto 1994).

A major parameter that needs to be considered is indeed the maturation status of DCs used for vaccination. Under steady state conditions, immature DCs induce and maintain tolerance (Steinman 2003). In fact, injection of antigen-pulsed immature DCs leads to the induction of regulatory T cells and inhibition of IFN-γ-producing T cells (Dhodapkar 2002, Dhodapkar 2001), whereas mature DCs loaded with antigen show enhanced immunogenicity and induce antigen-specific CTL responses (Jonuleit 2001, Slingluff 2003). In tumor therapy, DC

activation should be induced and optimized to generate mature DCs with the capacity to induce high-avidity CTLs in coordination with strong helper activity to elicit protective long-time memory responses (Berzofsky 2004). Maturation of MoDCs is frequently induced by addition of a combination of cytokines including TNF-α, IL-1ß, IL-6, and PGE_2 (Jonuleit 1997). Although MoDCs matured in such a way were reported to be unable to produce bioactive IL-12p70 (Kalinski 2001), they nevertheless induce Th1 and $CD8^+$ T cell responses (Lee 2002a, O'Neill 2004, Dhodapkar 1999).

A dominant issue in the setup of DC vaccination protocols is the choice of tumor antigens and the way of delivery. The target antigens have to be chosen carefully; preferentially antigens that are critical for tumor growth, since a directed immune responses will drive selection for tumor cells that lost expression of the target antigen and are thereby escaping immune depletion (O'Neill 2004). Antigens can be supplied to DCs in a variety of ways, including as peptides, whole proteins, or tumor lysates, as viral vectors or RNA (O'Neill 2004). The use of MHC-restricted peptides is disadvantageous, as the half-life of peptide: MHC complexes is short, and the selected peptide antigen has to be compatible with the patient's specific HLA type. On the contrary, application of whole protein enables the processing of different epitopes from the antigen's entire sequence (O'Neill 2004). Pulsing DCs with killed tumor cells, however, will additionally not only result in presentation of epitopes on MHC II, but also on MHC I molecules due to the special ability of DCs to cross-present antigens. This unique feature allows the development of specific CTLs through cross-priming and provides specific $CD4^+$ T cell help via antigen presentation on MHC II (Palucka 2007).

The route of administration of *ex vivo* generated DCs into the patient is a widely investigated point in DC vaccination, but the optimal procedure has not yet been established. DCs have been injected subcutaneously, intradermally, intravenously, intranodally, or directly into the tumor (Palucka 2007). Since the route of administration may affect the nature of T cell priming (Mullins 2003, Fong 2001b) injection of DCs into the lymph node may not be the best way to deliver the antigen-loaded DCs. It has been additionally suggested, that injection of large numbers of DCs into lymph nodes prevents coordinated positioning in lymphoid micro-structures, and therefore results in poor immune responses (Lesimple 2006).

Injections of DCs into skin require migration to the draining lymph node, but could be beneficial to induce immunity to cutaneous tumors (Mullins 2003). Migration of DCs from the site of antigen contact to lymphoid tissues is a crucial issue to induce anti-tumor responses. In early vaccination trials, *ex vivo* generated DCs did not leave the injection site due to their immobile phenotype (Morse 1999a). This problem was solved, when two studies reported that PGE_2 is essential during maturation to promote DC's ability to migrate (Scandella 2002, Luft 2002). Since for the generation of DCs for vaccination purposes, DCs have to be

cultivated and matured under serum-free conditions, addition of PGE_2 is required during maturation for the induction of a migratory phenotype.
The modulation of DC functions, like antigen capture and processing, maturation, and migration will allow us to improve the efficacy of DC-based immunotherapy. General studies on behaviour and manipulation of *ex vivo* generated DCs are necessary to understand the subtle balances of receptors and soluble mediators that will induce effective therapeutic immunity against cancer.

Prostaglandin E_2 in Dendritic cell Biology

Prostanoids belong to the class of biologically active small lipid molecules termed eicosanoids, which are products of the arachidonic acid metabolism. Prostaglandin E_2 (PGE_2), probably the most important mediator in the family of prostanoids, has multiple effects on immune cells and especially on dendritic cell functions (Gualde 2004). PGE_2 is used in combination with pro-inflammatory cytokines in DC-based vaccination protocols to generate mature DCs capable of inducing tumor-specific immune responses.

Prostanoids in Inflammation

Eicosanoids, comprising prostanoids and leukotrienes, are lipid mediators derived from C20 unsaturated fatty acids with a wide range of physiological functions by participation in cell-cell communications as in the central nervous system, and in inflammation and immunity (Serhan 1996). Various physiological and pathological stimuli can promote the production of prostanoids by cells of the innate immune system like macrophages, DCs and neutrophils. Prostanoids can be generated from arachidonic acids, which are released from membrane phospholipids by phospholipase A_2 in response to pro-inflammatory signals. Arachidonic acids can be converted by cyclooxygenase (COX) enzymes resulting in production of PGH_2, from which a series of prostaglandins (PG) can be generated (Harris 2002). Two isoforms have been described for the COX enzyme, COX-1 and COX-2. The constitutive form, COX-1, is produced by a variety of cell types, and is involved in maintenance of physiological homeostatic processes. (Smith 1995, Langenbach 1999, O'Banion 1999) COX-2 on the other hand is inducible by inflammatory stimuli like TNF-α, IL-1 and LPS, and promotes the production of large amounts of prostaglandins during inflammation (Ristimaki 1994, Yamamoto 1995, Norgauer 2003). Anti-inflammatory signals like IL-4 and IL-10 counteract prostaglandin production inhibiting COX-2 induction (Harizi 2005, Harizi 2004). Tissue-specific prostaglandin synthases convert PGH_2 into PGE_2, PGD_2 and other prostaglandins. The balanced expression of these enzymes can determine the profile of prostanoid

production. PGE_2 synthase has been reported to be inducible by pro-inflammatory stimuli (Filion 2001), and mouse bone marrow-derived DCs produce PGE_2 but not PGD_2 (Harizi 2004). PGD_2 in contrast is mainly produced by mast cells and exerts anti-inflammatory effects. In the process of wound healing, a shift of prostaglandin synthesis from PGE_2 during the acute inflammatory phase to dominant production of PGD_2 during the repair phase has been described recently (Kapoor 2007) and highlights the dynamic processes balancing immune responses.

Prostanoids are not stored within cells, but are immediately secreted after production. As prostanoids are chemically and metabolically instable, they act locally in the proximate microenvironment of their production. Therefore, prostanoids can act in an autocrine fashion, or paracrinely stimulate neighbouring cells (Narumiya 2003). The ability of cells to respond to the differential prostaglandin profile in their microenvironment is dependent on the expression profile of respective prostaglandin receptors. Whereas the production of prostaglandins is mainly restricted to cells of inflammation, receptors for prostaglandins are expressed on cells both of the innate and adaptive immune system. Signals mediated by prostaglandin receptors participate in the regulation of immune cell functions such as proliferation, migration and cytokine production providing a link between innate and adaptive immunity (Harizi 2005).

Prostaglandin E_2 Receptors in Immune cells

PGE_2 exerts its effects by binding to a group of G protein coupled receptors (GPCRs) with seven transmembrane domains, designated EP1-4, with different signal transduction pathways (Coleman 1994, Negishi 1995, Ichikawa 1996). Signaling through EP1 mediates elevation of free Ca^{2+} by a yet unidentified G protein. In mouse, EP3 exists in three alternatively spiced forms, which signal primarily via $G\alpha i$ and inhibition of adenylate cyclase (Sugimoto 2007). EP2 and EP4 are coupled to $G\alpha s$ and signal by stimulation of adenylate cyclase, which increases intracellular levels of cyclic adenosine monophosphate (cAMP) (Coleman 1995, Katsuyamo 1995).

The generation of mice with targeted deletion of each individual PGE_2 receptor provided new insights on the complex actions of PGE_2 in the regulation of immune responses. In the skin, PGE_2 is produced in substantial amounts during inflammation. Although Langerhans cells express all four PGE_2 receptor subtypes, migration to draining lymph nodes was only impaired in $EP4^{-/-}$ mice. PGE_2-mediated signals via EP4 are not only essential for migration of Langerhans cells, but also increased the expression of co-stimulatory molecules enhancing T cell stimulatory capacities (Kabashima 2003). Thus, EP4 receptor signaling promotes maturation and migration of Langerhans cells in mice and consequently facilitates initiation of skin immune responses.

Like murine Langerhans cells, resting bone marrow-derived DCs express all EP receptors on the surface, but stimulation with LPS dose-dependently up-regulates EP2 and EP4 expression, whereas EP1 and EP3 expression is not altered (Harizi 2003). Human MoDCs in contrast express only EP2 and EP4 (Baratelli 2004, Braun 2005), while the level of expression is regulated during maturation towards high expression of EP4 (Braun 2005, Scandella 2002).

Prostaglandin E_2 and Dendritic cell functions

Soluble mediators like cytokines, nitric oxide and prostanoids are involved in the inflammatory responses that drive DC maturation. Among prostanoids, PGE_2 has been most extensively studied for its regulatory effects on DCs. A lot of attention was attracted to PGE_2 when it was identified as the key regulator for DC migration (Scandella 2002, Luft 2002). PGE_2 is used in combination with pro-inflammatory cytokines to induce maturation of human DCs for vaccination purposes (Jonuleit 1997, Lee 2002a). It was in this setup, that PGE_2 was discovered to be a crucial factor for human DC migration in response to CCR7 ligands, as DC matured in the absence of PGE_2 were almost unresponsive to CCR7 ligands (Scandella 2002, Luft 2002). However, PGE_2-mediated signals are not required for migratory responsiveness of immature DCs to inflammatory chemokines like CCL3 (Chen 2004). Thus, there is evidence that migratory capacities of DCs are controlled by their status of activation. CCR7 expression is not sufficient for migration of mature DCs; migration towards CCR7 ligands reli on additional yet unidentified signals provided by PGE_2.

During maturation, DCs change their chemokine secretion profile as well as their responsiveness to chemokines (Sallusto 1998). The presence of PGE_2 during maturation inhibits expression of the pro-inflammatory chemokines CCL3 and CCL4 (Jing 2003, Jing 2004) regulating migration of immune cells into the site of infection.

PGE_2 has been described to cooperate with pro-inflammatory cytokines or TLR ligands to induce maturation of human DCs (Steinbrink 2000, Rieser 1997, Jonuleit 1997). Induction of maturation promotes the secretion of cytokines, which modulate the character of the immune response, dependent on the kind of cytokines as well as their quantity. PGE_2 was reported to contribute to the regulation of the cytokine expression profile of mature MoDCs, but with controversial outcome, promoting Th1 and Th2 responses. Whereas some studies provide evidence for a positive effect of PGE_2 on IL-12 production (Steinbrink 2000, Rieser 2007) at least in combination with TNF-α, other reports show a strong inhibition of IL-12 production in the presence of PGE_2 (Luft 2002, Kalinski 1997). Production of elevated levels of IL-10 was described in human MoDCs matured in the presence of PGE_2 (Kalinski 1997), while in other reports secretion of IL-10 was not increased, or even reduced (Jefford 2003, Jonuleit 1997, Scandella 2002). PGE_2-matured DCs show an enhanced potential to induce allogenic T cell

proliferation. If and in what direction the presence of PGE_2 during maturation of MoDCs biases T cell differentiation is not clear. Evidence for Th2 differentiation has been provided *in vitro* (Kalinski 1997, Kalinski 1998), while Th1 differentiation and the induction of CTL responses have been reported *in vitro* and *in vivo* (Steinbrink 2000, Jonuleit 1997, Lee 2002b, Ratzinger 2004, Schuler-Thurner 2002) using MoDCs matured in the presence of PGE_2. PGE_2 has also been described to induce the expression and release of IL-23 by DCs, which led to Th17 differentiation (Sheibanie 2004, Sheibanie 2007).

Since the available data on the impact of PGE_2 on human DCs functions is limited and controversial, we conducted several studies to characterize the regulatory role of PGE_2 on MoDCs under serum-free clinical relevant conditions. The understanding of the complex effects of PGE_2 on human DC functions will help to refine and improve DC-based vaccination protocols.

CHAPTER 2

Prostaglandin E_2 is generally required for human dendritic cell migration and exerts its effect via EP2 and EP4 receptors

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Abstract

The control of dendritic cell (DC) migration is pivotal for the initiation of cellular immune responses. Here we demonstrate that the migration of human monocyte-derived (Mo)DCs as well as of ex vivo peripheral blood (PB)DCs towards CCL21, CXCL12 and C5a is stringently dependent on the presence of the pro-inflammatory mediator prostaglandin (PG)E_2, although DCs expressed CXCR4 and C5aR on their surface and DC maturation was accompanied by CCR7 up-regulation independently of PGE_2. The necessity of exogenous PGE_2 for DC migration is not due to the suppression of PGE_2 synthesis by IL-4, which is used for MoDC differentiation, since maturation-induced endogenous production of PGE_2 cannot promote DC migration. Surprisingly, PGE_2 was absolutely required at early time points of maturation to enable MoDC chemotaxis, whereas PGE_2 addition during terminal maturation events was ineffective. In contrast to mouse DCs, which exclusively rely on EP4 receptor triggering for migration, human MoDCs require a signal mediated by EP2 or EP4 either alone or in combination. Our results provide clear evidence that PGE_2 is a general and mandatory factor for the development of a migratory phenotype of human monocyte-derived as well as for peripheral blood myeloid dendritic cells.

Introduction

Dendritic cells (DCs) are professional antigen-presenting cells operating as sentinels in peripheral tissues and lymphoid organs. DCs have the unique ability to take up, process, and prime also naïve T cells and are therefore critical for the induction of primary immune responses, for the induction of the immunological tolerance as well as for the regulation of the T cell-mediated immune response (Banchereau 1998, Banchereau 2000, Mellman 2001). Due to these features, DCs loaded with specific antigens are currently being used in vaccinations against tumors and infectious agents in numerous clinical trials (Schuler 2003, Figdor 2004, Paczesny 2003).

DC progenitors in the bone marrow give rise to circulating precursors that home to tissues, where they reside as immature cells. Thus, immature DCs are strategically located at portals of pathogen entry, such as the skin, the airways, or the gastrointestinal mucosa, and are particularly good at antigen ingestion through pinocytosis or receptor-mediated endocytosis and antigen processing (Lanzavecchia 1996). Exposure to pathogens triggers the maturation of DCs through recognition of the prototypic pathogen-derived macromolecules by Toll like receptors (Krutzik 2001, Takeda 2003, Mazzoni 2004). At the same time, DCs secrete large amounts of pro-inflammatory cytokines and chemokines, including CCL2, CCL3, CCL4, and CCL5, which in turn recruit other immature DCs, macrophages and monocytes to the inflamed tissue (Sallusto 1999). Along this line, immature DCs are also attracted by the complement component C5a and the bacterial peptide fMLP (Yang 2000, Sozzani 1995, Sallusto 1998). DC maturation terminates the ability of antigen uptake, whereas the capacity to stimulate T cells is enhanced through the up-regulation of co-stimulatory molecules (such as CD80, CD86), MHC molecules, and T cell adhesion molecules (e.g. CD48 and CD58), and the enhanced production of cytokines (IL-12, IL-2, TNF-α) (Banchereau 2000, Mellman 2001). Importantly, maturing DCs loose their responsiveness to inflammatory chemokines by either down-regulation or desensitization of the chemokine receptors CCR1, CCR2, and CCR5 on monocyte-derived DCs and CCR6 on Langerhans cells (Sallusto 1998, Sozzani 1998, Dieu 1998). Simultaneously, antigen-loaded DCs up-regulate surface expression of the homing chemokine receptor CCR7 and as a result acquire responsiveness to the chemokines CCL19 (ELC, Exodus-3, MIP-3ß, ¸CKß11) and CCL21 (SLC, Exodus-2, 6Ckine, TCA-4) (Sallusto 1998, Sozzani 1998, Dieu 1998, Yanagihara 1998, Chan 1999). The fact that CCR7 and its ligands are mandatory for homing was demonstrated in CCR7-deficient, and *plt/plt* mice lacking CCL19 and CCL21 (Forster 1999, Gunn 1999, Ohl 2004, Luther 2000, Vassileva 1999, Nakano 2001).

Recently, we and others found that maturation-induced up-regulation of CCR7 surface expression is not sufficient for monocyte-derived DCs (MoDCs) to migrate towards CCL19

and CCL21 ((Scandella 2002, Luft 2002, Jefford 2003). Indeed, MoDC migration towards CCL19 and CCL21 was readily observed upon maturation in the presence of the pro-inflammatory mediator prostaglandin (PG)E_2, albeit PGE_2 did not change the expression level of CCR7 on mature DCs (Scandella 2002, Luft 2002). CCR7 triggering in MoDCs matured in the presence of PGE_2 induced an enhanced PI3K-mediated phosphorylation of PKB/Akt (Scandella 2004). However, as PI3K inhibitors were not able to abrogate MoDC migration (Scandella 2004), the mechanism of how PGE_2 permits DC migration remains largely unknown.

PGE_2 is a lipid mediator of the eicosanoid family of oxygenated arachidonic acid and thus a potent modulator of immune responses in an autocrine and paracrine fashion. The production of prostaglandins is initiated by the liberation of arachidonic acid from plasma membrane phospholipids by phoshpolipases, such as cytosolic phoshpolipase A_2 ($cPLA_2$), in a variety of cell types during inflammation. Arachidonic acid is then metabolized into prostaglandin H_2 by the cyclooxygenases, i.e. the constitutively expressed COX-1 and the inducible COX-2 (Harris 2002, Gualde 2004, Funk 2001). Cell-specific prostaglandin synthases are responsible for the conversion of prostaglandin H_2 into different prostaglandins, including PGE_2. The prime mode of PGE_2 action is through signaling *via* four seven transmembrane domain, G protein-coupled receptors termed EP1-EP4 (Narumiya 2003, Narumiya 2001). Interestingly enough, in mice, the importance of PGE_2 for DC migration to draining lymph nodes *in vivo* has been demonstrated in *Ptger4*$^{-/-}$ animals lacking the PGE_2 receptor EP4 (Kabashima 2003). As MoDCs express the functional receptors EP2 and EP4 (Scandella 2002), it remains to be identified which of these PGE_2 receptors is responsible for the development of a migratory DC phenotype in humans.

MoDCs, that are most frequently used for DC-based immunotherapies, are differentiated from peripheral blood monocytes in the presence of granulocyte / macrophage colony stimulating factor (GM-CSF) and interleukin 4 (IL-4). IL-4, however, was shown to inhibit $cPLA_2$ thus limiting the endogenous production of PGE_2 in MoDCs (Zeller-Rieser 2002). As most MoDCs failed to leave the injection site after intradermal injection of patients undergoing an anti-tumor immunotherapy (Morse 1999a), Thurnher and colleagues therefore suggested to replace IL-4 by IL-13 for the generation of MoDCs, as IL-13 enhances cPLA2 (Thurnher 2001).

In the present study, we investigate whether PGE_2 is generally needed for immature and mature DCs to migrate towards chemokines and complement components. We also compare maturation and migratory capacities of human MoDCs generated in the presence of IL-4 or IL-13 in combination with GM-CSF. Furthermore, we assess the role of PGE_2 on the migration of peripheral blood myeloid $CD1c^+$ DCs, and we identify the PGE_2 receptors responsible for facilitating human MoDC chemotaxis.

Results

PGE_2 is required for the *ex vivo* migration of human myeloid DC

Human dendritic cells (DCs) are increasingly applied as vaccines for cancer patients. We and others have shown recently that prostaglandin E_2 (PGE_2) was required during maturation of monocyte-derived DCs (MoDCs) in order to permit migration in response to the lymph-node homing chemokines CCL19 and CCL21 (Scandella 2002, Luft 2002). In order to test whether the need for PGE_2 for DC migration is a phenomenon that is confined to *in vitro* differentiated MoDCs, we investigated whether PGE_2 has a similar effect on peripheral blood DCs. To this end, we isolated human peripheral blood myeloid DCs (PBDCs) by positive selection of $CD19^-$ $CD1c^+$ cells from fresh blood of healthy donors. Interestingly, *ex vivo* PBDCs under serum-free conditions either directly subjected to chemotaxis assays or cultured overnight in serum-free medium did not migrate in response to either CCL21 or CXCL12, which are known to attract mature DCs (Figure 1A, B). *Ex vivo* PBDCs kept in the presence of serum migrated in response to CXCL12, but barely to CCL21 (data not shown), in agreement with recent findings by Maraskovsky and colleagues (Luft 2002, Jefford 2003), which may indicate that serum contains substantial amount of PGE_2 (data not shown). Indeed, overnight addition of only PGE_2 in the absence of FCS, facilitated PBDC migration (data not shown). Stimulation of PBMC with poly I:C alone permitted only a few PBDCs to chemotax to CCR7 and CXCR4 ligands, whereas the further addition of PGE_2 to the culture medium induced a migratory phenotype (Figure 1A, B). Similarly, sCD40L matured PBDCs efficiently migrated only in the presence of PGE_2 (data not shown). The lack of responsiveness of *ex vivo* and poly I:C stimulated PBDCs was not due to the lack of CCR7 and CXCR4 surface expression. All *ex vivo* PBDCs expressed CCR7 and CXCR4 as measured by flow cytometry (Figure 1C). Stimulation with poly I:C led to an up-regulation of CCR7 and to a down-regulation of CXCR4, but PGE_2 had no significant effect on the surface expression of these receptors on matured PBDCs (Figure 1C). We further characterized the surface phenotype of PBDCs and found that freshly isolated $CD1c^+$ DCs expressed high levels of HLA-DR and the co-stimulatory molecule CD86, but not CD80 and CD83 (Figure 1C). Maturation of PBDCs by poly I:C led to a marked up-regulation of CD83, CD80, and CD86, independently of the addition of PGE_2. Since IL-4 as well as IL-13 have been used to differentiate DCs in vitro (Sozzani 1997, Chomarat 1998, Morse 1999b), we compared the effect of these two cytokines on the maturation of PBDCs. However, surface expression of chemokine receptors and maturation markers were similar (Figure 1C). Although the chemotactic responses to CCL21 and CXCL12 was higher in IL-4 PBDCs (Figure 1A) compared to IL-13 PBDCs (Figure 1B), in both cases, PGE_2 was mandatory for the efficient migration of peripheral blood myeloid DCs.

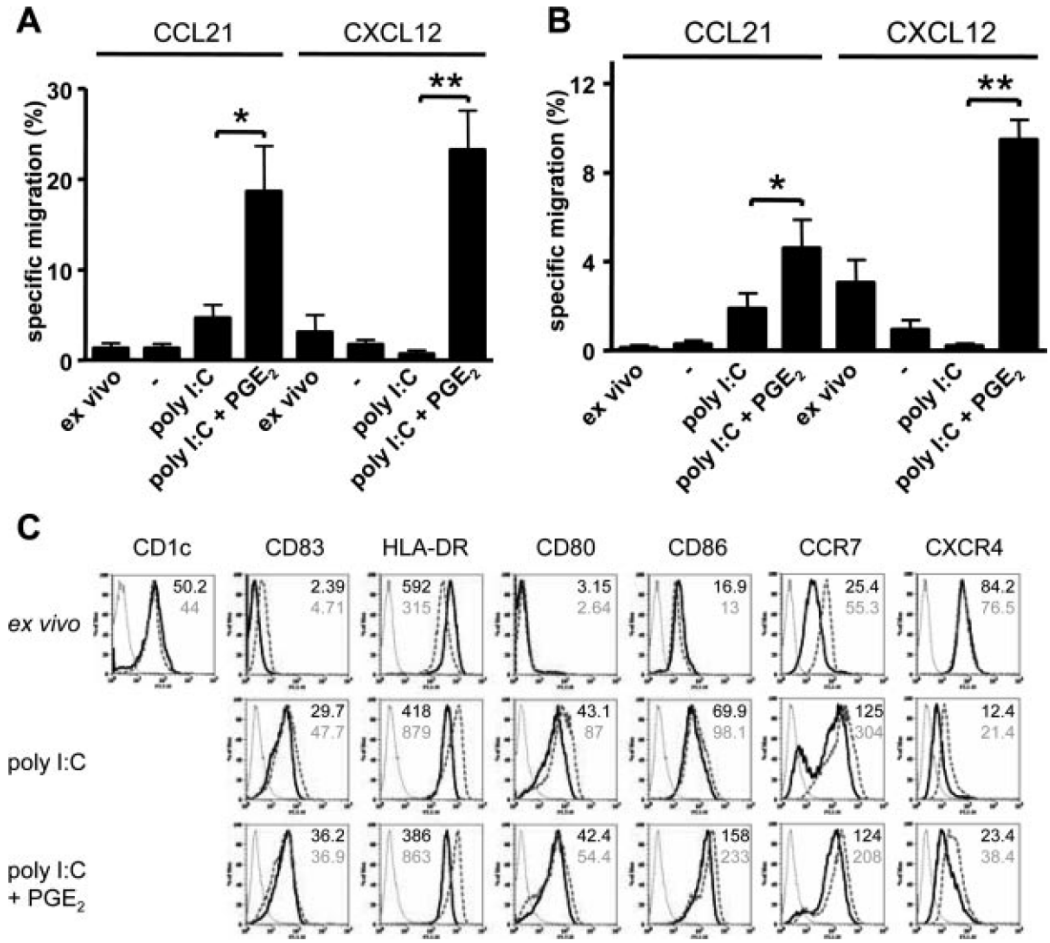

Figure 1. PGE_2 is required for human ex vivo myeloid DC migration in response to CCR7 and CXCR4 ligands. PBDCs were isolated from peripheral blood of healthy donors and directly analyzed for their migratory capacity towards the chemokines CCL21 and CXCL12 (ex vivo). Alternatively, PBDCs were cultured in serum-free medium containing GM-CSF and IL-4 (**A**) or GM-CSF and IL-13 (**B**) and stimulated or not with poly I:C and/or PGE_2 as indicated. Mean values and SEM of 5 (**A**) or 4 (**B**) independent experiments are shown. Asterisks indicate statistical significance with p values < 0.05 for * and p < 0.005 for **. (**C**) The surface expression of CD1c, CD83, HLA-DR, CD80, CD86, CCR7, and CXCR4 on ex vivo and cultured PBDCs was analyzed by flow cytometry. The solid line corresponds to PBDCs cultured in GM-CSF plus IL-4, whereas the dashed line represents PBDCs cultured in GM-CSF plus IL-13. Corresponding isotype controls are shown as thin gray line. Numbers indicated represent the mean fluorescence intensities for IL-4 (upper value) and for IL-13 (lower value).

Influence of IL-4 and IL-13 on endogenous PGE_2 production in DCs

To further characterize the role of PGE_2 on mediating chemotaxis, we used monocyte-derived DCs, which are most frequently applied for immunotherapies. First, we investigated the endogenous production of PGE_2 by MoDCs as well as by PBDCs. To this end, we collected culture supernatants of immature and poly I:C matured MoDCs generated with IL-4 or IL-13 in the presence of GM-CSF. Immature MoDCs cultured for 2 days with IL-4 secreted on average 10.6 ng/ml of PGE_2, similar to poly I:C matured MoDCs which produced 9.7 ng/ml of PGE_2 (Figure 2). Immature MoDCs differentiated with IL-13 and GM-CSF produced a comparable amount of PGE_2, namely 8.9 ng/ml (Figure 2). In contrast, a more than 7-fold increase of secreted PGE_2 (67.9 ng/ml) was measured in the supernatant of mature IL-13/GM-CSF MoDCs (Figure 2). Similarly, PBDCs cultured overnight in medium

supplemented with IL-13/GM-CSF produced on average 8 times more PGE_2 than PBDCs cultured in medium containing IL-4/GM-CSF, namely 11.9 versus 1.5 ng/ml of PGE_2 (Figure 2). This result is in agreement with the finding that IL-4 suppressed endogenous production of PGE_2 in matured MoDCs by inhibiting the cytoplasmatic form of phospholipase A_2 (Zelle-Rieser 2002).

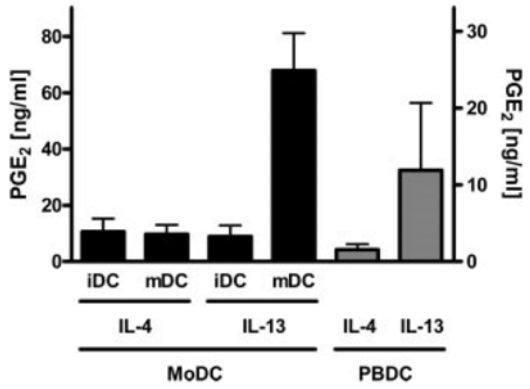

Figure 2. Mature DCs generated with GM-CSF and IL-13 release high amounts of PGE_2. Monocytes were differentiated into immature DCs with GM-CSF and IL-4 (IL-4) or GM-CSF and IL-13 (IL-13) and were either left immature (iDC) or were matured by the addition of poly I:C (mDC). *Ex vivo* PBDCs were cultured in medium containing GM-CSF and IL-4 or GM-CSF and IL-13. The release of PGE_2 into the supernatant of DC cultures was determined after 48 h for MoDCs, or 18-20 h for PBDCs by enzyme immunoassay. Mean values and SEM from supernatants derived from three to six donors are shown.

PGE_2 is generally required for MoDC migration

Next, we intended to analyze in detail the two media used to generate MoDCs under serum-free conditions used for clinical applications. To this end, monocytes were either cultured for 5 to 6 days in either IL-4/GM-CSF or IL-13/GM-CSF and matured by the addition of poly I:C for 2 days, both in the absence or presence of graded concentrations of PGE_2. Similar levels of CD83, HLA-DR, CD80, and CD86 were expressed on immature MoDCs irrespective of the presence of IL-4 or IL-13 (Figure 3). However, exogenous addition of PGE_2 to immature MoDCs generated in the presence of IL-4, in contrast to IL-13, substantially up-regulated CD83 and CD80 surface expression (Figure 3). The addition of 1 μg/ml of PGE_2 in conjunction with poly I:C for MoDC maturation had no effect on surface expression levels of the tested markers in both, IL-4 and IL-13, culturing conditions (Figure 3).

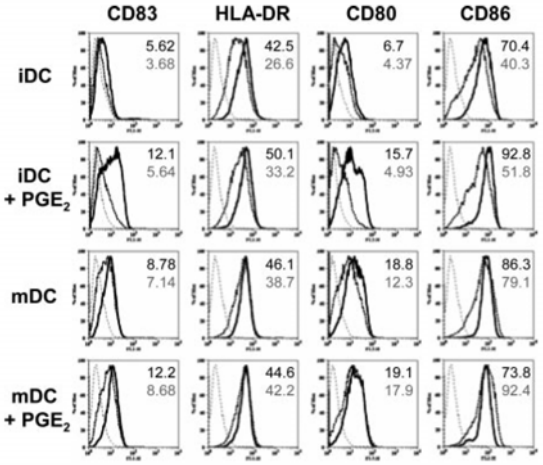

Figure 3. Influence of PGE_2 on phenotypic MoDC maturation. Human peripheral blood monocytes were cultured in serum-free medium containing either GM-CSF and IL-4 (solid line) or GM-CSF and IL-13 (dashed line) in the presence or absence of PGE_2 to differentiate into immature DCs (iDC). DCs were matured (mDC) by the addition of poly I:C (+/-PGE_2) for 2 days. Surface expression of CD83, HLA-DR, CD80, and CD86 was measured by flow cytometry. Corresponding isotype controls are depicted as thin dashed line. A representative experiment out of eleven for IL-4, respectively eight for IL-13, is shown. Numbers indicated represent mean fluorescence intensities for IL-4 (upper number) and for IL-13 (lower number).

Next, we investigated the chemotactic responses of MoDCs to various chemokines and chemoattractants. Therefore, we matured MoDCs generated in medium containing IL-4/GM-CSF with poly I:C in the presence of graded concentrations of PGE_2. Mature MoDCs in the absence of PGE_2 migrated neither in response to the chemokines CCL21 and CXCL12 nor to the chemoattractant C5a (Figure 4A). In contrast, exogenous addition of as little as 10 to 50 ng/ml of PGE_2 during maturation was sufficient to allow MoDCs to chemotax in response to CCL21, CXCL12 as well as to C5a (Figure 4A). The migration efficiency of mature MoDCs further increased with higher PGE_2 concentrations. To substantiate this finding, we subjected immature as well as mature MoDCs that were cultured in the presence or absence of PGE_2 to migration assays. Indeed, MoDCs generated in IL-4/GM-CSF medium migrated efficiently only upon incubation with PGE_2 (Figure 4B). The migration efficiency was highest for all attractants in PGE_2-treated mature MoDCs, but migration of immature MoDCs cultured in the presence of PGE_2 was also observed for all three ligands. In addition, similar numbers of migrated cells to CCL21 were observed for sCD40L or LPS matured MoDCs, and migration largely depended on the presence of PGE_2 during maturation (Figure 4C). Unexpectedly, under identical conditions, none of the MoDCs migrated in response to fMLP (data not shown). These results provide clear evidence that PGE_2 is generally required to develop a migratory DC phenotype.

As mature MoDCs generated in the presence of IL-13 and GM-CSF endogenously produced a similar amount of PGE_2 (Figure 2) as required for migration (Figure 4A), we analyzed the migratory capacity of IL-13/GM-CSF MoDCs in Transwell assays. Surprisingly, endogenous production of PGE_2 by IL-13/GM-CSF MoDCs was not sufficient to allow chemotaxis to any of the chemoattractants, but MoDCs did migrate when they were cultured in the presence of exogenous PGE_2 (Figure 4D). Strikingly, the PGE_2 effect on migration was even more pronounced. CXCL12- and C5a-mediated migration was exclusively observed in poly I:C/PGE_2-matured MoDCs. In agreement with IL-4/GM-CSF MoDCs, immature IL-13/GM-CSF MoDCs cultured in the presence of PGE_2 barely migrated towards CCL21, whereas they did not respond to CXCL12 and C5a at all (Figure 4D).

Taken together, we demonstrated that the necessity of PGE_2 for the migration of DCs is not due to the inhibitory effect of IL-4 on PGE_2 production. In fact, the maturation-induced endogenous production of PGE_2 is not sufficient for migration of MoDCs as previously suggested by Thurnher and co-workers (Zelle-Rieser 2002, Thurnher 2001). Moreover, our results clearly indicate that PGE_2 is a general and mandatory factor for the development of a migratory phenotype of human monocyte-derived as well as for peripheral blood myeloid dendritic cells.

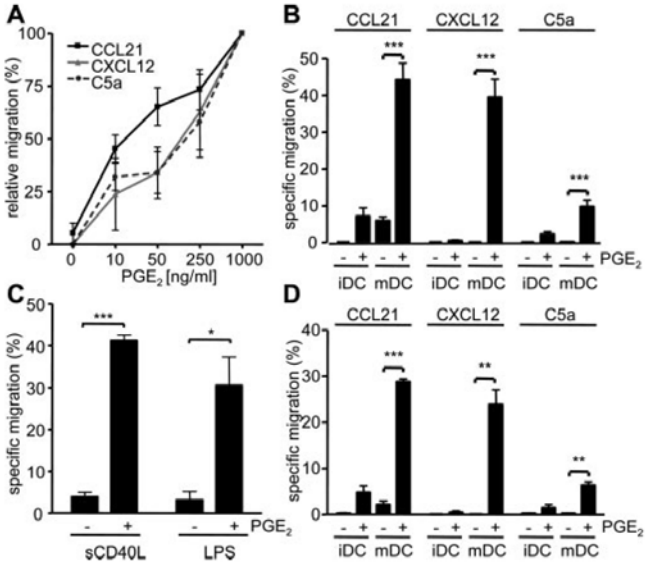

Figure 4. PGE_2 is generally required for MoDC migration. (**A**) MoDCs were generated in medium containing GM-CSF and IL-4 and matured with poly I:C in the presence of graded concentrations of PGE_2. After 2 days, the migration of mature DCs towards 250 ng/ml CCL21, 250 ng/ml CXCL12 and 10 nM C5a was measured in a Transwell chemotaxis assay. The number of migrated cells cultured with 1 μg/ml PGE_2 was set to 100% for each chemoattractant. Absolute average values for the migration towards CCL21, CXCL12, and C5a are 32.5, 16.0, and 7.4 %, respectively (n=3). Basal migration in the absence of chemoattractants was always below 0.5 %. Monocytes were differentiated into immature DCs (iDC) by culturing in medium supplemented with GM-CSF and IL-4 (**B**, **C**) or GM-CSF and IL-13 (**D**) in the presence or absence of PGE_2 and matured with poly I:C (**A**, **B**, **D**,) sCD40L or LPS (**C**) (+/-PGE_2) for 2 days. The migration of MoDCs was then analyzed by a Transwell chemotaxis assay in response to CCL21, CXCL12 and C5a. Mean values and SEM from four to six independent experiments of different donors are shown. Asterisks indicate statistical significance with p values < 0.05 for *, p < 0.01 for **, and p < 0.001 for ***.

Role of PGE_2 on CCR7, CXCR4, and C5aR expression on MoDCs

To exclude that the impaired DC migration in the absence of PGE_2 was simply due to a lack of receptor expression, we subjected MoDCs to flow cytometry analysis. As expected, immature IL-4/GM-CSF MoDCs did not express CCR7 (Figure 5A), which is in agreement with previous observations (Sallusto 1998, Sozzani 1998, Scandella 2002). Addition of PGE_2 lead to a marked up-regulation of CCR7 on immature MoDCs which was further increased upon maturation by poly I:C (Figure 5A). PGE_2 had no influence on CCR7 expression of mature MoDCs. CXCR4 was expressed on immature as well as on mature MoDCs and PGE_2 did not alter the expression level (Figure 5A). We were unable to detect C5aR surface expression by flow cytometry using two different commercially available antibodies (data not shown). Therefore, we performed real-time PCR to quantify mRNA levels of C5aR under the various MoDC culturing conditions. C5aR mRNA was present in immature and mature MoDCs (Figure 5B) and the amount of mRNA barely changed after maturation or after stimulation with PGE_2. Compared to immature MoDCs, we found on average a 6-, 2-, and 4-fold increase in mRNA levels after treatment with PGE_2, poly I:C, and poly I:C/PGE_2, respectively (Figure 5B). For comparison, we also quantified mRNA expression of CCR7. There, the up-regulation of CCR7 mRNA increased by 41-, 300-, and 378-fold compared to immature MoDCs (Figure 5B). In agreement with the unresponsiveness to fMLP, we found

neither surface expression nor mRNA for fMPL receptor under these conditions (data not shown). However, the influence of PGE_2 on the expression levels of CCR7, CXCR4, and C5aR was similar for MoDCs generated with IL-4 as compared to IL-13 (Figure 5). We conclude from these data that the migratory inability of MoDCs without PGE_2 is not due to the lack of CCR7, CXCR4 and C5aR expression; rather PGE_2 facilitated DC migration by a mechanism distinct from modulating the level of receptor expression.

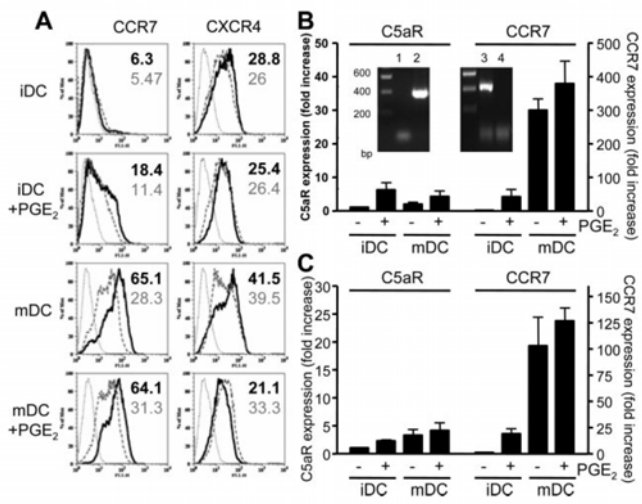

Figure 5. Impact of PGE_2 on the expression of CCR7, CXCR4, and C5aR by MoDCs. The chemokine receptor expression on immature and poly I:C matured MoDCs generated with GM-CSF and IL-4 (**A**, black solid line; B) or GM-CSF and IL-13 (**A**, gray dashed line; **C**) in the presence or absence of PGE_2 was measured by flow cytometry using CCR7 and CXCR4 specific antibodies (**A**). Numbers indicated represent mean fluorescence intensities for IL-4 (upper number) and for IL-13 (lower number). The mRNA expression of C5aR and CCR7 by MoDCs was examined by real-time PCR and normalized to the house-keeping gene GAPDH. Mean values and SEM from four independent experiments using different MoDC preparations are shown. Amplified transcripts (376 bp for C5aR and 430 bp for CCR7) were visualized on agarose gel electrophoreses (inlet of panel **B**, lane 2 and 3). The control PCR using H_2O as template and C5aR or CCR7 primers, respectively, are shown in lane 1 and 4 of the inlets.

PGE_2 is required at early time-points of MoDCs maturation to permit migration

For a better understanding of how PGE_2 permits DC chemotaxis, we incubated maturing MoDCs for different time periods with PGE_2. As shown before, marginal or no migration in response to CCL21 and CXCL12, respectively, was measurable for MoDCs matured with poly I:C alone. However, the co-stimulation of MoDCs with PGE_2 and poly I:C during the first 12 h of maturation, followed by a further incubation of 36 h in the presence of poly I:C alone, was almost as efficient as the stimulation with PGE_2 and poly I:C throughout the whole maturation period with respect to chemotaxis of MoDCs towards CCL21 and CXCL12 (Figure 6). Surprisingly, poly I:C-matured MoDCs that exclusively received PGE_2 for the terminal 12 h of maturation were not attracted by the chemokines (Figure 6). These data suggest that PGE_2 may induce the expression of so far unidentified genes, which enable DCs to sense a chemokine gradient. Further experiments are required to unravel such a putative mechanism.

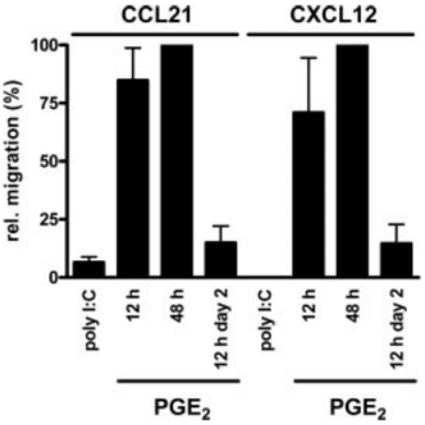

Figure 6. PGE_2 is required at early time-points of MoDCs maturation to permit cell migration. Immature DCs differentiated with GM-CSF and IL-4 were matured with poly I:C for 48 hours (poly I:C) and assessed for chemotaxis towards CCL21 (left) and CXCL12 (right). PGE_2 was added either for the whole period of maturation (48 h), for the initial 12 hours (12 h) or for the terminal 12 hours (12 h day 2) of maturation, respectively. In the case of adding PGE_2 at maturation initiation, MoDCs were incubated in medium containing 20 μg/ml poly I:C and 1 μg/ml PGE_2 for 12 h, washed extensively and seeded in fresh IL-4/GM-CSF medium containing 20 μg/ml poly I:C and cultured for another 36 h prior to functional analysis. Mean values and SEM of two to three MoDC preparations are shown. Absolute average values for the migration towards CCL21, and CXCL12 are 31.1, and 24.2 %, respectively.

In humans, the two PGE_2 receptors EP2 and EP4 can mediate MoDC migration

Gene targeting experiments in mice revealed that exclusively PGE_2 receptor EP4 was critical for Langerhans cells (LC) migration to draining lymph nodes *in vivo* (Kabashima 2003). Human MoDCs express two out of the four described PGE_2 receptors, namely EP2 and EP4 (Scandella 2002) and it remains to be determined which of these receptors can trigger human DC migration. To address this question, we made use of various specific EP2 and EP4 agonists. MoDCs were matured for two days with poly I:C and incubated either in the presence or absence of PGE_2, or in the presence of an EP2 or an EP4 agonist, or a combination thereof followed by testing the mobility of the DCs in a chemotaxis assay (Figure 7). In contrast to mouse LC, human MoDCs migrated readily in response to CCL21 upon maturation in the presence of the EP2 agonists butaprost and ONO-AE1-259-01 similar to MoDCs matured with poly I:C and EP4 agonists PGE_1-alcohol and ONO-AE1-329 (Figure 7A). Each agonist on its own and the combination of either butaprost and PGE_1-alcohol, or ONO-AE1-259-01 and ONO-AE1-329, were almost as potent as PGE_2 in facilitating DC migration in response to CCL21 (Figure 7A). Along this line, addition of a 4-fold excess of the EP4 antagonist ONO-AE3-208 over PGE_2 was unable to inhibit migration (Figure 7A). For CXCL12- and C5a- mediated chemotaxis, the EP2 as well as EP4 agonists permitted MoDCs to migrate, but the EP2 agonists were less effective (Figure 7B and C). In agreement with these findings, the EP4 antagonist was able to partially antagonize the effect of PGE_2. Thus, in contrast to mouse LCs, human MoDCs require a signal mediated by either EP2 or EP4 alone or the combination of both receptors to develop a migratory phenotype.

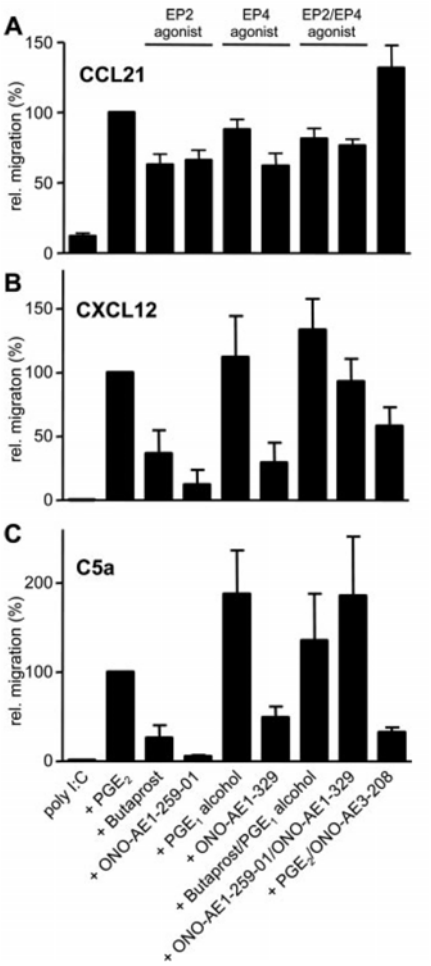

Figure 7. The PGE_2 receptors EP2 and EP4 can both trigger MoDC migration in response to CCL21, CXCL12, and C5a. Immature MoDCs (GM-CSF and IL-4) were matured with poly I:C and the chemotactic responses to CCL21 (**A**), CXCL12 (**B**) and C5a (**C**) was measured in Transwell assays. To the maturation stimuli, either 1μg/ml PGE_2, 1 μg/ml of specific EP2 agonists (Butaprost or ONO-AE1-259-01), 1 μg/ml of specific EP4 agonists (PGE_1-alcohol or ONO-AE1-329), or a combination of EP2 and EP4 agonists was added. In addition, 4 μg/ml of the EP4-specific antagonist ONO-AE3-208 was added to 1 μg/ml PGE_2 for the whole maturation procedure. Migration relative to MoDCs matured in the presence of PGE_2, which served as 100 % value, is shown. Mean values and SEM of up to six independent experiments are shown.

Discussion

The maturation of DCs is a key event in the initiation of a T cell response. DC maturation results in the up-regulation of CCR7, which directs migration into the T cell zone of draining lymph nodes. There, DCs will meet naïve T lymphocytes that were recruited by the same chemokine receptor. For the initiation of a T cell response, the up-regulation of CCR7 on DCs may represent a point of no return, and it is intriguing to observe that CCR7 surface expression is not enough to facilitate migration. Indeed, for a migratory phenotype, DCs require a second stimulus that permits chemokine receptor signaling and subsequent migratory response towards the chemokines. Recently, we and others have identified PGE_2 as a permissive factor that couples CCR7 on human DCs differentiated from peripheral blood monocytes by IL-4 and GM-CSF to signaling, e.g. protein kinase B activation, and ultimately DCs migrated towards the chemokines CCL19 and CCL21 (Scandella 2002, Luft 2002, Scandella 2004). Here we show that the effect of PGE_2 on MoDC migration is not restricted to CCR7. Indeed, immature as well as mature MoDCs migrated exclusively to the constitutive chemokine CXCL12 via its cognate receptor CXCR4, when PGE_2 was present in the culture

condition (Figure 4). The effect of PGE_2 stimulation on CXCR4- and CCR7-mediated migration was very similar with respect to the required dose of PGE_2 and the need for PGE_2 early during maturation. Moreover, PGE_2 did not affect the cell surface expression of neither of the two receptors on matured MoDCs (Figure 5). It is therefore likely, that the same PGE_2-triggered intracellular pathway determines the migratory capacity of CCR7 and CXCR4. In addition, we demonstrate that PGE_2 is also a mandatory factor for the development of a migratory DC phenotype for the complement fragment C5a, although the migration in response to C5a was rather inefficient compared to CCL21 and CXCL12 (Figure 4). Under our experimental setup MoDCs did not migrate in response to the bacterial chemoattractant fMLP, although fMLP was fully active (data not shown). This was rather unexpected as immature, but not mature, MoDC have been reported to migrate in response to fMLP and C5a (Sozzani 1995, Sallusto 1998), whereas Yang et al showed chemotaxis in immature as well as mature MoDCs (Yang 2000). However, at least the low portion of MoDCs that specifically migrated towards C5a was only able to do so when immature or maturing MoDCs were stimulated with PGE_2. Nevertheless, we can conclude that PGE_2 is a general permissive agent that controls MoDC migration in response to chemokines as well as complement chemoattractants.

Is the need for PGE_2 for DC migration restricted to *in vitro* generated MoDCs? Maraskovsky and colleagues reported that freshly isolated $CD1c^+$ PBDCs from human blood that were expanded *in vivo* with Flt-3 ligand, did not require sensitization with PGE_2 as the stimulation with CD40L *in vitro* sufficed to induce maturation and migration towards CCL19 under serum-containing conditions (Luft 2002, Jefford 2003). In this study we have re-investigated this issue by magnetically isolating $CD1c^+$ myeloid PBDCs and performing *in vitro* maturation and migration assays in serum-free conditions identical to MoDC preparations approved for clinical applications. *Ex vivo* PBDCs show a similar phenotype as immature MoDCs as they lack surface expression of CD83 and CD80, and express moderate levels of HLA-DR and CD86. Latter molecules are up-regulated upon maturation (Figure 1C). In contrast to immature MoDCs, PBDCs express a substantial amount of CCR7. These data indicate that peripheral blood DCs are similar to MoDCs in respect of migration but clearly represent two different DC populations. Under serum-free conditions, both CCR7 and CXCR4, however, are not functional, as the isolated PBDCs did not migrate to CCL21 and CXCL12 *ex vivo*. Strikingly, addition of 10 % FCS to *ex vivo* PBDCs during the chemotaxis assay was sufficient for PBDCs to migrate in response to CXCL12 (data not shown), thus confirming previous findings (Luft 2002, Jefford 2003), but demonstrating that the presence of serum has a major effect on DC migration. Indeed, FCS can contain sufficient concentrations of PGE_2 to trigger migration (P. Krause, unpublished). Maturation of PBDCs with poly I:C markedly up-regulated CCR7 expression and down-regulated CXCR4 expression but

facilitated only a minor population to migrate in response to CCL21, whereas no migration towards CXCL12 was observed (Figure 1A). But co-stimulation with PGE_2 and poly I:C or sCD40L resulted in substantial migration to both chemokines. It hence appears that the permissive function of PGE_2 for DC migration is not confined to MoDCs, but is also valid for PBDCs directly isolated from human blood under serum-free conditions. Taken together, our findings suggest that PGE_2 is a general mandatory factor for dendritic cell migration. In this context it is of interest that exogenous PGE_2 seem also to enhance the activities of monocytes to certain chemokines (Panzer 2004, Kurth 2001).

It has been speculated that the permissive role of PGE_2 for MoDC migration may be a consequence of the *in vitro* differentiation procedure. IL-4 has been reported to suppress the endogenous PGE_2 production of MoDCs (Zelle-Rieser 2002, Thurnher 2001) by down-regulating enzymes required for PGE_2 biosynthesis, such as phospholipase A_2 or cyclooxygenase-2 (Mehindate 1996). For the differentiation and maturation of MoDCs IL-4 may be replaced by IL-13 (Sozzani 1998, Chomarat 1998, Romani 1996), which rather enhances phospholipase A_2 expression at least in macrophages (Rey 1998). Thurnher and colleagues thus hypothesized that MoDCs generated with IL-13 may be able to produce PGE_2 which could potentially allow MoDCs to migrate (Thurnher 2001). To investigate whether IL-13-MoDCs indeed produce PGE_2 and whether endogenous production of PGE_2 by IL-13-MoDCs would facilitate migration, we generated MoDCs with IL-4 or IL-13 in the presence of GM-CSF. Indeed, we found that the concentration of endogenously produced PGE_2 in the supernatant of IL-4-treated mature MoDCs was very low (Figure 2). In contrast, mature MoDCs raised in IL-13 and GM-CSF secreted more than 70 ng/ml PGE_2 into the growth medium, which should suffice to trigger DC migration. Nevertheless, also IL-13-treated MoDCs required the addition of exogenous PGE_2 to permit migration (Figure 4D). Similarly PBDCs cultured in the presence of IL-13, compared to IL-4, secreted more PGE_2. Although IL-13-DCs endogenously produced PGE_2, they migrated for an unknown reason less efficiently than IL-4-DCs. For a better understanding of the permissive role of PGE_2 for MoDC migration, we investigated whether PGE_2 may act on the level of gene transcription or whether PGE_2 may trigger a signal transduction module mandatory for chemotaxis. As shown in Figure 6, PGE_2 was required during the first 12 h of maturation and could not facilitate migration when added during the last 12 h of the maturation period. In several, but not all, experiments it was even sufficient to add PGE_2 during the first 2 h of maturation (data not shown). We are therefore in favor of the hypothesis that PGE_2 may regulate chemotaxis by turning on or shutting off yet unknown genes required for migration. This may also explain why the endogenous secretion of PGE_2 even by IL-13-treated MoDCs was insufficient to accumulate enough PGE_2 during the initial period of maturation to facilitate migration. Although the conditions *in vitro* and *in vivo* are difficult to compare, it may well be that *in vivo*

the endogenous PGE_2 production by DCs is insufficient to trigger DC migration. Alternatively, it may be that blood DCs have had contact with PGE_2 during maturation at sites of inflammation. This may also explain why *ex vivo* PBDCs require a shorter period of PGE_2 contact to acquire a migratory phenotype, or why they migrated in response to CXCL12 in the presence of serum without PGE_2 supplementation (Luft 2002). Noteworthy, inflammation, which promotes DC migration, is associated with the rapid induction of arachidonic acid metabolism and PGE_2 production, thus leading to a local co-production of cytokines, such as TNF-α and IL-1ß and PGE_2 in inflamed lesions. The exogenous supply of PGE_2 may derive from IL-1ß, TNF-α- or LPS- stimulated macrophages or fibroblasts at sites of inflammation.

The essential role of PGE_2 for DC migration has been highlighted by Kabashima et al. in gene targeted mice lacking the PGE_2 receptor EP4 (Kabashima 2003). The morphology and density of class II-positive Langerhans cells (LC) in epidermal sheets were similar in *ptger4*$^{-/-}$ and *ptger4*$^{+/+}$ mice, indicating that EP4 deficiency does not affect LC generation or LC recruitment into the tissue. However, LC emigration from *ptger4*$^{-/-}$ skin explants and migration into the draining lymph node after FITC application in *ptger4*$^{-/-}$ mice was significantly reduced (Kabashima 2003). These experiments imply, that PGE_2 cannot be replaced by other agents that up-regulate cAMP levels in DCs. Moreover, they open an attractive therapeutic option for the pharmacological control of DC migration by the inhibition of the EP4 receptor. In human, we have recently shown that two of the four PGE_2 receptors, EP2 and EP4, are expressed on MoDCs (Scandella 2002). This is in accordance with the report by Luft et al. reporting that an agonist specific for EP2 and EP4 can trigger MoDC migration, but not an agonist specific for EP1 and EP3 (Luft 2002). In order to explore potential therapeutic options for the control of MoDC migration in humans, we used two specific agonists each for EP2 and EP4 as well as a specific antagonist of the EP4 receptor to dissect the role of these two PGE_2 receptors for MoDC migration *in vitro* (Figure 7). To our surprise, the EP2 *and* EP4 agonists were both equally competent in facilitating MoDC migration via CCR7. This finding was substantiated by the fact that the specific EP4 antagonist could not inhibit the effect of PGE_2 in enabling DC migration. Interestingly, for the migration towards CXCL12 and, even more pronounced, for C5a, EP4 agonists seemed to be more potent than EP2 agonists which correlated with a stronger inhibition of MoDC migration by the EP4 antagonist, although we observed substantial donor to donor variations. Nevertheless, there seems to be a clear difference between murine Langerhans cells and human MoDCs in the usage of PGE_2 receptors that can mediate migration. This result is pharmacologically relevant as we surmise that the treatment with EP2 *and* EP4 agonists or antagonists will most likely be required to interfere with DC migration in humans.

In summary, we show that PGE_2 is a general and mandatory factor for human MoDCs and PBDCs to migrate in response to the chemokines CCL21 and CXCL12 as well as to the

chemoattractant C5a. Thereby, endogenous production of PGE_2 by DCs was not sufficient for the development of a migratory phenotype. Furthermore and in contrast to mouse DCs, which exclusively rely on EP4 receptor triggering for migration, human MoDCs require a signal mediated by EP2 *or* EP4 either alone or in combination.

Material and Methods

Isolation of human peripheral blood myeloid DCs (PBDCs)

$CD1c^+$ myeloid DCs from peripheral blood of healthy donors were purified using the CD1c (BDCA-1) Dendritic Cell Isolation Kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's protocol. Briefly, peripheral blood mononuclear cells (PBMCs) were separated by centrifugation over a density gradient of Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden), depleted of $CD19^+$ cells and positively selected for CD1c. After isolation, *ex vivo* PBDCs were directly subjected to flow cytometric analysis and transwell chemotaxis assays. Alternatively, PBDCs were cultured at $1x10^6$ cells/ml in AIM-V medium (Gibco, Paisley, UK) supplemented with 50 ng/ml GM-CSF (Leukomax®, Novartis, Basel, Switzerland) and either 50 ng/ml IL-4 (PromoCell, Heidelberg, Germany) or 10 ng/ml IL-13 (PromoCell) and matured with 20 µg/ml poly I:C (Sigma, St Louis, MO) in the presence or absence of 1 µg/ml PGE_2 (Minprostin® E2, Pharmacia, Uppsala, Sweden). After 18-20 h, cells were harvested and analyzed for their migration capacity and maturation status by flow cytometry.

Generation of human monocyte-derived DCs (MoDCs)

Monocytes were positively selected from PBMCs of healthy donors using anti-CD14-conjugated magnetic microbeads (Miltenyi Biotec) as previously described (28). $CD14^+$ monocytes were cultured at $1x10^6$ cells/ml in AIM-V medium supplemented with 50 ng/ml GM-CSF and IL-4 (1:50 of the supernatant of an IL-4 producing J558 cell line) or 10 ng/ml IL-13 (Dr. Adrian Minty, Sanofi-Synthelabo, France). After 5 to 6 days, immature DCs were matured for 2 days by adding 20 µg/ml poly I:C, 0.5 µg/ml sCD40L (PromoCell), or 1 µg/ml LPS (Sigma), and, where indicated, 1 µg/ml PGE_2, 1 µg/ml of specific agonists for EP2 (Butaprost, Cayman Chemicals, Ann Arbor, MI; ONO-AE1-259-01, ONO Pharmaceutical, Osaka, Japan), EP4 (PGE_1-alcohol, Cayman Chemicals; ONO-AE1-329, ONO Pharmaceutical) or 4 µg/ml of the specific EP4-antagonist ONO-AE3-208 (ONO Pharmaceutical).

Chemotaxis assay

DCs ($2x10^5$ cells in AIM-V medium) were placed on a polycarbonate filter (5 µm pore size) of a 24-well Transwell plate (Corning Costar, NY) and allowed to migrate for 3 hours at 37°C/5% CO_2 to the lower chamber containing 250 ng/ml CCL21, 250 ng/ml CXCL12 (both PromoCell) or 10 nM C5a (Sigma), respectively. Input and migrated cells were counted by flow cytometry acquiring events of the appropriate size for 60 seconds. The number of spontaneously migrated cells towards AIM-V medium without chemoattractants was subtracted.

Flow cytometry

For flow cytometric analysis, MoDCs and PBDCs were stained at 4°C in PBS containing 0.5 % FCS and 0.1 % sodium azide using the following mouse anti-human mAb: anti-CD83-FITC (Immunotech, Marseille, France), anti-HLA-DR-FITC, anti-CD86-FITC, anti-CD80-PE, anti-CD88-PE (clone D53-1473), anti-fMLPR-PE (clone 5F1), anti-CXCR4 (mouse anti-human mAb 12G5) (all from Becton Dickinson Biosciences, San Jose, CA), anti-CD88-FITC (MCA1284F, Serotec, Oxford, UK), anti-CCR7 (rat anti-human mAb 3D12, kindly provided by Dr. R. Förster, Hannover), biotin-labeled anti-CD1c, anti-biotin-PE (Miltenyi Biotec), anti-rat IgG(Fab')$_2$-FITC (Jackson, West Grove, PA), and anti-mouse IgG_1-FITC (Silenus, Melbourne, Australia). Cell-associated fluorescence was measured using a FACScan II flow cytometer (Becton Dickinson Biosciences).

Quantitative Real-Time PCR

Total RNA of DCs was isolated using the NucleoSpin RNA II kit (Macherey Nagel, Düren, Germany) and transcribed into cDNA using the Reverse Transcription System Kit (Promega, Madison, WI) according to the manufacturer's protocols. Real-Time PCR was performed using a LightCycler together with the LightCycler FastStart DNA Master SYBR Green I kit (Roche, Mannheim, Germany) according to the manufacturer's recommendations. Briefly, the cDNAs were initially denatured for 10 min at 95°C. Specific DNA fragments were amplified with steps of 15 sec at 95°C, 5 sec at 60°C, and 11 sec at 72°C for 50 PCR cycles. The following oligonucleotide primers were used: for C5aR: 5'- CAG GAG ACC AGA ACA TGA ACT CC and 5'- TAC ATG TTG AGC AGG ATG AGG G, for CCR7: 5'- CCT GGG GAA ACC AAT GAA AAG C and 5'- GAG CAT GCC ACT GAA GAA GC, and for GAPDH (glyceraldehyde-3-phosphate dehydrogenase): 5'- GAA GGT GAA GGT CGG AGT C and 5'- GAA GAT GGT GAT GGG ATT TC. Optimal $MgCl_2$ concentrations were 3 mM for C5aR and CCR7, and 4 mM for GAPDH. The amount of amplified DNA fragments was normalized to GAPDH mRNA and the specificity of the PCR products was verified by determining the melting profiles and analyzed by agarose gel electrophoresis.

Quantification of PGE_2 by enzyme immunoassay (EIA)

Culture supernatants of immature and mature MoDCs were collected after two days of stimulation and the amount of secreted PGE_2 was determined using the PGE_2-EIA kit (Cayman, Ann Arbor, MI) according to the manufacturer's instructions.

Statistical evaluation

Differences between groups were assessed by the student's paired *t* test.

Acknowledgments

We thank ONO Pharmaceutical CO., LTD for the generous gift of the EP2 and EP4 agonists and the EP4 antagonist, Dr. Adrian Minty from Sanofi-Synthelabo Research for recombinant IL-13, and Dr. Reinhold Förster from the Institute of Immunology, Hannover Medical School for the anti-CCR7 antibody.

CHAPTER 3

Prostaglandin E_2 (PGE_2) inhibits expression of Sprouty2 and Spred1 in human monocyte-derived dendritic cells

Abstract

Monocyte-derived dendritic cells (MoDCs) are commonly used in immunotherapeutic DC-based cancer vaccination protocols. We have shown previously the pivotal role of Prostaglandin E_2 (PGE_2) for the development a migratory phenotype of mature MoDCs. While the enhancing effect of PGE_2 on chemotactic responses seems to apply to DCs in general, only DCs matured in the presence of PGE_2 showed chemotactic responses to various attractants. However, the mechanism by which PGE_2 facilitates migratory capacities of DCs has not been identified. We here identify two proteins, namely Sprouty2 and Spred1, which are negatively regulated by PGE_2 during MoDCs maturation. Sprouty2 and Spred1 are best described so far as negative regulators of the ERK/MAPK pathway in response to growth factor stimulation and have not been reported in DCs yet. Since the PGE_2-induced migratory phenotype of MoDCs correlates with the PGE_2-promoted down-regulation of Sprouty2 and Spred1 expression we investigated if Sprouty2 and Spred1 are involved in the regulation chemokine receptor signaling. Surprisingly, we found that over-expression of Sprouty2 or Spred1 inhibited CXCR4-mediated chemotaxis of MoDCs while migration via CCR7 was not affected. Our data imply a new regulatory mechanism for chemokine receptor-mediated signaling pathways.

Introduction

DCs are the main antigen-presenting cell type of the immune system. After they have taken up antigen in the periphery, which induces the process of maturation, DCs migrate to the draining lymph node to present the antigen to T and B lymphocytes. Clonal expansion of antigen-specific lymphocytes produces a pool of functional effector cells to cope with the infection. Since the induction of an adaptive immune response depends on the presentation of antigen in secondary lymphoid organs, the ability of DCs to migrate is an essential prerequisite and important to understand. Prostaglandin E_2 (PGE_2) is an inflammatory mediator of the prostanoid family and is produced by a variety of cells in response to physiological or pathological stimuli (Gualde 2004). The crucial role for PGE_2 during maturation of dendritic cells (DCs) in order to facilitate a migratory phenotype has been well established (Scandella 2002, Luft 2002, Legler 2006). However, the mechanism by which PGE_2 promotes migratory capacities remains unknown.

In mammals, Sprouty proteins have been described as antagonists in a variety of physiological processes like cell proliferation, cell motility, and receptor trafficking. Additionally, Sprouty is implied in the regulation of developmental branching processes like angiogenesis (Lee 2001, Zhang 2005) or lung development (Mailleux 2001). Sprouty proteins are conserved regulators of receptor tyrosine kinase (RTK) signaling, that were first identified in Drosophila (Hacohen 1998, Casci 1999). In human, four Sprouty homologs have been described so far, termed Sprouty1-4, (Minowada 1999, Tefft 1999) all of which share a unique, highly conserved cysteine-rich C-terminal region. Although the N-terminal region of Sprouty proteins is highly variable, it contains a conserved Src homology 2 (SH2) domain binding motif including a central tyrosine residue (Y55 in Sprouty2) (Guy 2003, Rubin 2003). The cysteine-rich C-terminal region of Sprouty proteins was thought to be unique until a highly homologous region was identified in Sprouty-related proteins with EVH1 domain (Spred), of which three mammalian isoforms exist (Wakioka 2001, Kato 2003). Beside the C-terminal Sprouty-like cysteine-rich domain, Spred family members contain a N-terminal EVH1 domain and a central c-Kit binding domain (Kato 2003, Wakioka 2001). Spred1 is primarily expressed in adult brain and some fetal tissues, and has been suggested therefore to play a role during development (Engelhardt 2004).

Whereas Sprouty is evenly distributed in the cytoplasm of unstimulated cells, ligand-dependent RTK activation leads to translocation of Sprouty to the leading edge of the plasma membrane (Impagnatiello 2001, Lim 2000, Lim 2002). Additionally, Sprouty2 has been reported to be localized to endosomes (Kim 2007) or microtubules (Lim 2000, Mason 2004). The testicular protein kinase 1 (Tesk1) has been recently identified as a negative regulator of

Sprouty2 function. Upon growth factor stimulation, co-expression of Tesk1 inhibits the translocation of Sprouty2 to membrane ruffles (Chandramouli 2008).

Like Sprouty, Spred proteins are best described so far as membrane-associated suppressors of growth factor-induced activation of the Ras/Raf/MAPK signaling pathway (Wakioka 2001, Hacohen 1998, Kramer 1999, Reich 1999, Miyajima 1992). After receptor activation Grb2 (growth factor receptor-bound 2) binds to phosphorylated tyrosine residues of the receptor or phosphorylated adaptor molecules. Forming a complex with SOS (son of sevenless), Grb2 activates Ras by exchange of bound GDP with GTP. GTP-bound Ras recruits Raf-1 which in turn is activated. The kinase MEK is phosphorylated and activated by Raf, which leads to phosphorylation and activation of ERK (Lowy 1993, Robinson 1997, Kerkhoff 2001). Activated ERK targets various cytoplasmatic and membrane-linked proteins for activation. Moreover, ERK is rapidly translocated into the nucleus where it activates transcription factors (Karin 1995, Hunter 2000). The ERK signaling pathway has been implied in several cellular processes like cell proliferation, differentiation and migration (Davis 2000, Kampen 2000).

Sprouty can affect RTK signaling at different levels which may be dependent on the cellular context (reviewed in Kim 2004). Inhibition of growth factor-mediated signaling by Sprouty has been described either at the level of Raf activation (Yusoff 2002, Sasaki 2003) or upstream of Ras by interference with the Grb:Sos complex (Lao 2006). The inhibitory effect of Spred has been suggested at the level of Ras-mediated activation of Raf (Wakioka 2001). Sprouty proteins may be very selective inhibitors, since they reportedly interfere with fibroblast growth factor- (FGF) mediated ERK activation while they do not affect epidermal growth factor (EGF)-induced signaling (Impagnatiello 2001, Sasaki 2001, Egan 2002, Wong 2001). Several interaction partners have been discovered for Sprouty including c-Cbl (cellular homologue of Casitas B lineage lymphoma proto-oncogene product), Grb2, Raf-1, Caveolin1, Gap1 (GTPase-activating protein), FRS2 (fibroblast growth factor receptor substrate 2), SHP-2 (Src homology 2 domain-containing protein-tyrosine phosphatase 2), Tesk1, PTP1B (protein tyrosine phosphatase 1B), and PTEN (Casci 1999, Sasaki 2003, Wong 2001, Impagnatiello 2001, Leeksma 2002, reviewed in Dikic 2003 and Kim 2004; Yigzaw 2003, Edwin 2006). Of these Raf-1 and Caveolin1 are also reported to bind to Spred (Sasaki 2003, Nonami 2005), and are considered to be necessary for inhibition of ERK phosphorylation by both Spry and Spred.

Phosphorylation of Sprouty proteins is crucial for their physiological function (Rubin 2003, Hanafusa 2002, Sasaki 2001, Tefft 2002, Mason 2004, Fong 2003). Sprouty2 can be phosphorylated at the N-terminal Y55 (Rubin 2003, Hanafusa 2002, Wong 2001, Fong 2003) or at C-terminal tyrosine residues, which plays a role in the specific Sprouty2-mediated inhibition (Rubin 2005). As recently discovered, phosphorylation of Sprouty2 can also occur

on conserved serine residues, which leads to the stabilization of the protein and is involved in its activation upon growth factor stimulation (Lao 2007, DaSilva 2006).
An inhibitory effect of Sprouty proteins on cell migration has been reported mainly in context with growth factor-induced signaling. The expression of human Sprouty2 in HeLa cells for example not only inhibits proliferation but also abrogates the migration-enhancing effect of growth factors (Yigzaw 2001). Additional data for the involvement of Sprouty in migratory processes come from embryonic development of *Xenopus laevis*, where Sprouty2 inhibits the FGF-mediated movement of mesodermal cells during gastrulation (Nutt 2001). The ability of human Sprouty2 to inhibit growth factor-mediated cell migration has been attributed to its C-terminal domain (Yigzaw 2001). Interestingly, blocking of ERK activity is no prerequisite for Sprouty2-mediated inhibition of migration. EGF-induced ERK activation is not altered by over-expression of human Sprouty2 in HeLa cells, although proliferation and EGF-mediated migration is markedly attenuated (Yigzaw 2001, Yigzaw 2003). The anti-migratory effect of human Sprouty2 has been suggested to be mediated by up-regulation of PTP1B activity, which is accompanied by reduced protein tyrosine phosphorylation (Yigzaw 2003).
In this study, we identified Sprouty2 and Spred1 to be negatively regulated by PGE_2 during maturation von MoDCs. Because PGE_2 promotes the migratory phenotype of mature DCs, we attempted to determine a new regulatory mechanism of chemokine receptor signaling involving Sprouty2 and Spred1.

Results

PGE_2 inhibits expression of Sprouty2 and Spred1 in mature MoDCs

PGE_2 is a crucial prerequisite during human DC maturation in order to induce a migratory phenotype (Scandella 2002, Luft 2002, Legler 2006). Although its key role is well established, the underlying mechanism by which PGE_2 enables chemotaxis is still unclear. To understand how PGE_2 increases responsiveness to chemokines and chemoattractants, we performed a global gene expression analysis. Therefore, we generated MoDCs and induced maturation in the absence or presence of PGE_2 using poly I:C or sCD40L. After 2 days of maturation cells were harvested and total RNA was extracted. The gene expression profiling was performed by Altana Pharma (Konstanz, Germany).
Results from the gene expression analysis showed two related gene products to be negatively regulated by PGE_2, namely Sprouty2 and Spred1. To corroborate these findings we analyzed MoDCs matured in the absence or presence of PGE_2 by quantitative real-time PCR. MoDCs were matured via TLR3 ligation using poly I:C or through stimulation of CD40. Regardless of the maturation pathway used, addition of PGE_2 leads to a 90% down-

regulation of Sprouty2 expression (Figure 1A). MoDCs matured in the presence of PGE_2 showed a 95% lower expression of Spred1 compared to MoDCs matured without PGE_2 (Figure 1B). Since we found a substantial donor to donor variation with poly I:C-matured MoDCs, it is not possible to directly compare the degree of inhibition induced by PGE_2 in MoDCs matured with different stimuli.

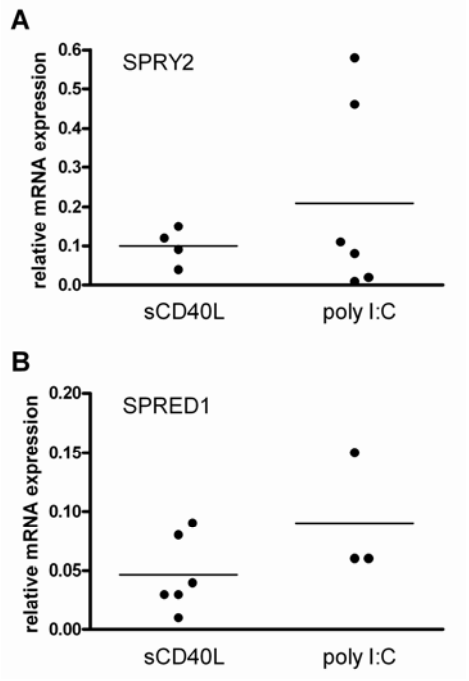

Figure 1. The presence of PGE_2 during maturation of MoDCs negatively regulates mRNA expression of Sprouty2 and Spred1. MoDCs were generated under serum-free conditions and maturation was induced using either sCD40L or poly I:C. Cells were allowed to matured for two days in the absence or presence of PGE_2. Expression levels of mRNA for Sprouty2 (**A**) and Spred1 (**B**) were analyzed by quantitative real-time PCR and normalized to two house-keeping genes. Specific mRNA expression of PGE_2-matured MoDCs was calculated relative to MoDCs matured in the absence of PGE_2. Relative expression of Sprouty2 (**A**) and Spred1 (**B**) mRNA is shown as PGE_2-induced change.

We next determined the expression of Sprouty2 on protein level. MoDCs were matured in the absence or presence of PGE_2 using sCD40L or poly I:C. Mature MoDCs were harvested and Sprouty2 expression was analyzed by Western Blotting. The down-regulation of Sprouty2 which was observed on mRNA level (Figure 1) was mirrored on protein level since PGE_2 strongly inhibited Sprouty2 protein expression in mature MoDCs (Figure 2). We could not analyze the expression of Spred1 on protein level due to unavailability of a specific anti-human Spred1 antibody.

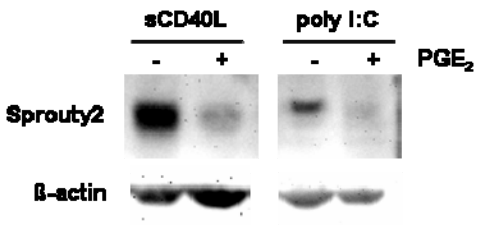

Figure 2. Sprouty2 protein expression is inhibited in MoDCs matured in the presence of PGE_2. MoDCs were generated and matured either with sCD40L (left panel) or with poly I:C (right panel) in the absence or presence of PGE_2. After two days of maturation protein content was analyzed by Western blotting. For the detection of Sprouty2 an anti-human Sprouty2 specific antibody was used. The blot was re-probed with anti-ß-actin antibody to ensure equal protein loading.

Since the mRNA expression of Sprouty2 was quantified between mature MoDCs as a function of the presence of PGE_2 (Figure 1), we additionally analyzed Sprouty2 expression profile of immature MoDCs and its development during maturation. Therefore, maturation

was induced in immature MoDCs using sCD40L in the absence or presence of PGE_2. Cells were harvested after 6h, 24h or 48h of maturation and analyzed for their content of Sprouty2 mRNA. Expression levels were compared to immature MoDCs from the same donor. Interestingly, Sprouty2 mRNA expression was induced early during maturation in the absence of PGE_2 (Figure 3A). However, the expression of Sprouty2 stabilized again on the same level as observed in immature MoDCs at later phases of maturation. The presence of PGE_2 during maturation inhibited Sprrouty2 mRNA expression even at early time-points and throughout maturation (Figure 3A).

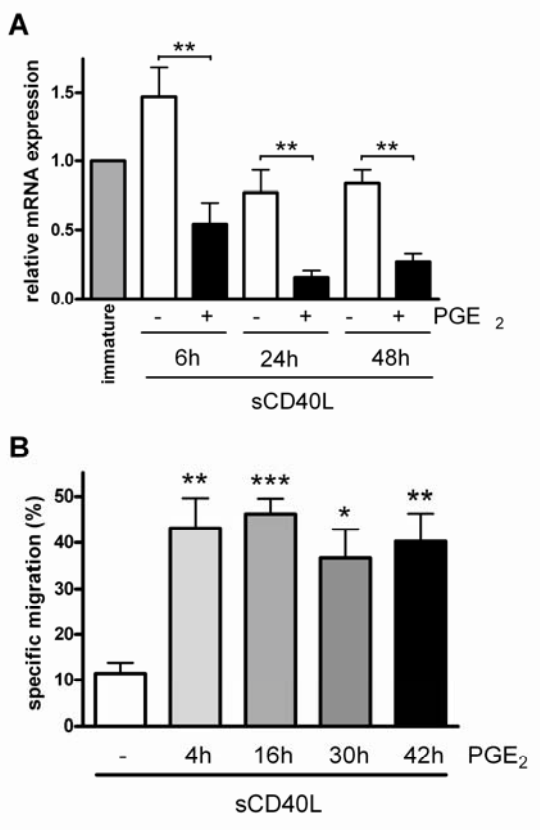

Figure 3. Kinetic effects of PGE_2 during MoDC maturation. (**A**) Expression of Sprouty2 mRNA in MoDCs during maturation and the effect of PGE_2. Immature MoDCs (grey bar) and MoDCs matured with sCD40L in the absence (white bars) or presence (black bars) of PGE_2 were analyzed for Sprouty2 expression on mRNA level by quantitative real-time PCR. Maturation was stopped and cells analyzed after 6h, 24h, or 48h of maturation. Expression of Sprouty2 mRNA was normalized to two housekeeping genes and calculated relative to Sprouty2 expression in immature MoDCs of the same donor. Mean values and SEM of six independent experiments with different donors are shown.
(**B**) Early and short time presence of PGE_2 during MoDCs maturation is sufficient to induce a migratory phenotype. MoDCs were matured via CD40 stimulation in the absence (white bar) or presence of PGE_2. Where indicated, PGE_2 was removed after 4h, 16h, or 30h of maturation and maturation continued in the absence of PGE_2. After 42h of maturation, MoDCs were analyzed in chemotaxis assays for their migratory behaviour towards 250 ng/ml CCL21. Specific migration was calculated relative to the number of input cells after subtraction of spontaneous migration to medium alone. Means and SEM of 6 independent experiments with different donors are shown. (**A**, **B**) Statistical significance was calculated using paired student's t-test with * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Because the addition of PGE_2 affected expression of Sprouty2 mRNA already after 6h of maturation, we determined the time period in which the presence of PGE_2 was necessary to establish migratory properties. To this end, MoDCs were matured through stimulation of CD40 while PGE_2 was added for the initial 4h, 16h or 30h of maturation, or for the whole time. Migratory capacities were analyzed in response to CCL21 after two days of maturation. Surprisingly, the addition of PGE_2 for only the first four hours of maturation was sufficient to induce full migration (Figure 3B). Hence, the early inhibitory effect of PGE_2 on Sprouty2 mRNA expression correlates with the full migratory ability of short-term PGE_2-treated MoDCs.

Effects of Sprouty2 over-expression in lymphoid cell lines

Since we found that the PGE_2-induced migratory phenotype of mature MoDCs correlates with a decreased expression of Sprouty2, we attempted to analyze the role of Sprouty2 in migration of DCs. In order to introduce strong Sprouty2 expression and monitor its effect on chemotactic behaviour of transfected cells, we cloned Sprouty2 into the pEGFP-N1 vector, thereby creating a Sprouty2-EGFP fusion construct. The GFP fusion allows determination of transfection efficiency and analysis of only the population carrying the Sprouty2 construct. Additionally, we created an EGFP fusion construct of a Sprouty2 mutant, termed SPRY2-Y55A-EGFP. In this mutant, which was a kind gift Dr. Akihiko Yoshimura (Kyushu University, Japan), the N-terminal tyrosine residue on position 55 is substituted by an alanine, disabling phosphorylation at this position.

We used lymphoid cell lines as model systems for the analysis of Sprouty2 in chemotactic processes of immune cells.

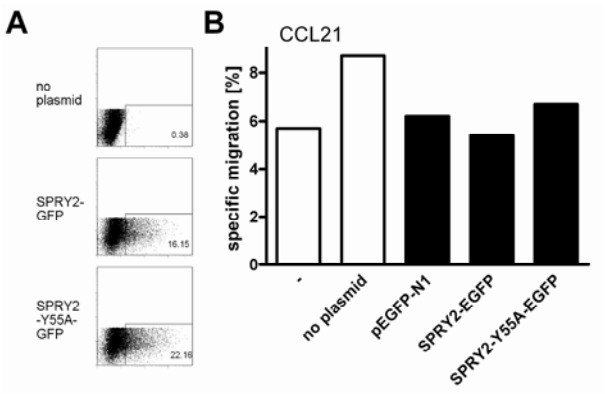

Figure 4. Over-expression of Sprouty2 does not affect CCR7-mediated migration of Raji cells. Raji cells were transfected with pEGFP-N1, SPRY2-EGFP, or a mutant Sprouty2 lacking the tyrosine residue at position 55 (SPRY2-Y55A-EGFP). As controls, cells were also transfected without plasmid. (**A**) Transfection efficiency was analyzed by flow cytometry. Percentage of living, GFP-expressing cells are given. (**B**) Transfected or non-transfected cells (-) were analyzed for migratory properties in response to CCL21 [500 ng/ml] using Transwell chemotaxis assays. Specific migration of living (white bars) or living, GFP-expressing cells (black bars) are shown from one experiment out of two performed.

The first cell line we used was Raji, a human lymphoblast-like cell line generated 1963 from an eleven year old male Burkitt's lymphoma patient. Raji cells could be transfected using Amaxa nucleofector technology. Transfection efficiency for Sprouty2-EGFP and Sprouty2-Y55A-EGFP fusion proteins yielded around 20% (Figure 4A). Although Raji cells express CCR7 and CXCR4 (data not shown), only a small proportion of cells migrated specifically towards CCL21 (5-8%, Figure 4B). Specific migration towards CXCL12 was even less (below 2%, data not shown). Expression of pEGFP-N1 alone did not alter the migratory capacity of Raji cells towards CCL21 (Figure 4B). However, over-expression of Sprouty2-EGFP as well as the Y55A-phosphorylation-deficient mutant had no effect on chemotactic responses of Raji cells (Figure 4B). Since transfection efficiency and specific chemotactic responses were very low, we sorted Raji cells one day after transfection to gain a pure population of cells highly expressing Sprouty2-EGFP and a control population negative for Sprouty2-expression. Although the procedures of transfection and sorting are very harsh, more than 80% of the cell population was still viable as tested by ToPro3 staining with flow cytometry

(Figure 5A). As depicted in Figure 5A, over 94% of the population selected for Sprouty2-EGFP-positive cells highly expressed Sprouty2-EGFP. When subjected to chemotaxis analysis, Sprouty2-EGFP expression had no effect on migration of Raji cells towards different concentrations of CCL21 (Figure 5B). In previous studies, Sprouty2 has been shown to inhibit growth factor-induced ERK activation (Martinez 2007). Therefore, we analyzed chemokine-induced ERK activation in addition to migration in the same sorted populations of Raji cells. In those cells that had been sorted for their lack of Sprouty2-EGFP expression, termed Sprouty2-EGFP-negative, ERK-phosphorylation could be induced with CCL21 as well as with CXCL12. In Raji cells over-expression of Sprouty2-EGFP had no effect on ERK activation. ERK was strongly phosphorylated in response to CXCL12 and could also be detected after CCR7 stimulation. Phosphorylation of ERK was weak after stimulation with CCL21 in Sprouty2-EGFP-positive cells as well as in Sprouty2-EGFP-negative cells.

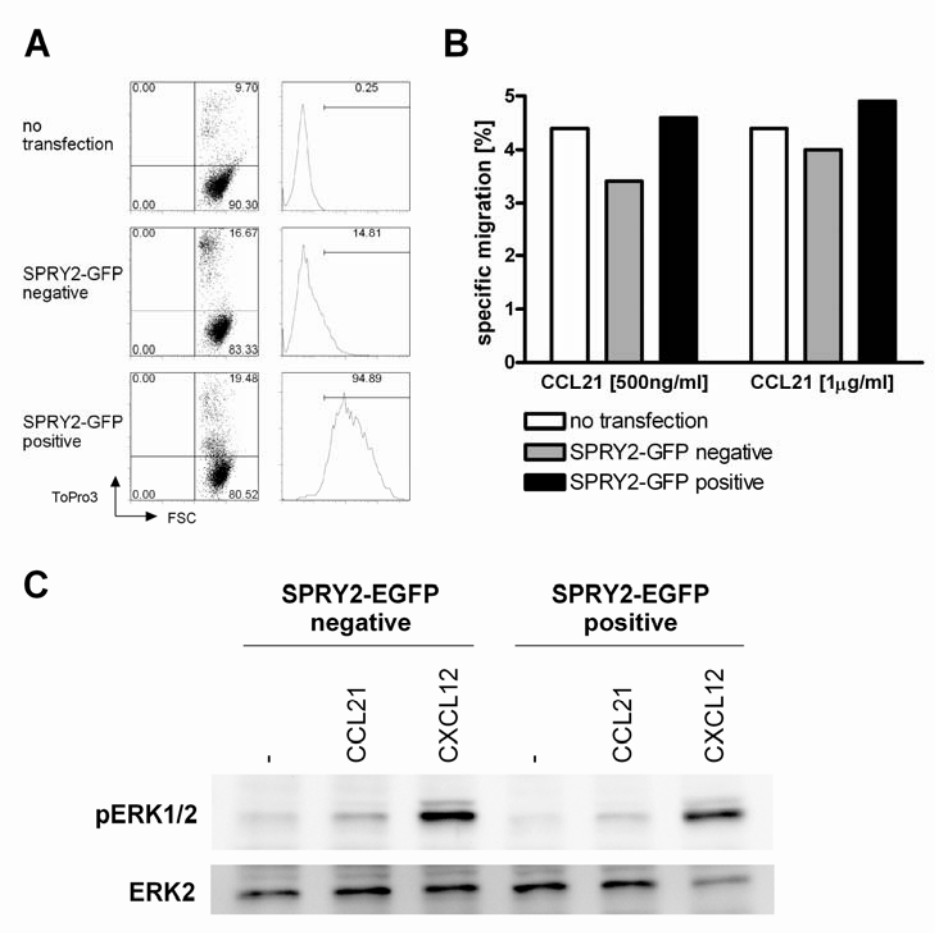

Figure 5. High expression of Sprouty2 does not alter Raji cell functions in response to chemokines. Raji cells were transfected with Sprouty2-EGFP using Amaxa nucleofector technology. One day later cells were sorted according to GFP expression into a high Sprouty-EGFP expressing population (Sprouty2-GFP positive) and a Sprouty2-GFP negative population (A, right panel). Dead cells were stained with ToPro3 (**A**, left panel). (**B**) Sorted transfected Raji cells were analyzed for chemotactic responses towards 500 ng/ml or 1μg/ml CCL21 using Transwell migration assays. As a control, untreated Raji cells were taken directly from culture (white bars). (**C**) Sorted Sprouty2-EGFP negative and positive populations were stimulated for two min with CCL21 [1 μg/ml] or CXCL12 [1 μg/ml], or left untreated (-). Cells were lysed, and activation of ERK was monitored by Western blotting using a specific anti-human phospho-ERK1/2 antibody. To control protein loading, blots were re-probed with an anti-human ERK2 antibody.

As a second cell line model we used CEM cells, which are human T lymphoblastoid cells. Analysis of the chemokine receptor profile on the surface of CEM cells revealed expression of CCR4, CCR7 (Figure 6A) and CXCR4 (Figure 6A), while CCR1, CCR5, CCR6, CCR9 and CXCR2 were absent (data not shown). Using Amaxa nucleofection technology CEM cells could be transfected with high efficiency, around 80% with pEFP-N1 (Figure 6B). CEM cells were transfected with Spry2-EGFP yielding nearly 70% positive cells (Figure 6B). To determine the effect of Sprouty2 on the migratory behaviour of CEM cells, we transfected CEM cells with either pEGFP-N1 or Sprouty2-EGFP and performed chemotaxis assays one day later. Cells carrying pEGFP-N1 migrated towards CCL21 (Figure 6C) and to a lesser extend towards CXCL12 (Figure 6D). As already observed in Raji cells, over-expression of Sprouty2-EGFP did not attenuate CCR7-mediated migration (Figure 6C). Interestingly, migration towards CXCL12, however, was strongly inhibited when Sprouty2 was expressed in CEM cells (Figure 6D). It could be possible that this inhibition of CXCR4-mediated chemotaxis is dependent on the phosphorylation of the N-terminal tyrosine residue at position 55. The Sprouty2-Y55A-EGFP mutant could be expressed with sufficient efficiency in only one experiment. However, over-expression of Sprouty2-Y55A-EGFP in this experiment had no effect on CXCR4-mediated migration, while Sprouty2-EGFP inhibited chemotaxis towards CXCL12 completely (data not shown).

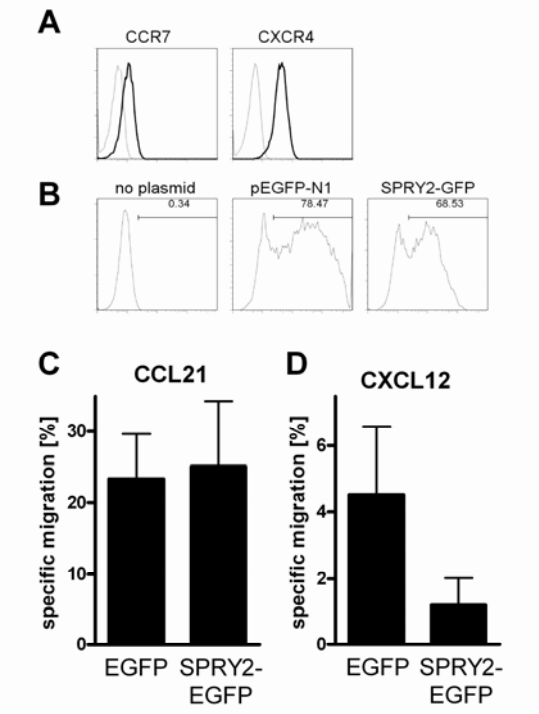

Figure 6. Sprouty2 over-expression in CEM cells abrogates CXCR4-mediated migration. (**A**) Surface expression of CCR7 (left, black line) and CXCR4 (right, black line) was analyzed on CEM cells by flow cytometry. Grey thin lines represent staining using an isotype control. (**B**) CEM cells were transfected with pEGFP-N1, Sprouty2-EGFP, or with no plasmid, and GFP signals were monitored one day after transfection by flow cytometry. Numbers indicate the percentage of living cells, which stained negative for ToPro3, expressing GFP. (**C**, **D**) Transfected CEM cells were analyzed for migratory properties towards CCL21 (**C**) or CXCL12 (**D**) in Transwell chemotaxis assays. Specific migration of only living, GFP expressing cells is presented. Results from three (**C**) and two (**D**) independent experiments are shown.

We also tried to induce over-expression of Spred1 in CEM cells. Since the transfection efficiency using the Spred1-EGFP construct was always very low, we can not draw general conclusions. However, in one experiment, in which 42% of living cells were positively

transfected with Spred1-EGFP, Spred1 expression did not inhibit CCL21-induced migration but completely blocked CXCR4-mediated chemotaxis (data not shown).
Although the migration experiments with Sprouty2- and Spred1-over-expressing cells need to be reproduced, there is a clear tendency suggesting a role for Sprouty2 and Spred1 in CXCR4-mediated but not CCR7-mediated chemotaxis.

Effects of Sprouty2 and Spred1 in chemokine-mediated responses of MoDCs

In order to analyze to role of Sprouty2 and Spred1 in MoDCs, we established a protocol for MoDCs transfection using Amaxa nucleofection. In this procedure, MoDCs are harvested two days after induction of maturation and transfected as described in the Materials and Methods section. To increase survival, MoDCs were transferred to RPMI containing serum after transfection. One day after transfection MoDCs were analyzed for transfection efficiency and migratory behaviour. Around 40% of mature MoDCs could be transfected with pEGFP-N1 (Figure 7A). Despite the harsh procedure of transfection and the prolonged culturing period, pEGFP-N1-transfected MoDCs still showed chemokine-directed migration (Figure 7B).

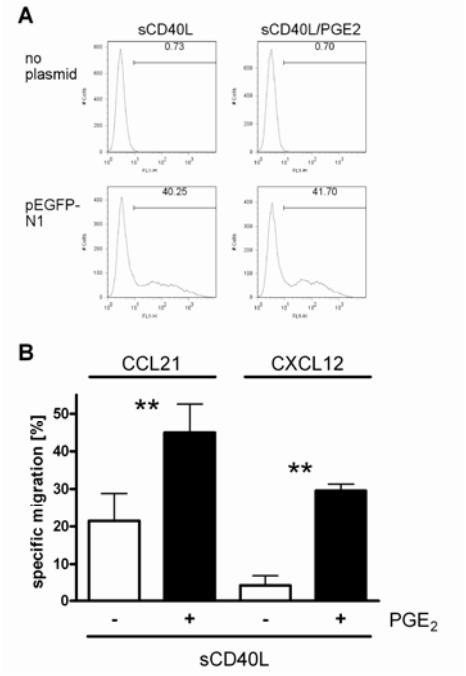

Figure 7. Transfection efficiency and migration of transfected MoDCs. MoDCs were generated and matured by ligation of CD40 in the absence or presence of PGE_2. After two days mature MoDCs were harvested and transfected with pEGFP-N1 (**A**, lower panel) or no plasmid (**B**, upper panel) using Amaxa nucleofector technology. (**A**) Transfection efficiency was analyzed one day after nucleofection by flow cytometry. Numbers indicate the percentage of EGFP expressing cells of all living MoDCs. Dead cells were excluded from analysis by staining with ToPro3. (**B**) MoDCs expressing pEGFP-N1 were analyzed for chemotactic responses towards 250 ng/ml CCL21 or 250 ng/ml CXCL12 in Transwell chemotaxis experiments. Only living, EGFP expressing cells were taken into analysis. Dead cells were stained with ToPro3 and excluded. Specific migration is presented as percentage of all living, EGFP expressing cells. Mean values and SEM of six (CCL21) or four (CXCL12) independent experiments with different donors are shown. Statistical significance was calculated using paired student's t-test with $p<0.01$ for **.

MoDCs that were matured in the presence of PGE_2 migrated more efficiently towards CCL21 and CXCL12 even though they were transfected with pEGFP-N1 (Figure 7B). By setting up a protocol for the introduction of expression vectors in MoDCs without changing migratory properties we were now able to analyze the effects of Sprouty2 and Spred1 over-expression in MoDCs. Although transfection efficiency varied between individual experiments, usually 15-45% of mature MoDCs expressed either pEGFP-N1 or Sprouty2-EGFP (Figure 8A). Expression of Spred1 was less efficient as already observed in CEM cells. To abrogate the

PGE_2-induced down-regulation of Sprouty2 and Spred1 expression in mature MoDCs we introduced Sprouty2-EGFP and Spred1-EGFP into MoDCs that were matured with sCD40L in the presence of PGE_2. As a control we also transfected pEGFP-N1. MoDCs matured in the presence of PGE_2 and expressing pEGFP-N1 showed stronger migration towards CCL21 and CXCL12 compared to pEGFP-N1-expression MoDCs that were matured without PGE_2 (Figure 8B, C). As observed in Raji cells and CEM cells, over-expression of Sprouty2 had no effect on CCR7-mediated migration of MoDCs. Expression of Spred1 could also not inhibit migration towards CCL21 (Figure 8B). However, when the PGE_2-induced down-regulation of Sprouty2 was abrogated by over-expression of Sprouty2-EGFP, the enhanced migratory capacity of PGE_2-matured MoDCs towards CXCL12 was strongly inhibited (Figure 8C). Over-expression of Spred1 seemed to have a similar effect, although the observed donor to donor variation demands reproduction of the experiment.

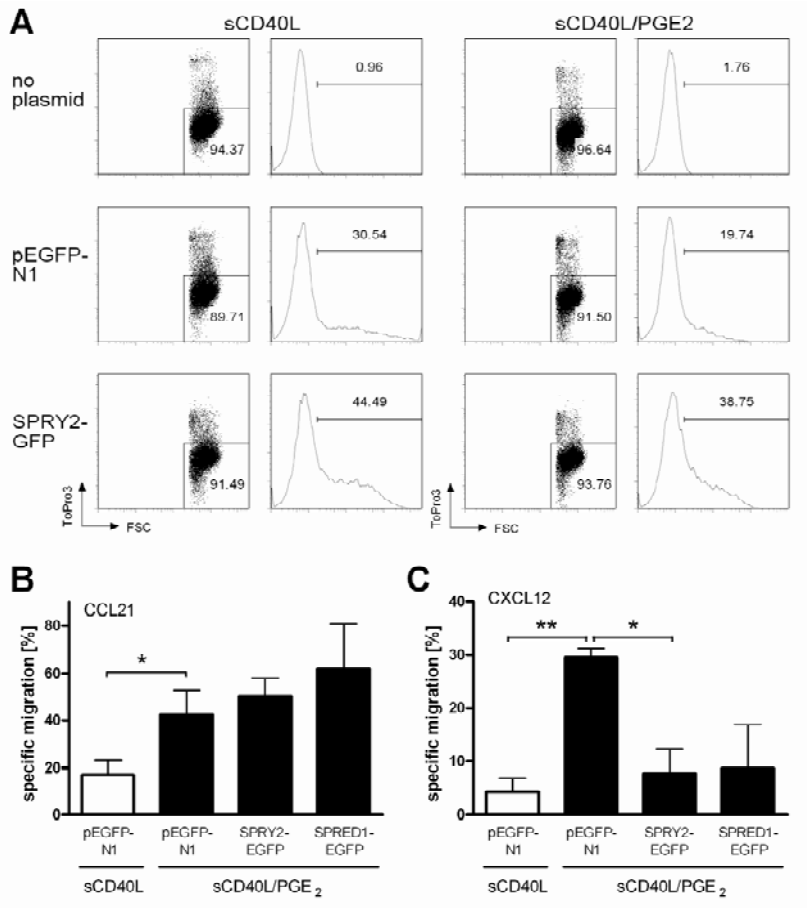

Figure 8. Over-expression of Sprouty2 and Spred1 inhibits CXCR4-mediated but not CCR7-mediated migration of MoDCs. MoDCs were matured using sCD40L in the absence or presence of PGE_2. Two days after induction of maturation MoDCs were transfected with pEGFP-N1, Sprouty2-EGFP, SPRED1-EGFP or without plasmid using Amaxa nucleofection technology. (**A**) One day later MoDCs were analyzed by flow cytometry. Dead cells were stained with ToPro3 and excluded from analysis. Transfection efficiency is presented as percentage of GFP expressing cells of all living cells. Transfected MoDCs were subjected to Transwell chemotaxis assays to monitor migratory properties in response to CCL21 (**B**) or CXCL12 (**C**). Specific migration of living, GFP expressing MoDCs is presented. Differences between groups were calculated by paired student's t-test with $p<0.05$ for * and $p<0.01$ for **. Mean values and SEM of four independent experiments with different donors are shown. Results for SPRED1-EGFP over-expression represent means of three independent experiments

As in CEM cells and partially in Raji cells over-expression of Sprouty2 and Spred1 in mature MoDCs seems to inhibit CXCR4-mediated migration while migration via CCR7 is not affected. These data suggest a specific role for Sprouty2 and Spred1 in regulation of chemokine-mediated responses.

In growth factor mediated signaling Sprouty2 exerts its inhibitory effect by inhibition of ERK phosphorylation. Since the expression levels of Sprouty2 and Spred1 are diminished in MoDCs matured in the presence of PGE_2, we analyzed ERK activation after chemokine receptor stimulation in mature MoDCs. Therefore MoDCs were matured using sCD40L in the absence or presence of PGE_2. After 2 days, cells were harvested and stimulated with CCR7 or CXCR4 ligands. Phosphorylation of ERK was monitored by Western blotting. Signaling via CCR7 induced activation of ERK in MoDCs irrespective of the presence of PGE_2 during the maturation process (Figure 9A). Although stimulation of CXCR4 induced stronger ERK phosphorylation in MoDCs matured under PGE_2-containing conditions (Figure 9B), ERK was also activated in MoDCs matured without PGE_2. The enhanced phosphorylation of ERK after CXCR4 stimulation in PGE_2-matured MoDCs correlates with the down-regulated expression of Sprouty2 and Spred1. This could point to a regulatory role of Sprouty2 and Spred1 in CXCR4-mediated responses.

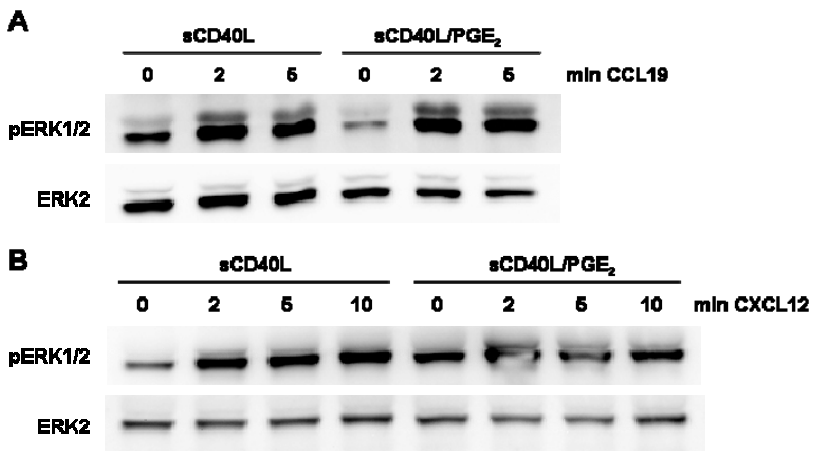

Figure 9. Effect of Sprouty2 over-expression on chemokine-mediated ERK activation in MoDCs. MoDCs were matured by ligation of CD40 in the absence or presence of PGE_2. Two days later mature MoDCs were stimulated for two, five, or ten minutes with 1 μg/ml CCL19 (**A**) or 1μg/ml CXCL12 (**B**). Activation of ERK was analyzed by Western blotting using a specific anti-human ERK1/2 antibody. Blots were re-probed with a specific anti-human ERK2 antibody to control protein loading.

It has been shown previously that the expression of human Sprouty2 increases the amount and activity of PTP1B, a phosphatase decreasing tyrosine phosphorylation of cellular proteins. In HeLa cells, over-expression of Sprouty2 led to a PTP1B-mediated decreased phosphorylation of p130Cas (Yigzaw 2003). The phosphorylation status of p130Cas modulates migratory responses of cells (Cary 1998, Klemke 1998, Ruest 2001) because p130Cas is a critical component of the focal adhesion complex (O'Neill 2000 Trends Cell Biol 10:111-119). Hence, the PTP1B-mediated decrease of p130Cas phosphorylation has been suggested to contribute to the anti-migratory actions of Sprouty2. Since PGE_2 down-regulates Sprouty2 in mature MoDCs, we tested if p130Cas phosphorylation takes place after stimulation of chemokine receptors. In MoDCs matured in the presence of PGE_2, stimulation of CXCR4 induced stronger phosphorylation of p130Cas than CCR7 stimulation (Figure 10). It still has to be determined, if p130Cas phosphorylation is inhibited by Sprouty2 expression in MoDCs matured in the absence of PGE_2.

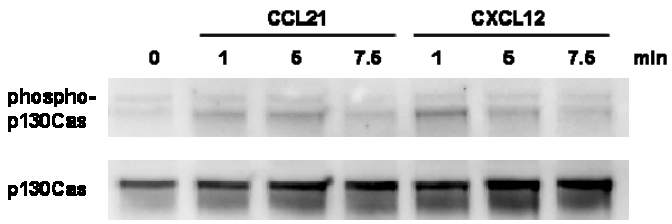

Figure 10. Chemokine-mediated activation of p130Cas in mature MoDCs. MoDCs were matured by ligation of CD40 in the presence of PGE_2. After two days of maturation cells were harvested and stimulated with CCL21 [1μg/ml] or CXCL12 [1μg/ml] for one, five or 7.5 min. Phosphorylation of p130Cas was analyzed in comparison to untreated MoDCs (0) by Western blotting using a specific anti-human phospho-p130Cas antibody (upper panel) . Blots were re-probed with an anti-human p130Cas antibody.

The inhibition of p130Cas phosphorylation could contribute to the chemotactic inability of MoDCs matured without PGE_2. Additionally, it has to be tested if the over-expression of Sprouty2 in PGE_2-matured MoDCs which inhibits CXCR4-mediated migration correlates with decreased phosphorylation of p130Cas.

Discussion

In mammals, the expression of Sprouty proteins and their role in the regulation of growth factor-mediated processes have so far been best described in embryonic tissues like lung, brain, heart, gut and muscles (reviewed in Kim 2004). The regulation of Sprouty2 in adult tissues however has not been well characterized. In adult mice Sprouty2 is highly expressed in brain, lung and heart (Tefft 1999). Sprouty2-deficient mice show a severe phenotype including hearing loss, enteric nerve hyperplasia and hypergangliosis, abnormal physiology of the digestive motility system and reduced body size, and Sprouty2 deficiency leads to premature death (Shim 2005, Taketomi 2005). Spred1 has been reported to be primarily expressed in adult brain and in some fetal tissues (Engelhardt 2004). Studies with knock out mice revealed that Spred1 is not vitally necessary for development and fertility, since adult mice deficient for Spred1 are viable and show no apparent defects (Bundschu 2005, Kuhnel 2004). However, loss of functional Spred1 revealed its involvement in growth regulation, since $Spred1^{-/-}$ mice show a dwarf phenotype (Inoue 2005).

In this study we are the first to describe expression of Sprouty2 and Spred1 in human MoDCs. Sprouty2 mRNA was expressed in immature MoDC, but was further induced early during the process of maturation. When maturation was initiated by sCD40L alone, the expression level of Sprouty2 mRNA returned to the same level as observed in immature MoDCs after 24 hours of maturation (Figure 3A). Addition of PGE_2 to the maturation cocktail not only inhibited Sprouty2 expression after 48 hours of maturation (Figure 1A), but affected expression of Sprouty2 already after six hours of maturation, since it counteracted the early maturation-induced enhanced expression of Sprouty2 (Figure 3A). We analyzed time-

dependent expression of Sprouty2 in comparison to immature MoDCs only after sCD40L-induced maturation; for that reason we can not conclude if the induction of Sprouty2 expression is a general feature of early maturation or rather a CD40-signaling-specific phenomenon. After growth factor-mediated stimulation Sprouty2 has been reported to be induced in mammalian fibroblasts and endothelial cells. This induction has been shown to be mediated by ERK activation and to serve as a negative feedback loop for the regulation of this pathway, as Sprouty2 inhibits the ERK/MAPK pathway (Ozaki 2001, Impagnatiello 2001, Sasaki 2001). Engagement of CD40 has been demonstrated to result in activation of the ERK signaling pathway through activation of Src kinases in human MoDCs (Vidalain 2000), and CD40-mediated signals induce Sprouty2 expression early during maturation of MoDCs. Hence, the early CD40L-mediated induction of Sprouty2 expression during maturation of MoDCs could be promoted by ERK activation. If Sprouty2 subsequently serves as a negative regulator of CD40-mediated ERK signaling needs to be determined. It is also not clear what effect the early expression of Sprouty2 might have on the process of maturation. CD40 signaling-mediated ERK activation controls the induction of certain cytokines like IL-1α and IL-1ß as well as the expression of the IL-1 receptor alpha chain (Vidalain 2000). Since the CD40L-induced expression of Sprouty2 in MoDCs is temporary and restricted to very early maturation, it is possible that in our system it does not play a role in the outcome of maturation after 48 hours, since expression of Sprouty2 on protein level is very high in MoDCs matured in the absence of PGE_2.

The presence of PGE_2 during two days of maturation results in MoDCs with strongly inhibited Spry2 expression; additionally we found the expression of Spred1 to be equally strong attenuated. The inhibitory effect of PGE_2 seems not to be dependent on the maturation stimulus, since we found PGE_2-induced down-regulation of Sprouty2 and Spred1 expression in sCD40L and poly I:C matured MoDCs. Moreover, PGE_2-mediated inhibition of Sprouty2 expression on mRNA level resulted in low level protein expression in both sCD40L- and poly I:C-matured MoDCs. However, maturation with poly I:C or sCD40L resulted in different expression levels of Sprouty2. MoDCs matured with poly I:C expressed Sprouty2 at lower levels compared to sCD40L-matured MoDCs. This was evident in real-time PCR, where there was less Sprouty2 mRNA in poly I:C-matured MoDCs (data not shown) and on protein level (Figure 2). However, regardless of how much Sprouty2 protein was present in mature MoDCs, PGE_2 strongly down-regulated Sprouty2 expression to the same level in poly I:C- and sCD40L-matured MoDCs (Figure 1A). The signaling pathway by which PGE_2 inhibits Sprouty2 and Spred1 expression needs to be determined.

Several studies demonstrated in a variety of cell types that over-expression of Sprout2 (Yigzaw 2001, Lee 2004, Zhang 2005, Edwin 2006) inhibits migration and proliferation in response to serum and growth factors. In contrast to Sprouty from *Drosophila melanogaster*

(Kramer 1999), mammalian Sprouty proteins do not only inhibit but can also potentiate RTK signaling (Cabrita 2003, Christofori 2003). In the case of Sprouty2, opposing effects of the C- and the N-terminus were described in the regulation of EGF-receptor signaling (Egan 2002). The actions of Sprouty2 are under regulation by Tesk1. Binding of Tesk1 inhibits the function of Sprouty2 to interfere with ERK phosphorylation (Chandramouli 2008). Additionally, the function of Sprouty2 can be controlled through its state of phosphorylation. Several studies described the necessity of phosphorylation for the physical functions of Sprouty2 (Rubin 2003, Hanafusa 2002, Sasaki 2001, Tefft 2002, Mason 2004, Fong 2003). While in earlier studies investigations focused on the crucial phosphorylation of the N-terminal tyrosine 55 (Rubin 2003, Hanafusa 2002, Wong 2001, Fong 2003), more recent reports show functional activity of Sprouty2 to be regulated by phosphorylation of C-terminal tyrosine residues (Rubin 2005) as well as serine residues (Lao 2007, DaSilva 2006). The activity of the Sprouty2 pool can thus be controlled by their phosphorylation status as well as by interactions with other proteins (Jarvis 2006). The complex actions of Sprouty2 and its regulation on multiple levels make it difficult to dissect the role of Sprouty2 in migration of MoDCs. Over-expression of Sprouty2-EGFP in MoDCs as well as in CEM cells inhibited CXCR4-mediated chemotaxis (Figure 8C, 6D). The artificial over-expression of Sprouty2 might however not mimic endogenous Sprouty2 expression, since posttranslational regulation mechanisms might interfere with regularly produced Sprouty2 but can not cope with artificial over-expression. Nevertheless, we found a correlation between the PGE_2-induced migratory capacity of MoDCs and the PGE_2-mediated down-regulation of Sprouty2 expression. MoDCs matured with sCD40L alone, which expressed a considerable amount of endogenously produced Sprouty2 protein (Figure 2), showed only very low migration towards CXCL12 (Chapter 2 Figure 4B).

Over-expression of Spred1 in MoDCs showed the tendency to strongly inhibit CXCR4-mediated migration (Figure 8C), whereas chemotaxis via CCR7 was not affected (Figure 8B). It should be pointed out that due to strong variations between donors and a limited number of donors tested, the results for Spred1 over-expression should be considered preliminary, until further supporting data is obtained.

We find that stimulation of CCR7 or CXCR4 leads to the phosphorylation of ERK in mature MoDCs independently of the presence of PGE_2 during maturation. However, although the experiment needs to be reproduced with more donors, ERK activation after CXCR4 stimulation seemed to be enhanced in MoDCs matured in the presence of PGE_2. However, the requirement of ERK activation for CXCR4-mediated migration is controversial. Interestingly, a very recent report described a role for CXCL12:CXCR4 in DC maturation and survival (Kabashima 2007b). Thus, the enhanced ability of PGE_2-matured MoDCs to induce

ERK activation after CXCR4 stimulation could have regulatory effects on cell functions rather than enhance migration.

Human Sprouty2 has been demonstrated to mediate its anti-migratory actions in part by increasing the amount of soluble protein tyrosine phosphatase 1B (PTP1B) and its activity (Yigzaw 2003). PTP1B dephosphorylates p130Cas in human cells (Yigzaw 2003), a critical component of the focal adhesion complex (O'Neill 2000). As the activity of p130Cas is controlled by its status of phosphorylation (Cary 1998, Klemke 1998, Ruest 2001), PTP1B action limits p130Cas activity. Hence, Sprouty2-induced PTP1B activity decreases p130Cas phosphorylation and migratory responses. Phosphorylation of p130Cas is involved in CXCR4-mediated signaling resulting in migration. In MoDCs that were matured in the presence of PGE_2, PGE_2 strongly decreased Sprouty2 expression, which may results in low activity of PTP1B and subsequently effective phosphorylation of p130Cas. Indeed, we found CXCR4-induced phosphorylation of p130Cas in MoDCs matured in the presence of PGE_2 (Figure 10). The PGE_2-promoted down-regulation of Sprouty2 could positively influence migratory capacity towards CXCR4-ligands by prevention of PTP1B activation, which allows p130Cas phosphorylation and thereby migration. Conversely, over-expression of Sprouty2 in MoDCs might induce PTP1B and dephosphorylation of p130Cas and thereby inhibition of migration. Analysis of the phosphorylation status of p130Cas in MoDCs over-expressing Sprouty2 will show, if this hypothesis is correct. Additionally, MoDCs that were matured without PGE_2 and express high levels of endogenous Sprouty2 should express larger amounts of soluble PTP1B than PGE_2-matured MoDCs that deactivates p130Cas and inhibits migration.

We are the first to show expression of Sprouty2 and Spred1 in human DCs. If the inhibitory effect of PGE_2 on Spry2 and Spred1 expression is specific for MoDC or applies to DCs in general has to be determined. As inflammatory mediator, PGE_2 is produced in large amounts during early processes of inflammation and therefore affects several cell types. It is possible that PGE_2-mediated signals affect Sprouty2 and Spred1 expression in other cell types and are involved in the modulation of inflammatory responses. The role of Sprouty and Spred proteins has been scarcely investigated in human immune cell population. Spred1 has been described recently as a negative regulator of mature late phase haematopoiesis. (Nonami 2004). Moreover, Spred-1 seems to play a role in the regulation of eosinophils during allergic asthma processes (Inoue 2005).

Sprouty and Spred proteins have been never before implied in chemokine receptor mediated processes. Over-expression of Sprouty2 and Spred1 in mature MoDCs inhibits migration towards CXCR4-ligand, and PGE_2-induced attenuation of Sprouty2 and Spred1 expression correlates with enhanced migratory capacity via CXCR4. The pathway, in which both Sprouty2 and Spred1 facilitate their negative effects on migration, seems not to be shared by

CCR7, since CCR7-mediated migration was not impaired after over-expression of those molecules. We show here for the first time, regulation of Sprouty2 and Spred1 expression in human DCs, which plays a role in chemokine-mediated signaling and migratory responses.

Materials and Methods

Cell lines

CEM cells and Raji cells were maintained in RPMI 1640 (Lonza, Verviers, Belgium) containing 10% FCS (Linaris, Wertheim-Bettingen, Germany), Penicilin/Streptomycin (100 IU/ml, Lonza) and 100 μM non-essential amino acid mix (Lonza).

Generation of monocyte-derived DCs (MoDCs)

Peripheral blood mononuclear cells (PBMC) were separated from peripheral blood of healthy donors by centrifugation on Ficoll Paque Plus (Amersham Biosciences, Uppsala Sweden). Monocytes were isolated by positive selection using anti-CD14 conjugated microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) and cultured at $1x10^6$ in AIM-V (Gibco, Paisley, UK) containing 50 ng/ml GM-CSF (Leukomax®, Novartis, Basel, Switzerland) and IL-4 (1:50 supernatant of an IL-4 producing J558 cell line). After 5-6 days of differentiation, immature MoDCs were harvested and maturation was induced by addition of 0.5 μg/ml trimeric sCD40L (PromoCell, Heidelberg, Germany) or 20 μg/ml poly I:C (Sigma, St Louis, MO) in the absence or presence of 1 μg/ml PGE_2 (Minprostin® E2, Pharmacia, Uppsala, Sweden). Maturation usually took place during two days. Where indicated, maturation was terminated at earlier time points to analyze mRNA content.

Quantification of SPRY2 and SPRED1 mRNA expression

MoDCs were harvested and total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany). Transcription into cDNA was performed using the Taqman® reverse transcription reagent (Applied Biosystems, Rotkreuz, Switzerland) with random hexamer primers according to the manufacturer's instructions.

Sprouty2 was amplified using the SYBR Green PCR Master Mix (Applied Biosystems) containing 200 nM forward (5' CGCGATCACGGAGTTCAGAT) as well as reverse primer (5' GGTGTTTCGGATGGCTCTGAT) according to the manufacturer's instructions using the Taqman 7700 (Applied Biosystems). For amplification of Spred1, 1 μl of cDNA was added to QuantiTect SYBR Green PCR Master Mix (Qiagen) containing 200 nM forward primer 5' GGAGAGCGACTCAGGGACAA and 200 nM reverse primer 5' ATCCTTGAGAAATATCCTCTATAGCTCTTC; the real-time PCR program was changed to:

denaturation at 95°C for 15 min followed by 40 cycles of 15 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C. Expression was normalized to two housekeeping genes β-2 microglobulin (β2M) and ubiquitin C (UBC) using 200 nM of 5'-GCTATCCAGCGTACTCCAAAGATTC and 5'-CAACTTCAATGTCGGATGGATGA for β2M or 5'-ATTTGGGTCGCGGTTCTTG and 5'-TGCCTTGACATTCTCGATGGT for UBC, respectively, and SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Relative mRNA expression was calculated by means of the ΔΔCt-method.

Generation of Sprouty2-EGFP, Sprouty2-Y55A-EGFP and Spred1-EGFP constructs

To generate a Sprouty2-EGFP fusion construct, Sprouty2 was cut from pSprouty2-RFP (kind gift from Dr. Tarun Patel, Loyola University of Chicago, IL) using *XhoI* and *KpnI* as restriction enzymes, and ligated into the pEGFP-N1 vector (Invitrogen). The Sprouty2 mutant Y55A was amplified from a plasmid provided by Dr. Akihiko Yoshimura (Kyushu University, Japan) using 5'-TATATACTCGAGATGGAGGCCAGAGCTCAG and 5'-ATATATGGTACCGTTGGTTTTTCAAAGTTC as primers. Sprouty2-Y55A was inserted into pEGFP-N1 using *XhoI* and *KpnI* as restriction enzymes. The mutation of tyrosine residue 55 to alanine was confirmed by sequencing. Spred1 was amplified from cDNA of total RNA of PBMCs using 5'- ATATGAATTCGCCACCATGAGCGAGGAGACGGCGA and 5'-TATAGGATCCCCAGCAGCTTTATGTTTCCCAC as primers, and *EcoRI* and *BamHI* as restriction enzymes for insertion to pEGFP-N1.

Transfection of MoDCs and cell lines

Mature MoDCs were harvested and transfected using Human Dendritic Cell Nucleofector Kit (Amaxa biosystems, Cologne, Germany). Therefore, $2x10^6$ MoDCs were resuspended in 100 μl nucleofector solution according to the manufacture's protocol. Usually, 10-30 μg of highly pure expression vector was added and cells were transferred into a nucleofector cuvette. MoDCs were transfected using nucleofector program U-002, and subsequently cultured in RPMI 1640 medium (Lonza, Verviers, Belgium) containing 10% FCS (Linaris, Wertheim-Bettingen, Germany). Transfection efficiency was analyzed the next day by flow cytometry (LSRII, BD Biosciences, Erembodegen, Belgium) using cells, which were nucleofected without plasmid, as negative control. Dead cells were stained with ToPro3 (Molecular Probes, Eugene, OR) or SytoxBlue (Molecular Probes). Raji cells and CEM cells were transfected using Amaxa nucleofector technology according to the manufacturer's instructions. Raji cells were sorted into GFP-negative and GFP-positive cells using the FACS Vantage SE (BD Biosciences).

Cell migration assay

Chemotaxis was analyzed using 24-well Transwell™ plates (Corning Costar, NY). 600 µl medium containing chemokine were placed in the lower well, while $1x10^5$ cells were placed on a polycarbonate filter with a pore size of 5 µm in the upper well. Cell were allowed to migrate into the lower well for three hours at 37°C/5% CO_2 and migrated cells were counted by flow cytometry using BD LSRII (BD). Specific migration was calculated by subtraction of spontaneously migrated cells towards medium alone without chemokine and is presented as percentage of the number of input cells. Optimal chemokine concentrations were tested for Raji and CEM cells. Raji cells migrated best towards 500 ng/ml CCL21, while for CEM cells 1 µg/ml CCL21 or 1 µg/ml CXCL12 were used. MoDCs migrated towards 250 ng/ml CCL21 or 250 ng/ml CXCL12.

Western blotting and activation of ERK- and p130Cas

Protein expression of Sprouty2 was analyzed by Western blotting using an anti-Sprouty2 specific antibody (kind gift of Prof. Gerhard Christofori, University of Basel, Switzerland). To ensure equal protein loading, the blot was re-probed with anti-ß-actin antibody (Abcam, Cambridge, UK).

For analysis of chemokine-induced activation of ERK or p130Cas, $1x10^6$ MoDC or $8x10^5$ Raji cells were stimulated with either 1 µg/ml CCL19 or CCL21 (Promocell), or 1 µg/ml CXCL12 (Promocell) at 37°C for indicated length of time. Cells were lysed in lysate buffer (1% NP-40, 50 mM Tris, 0.25% sodiumdesoxycholate, 150 mM NaCl, 1 mM NaF, 1 mM EGTA, 1 mM Na_3VO_4) and protease inhibitor (Roche) for 15 min and subsequently centrifuged for 10 min at 14000 x g. The supernatant was analyzed by Western blotting using a specific anti-human phospho-ERK1/2 antibody (Santa Cruz). Total expression of ERK2 was monitored by re-probing blots with an anti-human ERK2 antibody (Cell Signaling). Phosphorylation of p130Cas was determined using a specific anti-human phospho-p130Cas antibody (pY249, BD Pharmingen) and total p130Cas expression was visualized using an anti-human p130Cas antibody (BD).

Acknowledgements

We thank Prof. Gerhard Christofori (University of Basel, Switzerland) for the kind gift of anti-human Sprouty2 antibody, Dr. Tarun Patel (Loyola University of Chicago, IL) for the SPRY2-RFP construct, Dr. Akihiko Yoshimura (Kyushu University, Japan) for the pcDNA3-Sprouty2(Y55A) construct, and Dr. Eva-Maria Boneberg for helpful discussions, scientific advice and essential technical support.

CHAPTER 4

Role of prostaglandin E_2 (PGE_2)-induced regulator of G protein signaling 9 (RGS9) in chemokine-mediated functions of dendritic cells

Abstract

During the process of maturation induced by the up-take of antigen dendritic cells (DCs) develop the ability to migrate to the draining lymph node, where they elicit an antigen-specific immune response. Prostaglandin E_2 (PGE_2) has been shown to be the crucial factor during maturation of DCs for the induction of a migratory phenotype. Although PGE_2 is well established as the mandatory factor for chemotactic responses, the mechanism by which PGE_2 facilitates this effect is still unclear. By comparison of global gene expression profiles of human monocyte-derived DCs (MoDCs) matured in the absence or presence of PGE_2 we identified a member of the regulator of G protein signaling (RGS) family, namely RGS9, to be strongly induced by PGE_2 early during maturation of MoDCs. Both splice variants of RGS9 that were described so far in humans were expressed in MoDCs matured in the presence of PGE_2. To analyze the role of PGE_2-induced RGS9 in migratory responses of MoDCs, we generated different RGS9 constructs and over-expressed those in mature MoDCs. In addition, we analyzed the impact of RGS9 on migratory properties of DCs *in vivo* using RGS9-deficient ($RGS9^{-/-}$) mice. Surprisingly, DCs of $RGS9^{-/-}$ mice showed a strong migratory defect in an *in vivo* migration assay when compared to wild type mice. Consequently, $RGS9^{-/-}$ mice showed an impaired induction of functional antigen-specific cytotoxic T cell responses. We demonstrate here for the first time the expression of RGS9 in human DCs and a severe defect in DC migration in $RGS9^{-/-}$ mice *in vivo*.

Introduction

Chemokines and a complex network of modulators of chemokine receptor signaling control the migration of leukocytes into and out of inflammatory sites as well as the migration of developing lymphocytes and thus the organization of secondary immune anatomic structures (Springer 1994, Kunkel 2002, Rot 2004). Disturbed chemokine receptor signaling resulting in inappropriate migration, activation or survival of lymphocytes can lead to immunologic disorders (Balabanian 2005, Norman 2005).

Chemokine receptors belong to a large family of seven-transmembrane receptors that are coupled to heterotrimeric Gαßγ proteins (Marinissen 2001). Binding of the chemokine to its respective receptor catalyzes the exchange of guanosine 5'-diphosphate (GDP) by guanosine 5'-triphosphate (GTP) on the Gα subunit, which leads to the dissociation of Gα and the Gßγ complex (reviewed in Jung 1999, Hepler 1992, Neer 1995). Subsequently, both, GTP-bound Gα and Gßγ, activate multiple downstream signaling cascades, while Gßγ-induced signaling is essential for directional migration (Arai 1997, Neptune 1999). The duration that Gα remains bound to GTP controls the duration of signaling. Gα proteins possess an intrinsic guanosine triphosphatase (GTPase) activity that leads to hydrolysis of GTP and reassembly of the heterotrimeric G protein complex. Therefore, termination of a G protein coupled receptor (GPCR)-mediated signaling event is dependent on the GTPase activity of the Gα subunit. However, GTP hydrolysis by purified Gα proteins is to slow to account for deactivation rates of G protein-controlled processes like phototransduction (Arshavsky 1998) or ion channel regulation (Kurashi 1995).

The inactivation of Gα subunits can be modulated by a family of proteins that accelerate the GTPase activity of Gα, which iare termed regulators of G protein signaling (RGS) (Watson 1996a, Kehrl 1998, Siderovski 1999, Berman 1998, De Vries 2000, Ross 2000, Dohlman 1997). RGS proteins carry a conserved RGS domain of around 120 amino acids that serves as a GTPase activating protein (GAP) for Gα, thus controlling the response kinetics of various signalling processes (Koelle 1996, Hollinger 2002). By acceleration of transduction kinetics of receptor signals, RGS proteins enable cells to sense rapidly changing concentrations of signaling molecules. At least 20 RGS family members exist in humans, even more if proteins containing domains with weak homology to the RGS domain are taken into account (Kehrl 2006). Besides the RGS domain, which is sufficient for the GTPase stimulating action, many RGS proteins contain additional protein-binding domains, which mediate interactions with proteins other than Gα subunits, linking them to other signaling pathways (De Vries 1999, Zheng 1999). In eukaryotes, RGS proteins have been described to accelerate termination of G protein signaling in a variety of signaling pathways including pheromone signaling and transduction of photoresponse and olfactory response (Dohlman

1996, Chen 2000, Bruch 1996, Sinnarajah 2001). Moreover, RGS proteins have been implicated in the regulation of lymphocyte migration. For example, the over-expression of RGS1, RGS3, and RGS4 in B cell lines dramatically inhibits chemokine-mediated migration (Bowman 1998, Moratz 2000, Reif 2000). *In vivo*, transgenic T lymphocytes expressing RGS16 show reduced migration in response to CXCR4, CCR3, and CCR5 ligands, while CCR2- and CCR7-mediated migration is not impaired, suggesting chemokine receptor specificity of RGS protein actions (Lippert 2003). Along this line, the unresponsiveness of $CD4^+$ germinal center T cells to CXCL12 has been attributed to their expression of RGS13 and RGS16, which did not affect CXCR5-mediated migration (Estes 2004). Furthermore, the limited ability of regulatory T cells to migrate towards CCR7 ligands has been correlated with high expression of RGS1, RGS9 and RGS16 (Gavin 2002, Agenes 2005).

The modulation of migratory responses and sensitivity to selective chemoattractive signals can be controlled by RGS proteins implying them in the induction and regulation of immune responses. In recent years, the generation and analysis of several mutant mice deficient in one member of the RGS protein family revealed new insights into RGS protein functions. While targeted deletion of RGS14 resulted in early embryonic lethality due to a general role of RGS14 in mitosis (Martin-McCaffrey 2004), mice deficient for RGS4 in contrast developed without obvious defects (Grillet 2005), arguing for distinct roles of different members of the RGS protein family. T cells from RGS2 deficient mice showed a defect in proliferation and IL-2 production resulting in impaired antiviral immunity, but chemokine receptor signaling was not affected (Oliviera-dos-Santos 2000). Targeted deletion of RGS1, in contrast, results in disturbed chemokine receptor signaling (Moratz 2004a, Han 2005). B and T lymphocytes from RGS1 deficient mice exhibit augmented chemokine receptor signaling, leading to an enhanced migratory potential of B cells (Moratz 2004a, Kehrl 2006).

RGS proteins can be further classified into subfamilies dependent on their distinguished structural features. The R7 subfamily consists of four members unified by their N-terminal expression of a Gγ-like domain (GGL) and a dishevelled/EGL-10/pleckstrin domain (DEP) (Xie 2007). The best investigated R7 member is RGS9 that exists in two alternatively spliced forms, termed RGS9-1 and RGS9-2, which differ in their C-terminal tail (Granneman 1998, Rahman 1999, Zhang 1999). RGS9-1 and RGS9-2 were described to be selectively expressed in retina or neural tissues, respectively. In retina RGS9-1 is abundantly expressed and functions as GAP for transducin, the $G\alpha$ subunit specific for phototransduction, implicating a key role in the recovery phase of visual transduction (He 1998, Cowan 1998, Wensel 2002). The physiological function of RGS9-1 has been extensively studied in RGS9-deficient mice, which show extremely slowed photoresponse recovery from light flashes in rods and cones (Chen 2000, Lyubarsky 2001). Hence, RGS9-1 is essential for normal inactivation of phototransduction cascades enabling vision with high temporal resolution.

Less is known about RGS9-2, which is mainly expressed in the striatum (Granneman 1998, Rahman 1999, Thomas 1998). However, RGS9-2 has been described to negatively regulate dopamine and opioid receptor signaling (Garzon 2001, Rahman 2003, Zachariou 2003, Traynor 2005) as well as to be involved in motor coordination and working memory (Blundell 2008). No immunologic relevant phenotype has been reported for RGS9-deficient mice (Kehrl 2006)

Peripheral immature dendritic cells (DCs) are specialized to take up and process antigens in order to present them after maturation to T and B lymphocytes and thus induce an adaptive immune response (Banchereau 2000). The process of DC maturation is complex and involves the establishment of a migratory potential in response to lymph node-derived chemokines, like CCL21 and CCL19, which bind to the chemokine receptor CCR7. Since RGS proteins modulate responsiveness to chemokine receptor-mediated signals, expression of RGS proteins has been analyzed in DCs recently (Shi 2004). Maturation via toll like receptors (TLR) induced a change in the RGS protein expression profile of monocyte-derived DCs (MoDCs). While the expression of RGS14 and RGS18 was decreased, expression of RGS1, RGS16 and RGS20 was enhanced. Single over-expression of RGS1, for example, inhibited CCL21-induced migration of mature MoDCs (Shi 2004), although RGS1 is up-regulated in mature MoDCs, which normally have an enhanced potential to migrate in response to CCR7 signals. However, the overall balance of selective RGS proteins could contribute to the modulation of chemokine receptor responsiveness in mature DCs.

For dendritic cell based cancer immunotherapy, DCs can be generated from monocytes (MoDCs), loaded with tumor antigen and administered to the patient, where they will migrate to the draining lymph node and induce an antigen-specific anti-tumor immune response. The migration of MoDCs from the injection site into secondary lymphoid organs is a crucial step. We identified early prostaglandin E_2 (PGE_2) as the essential factor during maturation of DCs to facilitate a migratory phenotype (Scandella 2002, Luft 2002, Legler 2005). To determine the mechanisms by which PGE_2 induces migratory potential, we performed a global gene expression analysis with MoDCs mature in absence and presence of PGE_2 and compared gene transcription. Surprisingly, we found RGS9 to be one of the strongest regulated genes by PGE_2. In this study, we describe for the first time the expression of RGS9 mRNA in human DCs and aimed to determine the impact of RGS9 expression on migration of MoDCs. Additionally, we analyzed the role of RGS9 expression on migration *in vivo* using RGS9-deficient mice.

Results

PGE_2 induces RGS9-1 and RGS9-2 expression during maturation of MoDCs

We have described previously the essential role of PGE_2 during maturation of MoDCs for the induction of migratory capacities in response to lymph node-derived chemokines (Chapter 2). To discover the mechanism by which PGE_2 promotes the migratory phenotype of MoDCs, we performed a gene expression profiling in cooperation with Altana Pharma with MoDCs matured with sCD40L or poly I:C in absence or presence of PGE_2. Among the strongest regulated gene products was the RGS family member RGS9. To corroborate the results we performed quantitative real-time PCR analysis for detection of RGS9 mRNA in MoDCs matured in the presence or absence of PGE_2 while maturation was either induced by the TLR3 ligand poly I:C or sCD40L. When MoDCs were matured via TLR3 ligation, 1 μg/ml PGE_2 induced an average 20-fold increase of RGS9 on mRNA level (data not shown). The PGE_2-induced up-regulation of RGS9 mRNA was five times stronger (100-fold) when maturation was induced with sCD40L (data not shown). There are two isoforms of human RGS9 described, termed RGS9-1 and RGS9-2, which are the result of an alternative splicing process. To determine which splice variant is induced in MoDCs after maturation in the presence of PGE_2 we performed quantitative real-time PCR using primer pairs specially designed for the detection of only one splice variant. Surprisingly, we found both RGS9-1 and RGS9-2 to be induced after 48 h of maturation via CD40 in the presence of PGE_2 (Figure 1). To monitor RGS9 mRNA expression during the maturation process, we generated immature MoDCs and either left them immature or induced maturation using sCD40L in the absence or presence of PGE_2. Cells were harvested after 6 h, 24 h and 48h and mRNA levels of RGS9-1 and RGS9-2 were quantified relative to the respective expression level in immature MoDCs of the same donor. Neither splice variant of RGS9 could be detected at any time-point in MoDCs matured in the absence of PGE_2. However, PGE_2 induced RGS9-1 and RGS9-2 mRNA already after 6 h of maturation (Figure 1). In the case of RGS9-1, mRNA expression peaked at 24 h of maturation, but was still present at a high level after 48 h. RGS9-2-mRNA expression increased over time and peaked after 48 h of maturation in the presence of PGE_2. We tried to measure the PGE_2-mediated induction of RGS9 on protein level by Western Blotting. Due to the lack of a RGS9-specific antibody we could not detect RGS9 on protein level. We tried two commercially available antibodies with the same poor results. Both antibody detected unspecific background as tested by over-expression of RGS9 in CEM cells, where the antibodies detected the same pattern of proteins in transfected and non-transfected cells. Moreover, RGS9 could not be detected on protein level in brain lysates of C57Bl/6 mice with both antibodies.

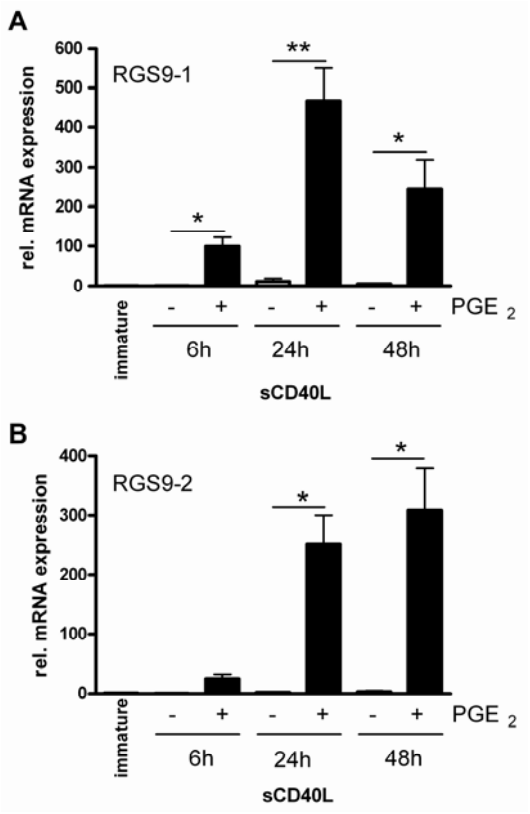

Figure 1. PGE_2 induces expression of RGS9 in mature MoDCs. Monocytes were isolated from human peripheral blood and differentiated into MoDCs. After 5-6 days immature MoDCs were harvested and maturation was induced by ligation of CD40 in the absence or presence of PGE_2. Cells were harvested six hours, 24 h or 48 h after induction of maturation. Expression levels of mRNA encoding for RGS9 splice variants, RGS9-1 (**A**) and RGS9-2 (**B**), were analyzed by quantitative real-time PCR. Expression was normalized to two housekeeping genes and calculated relative to RGS9-1 or RGS9-2 expression in immature MoDCs of the same donor. Mean values and SEM of six independent experiments with different donors are shown. Asterisks indicate statistical significance with $p<0.05$ for * and $p<0.005$ for **.

Effect of RGS9 over-expression in CEM cells

Since we found the PGE_2-induced expression of RGS9 in MoDCs that display a strong migratory phenotype, we investigated the effect of RGS9 expression on migration in CEM cells. CEM cells are human T lymphoblasts which are able to migrate towards CCR7 or CXCR4 ligands. For genetic manipulation of CEM cells a transfection strategy was established using Amaxa nucleofector technology. The RGS9-carrying vector pcDNA(+)RGS9L was introduced into CEM cells and after 24 h cells were analyzed for their migratory capacity towards CCL21 or CXCL12. Since RGS9 was untagged it was impossible to distinguish between transfected and untransfected cells. Therefore, migratory capacity of the whole population was measured. Transfection of untagged RGS9 into CEM cells had no significant effect on their migration towards CCL21 (Figure 2A), while migration towards CXCL12 was significantly reduced (Figure 2B). In order to analyze only migration of cells that are transfected, we cloned RGS9 into the pIRES2-EGFP vector, which allows additional transcription of EGFP without generating a RGS9-EGFP fusion protein. Transfection efficiency was high using pIRES2RGS9-EGFP (Figure 2C), while the introduction of the control vector without RGS9, pIRES2-EGFP, was less successful but sufficient (Figure 2C). Co-expression of EGFP together with RGS9 allowed us to monitor the migratory behaviour of only those cells over-expressing RGS9. Expression of RGS9 in CEM cells had no effect on CCL21-mediated migration (Figure 2D). Migration towards CXCL12 was slightly but not significantly reduced (Figure 2E).

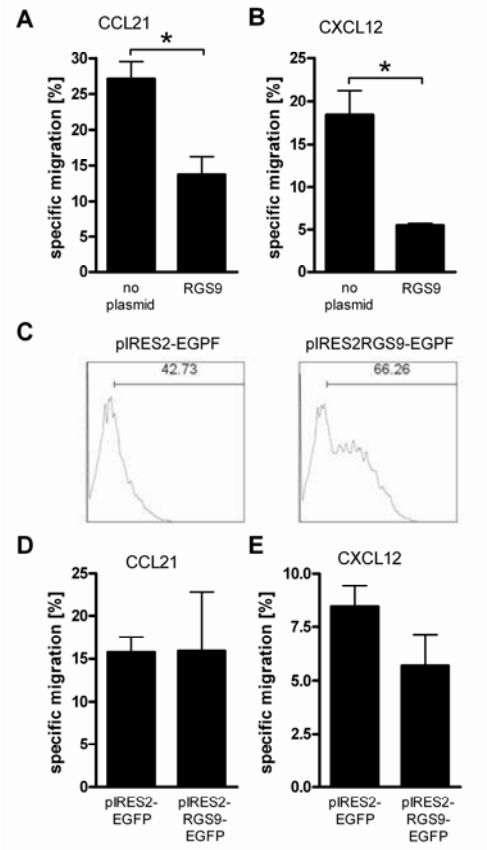

Figure 2. Effects of RGS9 over-expression on chemokine-mediated migration of CEM cells. CEM cells were transfected with two different vectors carrying RGS9 using Amaxa nucleofection technology. One day after transfection cells were analyzed for chemotactic behaviour in response to CCL21 (**A**, **D**), or CXCL12 (**B**, **E**). (**A**, **B**) CEM cells were transfected with pcDNA3.1(+)RGS9L or no plasmid as negative control. Migration of the whole living population was analyzed excluding dead cells by staining with ToPro3 or SytoxBlue. Mean values and SEM of five (**A**) or three (**B**) independent experiments are presented. Statistical significance was calculated using paired t-test with $p<0.05$ for *.
Over-expression of RGS9 was introduced in CEM cells using pIRES2RGS9-EGFP (**C**, **D**, **E**). Transfection efficiency was analyzed one day after transfection using flow cytometry. The result of one representative experiment is shown (**C**). As negative control pIRES2-EGFP was transfected. Chemotaxis of EGFP-expressing cells was analyzed in response to CCL21 (**D**) and CXCL12 (**E**). Dead cells were excluded from analysis by staining with ToPro3 or SytoxBlue. (**D**, **E**) Mean values and SEM from three independent experiments are presented.

Effects of RGS9 on migration of MoDCs

Since PGE_2 induces RGS9 expression in mature MoDCs, we analyzed the effect of RGS9 over-expression in MoDCs. If PGE_2-induced RGS9 expression is a mechanism by which PGE_2 facilitates the enhanced migratory capacity of MoDCs, over-expression of RGS9 should enhance migration in MoDCs that were matured in the absence of PGE_2. Therefore, MoDCs were generated and matured using sCD40L in the absence or presence of PGE_2. After two days of maturation MoDCs were transfected with pcDNA3.1(+)RGS9L using Amaxa nucleofector technology. Transfection of MoDCs was established and functionality of cells was analyzed as previously described (Chapter 3, Figure 4). Chemotactic properties of transfected MoDCs were analyzed one day after transfection. Transfection efficiency was controlled by transfection of pEGFP-N1 and analysis of GFP expression by flow cytometry. Over-expression of untagged RGS9 in sCD40L-matured MoDCs did not augment but rather inhibited migration towards CCL21 (Figure 3A). Additionally, CXCR4-mediated migration was inhibited in MoDCs matured in the absence of PGE_2 (Figure 3B). Transfection o RGS9 into PGE_2-matured MoDCs revealed the same inhibition of CCR7- and CXCR4-mediated migration (Figure 3). The inhibitory effect of RGS9 over-expression on CXCR4-mediated migration was not quite significant when calculated using student's paired t-test due to limited repetition and donor to donor variation.

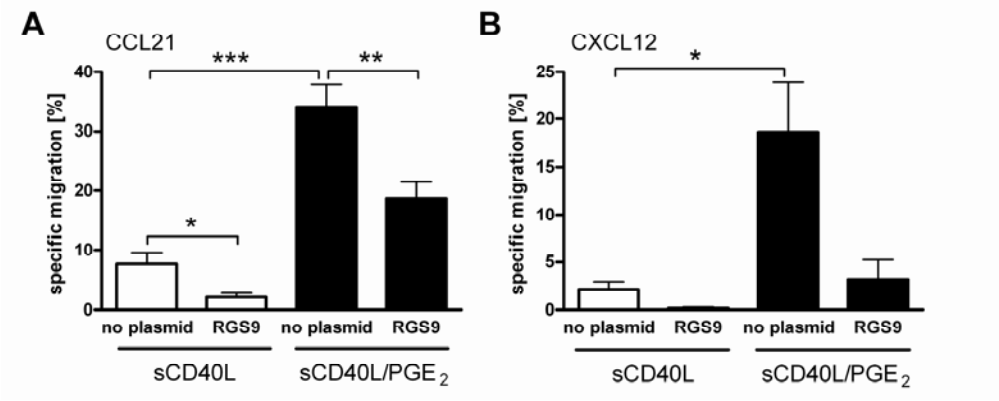

Figure 3. Effect of RGS9 over-expression on chemotactic responses of MoDCs. MoDCs were generated and matured using sCD40L in the absence or presence of PGE_2. Mature MoDCs were transfected with pcDAN3.1(+)RGS9L or no plasmid using Amaxa nucleofection technology. Migration was monitored in response to CCL21 (**A**) and CXCL12 (**B**) in Transwell chemotaxis assays, while dead cells were excluded from analysis by staining with ToPro3 or SytoxBlue. Mean values from eight (**A**) or four (**B**) independent experiment with different donors are shown. Asterisks indicate statistical significance calculated by student's paired t-test with $p<0.05$ for *, $p<0.005$ for ** and $p<0.0001$ for ***.

The over-expression of other members of the RGS family (RGS18, RGS1) has been shown to have similar effects on MoDCs migration (Shi 2004). The inhibition of migration was in this case correlated with the suppressed phosphorylation of ERK after stimulation with CCL19 and CXCL12. (Shi 2004) To determine the influence of RGS9 over-expression on ERK activation we stimulated mature MoDCs with CCL21 or CXCL12 and analyzed phosphorylation of ERK by Western Blotting. In MoDCs transfected with pEGFP-N1 stimulation with both CCL21 or CXCL12 induced phosphorylation of ERK. While RGS9 expression seems not to have an effect on ERK phosphorylation when MoDCs were stimulated with CCL21, stimulation with CXCL12 led to a reduced phosphorylation of ERK in MoDCs transfected with RGS9 (Figure 4). The inhibition of ERK phosphorylation was detected after RGS9 transfection in mature MoDCs that were matured in the absence of PGE_2 as well in those matured in the presence of PGE_2.

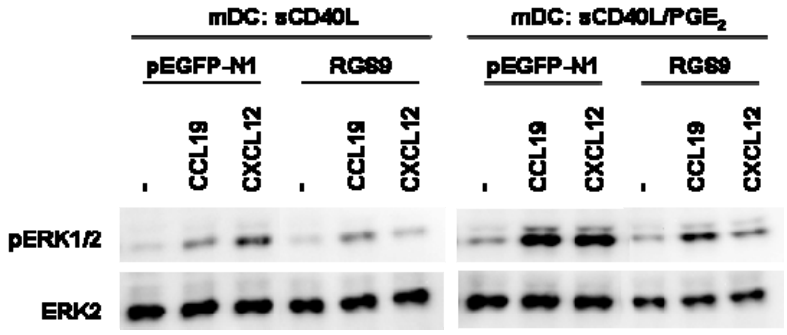

Figure 4. Role of RGS9 over-expression in chemokine-induced ERK activation of mature MoDCs. MoDCs were matured by ligation of CD40 in the absence or presence of PGE2 and transfected with pEGFP-N1 or pcDNA3.1(+)RGS9L using Amaxa nucleofection technology. One day after transfection MoDCs were harvested and stimulated for two minutes with CCL19 or CXCL12. Subsequently, cells were lysed and phosphorylation status of ERK1/2 was analyzed by Western blotting using an anti-human phospho-ERK1/2-specific antibody (upper panel). To monitor protein loading and content of total ERK, blots were re-probed with anti-human ERK2-specific antibody (lower panel)

Since the RGS9 construct used for all previously described experiments was untagged, we cloned RGS9 into the pIRES2-EGFP vector, which allows analysis of only RGS9-carrying cells by additional expression of EGFP. MoDCs were matured with sCD40L in the absence or presence of PGE_2 and transfected with pIRES2RGS9-EGFP or pIRES2-EGFP as a control. Transfection efficiency yielded around 40 percent (Figure 5A). When only positively transfected cells were analyzed for their chemotactic properties RGS9 over-expression did not inhibit migration, neither towards CCL21 (Figure 5B) nor towards CXCL12 (Figure 5C). Hence, by over-expressing RGS9 using the pIRES2-EGFP vector and thereby limiting migration analysis to transfected cells we could not reproduce the inhibitory effect of RGS9 on migration of MoDCs.

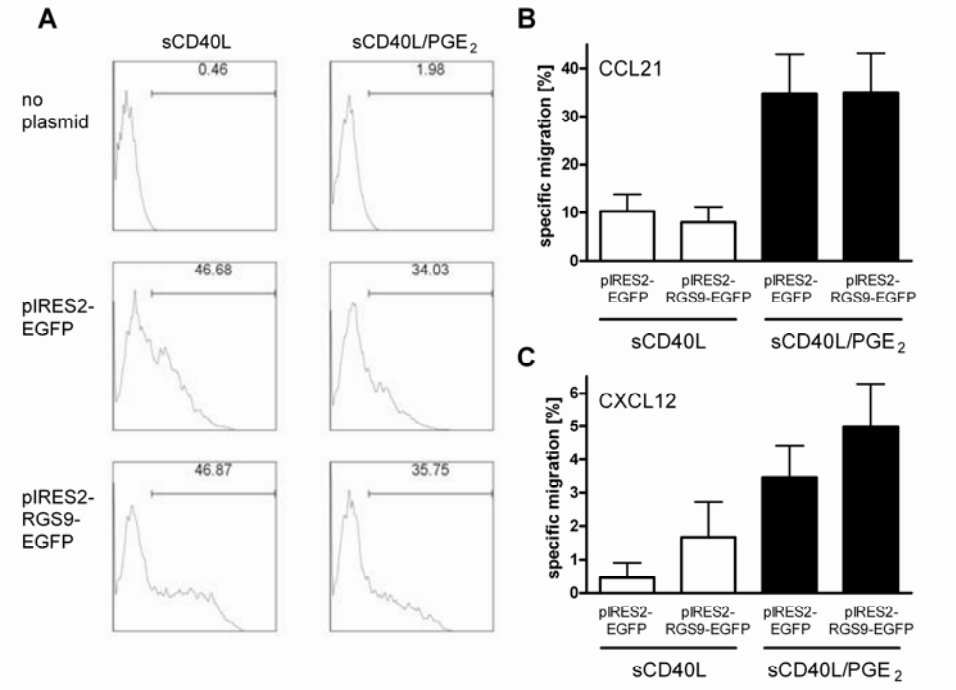

Figure 5. Effects of pIRES2RGS9-EGFP expression on MoDC chemotaxis. MoDCs were matured with sCD40L in the absence (white bars) or presence of PGE_2 (black bars), and transfected either without plasmid, with pIRES2-EGFP or pIRES2RGS9-EGFP. One day after transfection, expression of EGFP was monitored by flow cytometry (**A**) while dead cells were excluded from analysis by staining with ToPro3 or SytoxBlue. Chemotaxis of transfected cells was analyzed toward CCL21 (**B**), or CXCL12 (**C**). Only alive EGFP-expressing cells were taken into account.

Effects of RGS9 deficiency *in vivo*

To study the contribution of RGS9 in chemotactic processes, we analyzed migration of dendritic cells *in vivo* using RGS9 deficient mice ($RGS9^{-/-}$). $RGS9^{-/-}$ mice were a kind gift from Prof. J. Schwarz (University Leipzig) and were verified to be RGS9-deficient by amplification of the MC1 neopA cassette disrupting the *rgs9* gene (Chen 2000) (Figure 6A). Additionally, RGS9 mRNA expression was analyzed in brains of wild-type and $RGS9^{-/-}$ mice, since RGS9 has been described to be highly expressed in brain (Rahman 1999). Therefore, RNA was extracted from total brain lysates, transcribed into cDNA, and RGS9 was amplified by PCR.

As expected, RGS9 could only be amplified in wild-type mice while RGS9$^{-/-}$ mice did not express RGS9 transcripts (Figure 6B).

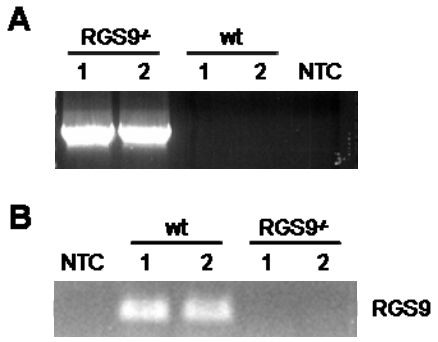

Figure 6. Analysis of RGS9-deficiency in RGS9$^{-/-}$ mice. (**A**) Tail biopsies were taken from two wild type C57Bl/6 mice (wt) and two RGS9$^{-/-}$ mice and analyzed by PCR for the disruption of the RGS9 gene by insertion of the neomycin resistance cassette.
(**B**) RNA was extracted from brain of two wild type and two RGS9$^{-/-}$ mice and analyzed for RGS9 mRNA expression by real-time PCR. Samples were loaded onto a 1% agarose gel to monitor amplification of a 300 bp fragment of RGS9. (NTC= no template control)

RGS9 has been described to be mainly expressed in retina and brain, but low expression of mRNA has also been reported in mouse lymph nodes and spleen (Moratz 2004b Methods Enzymol 389) as well as in human lymphocytes (Larminie 2004). We analyzed RGS9 expression in T cells and DCs isolated from spleens of C57Bl/6 mice and used RGS9 expression in brain as positive control. Interestingly, we found RGS9 to be expressed in T cells and DCs. RGS9 could therefore play a role in the regulation of leukocyte functions.

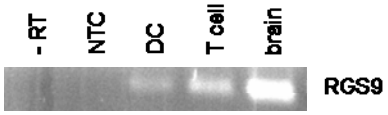

Figure 7. Expression of RGS9 in mouse leukocytes. T cells and DCs were isolated from spleen of wild type C57Bl/6 and RGS9$^{-/-}$ mice. RNA was isolated and transcribed into cDNA. RGS9 was amplified and visualized as a 300 bp fragment on a 1% agarose gel. Expression of RGS9 in brain was used as a positive control. To exclude amplification of residual genomic DNA, RNA without reverse transcriptase from the same preparation was used as template (-RT). (NTC= no template control)

To monitor migration of DCs and the impact of RGS9-deficiency *in vivo* we performed skin painting experiments using RGS9$^{-/-}$ mice and age- and sex-matched wild-type mice (C57Bl/6). In this *in vivo* migration experiment, FITC is applied to the skin and is taken up by peripheral DCs which in response migrate to the draining lymph node. One day after FITC was applied to the shaved abdomen, mice were sacrificed and the proportion of FITC-positive CD11c^{+} DCs was analyzed in inguinal lymph nodes. Whereas in wild-type mice an average of 16.5% of living, CD11c^{+} DCs were also FITC^{+}, only 2.7% were FITC^{+} in RGS9$^{-/-}$ mice (Figure 8). Since less peripheral DCs were able to migrate to the draining lymph node in RGS9-defincient mice after one day of stimulation, we addressed the question whether this defect inhibited the induction of an antigen-specific immune response in RGS9$^{-/-}$ mice. To answer this question, we subcutaneously injected PLGA microspheres carrying OVA and CpG into RGS9$^{-/-}$ and matched wild-type control mice. After six days, mice were sacrificed and spleens were removed. Spleen cell suspensions were re-stimulated with OVA-peptide (SIINFEKL) for five hours and IFN-γ production of OVA-specific CD8^{+} T cells was measured

by flow cytometry. In line with our finding that RGS9$^{-/-}$ DCs are inhibited in leaving the periphery, RGS9-deficient mice produced less antigen-specific IFN-γ secreting CD8^{+} T cells in response to OVA (Figure 9). Our data suggest that, due to a migratory defect of DCs, RGS9$^{-/-}$ mice are limited in the induction of an antigen-specific immune response.

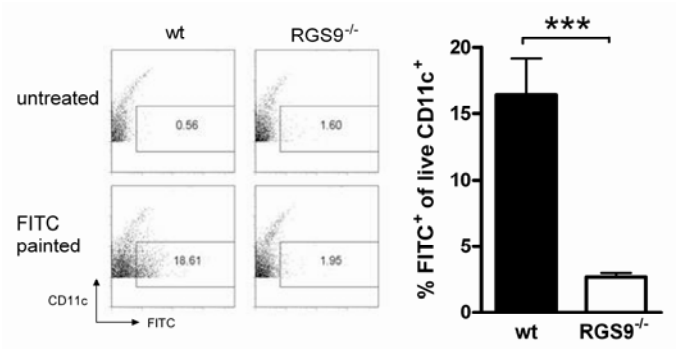

Figure 8. DCs of RGS9$^{-/-}$ mice display a migratory defect *in vivo*. FITC was applied to the shaved abdomen of wild type C57Bl/6 mice or RGS9$^{-/-}$ mice. One day later, inguinal lymph nodes were removed, and the number of FITC-positive CD11c+ DCs was quantified by flow cytometry. Results from one representative experiment are shown in dot plots (left). Means and SEM of two independent experiment with a total of nine (wt) and eleven (RGS9$^{-/-}$) mice are shown. Statistical significance was calculated using student's unpaired t-test with p<0.0001 for ***.

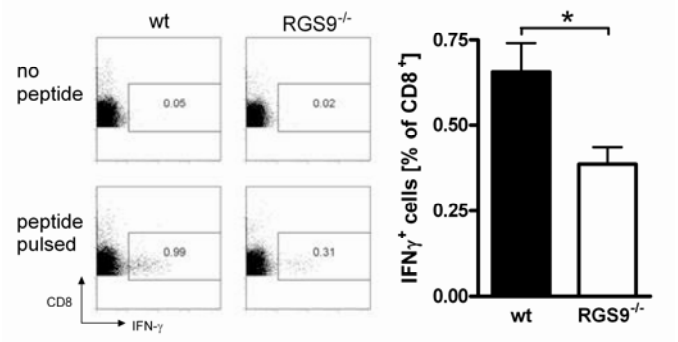

Figure 9. Antigen-specific induction of CTL-response is inhibited in RGS9-deficient mice. PLGA microspheres containing OVA and CpG were injected subcutaneously into wild type or RGS9$^{-/-}$ mice. After six days spleens were analyzed for their content of OVA-specific IFN-γ-producing CD8^{+} T cells. One representative experiment is shown in dot plots (left). Means and SEM of two independent experiment with a total of eight (wt) and nine (RGS9$^{-/-}$) mice are shown. Statistical significance was calculated using student's unpaired t-test with p<0.05 for *.

Discussion

We have previously reported that PGE_2 is the key factor during the maturation of human MoDCs for the establishment of a migratory phenotype (Chapter 2, Scandella 2002). By gene expression analysis of MoDCs that were matured in the absence or presence of PGE_2 we found RGS9 to be induced by PGE_2, as well as other RGS proteins, which were regulated by PGE_2. Human MoDCs have been reported to express a distinguished RGS protein profile depending on their differentiation status. While immature MoDCs constitutively expressed RGS2, RGS10, RGS14, RGS18 and RGS19, maturation via TLR signaling markedly induced RGS1, RGS16 and RGS20, and down-regulated RGS14 and RGS18 (Shi 2004). Interestingly, the described changes in RGS expression are not specific events following TLR signaling. Maturation through ligation of CD40 induced RGS1 and RGS16 mRNA expression, whereas RGS14 and RGS18 were down-regulated (data not shown), suggesting that alteration in the RGS expression profile is a general feature of DC maturation. However,

under serum-free conditions we found that a long-lasting maturation-induced down-regulation of RGS14 and RGS18 mRNA is dependent on the presence of PGE_2 (data not shown). Moreover, the induction of RGS1 expression during maturation is strongly and significantly enhanced in the presence of PGE_2 (data not shown). Contra-intuitively, MoDCs matured in the presence of PGE_2 show a dramatically augmented potential to migrate towards CCR7 and CXCR4 ligands, although concurrent RGS1 expression is induced, which has been shown to inhibit CCR7- and CXCR4-mediated migration (Shi 2004, Moratz 2000). Like RGS1, RGS16 has been described as a negative regulator of chemokine-induced migration (Lippert 2003, Berthebaud 2005). We found RGS16 mRNA expression induced six hours after stimulation with sCD40L, but the addition of PGE_2 counteracted this induction (data not shown). The negative impact of PGE_2 on RGS16 expression only played a role at very early stages of maturation, since at later time points RGS16 expression returned to the same level as observed in immature MoDCs. For that reason it is not possible that the PGE_2-dependent down-modulation of RGS16 expression antagonizes the PGE_2-mediated enhanced RGS1 expression. However, PGE_2 also strongly inhibited expression of RGS14 and RGS18, which negatively regulates MoDCs migration towards CCL21 and CXCL12 (Shi 2004), and could thereby circumvent the consequence of augmented RGS1 expression.

Most RGS proteins have been shown to bind and regulated several members of the Gi subfamily of $G\alpha$ proteins with minimal discrimination between them. Some RGS proteins are not even selective for different G protein subfamilies showing GAP activity toward members of the Gq subfamily as well (Natochin 1998, De Vries 2000). This lack of specificity, however, has been mainly studied in *in vitro* GAP assays. In a cellular environment, higher specificity between particular RGS proteins and G proteins was suggested, since lipid modifications and expression of interaction domains can control the spatial distribution of RGS proteins and thereby target them to certain subcellular locations (De Vries 1996, Martemyanov 2003). The regulation of the localization and membrane interaction of RGS proteins seems to be vitally important for their specificity and activities in controlling G protein signaling pathways. Anchoring proteins have been described to target RGS proteins to the plasma membrane (Hu 2002, Lishko, Drenan 2005). We found MIR16, the membrane interacting protein of RGS16 (Zheng 2000), to be down-regulated by PGE_2 in a global gene expression profiling comparing MoDCs matured in the absence or presence of PGE_2. MIR16 also shows weak interactions with other RGS proteins via their RGS domain. Since MIR16 is an integral membrane protein localizing to intracellular membranes as well as to the plasma membrane, it has been suggested to act as an anchoring protein for RGS proteins (Zheng 2000). The balance between specific RGS protein expression and the availability of RGS interacting proteins could play a role in the modulation of chemokine receptor responsiveness in MoDCs. Receptors themselves may contribute to selective RGS protein interactions with G

proteins, as GPCRs alone or in cooperation with their linked G protein can selectively recruit specific RGS proteins to the plasma membrane, thereby controlling their signaling functions (Roy 2003). Several RGS proteins have been described to regulate signaling of one chemokine receptor but not that of another *in vitro* and *in vivo*. For example, RGS16 inhibits CXCR4-mediated migration, but does not affect migration towards CXCL13, CCL21 and CCL7 (Estes 2004, Lippert 2003, Berthebaud 2005). Therefore, the specific expression pattern of chemokine receptors as well as RGS proteins could determine signaling modulation. In DCs, the maturation-induced alteration of chemokine receptor expression on the surface and importantly of the intracellular RGS protein profile may contribute to the establishment of the unique migratory properties of mature DCs. PGE_2 seems to play a role in regulating the expression of a specific RGS protein pattern that might contribute to the PGE_2-promoted enhanced migratory capacity of MoDCs.

We found RGS9 to be induced by PGE_2 during maturation of MoDCs. RGS9 exists in two splice variants, RGS9-1 and RGS9-2, which are reported to be selectively expressed in retina and striatal brain regions, respectively (Zhang 1999, Rahman 1999). However, RGS mRNA profiling in human tissues revealed moderate expression of RGS9 mRNA in lymphocytes (Larminie 2004). Moreover, RGS9 expression has been reported in regulatory T cells (Tregs). The limited migratory capacity of Tregs towards CCR7 ligands has been correlated with enhanced expression of RGS1, RGS16, and RGS9. Unfortunately, RGS9 was not further specified as to which splice variant was expressed, and RGS9 expression was not analyzed on protein level (Agenes 2005).

In MoDCs we show the PGE_2-dependent induction of RGS9 mRNA during maturation independently of the maturation pathway (Figure 1 and data not shown). Due to the lack of a RGS9-specific antibody we could not detect RGS9 on protein level. To determine the impact of RGS9 expression on migration of mature MoDCs we over-expressed RGS9. Although over-expression of untagged RGS9 seemed to inhibit CCR7- and CXCR4-mediated migration of CEM cells (Figure 2A, B) and MoDCs (Figure 3), co-expression of EGFP that allowed to limit analysis of migration to only transfected cells revealed no effect of RGS9 expression on migration (Figure 2D, E; Figure 5). These data are inconclusive, since over-expression of RGS9 protein itself could not be controlled.

Besides RGS6, RGS7 and RGS11, RGS9 belongs to the R7 subfamily of RGS proteins, which are generally reported to be poorly expressed in lymphoid cells and tissues (Kehrl 2006). All members of the R7 subfamily contain a Gγ-like (GGL) domain between N-terminus and RGS domain (Xie 2007), which allows them to associate with an atypical G protein ß subunit (Snow 1998, Makino 1999, Sow 1999, Levay 1999). The family of G protein ß subunits consists of 5 members. Whereas Gß1-4 are highly homologous with 80-90% sequence identity, Gß5 is different in structure and cellular localization sharing only 50%

sequence identity with Gß1-4 (Watson 1996b, Watson 1994). Gß5 is alternatively spliced and can exist in a long form, Gß5-L, which is exclusively found in the outer segment of retinal photoreceptors (Watson 1996b, Zhang 2000), and a short form, Gß5-S, which is expressed in brain and the inner retina (Watson 1994). Importantly, RGS proteins of the R7 family form complexes with Gß5 over their GGL domain but do not interact with Gß1-4, indicating a selective role for Gß5 binding for R7 functionality (Snow 1998, Levay 1999). Besides association with GGL domain-containing RGS proteins, Gß5 can bind to certain G protein γ subunits like Gγ2 (Watson 1994, Jones 1999).

The interaction between Gß5 and the GGL domain is essential for the stability of both the Gß5 and the RGS protein (Chen 2003). Retinas of RGS9-deficient mice do not express Gß5-L protein, although mRNA encoding Gß5 is abundantly present (Chen 2000). Conversely, Gß5-deficiency results in very low levels or absence of RGS proteins of the R7 subfamily from retina, despite mRNA expression (Chen 2003). In normal retina, RGS9-1 accelerates the GTPase activity of transducin, the Gα subunit of phototransduction, while RGS9-1 is associated with Gß5 (Arshavsky 2002, Cowan 2000). Consequently, photoreceptors lacking RGS9 or Gß5 show abnormally slow recovery rates of light responses (Chen 2000, Krispel 2003). Most importantly, it has been reported that elevation of RGS9-1 mRNA by transgenic expression did not increase RGS9-1 protein in retina, indicating that the amount of RGS9-1 protein in not controlled by the level of its transcript but rather by the availability of Gß5, which limits RGS9-1 protein expression (Chen 2003). Therefore, it has been concluded, that RGS9 needs to associate with Gß5 in order to maintain its stability and perform its functions (Makino 1999, Witherow 2000, Kovoor 2000, Chen 2003).

To our knowledge Gß5 is not expressed in immune cells (Zhang 2000). Therefore, it is possible, that PGE_2-induced RGS9-1 mRNA expression in mature MoDCs does not translate to RGS9 protein expression. Furthermore, introduction of plasmids carrying RGS9 need not necessarily lead to RGS9 protein over-expression due to the lack of concurrent Gß5 expression. Since we were unable to monitor RGS9 protein expression, it can not be excluded, that RGS9-1 mRNA is not translated to protein in human DCs. However, gene expression profiling of human tissues, revealed abundant expression of Gß5 in $CD19^+$ B cells (*www.symatlas.gnf.org*, Kehrl 2006), although GGL domain expressing RGS proteins are absent (Kehrl 2006). The expression of Gß5 in B cells seems to be specific for humans, as mouse B cells do not express Gß5 (*www.symatlas.gnf.org*). Although we are not aware of a report showing Gß5 protein expression in B cells so far, it would be astonishing to find such high Gß5 mRNA expression without functional translation to protein. Thus, a potential mechanism could exist, which stabilizes Gß5 protein in leukocytes. Analysis of Gß5 expression in human MoDCs still has to be performed. All *in vitro* data obtained in this study

concerning RGS9-1, however, should be considered carefully due to the lack of protein expression data.

In photoreceptors, the RGS9-1:Gß5 complex is additionally tightly associated with the transmembrane RGS9 anchor protein (R9AP). By binding to the N-terminal domain of RGS9-1, R9AP mediates the anchoring of RGS9-1 to the disc membranes of the outer segment of photoreceptors (Hu 2002). R9AP mRNA has been described to be exclusively expressed in retina, whereas the protein only exists in photoreceptors (Hu 2002, Keresztes 2003). The stability of RGS9-1:Gß5 is determined by R9AP in photoreceptors, and consequently R9AP dramatically augments the functionality of RGS9-1:Gß5 (Lishko 2002, Hu 2003, Martemyanov 2003). R9AP has therefore been suggested to be an essential component of the GTPase activating complex for transducin (Keresztes 2003). Importantly, R9AP coordinates the delivery of the RGS9-1:Gß5 complex to the membrane (Martemyanov 2003). Taken together the knowledge about RGS9-1 and its dependence on specific protein binding, which are very selectively expressed, it seems questionable if RGS9-1 is expressed in human MoDCs on protein level and that its PGE_2-induced expression plays a role in regulation of chemokine receptor signaling.

Both RGS9 variants, RGS9-1 and RGS9-2, arise from alternative splicing of the same gene transcript (Zhang 1999), which is induced by PGE_2 during maturation of MoDCs. Although RGS9-1 mRNA may not be translated to protein, or the protein may be rapidly degraded due to the lack of interacting proteins, RGS9-2 mRNA could still be made into protein. RGS9-2 has been described so far to be expressed predominantly in brain (Granneman 1998, Rahman 1999). In the central nervous system, RGS9-2 has been shown, like RGS9-1 in retina, to associate with to proteins, one of which is again Gß5 (Chen 2003, Martemyanov 2005) and the other the syntaxin family protein R7BP (Drenan 2005, Martemyanov 2005). Gß5 and R7BP contribute the folding and stability of RGS9-2 as well as to its targeting to the membrane (Chen 2003, Song 2006, Anderson 2007). However, in a recent study, RGS9-2 could be expressed, and exerted regulatory functions in HEK293 cells without concurrent expression of Gß5. Additionally, over-expression of Gß5 in this system did not affect regulatory responses of RGS9-2 (Bouhamban 2006). Since RGS9-2 also binds to the cytoskeleton protein α-actinin-2 (Bouhamban 2006) and might be stable without Gß5 in certain environments, it could possibly be expressed in DCs and contribute to the modulation of chemokine receptor-mediated migration. Again, only analysis of RGS9 expression on protein level in MoDCs will tell.

In order to determine the impact of RGS9 expression on migration of immune cells *in vivo*, we analyzed RGS9-deficient mice. In an *in vivo* migration assay, in which migration of skin-resident DC to the draining lymph node was monitored, we found significantly less $CD11c^+$ $FITC^+$ DCs in the lymph nodes of $RGS9^{-/-}$ mice compared to wild-type controls one day after

application of FITC to the abdomen (Figure 8). Due to the lack of a specific antibody against RGS9, we could not detect RGS9 protein expression in DCs of wild-type mice, but RGS9 mRNA was indeed produced (Figure 7). Murine DCs could therefore express a form of RGS9, which plays a role in the regulation of DC cell functions, since the lack of RGS9 resulted in a profound defect. Besides a strong decrease of migrated DCs, we found a attenuated potential to induce an antigen-specific immune response in RGS9$^{-/-}$ mice (Figure 9). The reduced ability of RGS9-deficient mice to mount an antigen-specific CD8^{+} response could be directly linked to a migratory defect of DCs in these mice, because we used a model of infection that relies on migration of DCs from the periphery to secondary lymphoid organs. However, DCs from RGS9$^{-/-}$ mice have to be further analyzed. It is unclear, if the same number of DC precursor is produced, immigrate and settle in the periphery in RGS9$^{-/-}$ compared to wild-type mice. Moreover, the process of DC maturation has to be compared, as the lack of RGS9 could influence signaling transduction involved in maturation, without directly affecting chemokine receptor responsiveness, but nevertheless resulting in a migratory defect. As we found RGS9 mRNA to be also expressed in T cells, the impact of RGS9 deficiency should be analyzed additionally on signal transduction and migration of T cells. The organization of secondary lymphoid organs depends on the migration of developing lymphocytes into lymphoid tissues. If the chemotactic defect of RGS9$^{-/-}$ cells would also affect lymphocytes of the T and B lineage, the microorganization of secondary lymphoid organs could be disturbed. Mice deficient in RGS1 show disturbed architectural organization of secondary lymphoid organs, which affects the outcome of humoral immune responses (Moratz 2004a). Analysis of lymph nodes using immunohistochemistry will reveal, if the lymph node organization is disturbed in RGS9$^{-/-}$ mice. A disturbed organization of lymphoid tissues could play a role in the observed inhibited induction of antigen-specific IFN-γ producing CD8^{+} T cells in RGS9$^{-/-}$ mice. We analyzed the content of CD4^{+} and CD8^{+} T cells in lymph nodes of wild-type and RGS9$^{-/-}$ mice (data not shown) and found that the proportion between CD4^{+} and CD8^{+} T cells was shifted towards CD4^{+} T cells in RGS9$^{-/-}$ (CD4:CD8 = 1.32 in wt; 2.14 in RGS9$^{-/-}$).

Compared to other RGS-deficient mice the observed phenotype of RGS9$^{-/-}$ was rather unexpected, as targeted deletion of other RGS proteins resulted in enhanced migration of lymphocytes. For example, the lack of RGS1 leads to hyperresponsiveness of B cells to chemokine receptor-mediated signals *in vivo*. RGS1-deficient B cells showed improved adherence to high endothelial venules and homing to lymph nodes, while migratory speed within lymph node follicles was accelerated (Han 2005 Immunity 22(3):343-54). *In vitro*, RGS1 and RGS13 mRNA silencing enhances the responsiveness of human B cells to CXCL12 and CXCL13 (Han 2006).

Further detailed immunological analysis of RGS9-deficient mice will be necessary to illuminate the impact of RGS9 in regulatory processes of leukocytes.

Material und Methods

Generation of MoDCs

Peripheral blood mononuclear cells were separated from peripheral blood of healthy donors by density gradient centrifugation on Ficoll Paque Plus (Amersham Biosciences, Uppsala Sweden), subsequently monocytes were positively selected using anti-CD14 conjugated microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany). Monocytes were cultured at $1x10^6$/ml in AIM-V (Gibco, Paisley, UK) supplemented with 50 ng/ml GM-CSF (Leukomax®, Novartis, Basel, Switzerland) and IL-4 (supernatant of an IL-4 producing J558 cell line). After 5-6 days, immature MoDCs were harvested, plated at $1x10^6$ in fresh AIM-V, and maturation was induced by addition of 0.5 μg/ml trimeric sCD40L (PromoCell, Heidelberg, Germany) or 20 μg/ml poly I:C (Sigma, St Louis, MO). Where indicated, 1 μg/ml PGE_2 (Minprostin® E2, Pharmacia, Uppsala, Sweden) was added. Usually, maturation was allowed to occur during two days. Where indicated, maturation was terminated at earlier time points to analyze mRNA content.

Transfection

Mature MoDCs were transfected using Amaxa nucleofector technology according to the manufacturer's instructions. Briefly, $2x10^6$ mature MoDCs were resuspended in 100 μl nucleofector solution provided with the Human Dendritic Cell Nucleofector Kit (Amaxa Biosystems, Cologne, Germany) and mixed with 30 μg plasmid DNA. For MoDCs the nucleofector program U-002 was used. After transfection procedure, MoDCs were cultured in RPMI 1640 medium (Lonza, Verviers, Belgium) supplemented with 10% FCS (Linaris, Wertheim-Bettingen, Germany).

CEM cells were maintained in RPMI 1640 (Lonza, Verviers, Belgium) containing 10% FCS (Linaris, Wertheim-Bettingen, Germany), Penicilin/Streptomycin (100 IU/ml, Lonza) and 100 μM non-essential amino acid mix (Lonza). For transfection, $2x10^6$ CEM cells were resuspended in 100 μl Amaxa Nucleofector Solution V, mixed with 30 μg plasmid DNA and processed according to the manufacturer's protocol. Nucleofector program X-001 was used for CEM cells.

Transfection efficiency was analyzed by flow cytometry one day after transfection. Dead cells were stained with ToPro3 (Molecular Probes, Eugene OR) or SytoxBlue (Molecular Probes), and excluded from analysis.

Quantitative real-time PCR

Total RNA was isolated from human and mouse cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol, including an additional digest with DNase I (Qiagen). cDNA was synthesized from 1 μg total RNA according to the manufacturer's instructions using random hexamer primer with the TaqMan® Reverse Transcription Reagents (Applied Biosystems). RGS9-1 and RGS9-2 were quantified by real-time PCR using 5'-TGCTCATGAAGAAGGATTCTTATGC and 5'-CCTGCGGTCCAGCTTGCT; or 5'-CCTGTCTTTGCCAGGCTCTCA and 5'-TCCTCCGAGTCCATCAAGCA, respectively. RGS9-1 and RGS9-2 mRNA expression was normalized to two housekeeping genes β-2 microglobulin (β2M) and ubiquitin C (UBC) using 5'-GCTATCCAGCGTACTCCAAAGATTC and 5'-CAACTTCAATGTCGGATGGATGA for β2M and 5'-ATTTGGGTCGCGGTTCTTG and 5'-TGCCTTGACATTCTCGATGGT for UBC, respectively. Reactions were performed with SYBR Green PCR Master Mix (Applied Biosystems) containing 200 nM forward as well as reverse primers according to the manufacturer's instructions using the Taqman 7700 (Applied Biosystems).

Cloning of RGS9 constructs

A vector containing RGS9L (pcDNA3.1(+)RGS9L) was purchased from Missouri S&T cDNA Resource Center. RGS9L was excised from pcDNA(+)RGS9L using *NheI* and *XhoI* restriction sites and inserted into pIRES2-EGFP (Clontech).

ERK activation

For analysis of chemokine-induced ERK activation, $1x10^6$ MoDC were stimulated for two minutes with either 1 μg/ml CCL19 (Promocell) or 1 μg/ml CXCL12 (Promocell) in 50 μl AIM-V medium at 37°C. Cells were lysed on ice in lysate buffer (1% NP-40, 50 mM Tris, 0.25% sodiumdesoxycholate, 150 mM NaCl, 1 mM NaF, 1 mM EGTA, 1 mM Na_3VO_4) and protease inhibitor (Roche) for 15 min and subsequently centrifuged for 10 min at 14000 x g. The supernatant was analyzed by Western blotting using a specific anti-human phospho-ERK1/2 antibody (Santa Cruz). Total expression of ERK2 was monitored by re-probing blots with an anti-human ERK2 antibody (Cell Signaling).

Migration assays

Chemotaxis of MoDCs and CEM cells was determined using Transwell™ plates (Corning Costar, NY) with a membrane pore sizes of 5 μm. Therefore, $1x10^5$ cells were placed in the upper well and allowed to migrate to the lower well containing medium supplemented with chemokine for 3 h at 37°C/5%CO_2. MoDCs migrated in response to 250 ng/ml CCL21 and 250 ng/ml CXCL12. For CEM cells 1 μg/ml CCL21 or 10 ng/ml CXCL12 was used. The

number of cells that migrated through the membrane was counted by flow cytometry using the BD LSRII (BD Biosciences). Specific migration was calculated as percentage of migrated cells from total input cells after subtraction of spontaneous migration towards medium alone. In experiments with cells expressing a GFP-tagged construct, analyses of migration was constricted to GFP-expressing cells; additionally dead cells were excluded from analysis by positive staining with ToPro3 or SytoxBlue.

Migratory properties of DCs *in vivo* were determined by skin painting assay. Therefore, 25 µl FITC (1:1 in dibuthylphtalate:acetone) were applied to the shaved abdomen of female, age-matched C57Bl/6 or RGS9$^{-/-}$ mice. After one day, inguinal lymph nodes were taken and disrupted on sterile wire meshes. DCs in cell suspensions of lymph nodes were stained with PE-conjugated anti-mouse CD11c antibody (BD). FITC^{+}CD11c^{+} cells were quantified relative to all CD11c^{+} cells, whereas dead cells were excluded by staining with ToPro3.

Analysis of RGS9 deletion in RGS9$^{-/-}$

RGS9-deficient mice were kindly provided by Prof. J. Schwarz (University Leipzig). DNA was extracted from tail biopsies of C57Bl/6 and RGS9$^{-/-}$ mice using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. Disruption of the rgs9 gene was determined by PCR using 5'-TCGCCGCTCCCGATTCGCAGCGCA as forward primer binding inside the MC1neopA cassette and 5'-GAGAAAAGGATCCAGGAACCTGTAG as reverse primer binding outside the 1.3-kb short arm (Chen 2000).

To determine expression of RGS9 in mouse brain, T cells and DCs, mRNA was extracted from respective tissue or cells and transcribed into cDNA. RGS9 was amplified using TaqMan® PCR master Mix (Applied Biosystems) according to the manufacturer's instructions with 5'-CCCAAGAATCTCATCCTCAAGC and 5'-TGGCAGTCCAGCGCGTA as primers.

Antigen-specific immune response *in vivo*

Wild type C57Bl/6 or RGS9$^{-/-}$ mice were injected subcutaneously with 5 mg of poly(lactic-co-glycolic acid) (PGLA) microspheres containing ovalbumin and CpG, which were kindly provided by Dr. E. Schlosser (University of Konstanz). Mice were sacrificed after six days, and spleen cell suspensions were re-stimulated with 10^{-6}M ovalbumin peptide (SIINFEKL) for five hours in the presence of 10 µg/ml Brefeldin A. CD8^{+} T cells were stained with APC-Cy7-conjugated anti-mouse CD8 antibody (BD Biosciences) and intracellular IFN-γ was stained using a FITC-labeled anti-IFN-γ antibody (kindly provided by Dr. E. Schlosser). IFN-γ production by CD8^{+} cells was analyzed by flow cytometry using the BD LSRII.

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CHAPTER 5

Prostaglandin E_2 is a key factor for monocyte-derived dendritic cell maturation: enhanced T cell stimulatory capacity despite IDO

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Abstract

The exclusive ability of DCs to stimulate primary and secondary immune responses favours the use of antigen-loaded human monocyte-derived DCs (MoDCs) in vaccinations against tumors. Previous studies demonstrated that prostaglandin E_2 (PGE_2) is fundamental during MoDC maturation to facilitate migration towards lymph node-derived chemokines. A recent study challenged the use of PGE_2, as PGE_2 induced indoleamine-2,3-dioxygenase (IDO) in mature MoDCs. In MoDCs compatible for clinical use, we now demonstrate that PGE_2 is responsible for IDO induction if matured by sCD40L, LPS, or cytokines. In contrast, IDO expression in MoDCs matured by TLR3 triggering occurs independently of PGE_2. Surprisingly, despite active IDO protein, MoDCs matured with PGE_2 display a greater potential to stimulate naïve $CD4^+$ and $CD8^+$ T cell proliferation, which is not further increased by IDO inhibition. Moreover, we found elevated levels of tryptophanyl-tRNA-synthetase (TTS) in T cells cultured with PGE_2-matured MoDCs. Our data demonstrate that PGE_2 induces IDO in MoDCs, but that T cell stimulating capacities of PGE_2-matured MoDCs overcome IDO activity probably through TTS induction. As PGE_2 is critical for DC migration and enhances the capability of MoDCs to induce T cell proliferation, we highly recommend supplementing DC maturation stimuli with PGE_2 for use in clinical trials.

Introduction

Dendritic cells (DCs) are key regulators of the immune system and have the unique ability to initiate naïve T cell responses. Immature DCs are present in peripheral tissues, where they are poised to capture antigens from incoming pathogens. Up-take and processing of antigens in conjunction with stimulation with inflammatory cytokines leads to the maturation of DCs. One characteristic of this differentiation process is the change of the chemokine receptor expression profile, resulting in the up-regulation of the chemokine receptor CCR7 (Sallusto 1998, Sozzani 1998). CCR7 expression renders DCs responsive to the chemokines CCL19 (ELC, Exodus-3, MIP-3ß) and CCL21 (SLC, Exodus-2, 6Ckine, TCA-4), which directs their homing to the T cell zone of draining lymphoid organs (Ohl 2004). There, DCs present their peptide antigens to naïve T cells, thereby inducing an immune response involving cytotoxic T cells, T helper cells, B cells, as well as NK cells (Banchereau 2000, Mellman 2001).

The exclusive ability to stimulate primary and secondary immune responses prompted the use of antigen-loaded human monocyte-derived DCs (MoDCs) in vaccinations against tumors in numerous clinical studies (Schuler 2003, Cerundolo 2004, Banchereau 2005). However, DC-based immunotherapy as currently applied has not yet proven to be clinically successful (Schadendorf 2006). Nevertheless, the question is not whether immunotherapies using DCs work, but how to refine the immunological and clinical parameters of vaccination with DCs in order to improve the efficacy (Banchereau 2005). One major problem was that antigen-loaded DCs failed to leave the injection site (Morse 1999a). This drawback was solved by the discovery that addition of the pro-inflammatory mediator prostaglandin E_2 (PGE_2) to any classical maturation stimulus of MoDCs facilitates migration towards CCR7 ligands (Scandella 2002, Luft 2002, Scandella 2004, Legler 2006). Indeed, PGE_2 is a general and mandatory factor, as human immature and mature MoDCs as well as *ex vivo* DCs require PGE_2 during maturation to migrate in response to chemokines and chemoattractants (Legler 2006). Interestingly, the PGE_2 signal facilitating migration can be mediated by either EP2 or EP4 receptor triggering on human polyI:C matured MoDCs (Legler 2006), but seems to be restricted to EP4 in mouse Langerhans cells (Kabashima 2003). Furthermore, PGE_2 was also shown to enhance DC maturation and their stimulatory capacity to prime naïve T cells and to modulate chemokine and cytokine production of DCs through a yet unknown mechanism (Scandella 2002, Jonuleit 1997, Rieser 1997, Jefford 2003, Rubio 2005).

A recent study by Braun et al., challenges the use of PGE_2 for DC maturation describing that the addition of PGE_2 to maturation stimuli strongly up-regulated indoleamine 2,3-dioxygenase in MoDCs (Braun 2005). Indoleamine 2,3-dioxygenase (IDO) is an enzyme that degrades tryptophan and, expressed by DCs, can suppress T cell proliferation and survival and may

promote tolerance (Mellor 2004, Grohmann 2003). Interestingly, PGE_2 was shown to induce IDO on both mRNA and protein level via EP2 receptor activation, but a second signal through TNF receptors or Toll-like receptors was crucial for the enzyme activity (Braun 2005). In their hands, induction of IDO strictly depended on PGE_2, as DC maturation stimuli alone did not induce IDO expression (Braun 2005).

As *ex vivo* generated MoDCs are widely used in clinical trials and a comprehensive study on IDO expression, cell migration and T cell priming by MoDCs that are generated under clinically approved conditions is missing, we decided to investigate on recent concerns about the use of PGE_2 and its effects on MoDCs under clinically relevant, serum-free conditions. Thus, we used different stimuli in combination with PGE_2 and specific PGE_2 receptor agonists for MoDC maturation and investigated on IDO expression and activity, cell migration, and examined the capacity of MoDCs to stimulate T cell proliferation in relation to IDO expression.

Results

Influence of PGE_2 on IDO expression in human mature MoDCs

Several protocols exist to generate mature dendritic cells *ex vivo*. One of which, using TNF-α together with PGE_2 as maturation stimuli, was recently shown to induce the expression of IDO (Braun 2005), an enzyme involved in the degradation of tryptophan and thought to induce immune tolerance (reviewed by Mellor 2004, Grohmann 2003). In order to test whether IDO mRNA is expressed on human MoDCs generated under serum-free conditions compatible for clinical trials, we isolated human monocytes from peripheral blood of healthy donors by positive selection using CD14 microbeads. Monocytes were differentiated into immature MoDCs by incubation for 5 to 6 days in serum-free medium containing IL-4 and GM-CSF. Immature MoDCs were then matured for 2 days with either poly I:C or soluble trimeric CD40L (sCD40L) in the absence or presence of PGE_2, which is essential to generate a general migratory DC phenotype Legler 2006). IDO mRNA from mature MoDCs was quantified by real-time RT-PCR. As shown in Figure 1A, PGE_2 provoked a dramatic increase of IDO mRNA in MoDCs matured with sCD40L, similar to MoDCs matured with TNF-α in combination with PGE_2 (Braun 2005). In striking contrast, MoDCs matured by poly I:C constitutively expressed high levels of IDO mRNA independently of PGE_2 (Figure 1A). The induction of IDO mRNA on sCD40L matured MoDCs by PGE_2 was on average 113-fold, ranging from 17 to 270-fold, depending on the donor (n=7), whereas PGE_2 had virtually no effect (less than 1.5-fold, n=6) on IDO expression in poly I:C matured MoDCs (Figure 1B).

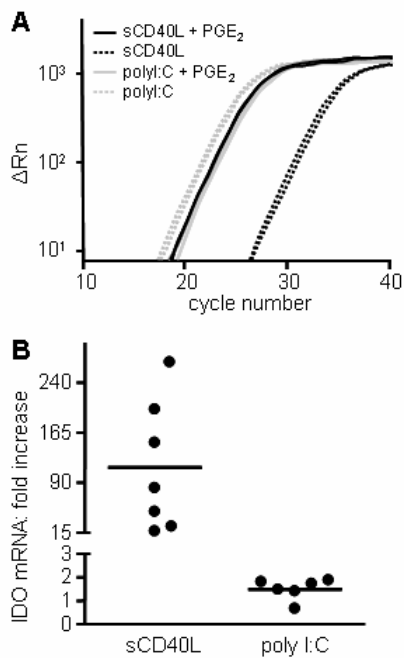

Figure 1. PGE_2 enhances IDO mRNA expression in human MoDCs matured with sCD40L but not with poly I:C. MoDCs generated under serum-free, clinically relevant conditions were matured for 48 h with either trimeric sCD40L or poly I:C in the presence or absence of PGE_2. Expression of IDO mRNA was analysed by real-time RT-PCR. (**A**) Amplification plots of IDO mRNA expression in MoDCs of a representative donor in duplicates matured with sCD40L (black lines) and poly I:C (grey lines), respectively, are shown. Dashed lines represent MoDCs matured in the absence of PGE_2, whereas solid lines correspond to MoDCs matured in the presence of PGE_2. (**B**) Induction of IDO mRNA expression by PGE_2 in mature MoDCs of multiple donors was quantified by real-time RT-PCR and expressed as fold increase induced by PGE_2. Each closed circle represents the mean of a duplicate analysis of a single donor (n=7 for sCD40L and n=6 for polyI:C).

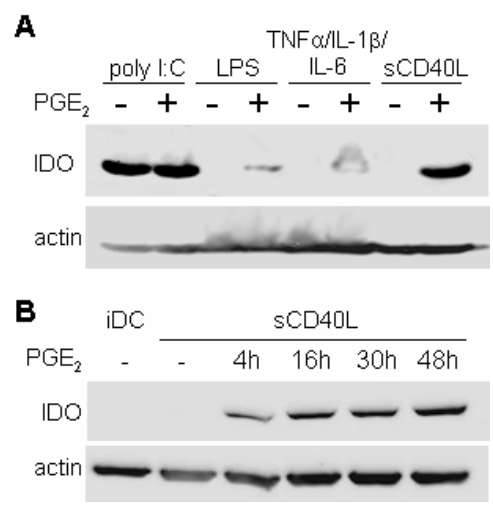

Figure 2. PGE_2 is not generally required for IDO protein expression in human MoDCs. IDO protein expression was analyzed by Western blotting using an IDO-specific antibody in total cell lysates of MoDCs matured with either sCD40L, poly I:C, LPS or a combination of IL-1ß, IL-6 and TNF-α in the presence or absence of PGE_2 (**A**). The blot was re-probed with ß–actin antibody to ensure equal protein loading. (**B**) Expression of IDO protein was analyzed in immature (iDC) or sCD40L matured MoDCs, which were cultured in the absence or presence of PGE_2 for the initial 4, 16 or 30 h of maturation or for the full maturation period (48 h).

Next, we investigated IDO expression on protein level. Again, we found substantial IDO expression in poly I:C matured MoDCs independently of PGE_2 supplementation (Figure 2A). In contrast, IDO expression was only detected in sCD40L matured MoDCs if PGE_2 was present during the maturation process (Figure 2A). In the latter case, the level of IDO was similar to poly I:C matured MoDCs, confirming our data obtained on mRNA level. Furthermore, we also matured MoDCs with LPS or a cocktail of cytokines including TNF-α, IL-1ß and IL-6, in the presence or absence of PGE_2. Under these conditions, IDO expression was restricted to MoDCs matured in the presence of PGE_2 (Figure 2A). These data clearly suggest that PGE_2 is responsible for the induction of IDO on MoDCs matured with sCD40L, LPS or cytokine cocktail, whereas TLR3 triggering alone was sufficient for a high expression of the tryptophan degrading enzyme. We have recently demonstrated that the addition of PGE_2 to DC maturation cocktail has to occur at the beginning of the maturation period to induce a migratory phenotype, but can be omitted at later time points (Legler 2006). Thus,

we investigated whether IDO up-regulation in maturing MoDCs happens also at early time points and whether short term incubation of DCs with PGE_2 may prevent IDO expression. Therefore, we co-incubated MoDCs, that were matured with sCD40L for 48 h, with PGE_2 during the initial 4 h, 16 h or 36 h, and for the entire 48 h of maturation, respectively (Figure 2B). In fact, IDO protein levels increased the longer PGE_2 was present during MoDC maturation, but was already detectable when maturing DCs were treated with PGE_2 exclusively for the initial 4 h of maturation followed by a further incubation of 36 h in the presence of sCD40L alone (Figure 2B).

PGE_2 is not obligatory for IDO activity in MoDCs matured with TLR3 ligands

As IDO expression does not necessarily correlate with its activity (Braun 2005, Mellor 2004, Grohmann 2003), we analyzed PGE_2-induced IDO for its activity to degrade tryptophan to kynurenine. To this end, MoDCs were matured with sCD40L or poly I:C in the presence or absence of PGE_2 for 2 days and mature cells were incubated with tryptophan. To determine tryptophan degradation, we quantified residual tryptophan concentration as well as generated kynurenine in culture supernatants by HPLC. As expected, MoDCs matured with

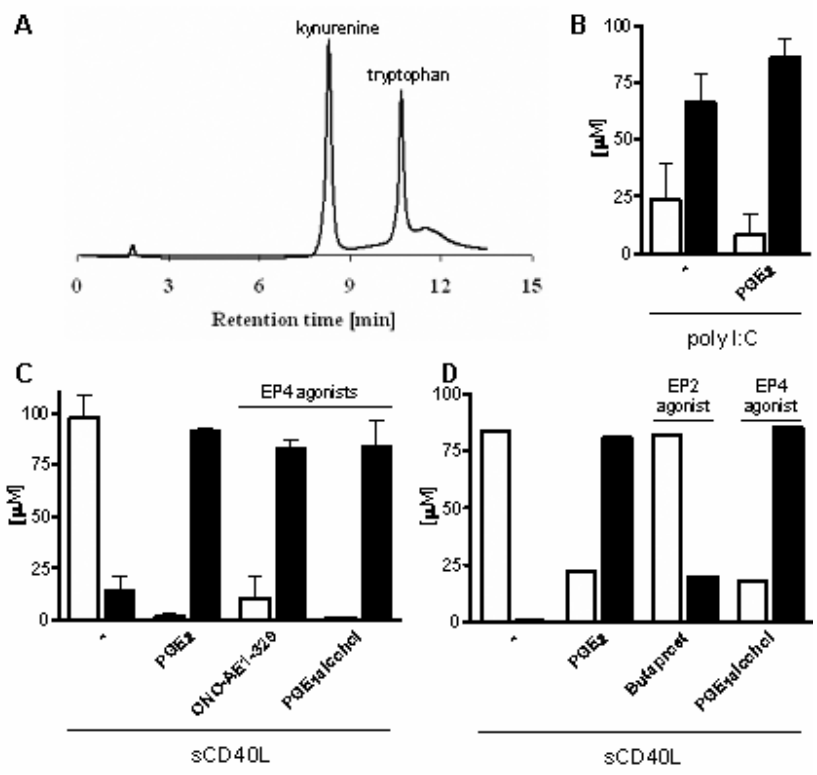

Figure 3. PGE_2 induces active IDO protein primarily through EP4 receptor signalling, but is not essential for IDO activity during maturation via TLR3. IDO activity was assessed by quantification of tryptophan degradation to kynurenine using HPLC analysis (**A**). MoDCs were matured for 48 h by polyI:C (**B**) or sCD40L (**C**, **D**) in the presence or absence of PGE_2, EP2- (butaprost) or EP4- (ONO-AE1-329, PGE_1-alcohol) specific agonists. Cells were washed and incubated in HBSS in the presence of 100 µM of tryptophan for 4 hours. Supernatants were subjected to HPLC analysis to assess IDO mediated degradation of tryptophan (white bars) to kynurenine (black bars). Mean values and SEM of seven independent experiments (**B**, **C**) of individual donors are shown.

sCD40L alone, showing undetectable IDO expression, did not degrade tryptophan (Figure 3B). In contrast, IDO from the supernatant of MoDCs matured with sCD40L and PGE_2 readily degraded tryptophan to kynurenine (Figure 3B). Correlating with IDO expression, MoDCs matured with TLR3 ligand poly I:C produced active IDO independently of PGE_2, since more than 70% of tryptophan was converted to kynurenine in the presence as well as absence of PGE_2 during the maturation process (Figure 3A). PGE_2 has been suggested to be a mandatory factor for active IDO expression in mature MoDCs (Braun 2005). Surprisingly, we found this statement not to hold true in general, as certain maturation pathways, such as TLR3-mediated maturation, seem to circumvent the obligatory signal by PGE_2.

PGE_2-induced IDO activity is mediated primarily by EP4 signaling

The effect of PGE_2 on human MoDCs can be mediated by two receptors, namely EP2 and EP4. We have shown previously that activation of EP2 and EP4 either alone or in combination is mandatory to induce a migratory phenotype of DCs (Legler 2006). A migratory DC phenotype is essential to ensure homing of antigen-loaded MoDCs to secondary lymphoid organs and thereby triggering of antigen-specific T cell proliferation. Since PGE_2 also induces active IDO production, which may inhibit T cell proliferation, it has been suggested to prevent IDO expression but simultaneously to induce a migratory phenotype by

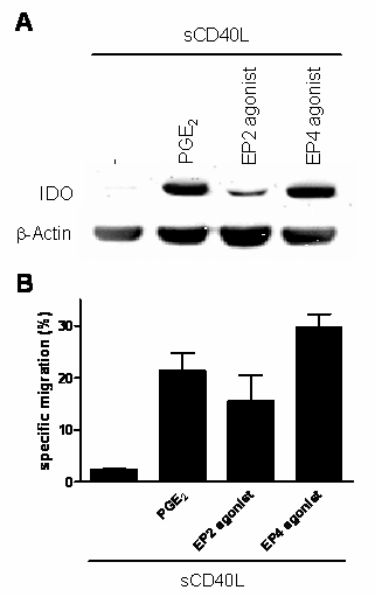

Figure 4. PGE_2 induces IDO protein expression primarily via EP4. IDO protein expression (**A**) and migratory behaviour (**B**) of MoDCs matured with sCD40L in the absence or presence of PGE_2, EP2 (butaprost) or EP4 (PGE_1-alcohol) receptor agonist was analyzed. (**A**) IDO protein expression was analyzed by Western blotting using an anti-IDO specific antibody. The blot was re-probed with a ß–actin antibody to ensure equal protein loading. One representative experiment out of eight is shown. (**B**) To prove functionality of EP receptor agonists, migratory capacity of MoDCs was analyzed in Transwell™ chemotaxis assay in response to CCL21. Mean values of two independent experiments with different donors are shown.

substituting PGE_2 with a specific EP4 agonist (Braun 2005). To analyze IDO induction through EP4 receptor signaling under conditions compatible for clinical trials, we matured MoDCs with sCD40L in the absence or presence of PGE_2 or two specific EP4 receptor agonists, ONO-AE1-329 and PGE_1-alcohol. Surprisingly, under these conditions, IDO protein was induced und fully active if MoDCs were matured in the presence of either of the two EP4

agonists and was comparable to PGE_2 (Figures 4A, 3B). Unexpectedly, EP4 receptor induced IDO activity was even higher than EP2 receptor mediated IDO induction by butaprost (Figure 3C). The ineffective degradation of tryptophan by MoDCs matured with sCD40L in the presence of butaprost correlated with low induction of IDO protein expression (Figure 4A). In order to ensure functionality of the EP2 agonist, we analyzed the migratory behavior of MoDCs. We matured MoDCs with sCD40L in the absence or presence of PGE_2, the EP2 agonist butaprost or the EP4 agonist PGE_1-alcohol and subjected the cells to Transwell™ chemotaxis assays. As noted for poly I:C (Legler 2006), sCD40L-matured MoDCs migrated in response to the homing chemokine CCL21 only if MoDCs were matured in the presence of either PGE_2, EP2 or EP4 agonists, demonstrating that butaprost was biologically active (Figure 4B). Although the EP2 agonist butaprost did not induce high levels of IDO, it provoked a migratory capacity similar to PGE_2 and EP4 agonist.

PGE_2 enhances the stimulatory capacity of MoDCs

Supplementation of PGE_2 to MoDC maturation stimuli (cytokine cocktail, sCD40L, or poly I:C) was shown to enhance their capacity to promote $CD4^+$ and $CD8^+$ T cell proliferation (Scandella 2002, Rubio 2005). To investigate the effect of PGE_2 on stimulatory properties of MoDCs compatible for clinical trials, we differentiated MoDCs from monocytes by addition of IL-4 and GM-CSF in serum-free medium. MoDCs were matured with sCD40L in the absence or presence of PGE_2 for 2 days and subsequently co-cultured with alloreactive naïve $CD45RO^-CD4^+$ or $CD45RO^-CD8^+$ T cells. T cell proliferation was assessed after 5 days of stimulation using a BrdU cell proliferation ELISA. As depicted in Figure 5, IDO positive MoDCs matured in the presence of PGE_2 were significantly more efficient to induce T cell proliferation of both $CD4^+$ and $CD8^+$ subpopulations.

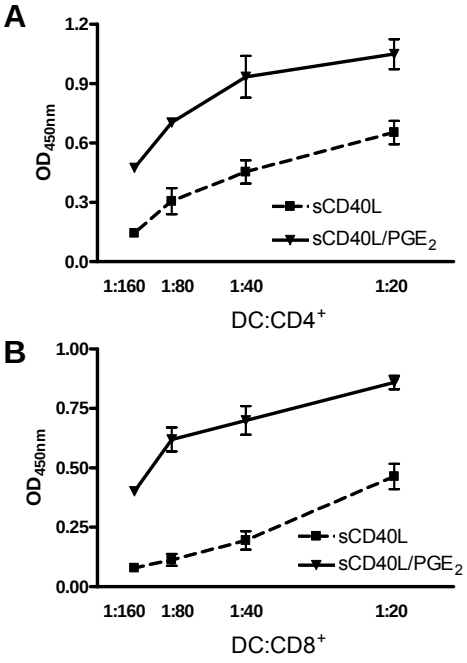

Figure 5. PGE_2 enhances the stimulatory capacity of MoDCs. MoDCs were matured with sCD40L in the presence (solid lines) or absence (dashed lines) of PGE_2 and co-cultured with alloreactive naïve $CD4^+$ (**A**) or $CD8^+$ (**B**) T cells. After 5 days of stimulation, T cell proliferation was assessed by BrdU cell proliferation ELISA. Mean values and SEM of a representative experiment out of six are shown.

PGE_2-induced IDO does not limit the capacity of MoDCs to stimulate T cell proliferation

As addition of PGE_2 to sCD40L for maturation of MoDCs promotes the expression of active IDO, we investigated whether active IDO interfered with MoDC-induced T cell proliferation. To this end, we matured MoDCs with sCD40L in the absence and presence of PGE_2 and co-cultured mature MoDCs with freshly isolated alloreactive naïve $CD45RO^-CD4^+$ or $CD45RO^-CD8^+$ T cells as above. IDO activity was inhibited by addition of 20 μM 1-methyltryptophan (1-MT). As 1-MT is solved in HCl, a solvent control was included to ensure specific IDO inhibition and exclude solvent dependent effects. $CD4^+$ T cell proliferation was enhanced by PGE_2 matured MoDCs and could not be further augmented by addition of 1-MT (Figure 6A). Even higher concentrations of 1-MT (200 μM, 2 mM) did not improve T cell proliferation (data not shown). In addition, IDO inhibition by 1-MT did also not enhance alloreactive $CD8^+$ T cell proliferation stimulated by MoDCs matured in the presence of PGE_2 (Figure 6B).

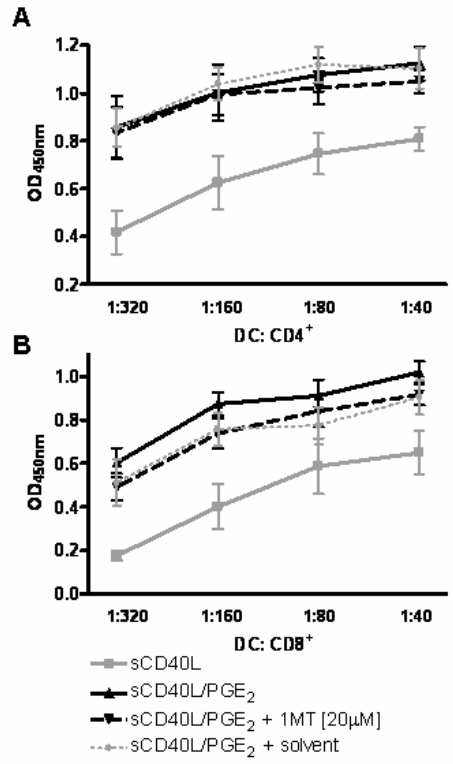

Figure 6. PGE_2 induced IDO activity does not limit the enhanced stimulatory capacity of PGE_2 matured DCs. MoDCs were matured with sCD40L in the presence (black solid lines) or absence (grey solid lines) of PGE_2 and co-cultured with alloreactive naïve $CD4^+$ (**A**) or $CD8^+$ (**B**) T cells for 5 days. To inhibit IDO activity, 1-methyltryptophan (1-MT, black dashed lines) was added for the entire co-culture. To ensure specificity, HCl, the solvent of 1-MT, was added at the same concentration (grey dotted line). Mean values and SEM of 4-6 independent experiments with different donors are shown.

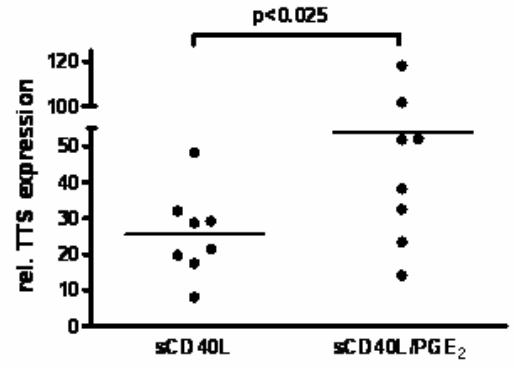

Figure 7. Elevated expression of tryptophanyl-tRNA-synthetase (TTS) in T cells co-cultured with PGE_2-matured MoDCs. MoDCs matured with sCD40L in the presence or absence of PGE_2 were co-cultured with naïve allogenic $CD4^+$ T cells. After 4 days, TTS mRNA of sorted T cells from the co-culture was quantified by real-time RT PCR. Relative TTS expression was normalized to TTS mRNA in unstimulated T cells from the same donor. Each closed circle represents the mean of a duplicate analysis of a single donor (n=8).

Elevated levels of tryptophanyl-tRNA-synthetase (TTS) in T cells co-cultured with PGE_2-matured MoDCs

TTS is another important enzyme of the tryptophan metabolism. TTS is responsible for the association of tryptophan with its specific tRNA. This tryptophan-tRNA complex generates a reservoir of tryptophan that is available for protein *de novo* synthesis (Fleckner 1995, Rubin 1991). Elevation of the TTS expression level can thus serve as counter-mechanism to protect IDO-mediated tryptophan deprivation. To investigate whether this may explain why T cells can proliferate in the presence of active IDO, we co-cultured naïve $CD4^+$ T cells with allogenic MoDCs matured with sCD40L in the presence or absence of PGE_2. Four days after stimulation, T cells were isolated and TTS mRNA was quantified by real-time RT-PCR. In fact, T cells derived from co-cultures with sCD40L-matured MoDCs express on average 25-fold more TTS compared to naïve T cells from the same donor (Figure 7). Moreover, the TTS levels further increase by a factor of two if PGE_2 was added to the MoDC maturation stimulus (Figure 7). These data provide clear evidence that PGE_2-induced IDO expression in MoDCs is compensated and nullified with enhanced expression of TTS in the proliferating T cells.

Discussion

The aim of efficient vaccines against cancer is to induce tumor-specific effector T cells that eliminate the cancerous tissue in conjunction with establishing a pool of tumor-specific memory T cells that control eventual tumor relapse (Banchereau 2005). DCs are key players in launching an efficient T cell-mediated immunity owing to their unique capacity to acquire, process and present antigens to all subsets of T cells. The first clinical study of a DC vaccine was published 10 years ago in which follicular B cell lymphoma patients were treated with peripheral-blood derived DCs loaded with recombinant tumor-specific idiotype proteins (Hsu 1996). This hallmark-study initiated a whole series of experimental studies and clinical trials using antigen-loaded DCs as vaccines to augment tumor-specific T cell responses in cancer patients (Schuler 2003, Cerundolo 2004, Banchereau 2005, Davis 2003, Figdor 2004). One important aspect was the development of a protocol to generate large amounts of monocyte-derived DCs (Sallusto 1994). Most clinical studies nowadays use such MoDCs, in which monocytes are isolated from peripheral blood and cultured in the presence of IL-4 and GM-CSF for several days to differentiate into immature DCs. These cells can be further matured with different stimuli, such as poly I:C, sCD40L, LPS, or a combination of the cytokines IL-1ß, IL-6 and TNF-α. These matured MoDCs, when loaded with antigens, are capable to propagate antigen-specific T cells *in vitro* and show cytotoxic activity for antigen-bearing target cells in the case of $CD8^+$ T cells. However, despite a robust activation of the adaptive

immune response against the targeted tumor antigen, DC-based immunotherapies often showed only a marginal clinical anti-tumor activity (Schadendorf 2006). This drawback is easily explained by the fact that *in vitro* generated, antigen-loaded, radioactively-labeled mature DCs could not be detected in lymph nodes of the patients (Morse 1999a). Migration of mature DCs to the T cell zone of secondary lymphoid organs is guided by the homing chemokines CCL19 and CCL21 (Ohl 2004, Forster 1999). We and others have realized that CCR7 expression, the chemokine receptor for CCL19 and CCL21, alone is not sufficient to facilitate migration of clinical-grade MoDCs (Scandella 2002, Luft 2002, Scandella 2004, Legler 2006). In fact, whatever stimuli may be used to mature clinical-grade MoDCs, supplementation of the maturation cocktail with the pro-inflammatory mediator prostaglandin E_2 is fundamental for the development of a migratory MoDC phenotype (Scandella 2002, Luft 2002, Scandella 2004, Legler 2006, van Helden 2006). Moreover, also *ex vivo* peripheral blood DCs rely on PGE_2 addition for efficient migration (Legler 2006). The crucial role of PGE_2 for DC migration has been further substantiated by the finding that skin-derived Langerhans cells derived from *ptger4* null mice, which lack the PGE_2 receptor EP4, showed impaired homing to draining lymph nodes, whereas $EP2^{-/-}$ animals had no such phenotype (Kabashima 2003). In contrast, in human, PGE_2 triggering can be mediated through either EP2 or EP4 to give rise to migratory DCs (Figure 4B and Legler 2006).

A recent study by Braun and coworkers now challenged the use of PGE_2 supplementation for MoDC maturation as they found that PGE_2 was responsible for the induction of IDO (Braun 2005). IDO is the initial and rate-limiting enzyme which converts tryptophan to kynurenine; and tryptophan is an essential amino acid important for protein synthesis, cell survival, and proliferation (Taylor 1991, Murray 2003). Hallmark-studies by Munn and Mellor discovered that IDO was able to prevent rejection of the fetus during pregnancy (Munn 1998, Mellor 2001). IDO expression in DCs depends on IFN-γ stimulation and correlates with the inhibition of T cell proliferation which can be prevented with the IDO inhibitor 1-MT (Hwu 2000, Munn 2002, Terness 2005). Moreover, the tryptophan-derived catabolites kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid can induce activation induced T cell death (Frumento 2002, Terness 2002). Braun and collaborators now described that the presence of PGE_2 during TNF-α or LPS induced maturation of human MoDCs induces active IDO protein (Braun 2005). In the absence of PGE_2, however, they found no IDO protein. The authors therefore suggested reconsidering the use of PGE_2 in DC-based immunotherapy protocols. Using human MoDCs compatible for clinical trials we now describe that the addition of PGE_2 to LPS, sCD40L, or a cocktail of cytokines for MoDC maturation provoked up-regulation of IDO on mRNA and protein level (Figure 1, 2A) confirming and extending the observations by Braun, *et al.* (Braun 2005). However, we demonstrated that TLR3-mediated MoDC maturation using poly I:C induced IDO expression independently of PGE_2 (Figure 1, 2A) and

poly I:C induced IDO protein was fully active (Figure 3A). In our hands, IDO protein expression strictly correlated with the enzyme's activity. Thus, PGE_2 is not a general pre-requisite for IDO expression in mature MoDCs, as implied previously (Braun 2005).

In humans, the PGE_2 promoted enhanced migratory capacity of MoDCs is mediated through the PGE_2 receptors EP2 and EP4 (Legler 2006), in contrast to mice, where the effect is accredited to EP4 alone (Kabashima 2003). Braun and colleges demonstrated that PGE_2-induced active IDO in human MoDCs is mediated exclusively through EP2 triggering. As a consequence, they suggested replacing PGE_2 with a specific EP4 agonist in maturation protocols for DC-based immunotherapies to induce a migratory phenotype but to prevent expression of active IDO (Braun 2005). In striking contrast, using two independent specific agonists we clearly demonstrate that under serum-free, clinically relevant conditions, PGE_2 induced IDO protein expression and activity is primarily mediated by the PGE_2 receptor EP4 (Figure 4A, 3B). EP2 triggering also induced IDO activity, but to a much lower level (Figure 4A), which correlated with lower IDO protein induction (Figure 4A). The addition of PGE_2, EP2 or EP4 agonists during MoDC maturation is not only essential for the development of a migratory phenotype it also induces active IDO protein. Interestingly, IDO may even be critical for DC activation and chemotaxis, as incubation of maturing DCs with the IDO inhibitor 1-MT impaired maturation-induced regulation of chemokine receptor expression (Hwang 2005). With respect to DC-based immunotherapy, antigen-loaded DCs must acquire a migratory phenotype to reach the draining lymph node to initiate an efficient immune response. Initial studies using MoDCs that were matured in the absence of PGE_2 showed a clear deficit in migration (Morse 1999a). These data clearly argue for the use of PGE_2 (or receptor agonists) for the generation of clinical-grade DCs. The induction of IDO which may inhibit antigen-specific T cell proliferation within the lymph node now challenges the use of PGE_2. Unexpectedly, despite active IDO expression, PGE_2-matured MoDCs showed an enhanced capacity to induce allogenic $CD4^+$ and $CD8^+$ (Figure 5, 6), as well as antigen-specific (Rubio 2005) T cell proliferation compared to MoDCs matured in the absence of PGE_2. In order to test whether the augmented capacity of PGE_2-matured MoDCs was nevertheless limited by active IDO expression, we inhibited IDO activity with 1-MT. Strikingly, we were unable to increase the number of proliferating T cells by PGE_2-matured MoDCs when IDO activity was blocked (Figure 6). The robust T cell activation of both T helper as well as cytotoxic T cell subpopulations induced by PGE_2-matured MoDCs seems not to be restrained by IDO expression. The fact that efficient T cell proliferation can be induced by IDO-positive DCs was discovered recently. Terness and colleagues found that the proliferation of neither OKT3-stimulated human T cells of healthy donors, nor myelin-basic-protein-specific T cells of patients with multiple sclerosis was impaired by autologous MoDCs expressing IDO (Terness 2002). Moreover, resistance of synovial T cells of rheumatoid

arthritis patients to IDO-mediated tryptophan deprivation was shown to be associated with enhanced expression of the enzyme tryptophan-tRNA-synthetase (TTS) (Zhu 2006). TTS protects T cells from IDO activity in two ways (Zhu 2006, Boasso 2005): One includes the formation of tryptophane-tRNA-complexes, which act as a reservoir of tryptophan for protein synthesis thus overcoming the tryptophan-deprivation effect of IDO. The other way is the reduction of tryptophan catabolites, which can induce cell death. We discovered that T cells that are co-cultured with MoDCs expressed substantial amount of TTS. Interestingly, we found even higher amounts of TTS in proliferating T cells that were engaged by MoDCs matured in the presence of PGE_2 (Figure 7). This novel finding can easily explain why MoDCs matured with PGE_2 can still prime T cells despite IDO expression. Interestingly enough, IDO as well as TTS, are both induced by IFN-γ (Fleckner 1995). Thus, it is conceivable that under pathological conditions, where IFN-γ is produced, TTS activity of T cells that are recruited along with IDO-positive DCs to the lymph node at an early phase is capable to silence the tolerogenic potential of IDO. However, in order to regulate the immune response, T cell proliferation within the lymph node needs to be stopped at later time points, e.g when IFN-γ is no longer produced to induce TTS, whereas the homed DC may still express functional IDO.

In summary, we demonstrate that PGE_2 induces active IDO expression in DCs generated under clinically relevant conditions early during maturation. However, PGE_2 is not fundamental for IDO induction, as functional IDO expression is observed in TLR3 mediated MoDC maturation in the absence of PGE_2. Since under serum-free conditions, IDO expression by PGE_2 is mediated primarily through EP4 receptor, the proposed replacement of PGE_2 by a specific EP4 agonist (Braun 2005) for generation of migratory and IDO negative MoDCs can no longer be recommended. Although PGE_2 induces functional IDO in MoDCs, the T cell stimulating capacity is enhanced. In addition, we demonstrate that T cells activated by PGE_2-matured MoDCs express high levels of TTS, which protect them from tryptophan deprivation by IDO-expressing DCs. Overall, PGE_2 is not only fundamental for the development of a migratory phenotype but enhances T cell stimulatory capacities of mature MoDCs.

Materials and Methods

Generation of human MoDCs

Monocytes were isolated from peripheral blood monocytes (PBMCs) as previously described (Scandella 2004, Legler 2006). Briefly, PBMCs were isolated from whole blood of healthy donors by density gradient centrifugation on Ficoll Paque Plus (Amersham Biosciences,

Uppsala Sweden). Monocytes were positively selected from PBMCs using anti-CD14-conjugated magnetic microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) and cultured at $1x10^6$ cells/ml in AIM-V medium supplemented with 50 ng/ml GM-CSF (Leukomax®, Novartis, Basel, Switzerland) and IL-4 (supernatant of an IL-4 producing J558 cell line). Immature DCs were harvested after 5 to 6 days, and maturation was induced for 2 days by adding 0.5 μg/ml trimeric sCD40L (PromoCell, Heidelberg, Germany), 20 μg/ml poly I:C (LPS-free; Sigma, St Louis, MO), 1 μg/ml LPS (*Salmonella abortus equi*, Sigma) or a cocktail of pro-inflammatory cytokines (20 ng/ml TNF-α, 10 ng/ml IL-1β, 20 ng/ml IL-6, all purchased from PromoCell, Heidelberg, Germany). Where indicated, 1 μg/ml PGE_2 (Minprostin® E2, Pharmacia, Uppsala, Sweden), 1 μg/ml of specific agonists for EP2 (butaprost, Cayman Chemicals, Ann Arbor, MI) or EP4 (PGE_1-alcohol, Cayman Chemicals; ONO-AE1-329, ONO Pharmaceutical, Osaka, Japan) was added.

Cell migration assay

To measure chemotaxis, $1x10^5$ DCs were placed on a polycarbonate filter with a pore size of 5 μm in a 24-well Transwell™ plate (Corning Costar, NY). Cells were allowed to migrate towards 250 ng/ml CCL21 (PromoCell, Heidelberg, Germany) for 3 hours at 37°C/5% CO_2. Migrated cells were counted by flow cytometry (LSRII, BD Biosciences, Erembodegen, Belgium) and specific migration was calculated as percentage of the number of input cells after subtraction of spontaneous migration toward AIM-V medium without chemokine.

MoDC induced T cell proliferation

Human peripheral blood T cells were isolated from PBMCs of healthy donors using the pan T cell isolation kit (Miltenyi Biotec) according to the manufacturer's protocol. Naïve T cells were negatively sorted using anti-CD45RO conjugated magnetic microbeads (Miltenyi Biotec), resulting in a pure population of $CD45RA^+$ expressing cells. Naïve T cells were further separated into $CD4^+$ and $CD8^+$ populations using anti-CD4 conjugated magnetic microbeads (Miltenyi Biotec). Naïve $CD4^+$ and naïve $CD8^+$ cells, respectively, were co-cultured with mature MoDCs in graded ratios in RPMI 1640 medium containing 10% FCS. Where indicated, 20 μM of 1-methyltryptophan (Sigma) was added. T cell proliferation was measured after 5 days of co-culture using BrdU cell proliferation ELISA kit (Roche, Indianapolis, IN) according to the manufacturer's protocol.

IDO mRNA and protein expression

Total RNA of mature MoDCs was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) and transcribed into cDNA using the Taqman® reverse transcription reagent (Applied Biosystems, Rotkreuz, Switzerland) with random hexamer primers according to the

manufacturer's instructions. For amplification of IDO, 1 μl of cDNA was added to QuantiTect SYBR Green PCR Master Mix (Qiagen) containing 200 nM forward primer 5'-TGTCCGTAAGGTCTTGCCAAGA and 200 nM reverse primer 5'-CACCAATAGAGAGACCAGGAAGAATC. Real-time PCR was performed with an initial step of denaturation at 95°C for 15 min followed by 40 cycles of 15 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C using the Taqman 7700 (Applied Biosystems). IDO mRNA expression was normalized to two housekeeping genes ß-2 microglobulin (β2M) and ubiquitin C (UBC) using the primers 5'-GCTATCCAGCGTACTCCAAAGATTC and 5'-CAACTTCAATGTCGGATGGATGA for β2M and 5'-ATTTGGGTCGCGGTTCTTG and 5'-TGCCTTGACATTCTCGATGGT for UBC, respectively, and SYBR Green PCR Master Mix (Applied Biosystems) containing 200 nM forward as well as reverse primer according to the manufacturer's instructions. Relative mRNA expression was calculated with the ΔΔCt-method. IDO protein expression was analyzed by Western blotting using an anti-IDO specific antibody (Upstate, Charlottesville, VA). The blot was re-probed with a ß–actin antibody (Abcam, Cambridge, UK) to ensure equal protein loading.

Detection of active IDO protein in MoDCs

Mature MoDCs were harvested and washed 3 times in Hanks balanced salt solution (HBSS). $2x10^6$ cells were resuspended in 1 ml HBSS containing 100 μM L-tryptophan (Sigma) and incubated for 4 h at 37°C/5% CO_2. Supernatants were collected and subjected to HPLC analysis after addition of 200 mM H_2SO_4. Tryptophan and kynurenine were analyzed using a HPLC system consisting of two high-pressure pumps (Shimadzu LC-10ATvp), an autoinjector (Gilson 234), a C18 reversed-phase column (Grom-Sil 120 ODS-5ST, 5 µm, 150x4.6 mm, Grom, Herrenberg, Germany), and a UV-VIS diode array detector (Shimadzu SPD-M 10). Analysis was performed at RT using a gradient program with a mobile phase comprising a mixture of 100 mM ammonium phosphate buffer pH 2.6 and methanol at a flow rate of 1 ml/min with a detection wavelength of 227 nm. The solvent phase (5% (v/v) methanol) was initially held for 1 min, then the concentration was increased to 45% over a period of 6 min and lowered to 5% within 0.5 min and held for an additional 7 min. Amounts of tryptophan and kynurenine in MoDC supernatants were quantified with the CLASS-VP software (Shimadzu) on the basis of standard solutions of 20 μM, 50 μM and 100 μM of tryptophan and kynurenine (Sigma), respectively.

Quantification of tryptophanyl-tRNA-synthetase (TTS)

MoDCs matured in the presence or absence of PGE_2 were co-cultured with naïve allogenic $CD4^+$ T cells as described above. After 4 days, T cells from the co-culture were positively selected using an anti-CD3 antibody and magnetic nanoparticles (StemCell Technologies,

Vancouver, BC), RNA was extracted and transcribed into cDNA. TTS mRNA was quantified by real-time PCR as described previously (Zhu 2006). TTS expression was normalized to two house-keeping genes (β-2 microglobulin and ubiquitin C) and calculated relative to unstimulated $CD4^+$ T cells from the same donor.

Acknowledgements

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CHAPTER 6

Prostaglandin E_2 enhances T cell proliferation by inducing the co-stimulatory molecules OX40L, CD70 and 4-1BBL on dendritic cells

Abstract

Dendritic cell-based immunotherapy of malignant and infectious diseases relies on two critical parameters: antigen transport from the periphery to draining lymph nodes and efficient priming of primary and secondary immune responses. Prostaglandin E_2 (PGE_2) signaling has been shown to be pivotal for the development of a migratory DC phenotype. In fact, human monocyte-derived dendritic cells (MoDCs), as used in clinical trials, migrate in response to lymph node derived chemokines exclusively after exposure to PGE_2. Here, we demonstrate that PGE_2 also enhances the ability of MoDCs to induce antigen-specific primary and memory T cell responses. The augmented T cell stimulatory capacity of MoDCs matured in the presence of PGE_2 correlates with the induction of co-stimulatory molecules of the TNF super family, namely OX40L, CD70 and 4-1BBL. While the expression of OX40L and CD70 occurs independently of the maturation pathway, it is dependent on the presence of PGE_2 during the initial phase of maturation. Blocking OX40/OX40L signaling using neutralizing antibody impaired the enhanced T cell proliferation induced by PGE_2-matured MoDCs. Moreover, MoDCs matured in the presence of PGE_2 induced the expression of OX40, OX40L, and CD70 on T cells facilitating T cell-T cell interactions that warrant a long lasting co-stimulation. Our data clearly show the PGE_2-dependent expression of co-stimulatory molecules of the TNF super family that play a role in the enhanced ability of PGE_2-matured MoDCs to induce T cell proliferation.

Introduction

Dendritic cells (DCs) are key players in defence to pathogens due to their capacity to induce primary and secondary T cell responses. Optimal T cell activation needs two signals, both of which are provided by mature DCs. Firstly, the recognition of the specific peptide bound to self-MHC and secondly ligation of co-stimulatory molecules, that provide signals essential for proliferation, survival and cytokine production. The most important co-stimulatory receptor for early proliferation of naïve T cells is CD28 (Lenschow 1996). However, for T cell proliferation and survival at later phases and the development of memory T cells additional co-stimulatory molecules were described to deliver essential signals. Among these molecules, which belong to the TNF/TNFR superfamily, are OX40L/OX40 (CD134), 4-1BBL/4-1BB (CD137) and CD70/CD27 (Watts 2005).

OX40 is induced on activated T cells and provides an essential signal for optimal $CD4^+$ T cell function (Gramaglia 1998, Kopf 1999, Chen 1999, Murata 2000) as well as for the generation of memory T cells (Gramaglia 2000, Rogers 2001, Hendriks 2005) by promoting prolonged survival of effector T cells (Rogers 2001). Signaling through OX40 is controlled *in vivo* by the availability of the ligand OX40L. The expression of OX40L is tightly regulated but can be induced on APCs such as DCs (Ohshima 1997, Brocker 1999), B cells (Stuber 1995) and microglia (Weinberg 1999). The lack of OX40L on APCs results in a marked reduction of cytokine production and proliferation of $CD4^+$ T cells (Chen 1999, Murata 2000). Murine DCs transfected with mRNA encoding OX40L induce enhanced anti-tumor activity *in vivo*, while transfection of OX40L mRNA into human MoDCs improves the induction of antigen-specific CTLs *in vitro* (Danull 2005).

CD27 is expressed on naïve $CD4^+$ and $CD8^+$ T cells (Borst 2005) and on primed B cells (Xiao 2004) and signaling through CD27 supports T cell survival. In transgenic mice constant activation of CD27 by persistent expression of CD70 on B cells leads to excessive T cell proliferation (Arens 2001, Tesselaar 2003). The expression of CD70 is tightly regulated and can be transiently induced on T cells, B cells and DCs after antigen receptor engagement or TLR stimulation, respectively (Bowman 1994, Tesselaar 1997, Tesselaar 2002). For expansion and survival of primed $CD8^+$ T cells expression of CD70 on activated DCs has been shown to play an important role in mice (Schildknecht 2007, Taraban 2004, Laouar 2005). In addition to DCs, OX40L and CD70 are inducible on T cells, thus providing a mechanism by which co-stimulatory survival signals can be delivered through T cell-T cell contact after those signals are no longer available on APCs (Soroosh 2006).

Since DCs are very rare in peripheral blood, the most commonly used procedure to generate mature DCs for immunotherapeutic purposes involves the differentiation of monocytes into immature DCs (Sallusto 1994, Peters 1993). Maturation can be induced by addition of a pro-

inflammatory cytokine cocktail consisting of TNF-α, interleukin (IL)-1ß, IL-6 and Prostaglandin E_2 (PGE_2) (Jonuleit 1997). PGE_2 is a metabolite from the arachidonic acid cycle and has been shown to be the key factor during DC maturation for the acquisition of a migratory phenotype (Kabashima 2003, Legler 2006, Scandella 2002, Luft 2002). Since chemotactic responsiveness of mature DCs is promoted by PGE_2, the transport of antigen from the periphery to the draining lymph nodes and thus the induction of an antigen-specific immune response is PGE_2-dependent. In addition to promoting migratory capacities of DCs, PGE_2 provides monocyte-derived DCs (MoDC) with the enhanced ability to induce allogenic as well as antigen-specific T cell proliferation (Scandella 2002, Krause 2007, Rubio 2005). Moreover, using mice deficient in the PGE_2 receptor EP4 Kabashima et al. showed that PGE_2-EP4 signaling enhances the T cell stimulatory capacity of Langerhans cells (Kabashima 2003). The mechanism by which PGE_2 induces enhanced T cell stimulatory capacities in mature MoDCs is still unclear.

In this study, we show a strictly PGE_2-dependent expression of OX40L and CD70 in mature MoDCs, which increase the capacity of those DCs to induce antigen-specific T cell proliferation.

Results

PGE_2 potentiates the ability of human MoDCs to induce T cell proliferation

It is well established that PGE_2 is the crucial factor during MoDC maturation in order to generate DCs with a migratory phenotype (Scandella 2002, Luft 2002, Legler 2006). Additionally, it has been shown previously, that MoDCs matured in the presence of PGE_2 under serum-free conditions initiate improved allogenic T cell activation (Krause 2007, Scandella 2002). To determine whether this effect also applies to antigen-specific T cell stimulation, we generated MoDCs matured with sCD40L in the absence or presence of PGE_2 and co-cultured them with autologous CFSE-labeled PBMCs. During maturation MoDCs were pulsed either with tetanus toxoid (TT) or keyhole limpet hemocyanin (KLH). TT could induce memory T cell proliferation, since all donors had multiple vaccinations with TT. KLH, however, should induce a primary immune response, as immune cells have never before been confronted with this antigen under normal circumstances. Immature antigen-pulsed MoDCs induced only minor T cell proliferation after six days (Figure 1A, B), which is expected since they were unable to provide co-stimulatory signals due to the lack of expression of CD83 and weaker expression levels of CD80 and CD86 compared to mature MoDCs (Figure 1C). Surprisingly, maturation through CD40 signaling did not strongly improve the ability to induce T cell proliferation (Figure 1A, B), despite high surface

expression of CD83, CD80 and CD86 (Figure 1C). Only MoDCs matured in the presence of PGE_2 were able to promote strong antigen-specific primary and memory T cell proliferation (Figure 1A, B). The surface expression of co-stimulatory molecules like CD83, CD80 and CD86 on mature MoDCs is not PGE_2-dependent (Figure 1C); nevertheless PGE_2 has a crucial impact on the capacity of mature MoDCs to stimulate T cell proliferation. The augmented stimulatory capacity of PGE_2-matured MoDCs was not limited to one T cell subset, but affects both $CD4^+$ and $CD8^+$ T cells. MoDCs matured in the presence of PGE_2 could induce moderate T cell proliferation in the absence of antigen (data not shown).

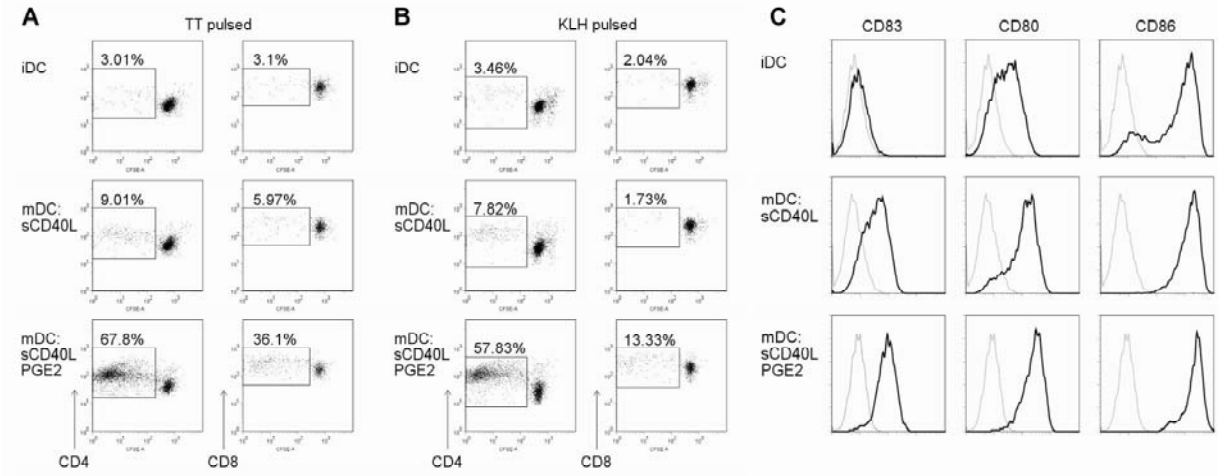

Figure 1. PGE_2 enhances T cell stimulatory capacities of human MoDCs. MoDCs were generated and left immature (iDC) or matured (mDC) using sCD40L in the absence or presence of PGE_2 and pulsed with tetanus toxoid (TT) (**A**) or Keyhole limpet hemocyanin (KLH) (**B**). (**A, B**) Antigen-pulsed MoDCs were cultured together with autologous CFSE-labeled PBMCs and T cell proliferation was analyzed after 6 days by flow cytometry. Percentages of alive (Sytox Blue negative), divided ($CFSE^-$), $CD3^+CD4^+$ or $CD3^+CD8^+$ cells are shown for a representative out of at least five experiments.(**C**) Immature (iDC) or matured MoDCs (mDC) were analyzed for expression of co-stimulatory molecules by flow cytometry. Maturation was induced by addition of trimeric soluble CD40L in the absence or presence of PGE_2. Grey thin lines represent isotype controls while black bold lines represent specific staining for CD83, CD80 or CD86 as indicated.

PGE_2 promotes expression of co-stimulatory molecules of the TNF superfamily on MoDCs

To investigate the mechanism responsible for the enhanced T cell activating properties of PGE_2-matured MoDCs we performed a gene expression analysis on MoDCs matured using soluble CD40L (sCD40L) in the absence or presence of PGE_2. Analyses revealed up-regulation of three non-classical co-stimulatory molecules of the TNF superfamily, namely TNFSF4 (OX40L, CD134), TNFSF7 (CD70, CD27L) and TNFSF9 (4-1BBL, CD137L). The results were corroborated by quantitative real-time PCR, showing a 10- to 30-fold higher expression on mRNA level induced by PGE_2 (Figure 2A). Since it was suggested that at least CD70 is regulated on posttranslational as well as transcriptional level (Tesselaar 2002), surface expression was assessed by flow cytometry after one and two days of maturation. Neither OX40L nor CD70 were expressed by immature MoDCs and were not induced by PGE_2 alone (Fig 2B, C). After 24 hours of maturation via CD40 ligation, both molecules were

absent from the surface. However, after 48 h OX40L and CD70 could be detected on the surface of MoDCs matured in the presence of PGE_2, while both molecules were absent on those MoDCs matured without PGE_2 (Figure 2B, C). The induction of 4-1BBL on mRNA level (Figure 2A) could not be confirmed for cell surface expression, which might be due to poor antibody quality. Lee et al. reported only weak expression of 4-1BBL on MoDCs after stimulation with LPS for 48 hours, which was further increased after 72 hours of maturation. Hence, 4-1BBL surface expression may be regulated later during maturation than OX40L and CD70.

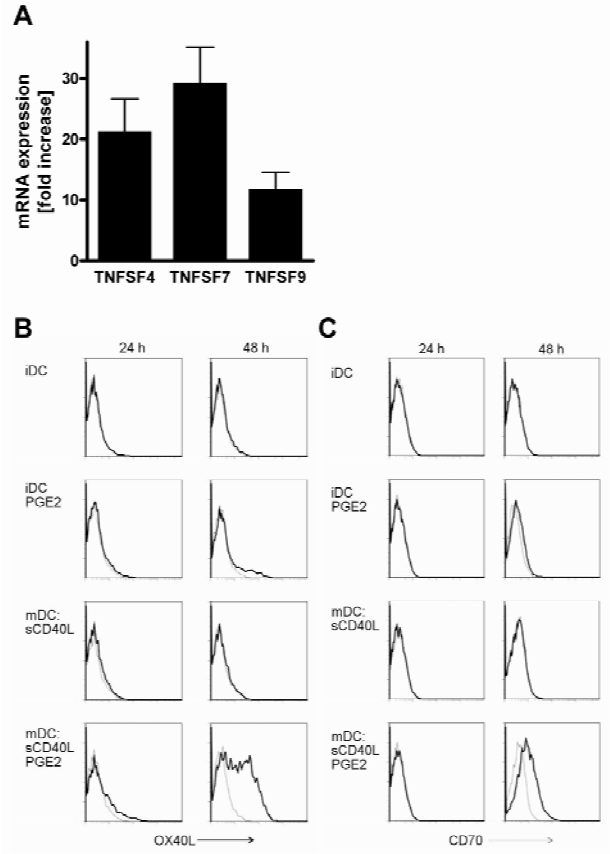

Figure 2. Co-stimulatory molecules of the TNF superfamily are induced on MoDCs by PGE_2. (**A**) Real-time RT-PCR analysis of TNFSF4, TNFSF7 and TNFSF9 mRNA expression in MoDCs that were matured in the absence or presence of PGE_2. The means and SEM of PGE_2-induced fold increase of specific mRNA expression is shown (n=7). (**B**, **C**) Surface expression of OX40L (**A**) or CD70 (**B**, black bold lines) on immature MoDCs (iDC) or sCD40L-matured DCs in the absence or presence of PGE_2 after 24 h or 48 h of culture was analyzed by flow cytometry. Grey thin lines represent isotype control staining.

Expression of OX40L and CD70 on mature MoDCs is PGE_2 dependent

A previous study has shown that expression of OX40L on human dendritic cells is primarily controlled by CD40 signaling, but can also be induced after TNF-α activation (Ohshima 1997). By triggering maturation pathways via TLR4 with LPS or TLR3 signaling with poly I:C, or using a cytokine cocktail containing TNF-α, IL-1ß, IL-6, and PGE_2, we found that PGE_2 induces expression of OX40L on MoDCs when applied in combination with each of these maturation stimuli (Figure 3A). None of the tested maturation stimuli was able to induce OX40L expression in the absence of PGE_2. Under serum-free conditions OX40L expression on mature human MoDCs seems to be independent of the maturation stimulus but is dependent on the presence of PGE_2. In mice, CD70 expression has been reported to be

inducible on $CD11c^+$ bone marrow derived DCs after CD40 or TLR4 stimulation (Tesselaar 2002). In human MoDCs, expression of CD70 was detectable in the presence of PGE_2 when maturation was induced via CD40 or TLR4 or using TNF-α, IL-1ß and IL-6 (Figure 3B), although the PGE_2-induced expression was very low after LPS stimulation. Maturation via TLR3 signaling led to low expression of CD70, which was strongly enhanced in the presence of PGE_2 (Figure 3B). Peripheral DCs will mature in the presence of PGE_2 when encountering pathogens *in vivo,* because PGE_2 can be produced in large quantities by monocytes, macrophages, fibroblast and keratinocytes under inflammatory conditions (Sato 1997, Endo 1998, Kanekura 1998). As maturing DCs will leave the site of infection very rapidly, their exposure to high concentration of PGE_2 in the inflamed tissue is temporary. We therefore determined if a short stimulation with PGE_2 during the initial hours of maturation was sufficient to promote OX40L and CD70 expression on mature MoDCs. As shown in Figure 3C, addition of PGE_2 to the maturation stimulus for the initial 3 hours of maturation was sufficient to induce expression of OX40L and CD70 after 48 hours of maturation. A prolonged presence of PGE_2 could further increase surface expression of both co-stimulatory molecules in some donors, but was dispensable in others.

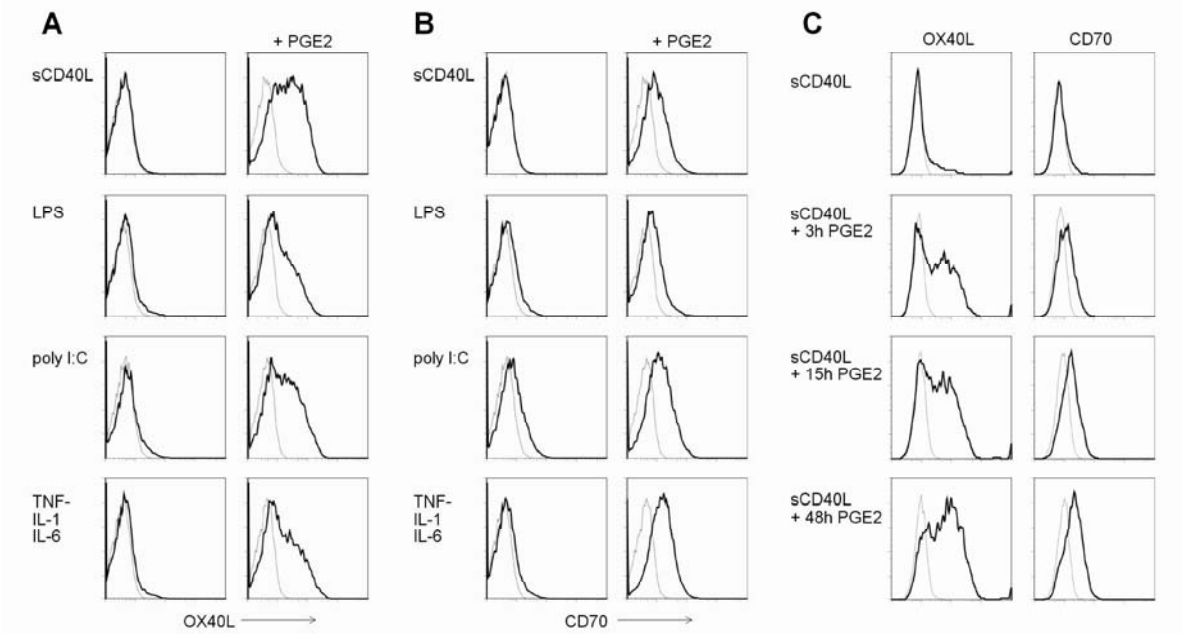

Figure 3. PGE_2 induces OX40L and CD70 expression on mature MoDCs independently of the maturation pathway. (**A**) MoDCs were matured for 48 h using sCD40L, LPS, poly I:C or a cytokine cocktail consisting of TNF-α, IL-1ß and IL-6, in the absence or presence of PGE_2 and analyzed for the expression of OX40L (**A**, black heavy line) and CD70 (**B**, black heavy line) by flow cytometry. (**C**) MoDCs were matured with sCD40L while PGE_2 was present for the first 3 h or 15 h, or for the full period of maturation. Expression of OX40L and CD70 (black heavy lines) was assessed by flow cytometry after 48 h of maturation. The isotype control staining is presented as grey thin line. A representative out of four independent experiments with different donors is shown.

PGE_2-induced augmentation of T cell stimulatory capacities of MoDCs can be inhibited by OX40L blockade

Transfection of mRNA encoding OX40L into mouse DCs has been shown to enhance their ability to induce anti-tumor immunity (Danull 2005). To determine the impact of PGE_2-induced OX40L expression on human MoDCs we cultured antigen-pulsed mature MoDCs generated in the absence or presence of PGE_2 together with autologous PBMCs while blocking OX40L:OX40 interaction using a neutralizing goat anti-human OX40L antibody. After six days of culture OX40L blockage inhibited concentration-dependently the enhanced memory $CD4^+$ and $CD8^+$ T cell proliferation promoted by TT-pulsed MoDCs matured in the presence of PGE_2 (Figure 4A, B). As a control we also added normal goat IgG which had no influence on T cell proliferation. Since OX40 provides an anti-apoptotic signal leading to survival of proliferating T cells (Rogers 2001), it is not surprising that the effect of OX40L blockage is more profound at day 12 (Figure 4C, D) compared to day six. The capacity of PGE_2-matured MoDCs to potentiate T cell proliferation could be partially reversed by OX40L blockage not only for memory, but for primary T cell responses as well (Figure 5).

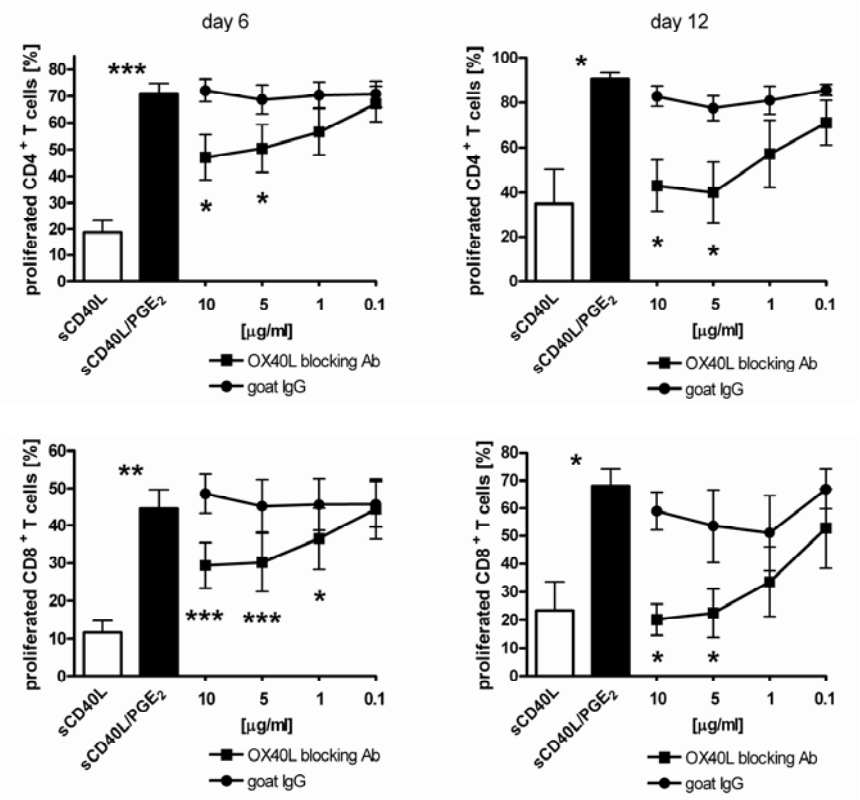

Figure 4. Blockade of OX40L can partially reverse the PGE_2-induced enhanced capacity of MoDCs to stimulate memory T cell proliferation. MoDCs were matured with sCD40L in the absence (white bars) or presence (black bars) of PGE_2, pulsed with tetanus toxoid (TT) and cultured together with autologous CFSE-labeled PBMCs for 6 (**A**, **B**) or 12 days (**C**, **D**). In the co-cultures containing sCD40L/PGE_2-matured MoDCs different amounts of a goat anti-human OX40L neutralizing antibody (■) or normal goat IgG (◆) were added. T cell proliferation was analyzed by CFSE-dilution of live (SytoxBlue negative), $CD3^+CD4^+$ (**A**,**C**) or $CD3^+CD8^+$ (**B**, **D**). Percentages of dividing ($CFSE^-$) $CD3^+CD4^+$ or $CD3^+CD8^+$ cells from all alive, $CD3^+CD4^+$ or $CD3^+CD8^+$ cells are presented. Means and SEM of at least four independent experiments with different donors are shown. Statistical differences were assessed in comparison to MoDCs matured with sCD40L/PGE_2. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

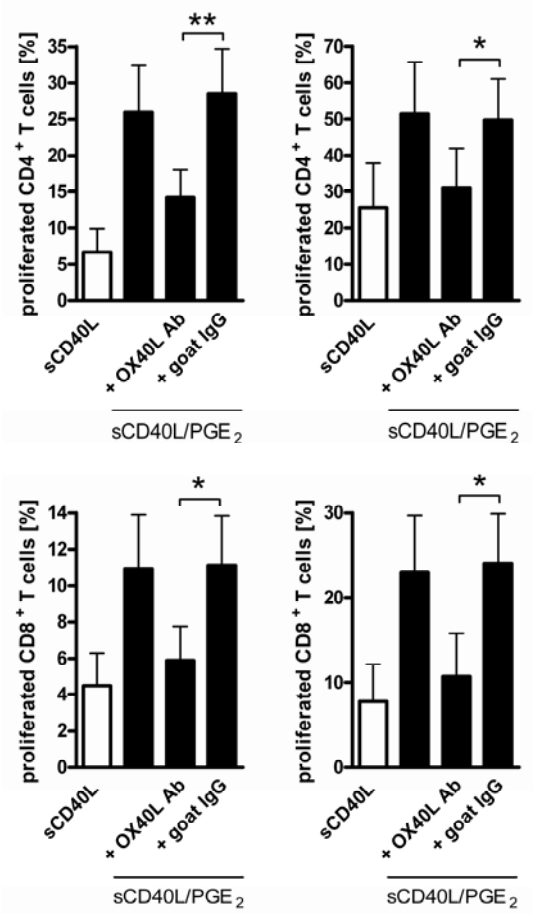

Figure 5. The enhanced primary T cell response induced by PGE_2-matured MoDCs can be inhibited by OX40L-blockade. MoDCs were matured with sCD40L in the absence (white bars) or presence of PGE_2 (black bars) and pulsed with KLH for 48 h. Freshly isolated autologous PBMC were labelled with CFSE and cultured together with MoDCs at a ratio of 10:1 (PBMC:MoDC). Where indicated 5 μg/ml goat anti-human OX40L neutralizing antibody or normal goat IgG were added. After six (**A**, **B**) or 12 (**C**, **D**) days of culture T cell proliferation was analyzed by flow cytometry. T cell proliferation is presented as percentage of alive (Sytox Blue negative), dividing ($CFSE^-$) $CD3^+CD4^+$ (**A**, **C**) or $CD3^+CD8^+$ (**B**, **D**) cells. Means and SEM of seven (**A**, **B**) or four (**C**, **D**) independent experiments with different donors are shown. * $p<0.05$, ** $p<0.01$

PGE_2-matured MoDCs induce expression of OX40L, OX40 and CD70 in T cells

Co-stimulatory molecules are involved in contacts between T cells. It has been previously reported that the expression of OX40L by activated $CD4^+$ T cells can provide a signal through OX40 on adjacent T cells sustaining cell survival by T cell-T cell contacts. We therefore measured the expression of OX40L and OX40 on $CD4^+$ T cells after two and six days of co-culture of PBMCs and autologous antigen-pulsed MoDCs, that had been previously matured using sCD40L in the absence or presence of PGE_2. Already after two days but more profoundly after six days of culture a greater percentage of $CD4^+$ T cells expressed OX40L when stimulated with PGE_2-matured MoDCs compared to those cultured with MoDCs matured in the absence of PGE_2 (Figure 6A, B). OX40 is induced in $CD4^+$ T cells after activation (Croft 2003 review) and its expression is necessary for T cells to become susceptible to OX40L-mediated survival signals. As shown in Figure 6C, MoDCs pulsed with TT and matured in the presence of PGE_2 induced high numbers of OX40-expressing $CD4^+$ T cells after six days. In addition, significantly more T cells reacting to the primary antigen KLH expressed OX40, when the antigen was presented by MoDCs matured in the presence of PGE_2 (Figure 6D). Since the expression of CD70 can be induced on T cells after TCR engagement, we determined the surface expression level of CD70 on $CD4^+$ and $CD8^+$ T cells

after six days of stimulation by TT-pulsed (Figure 6E) or KLH-pulsed (Figure 6F) MoDCs matured in the absence or presence of PGE_2. MoDCs matured in the presence of PGE_2 promoted CD70 expression in a substantial percentage of $CD4^+$ as well as $CD8^+$ T cells.

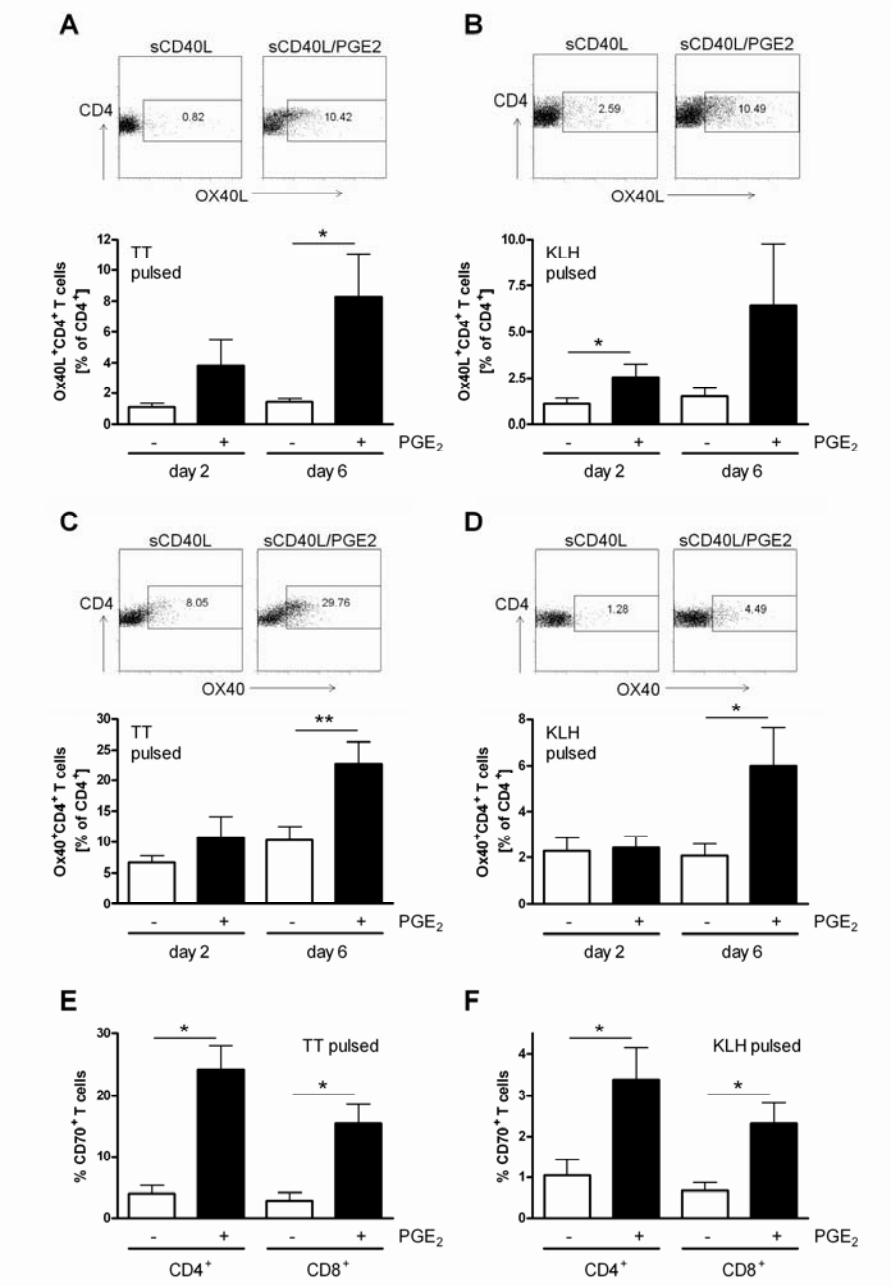

Figure 6. Induction of OX40L, OX40 and CD70 expression on T cells is enhanced by MoDCs matured in the presence of PGE_2. MoDCs were mature with sCD40L in the absence or presence of PGE_2, pulsed with TT (**A**, **C**, **E**) or KLH (**B**, **D**, **F**) and cultured together with autologous unlabeled PBMCs. After 6 days expression of OX40L (**A**, **B**), OX40 (**C**, **D**) and CD70 (**E**, **F**) was analyzed on $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells by flow cytometry. (**A**-**D**) One representative experiment on day six is shown in dot plots, while diagrams display means and SEM for at least five independent experiments with different donors. (**E**, **F**) Means and SEM of at least four independent experiments are presented. * $p<0.05$, ** $p<0.01$

To dissect the relation between proliferation and expression of co-stimulatory molecules on $CD4^+$ T cells, we co-cultured TT-pulsed MoDCs matured in the absence or presence of PGE_2 with PBMCs, which were labeled with the fluorescent tracer Cell Trace Far Red. After six

days of stimulation the expression of OX40L and CD70 were analyzed on proliferated (Cell Trace Far Red $^{-}$) and non-proliferated (Cell Trace Far Red $^{+}$) $CD4^+$ T cells. Most dividing $CD4^+$ T cells did not express OX40L (Figure 7); hence the higher percentage of $CD4^+$ T cells expressing OX40L induced by PGE_2-MoDCs (Figure 6A) can not be attributed to the greater number of divided T cells. Expression of OX40L was also induced in a small proportion of non-dividing $CD4^+$ T cells (Figure 7). CD70 expressing $CD4^+$ T cells were detected among the non-proliferated as well as the proliferated population. Additionally there was a population of dividing T cells, which did not express CD70.

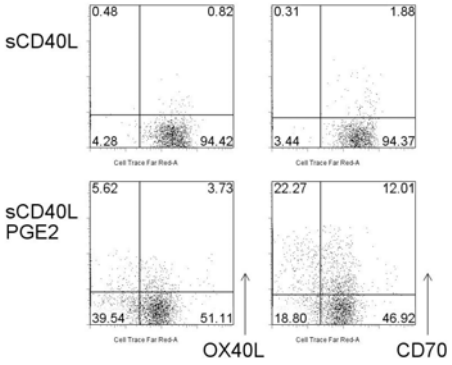

Figure 7. PGE_2-matured MoDCs induce OX40L and CD70 expression in non-proliferating as well as proliferating T cells. PBMCs were labeled with Cell Trace Far Red and co-cultured for 6 days with autologous MoDCs that were matured with sCD40L in the absence or presence of PGE_2 and pulsed with TT. T cell proliferation and surface expression of OX40L and CD70 were analyzed by flow cytometry. Gates were set on $CD3^+CD4^+$ T cells and Cell Trace Far Red is presented on the x axes. One representative experiment out of three is shown.

In summary, PGE_2-matured MoDCs are superior to those matured in the absence of PGE_2 in inducing surface expression of the co-stimulatory molecules OX40L and CD70 in T cells. Although the mechanism by which this is achieved remains unclear, the emerging T cell population might gain the competency to provide survival signals through T cell-T cell contact when expression of co-stimulatory ligands on APCs has diminished.

Discussion

The exceptional ability of dendritic cells to induce antigen-specific immune responses has brought them into the focus of cell-based immunotherapy of malignant and infectious diseases. Since human DCs are hard to purify in sufficient numbers, MoDC are used as the source of DCs in most clinical studies. Large numbers of monocytes can be isolated from peripheral blood of patients and differentiated *in vitro* under serum-free conditions into immature MoDCs, which are competent to take up and process antigens. The pulsing with tumor-antigen and the induction of maturation results in antigen-presenting DCs, which can potently activate tumor antigen-specific immune responses. We and others have shown previously, that PGE_2 is required during maturation to allow MoDCs to develop a migratory phenotype. The presence of PGE_2 in the initial phase of maturation is essential to promote migration towards the lymph node-derived chemokines CCL21 and CXCL12 (Legler 2006).

Interestingly, PGE_2 has also an effect on the T cell stimulatory properties of MoDCs, as allogenic T cell proliferation is enhanced in MLR (mixed lymphocyte reaction) when MoDCs were matured in the presence of PGE_2 (Scandella 2002, Krause 2007). Here we provide evidence, that PGE_2-matured MoDCs have an increased potential to induce antigen-specific T cell proliferation via enhanced expression of co-stimulatory molecules of the TNF superfamily. The expression of OX40L on mature DCs promotes the functionality and survival of OX40-bearing $CD4^+$ T cells at the end of the primary immune response (Gramaglia 1998, Rogers 2001). Because signals for prolonged T cell survival are delivered through OX40, consequently a greater number of memory T cells can develop. The lack of OX40L expression on mature DCs leaves mice unable to mount a specific cellular anti-tumor response (Zaini 2007). Conversely, transfection of murine DCs with mRNA encoding OX40L augmented the induction of an anti-tumor response (Danull 2005). Induction of rather weak OX40L expression on human MoDCs has been described after TNF-α and CD40L stimulation (Ohshima 1997). In this study, we identify PGE_2 as a key factor for expression of OX40L and CD70 on the surface of mature MoDCs (Figure 2). In the presence of PGE_2, OX40L and CD70 are both expressed independently of the provided maturation stimulus (Figure 3A, B). The enhanced proliferation of antigen-specific T cells induced by MoDCs matured in the presence of PGE_2 could be partially reversed by antibody-mediated blockage of Ox40L (Figure 4, 5). Hence, PGE_2-induced OX40L on mature MoDCs plays a role in the enhanced T cell stimulatory capacity of PGE_2-matured MoDCs. By blocking OX40L we observed not only inhibition of $CD4^+$ T cell proliferation but also a strong reduction of $CD8^+$ T cell expansion (Figure 4B, D, 5B, D) even though OX40 is primarily described to affect $CD4^+$ T cell expansion. However, we could detect expression of OX40 on 2-15% of $CD8^+$ T cells after 6 days of co-culture with PGE_2-matured MoDCs, while OX40 expression was mostly absent when MoDCs were matured in the absence of PGE_2 (data not shown). The inhibitory effect of OX40L-blockage on $CD8^+$ T cell proliferation is nevertheless more likely to be indirect, since $CD4^+$ T cells can modulate $CD8^+$ T cell responses through multiple mechanisms including cytokine production and supply of co-stimulatory signals. Consequently, the block of OX40 signals in our system inhibited $CD4^+$ T cells, which in turn could not provide sufficient help for $CD8^+$ T cell expansion. Although OX40 signaling can co-stimulate $CD8^+$ T cells, the signal is weaker than that delivered by 4-1BB (Taraban 2002). In the observed enhanced ability of PGE_2-matured MoDCs to stimulate $CD8^+$ T cell responses, 4-1BBL:4-1BB interaction could play a role. We found 4-1BBL mRNA to be induced on mature MoDCs generated in the presence of PGE_2 (Figure 2A). Expression of CD70 on activated DCs contributes to the expansion of $CD8^+$ T cells and leads to the generation of functional $CD8^+$ memory T cells even in the absence of $CD4^+$ T cells (Bullock 2005). In fact, binding of CD70 to its receptor, CD27, promotes survival of activated T cells, rather than

augmenting cell cycle progression (Hintzen 1995, Hendriks 2003). Taraban et *al*. showed a complete abrogation of $CD8^+$ T cell proliferation in the absence of CD70 (Taraban 2004). Recently, it has been reported that despite the expression of CD70 on different cells types, CD70 on DCs is essential for the priming of $CD8^+$ T cells by pathogens *in vivo* (Schildknecht 2007). The finding, that antigen-induced expansion of $CD8^+$ T cells is dependent on CD70 expression by DCs, highlights the importance of our results. In human MoDCs the expression of CD70 is dependent on the presence of PGE_2 during maturation (Figure 3B). Additionally, we found that PGE_2-matured MoDCs induced CD70 expression on $CD4^+$ and $CD8^+$ T cells (Figure 6E, F). This CD70 expression was not limited to proliferating T cells but could also be measured on non-proliferating T cells (Figure 7). Interactions of T cells mediated by CD70 and CD27 can occur after T cell priming relatively early during the immune response (Lens 1997), and the CD70-mediated stimulation of CD27 has been shown to play a critical role not only in T cell activation but also in T cell-dependent modulation of B cell functions (reviewed in Borst 2005). Similar to CD70, OX40L expression was induced on $CD4^+$ T cells which were stimulated with MoDCs matured in the presence of PGE_2 (Figure 6A, B). OX40L expressed on T cells has previously been shown to provide a potent co-stimulatory signal, which augmented $CD4^+$ T cell proliferation and sustained $CD4^+$ T cell longevity (Mendel 2006, Soroosh 2006). The contact between OX40L-expressing T cells and adjacent $OX40^+$ T cells was suggested to occur in T cell zones of lymph nodes or spleen when expression of OX40L on APC had diminished (Mendel 2006). Therefore it is possible that the induction of OX40L and CD70 on T cells, which is induced by PGE_2-matured MoDCs, ensures maintenance of survival signals through T cell-T cell contact that can sustain and enhance an antigen-specific immune responses.

Interactions of co-stimulatory molecules between APC and T cell or between T cells may provide signals for differentiation into a specific T cell subset or survival of certain T cell populations (Borst 2005). The nature of cytokines produced during differentiation of T cells strongly influences the outcome of a T cell-mediated immune response. The production of IFN-γ by $CD4^+$ T cells characterizes them as Th1 cells which play a major role in the defence against intracellular pathogens (Weaver 2007) and tumors (Shankaran 2001). In contrast, $CD4^+$ T cells which produce IL-4, IL-5 and IL-13 are termed Th2 cells and mediate immunity against helminth infection. The expression of OX40L and CD70 on DCs and T cells has been reported to modulate the Th1/Th2 differentiation of $CD4^+$ T cells. For example, a recent study identified CD70 as the crucial factor on a murine $CD205^+$ DCs subset which induced Th1 $CD4^+$ T cells *in vivo* independently of IL-12 (Soares 2007). There are controversial data regarding the effect of OX40 engagement on T cell differentiation. Several studies reported that signaling through OX40 enhanced IL-4 production of T cells resulting in the development of Th2 effector cells (Ohshima 1998, Flynn 1998, Mendel 2006). In contrast, OX40L:OX40

interactions were shown to be involved in the generation of Th1 responses in disease models like inflammatory bowel disease and experimental allergic encephalomyelitis (Higgins 1999, Weinberg 1999). Moreover, engagement of OX40L provides a signal to APCs leading to enhanced IL-12 production by DCs (Ohshima 1997) which would also favour Th1 differentiation. Along these lines, MoDCs matured in the presence of PGE_2 have been shown to induce differentiation of Th1 cytokine producing $CD4^+$ T cells (Rubio 2005). Nevertheless, interactions of OX40L and OX40 might not directly bias T cell differentiation towards Th1, but rather mediate survival of activated T cells, which then differentiate according to the balance of a variety of available mediators.

An anti-tumor immune response is usually weaker than a response to foreign pathogens due to the lack of danger signals. Moreover, the tumor environment can be immunosuppressive resulting in antigen-specific tolerization of T cells. Stimulation of OX40 has been reported to restore antigen-specific proliferation and cytokine production of anergic $CD4^+$ T cell, suggesting that OX40 signals can reverse established tolerance. In line with this finding OX40 signaling has been shown to enhance anti-tumor immune responses leading to tumor rejection in a variety of tumor models (Weinberg 2000, Pan 2003, Morris 2001). Breaking tumor-specific tolerance is crucial for any immunotherapeutic strategy; therefore the expression of OX40L on DCs could be of essential relevance in DC-based immunotherapy. Overcoming tolerance is not the only challenge immunotherapy is facing. The presence and induction of regulatory T cells present another problem. Even in this case the expression of OX40L on MoDCs is of high importance, since OX40 stimulation on T cells leads not only to high frequencies of antigen-specific effector and memory T cells, but also opposes regulatory T cell (T reg)-mediated suppression (Takeda 2004). When exposed to OX40L-bearing cells, $CD25^-CD4^+$ T cells became insusceptible to Treg-mediated suppression (Takeda 2004). In more recent studies OX40 was identified to provide a negative signal for Foxp3 expression. Consequently, OX40 co-stimulation promoted the generation of effector T cells while inhibiting the formation of $Foxp3^+$ Tregs (So 2007, Vu 2007). In addition, OX40 signals abrogated suppressor functions of natural $Foxp3^+$ Tregs (Vu 2007). Besides their role in the initiation of an adaptive immune response, DCs also play a role in the innate immune system. A recent study described the anti-tumor activity of NKT cells to be dependent on the expression of OX40L on DCs (Zaini 2007).

Taken together it seems to be clear, that signals induced by co-stimulatory molecules like OX40L and CD70 have beneficial effects on immunotherapeutic outcomes by facilitating protective effector and memory T cell responses, breaking of existing tolerance and preventing suppressor activity of Tregs. For this reason, the PGE_2-dependent expression of OX40L and CD70 on mature human MoDCs that we describe in this study is a new parameter that will help to further optimize DC-based immunotherapy.

Materials and Methods

Generation of monocyte-derived dendritic cells (MoDCs)

Human monocytes were positively selected from whole blood of healthy donors as previously described (Scandella 2002). Briefly, peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Ficoll Paque Plus (Amersham Biosciences, Uppsala Sweden), and $CD14^+$ monocytes were further purified using anti-CD14 conjugated microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany). Monocytes were cultured at $1x10^6$ cells/ml in AIM-V medium (Gibco, Paisley, UK) supplemented with 50 ng/ml GM-CSF (Leukomax®, Novatis, Basel, Switzerland) and IL-4 (1:50 supernatant of an IL-4 producing J558 cell line). After 5-6 days, immature DCs were harvested and matured for 2 days by addition of 0.5 μg/ml trimeric soluble CD40L (sCD40L, PromoCell; Heidelberg, Germany), 20 μg/ml poly I:C (Sigma, Saint Louis, MO), 10 μg/ml LPS (*Salmonella abortus equi*; Sigma) or a combination of 20 ng/ml TNF-α, 10 ng/ml IL-1β and 20 ng/ml IL-6 (all from PromoCell). Maturation occurred in the absence or presence of 1 μg/ml PGE_2 (Minprostin® E2, Pharmacia, Uppsala, Sweden). For antigen loading 10 μg/ml KLH (Sigma) or 10 μg/ml TT (Berna Biotech AG, Berne, Switzerland) were added during maturation.

Co-cultures and T cell proliferation assay

PBMCs were isolated from peripheral blood by density gradient centrifugation and labeled with the fluorescent cell tracers CFSE (Molecular Probes, Eugene, OR) or Cell Trace Far Red (Molecular Probes) according to the manufacturer's protocol. Previously prepared $1x10^5$ MoDCs were cultured together with $1x10^6$ PBMCs in 1 ml AIM-V. Where indicated graded amounts of a goat anti-human OX40L neutralizing antibody (R&D Systems, Minneapolis MN) or normal goat IgG (R&D Systems) were added. After 6 or 12 days of culture T cell proliferation was assessed by dye dilution due to cell division using flow cytometry (BD LSRII, BD Biosciences).

Flow cytometry

Cell surface expression of co-stimulatory molecules on MoDCs was analyzed using anti-CD83-PE, anti-CD80-PE, anti-CD86-PE, anti-OX40L-PE, anti-CD70-PE and IgG-PE isotype control antibodies (all purchased from BD Pharmingen, San Diego, CA). T cell proliferation was assessed by CFSE dilution assays of cells stained with anti-CD3-APC-Cy7, anti-CD4-PE-Cy7, anti-CD8-APC (all BD Pharmingen). Dead cells were excluded by Sytox Blue staining (Molecular Probes). For analysis of proliferation and surface expression of T cells labeled with Cell Trace Far Red T cells were stained with anti-CD4-PacificOrange (Caltag, Carlsbad, CA) and anti-OX40L-PE or anti-CD70-PE. In cultures with non-labeled PBMCs

surface expression of co-stimulatory molecules on T cells was stained using anti-CD3-APC-Cy7, CD4-PE-Cy7, CD8-PacificBlue in combination with either anti-OX40L-PE and anti-OX40-FITC or anti-CD70-PE (all purchased from BD Pharmingen).

Quantitative real-time PCR

For quantification of mRNA expression levels of TNFSF4, TNFSF7 and TNFSF9, total RNA was isolated from MoDCs matured with sCD40L in the absence or presence of PGE_2 using the RNeasy mini kit (Qiagen, Hilden, Germany). Using random hexamer primers, total RNA was transcribed into cDNA with the Taqman® reverse transcription reagent (Applied Biosystems, Rotkreuz, Switzerland) according to the manufacture's instructions. Amplification of TNFSF4, TNFSF7 and TNFSF9 mRNA was preformed using the QuantiTect SYBR Green PCR Master Mix (Qiagen) and the Taqman 7700 (Applied Biosystems). PCR program contained an initial denaturation step at 95°C for 15 min followed by 40 cycles of 15 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C. Forward and reverse primers were used at 200 nM each and sequences were as follows: TNFSF4 5'-CACCTACATCTGCCTGCACTTCT, 5'-GTTGTTCTGCACCTTCATGATTTC; TNFSF7: 5'-CCTCGTGGTGTGCATCCA, 5'-ATGCCATCACGATGGATACGTA; TNFSF9: 5'-GAGCTTTCGCCCGACGAT, 5'-GCAGCTCTAGTTGAAAGAAGACATAGTAGA. Expression of mRNA was normalized to ß-2 microglobulin (ß2M) and ubiquitin C (UBC) using SYBR Green PCR Master Mix (Applied Biosystems) containing 200 nM forward and reverse primer (ß2M: 5'-GCTATCCAGCGTACTCCAAAGATTC and 5'-CAACTTCAATGTCGGATGGATGA; UBC: 5'-ATTTGGGTCGCGGTTCTTG and 5'-TGCCTTGACATTCTCGATGGT). Relative mRNA expression was calculated with the $\Delta\Delta$Ct-method.

Statistical evaluation

Differences between groups were assessed by the student's paired *t* test.

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CHAPTER 7

General discussion

The immune system can not only fight foreign pathogens that invade the body, but can also fight malignant changes of body cells which give rise to tumors. Evidence that the immune system can recognize and react to cancer comes from multiple observations like the increased incidence of tumors in immunodeficient patients or mice and the immune-mediated rejection of experimental tumors (Palucka 2007). Since dendritic cells (DCs) are most effective in the induction and regulation of immune responses, they present an interesting target for cellular immunotherapy. The manipulation of DCs to induce an effective and long-time protective anti-tumor response is a promising therapeutic strategy, because of the natural capacity of DCs to induce adaptive immune responses including long-lasting memory, and to break established peripheral tolerance. In experimental tumor models, DCs loaded with tumor antigens can induce protective immune response (Grabbe 1995). In human, large amounts of DCs can be generated *ex vivo* by differentiation from monocytes. Therefore, monocytes are isolated from peripheral blood of patients, and cultured in medium containing IL-4 and GM-CSF to differentiate into immature DC. *Ex vivo* generation of DCs allows manipulation and characterization of a uniform cell population, which can be pulsed with tumor-antigen and administered back to the patient to induce anti-tumor immunity. As immature DC are ineffective to facilitate effective immune responses (Dhodapkar 2002, Dhodapkar 2001, Hawiger 2001) and promote induction of peripheral tolerance, it is necessary to provide activation signals resulting in mature DCs with the potential to stimulate adaptive immune responses. Migration of mature DCs from the periphery into secondary lymphoid organs is a crucial process for the transport of antigen and initiation of immune responses. The capacity to migrate is therefore essential for DCs intended for vaccination purposes. In early studies, *ex vivo* generated DCs could not leave the injection side and failed to enter draining lymph nodes (Morse 1999a). Mature DCs up-regulate CCR7 expression, the chemokine receptor that guides migration to secondary lymphoid organs. However, CCR7 surface expression is not sufficient for migration. The presence of the lipid mediator prostaglandin E_2 (PGE_2) positively renders the responsiveness of DCs to CCR7 mediated signals allowing efficient migration (Scandella 2002, Luft 2002). For vaccination, DCs are most commonly matured using a combination of pro-inflammatory cytokines and PGE_2 (Jonuleit 1997, Lee 2002). To characterize the role of PGE_2 in the maturation process of DCs we conducted several studies providing new insights into modulation of DC functions.
When we analyzed migratory behaviour of mature MoDCs, we found that the presence of PGE_2 is a general prerequisite for migration (Chapter 2). The requirement of PGE_2 for migratory responsiveness of mature DCs is not limited to migration via CCR7, but extends to

migration via CXCR4 and C5aR. Therefore, it is tempting to speculate that the induction of homing potential of maturing DCs to lymph nodes is controlled by cells of the innate immune system, which produce PGE_2 in inflammatory situations at the site of infection. PGE_2 could be considered as a control factor of DC migration. During the initial phase high amounts of PGE2 are found at the site of infection allowing DCs to leave the periphery and migrate to the draining lymph nodes. Once an adaptive immune response is induced and the infection can be cleared, production of PGE_2 at sites of infection ceases, and migration of additional maturing DCs is paused to prevent continuous T cell priming in draining lymph nodes. The finding that endogenously produced PGE_2 by DCs is not sufficient to facilitate migration (Chapter 2) could support the theory of innate immune cells controlling migratory responses of maturing DCs by supply of PGE_2.

Immature DCs do not require PGE_2-mediated signals for migration towards pro-inflammatory chemokines (Chen 2004). However, under steady state conditions, immature Langerhans cells have been reported to migrate into cutaneous lymph nodes, but migration is slow and less efficient compared to mature DCs (van den Broek 2007). Langerhans cells adhere to surrounding keratinocytes through the adhesion molecule E-cadherin. Immature DCs can up-regulate CCR7 expression in response to disruption of E-cadherin-mediated interactions, which results in mature DCs that do not induce effector functions, but rather promote T cell tolerance (Jiang 2007). DC migration towards lymph node-derived chemokines could be generally controlled by PGE_2 to limit excessive migration of immature peripheral DCs, which induce tolerance.

In the attempt to identify the mechanism by which PGE_2 facilitates migratory capacities of mature DCs, we identified Sprouty2 and Spred1 to be negatively regulated by PGE_2 in mature MoDCs (Chapter 3). For the first time, we show expression of Sprouty2 and Spred1 in DCs, and their potential contribution to chemokine receptor signaling regulation. The inhibitory effect of PGE_2 on Sprouty2 and Spred1 expression correlates with the enhanced migratory potential of MoDCs matured in the presence of PGE_2. In over-expression studies we identified a potential mechanism by which Sprouty2 could contribute to regulation of CXCR4-mediated signaling. As Sprouty2 and Spred1 have never before been implied in chemokine receptor signaling, further studies will be required to characterize their function. However, Sprouty2 could regulate CXCR4-mediated migration of DCs by inhibition of p130Cas (Figure 1). Therefore, Sprouty2 and Spred1 could present new targets to modulate and interfere with chemokine receptor signaling.

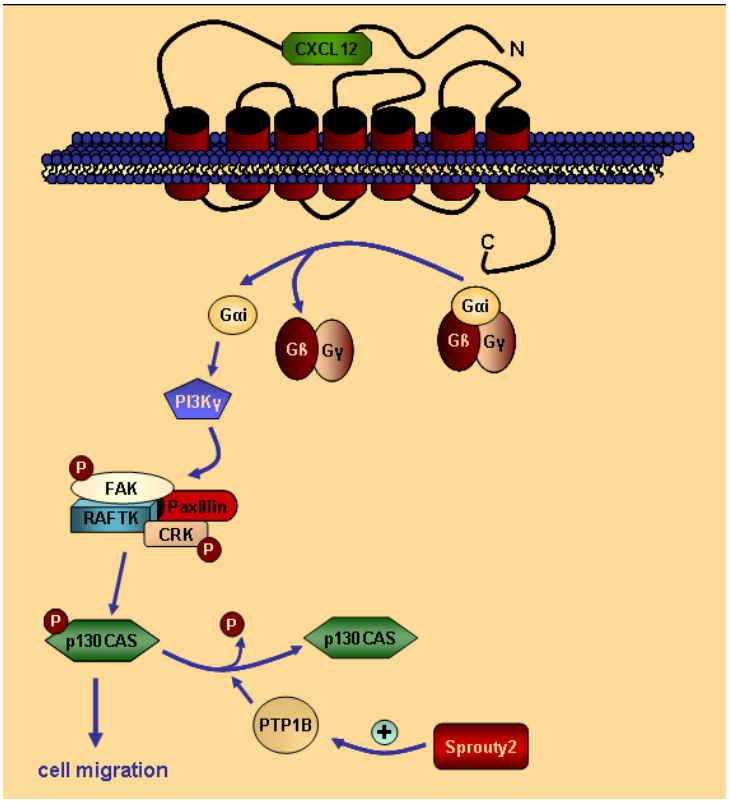

Figure 1. Potential contribution of Sprouty2 in CXCR4-mediated signaling. CXCR4 signaling is mediated via heterotrimeric G proteins. After CXCL12 binding the Gα subunit dissociates from the Gßγ complex. The active Gαi subunit induces a signaling cascade that leads to phosphorylation of p130Cas (Crk-associated substrate). Phosphorylated p130Cas promotes cell migration as a critical component of the focal adhesion complex. p130Cas is dephosphorylated and thereby inactivated by protein tyronsine phosphatase 1B (PTP1B). The activity of PTP1B can be enhanced by Sprouty2. Sprouty2 can therefore mediate its anti-migratory effects by augmenting inactivation of p130Cas. As PGE_2-mediated signals down-regulate Sprouty2 expression in mature MoDCs, we propose that the lower expression of Sprouty2 in PGE_2-matured MoDCs contributes to their enhanced migratory capacities. Conversely, low migratory responsiveness of MoDCs matured in the absence of PGE_2 could be attributed to high expression levels of Sprouty2. (graphic designed by Karin Schäuble)

Besides induction of a migratory phenotype, we identified additional effects of PGE_2 that could be beneficial for the maturation of MoDCs generated for vaccination purposes. Most prominently, we found the PGE_2-dependent induction of OX40L and CD70 on mature MoDCs (Chapter 6). From the same family, we also found 4-1BBL to be inducible by PGE_2, although protein expression could not be addressed due to poor antibody quality. All three molecules belong to the TNF superfamily, and play a role in T cell survival and memory T cell generation. MoDCs matured in the presence of PGE_2 initiated enhanced T cell proliferation, despite expression of IDO (Chapter 5). To prove a contribution of OX40L, CD70 and 4-1BBL in the elevated T cell stimulatory capacity of PGE_2-matured MoDCs, we cloned and produced soluble Fc-fusion proteins of OX40, CD27 and 4-1BB, which however had no inhibitory effect on T cell proliferation. Since only for OX40L a neutralizing antibody was commercially available, we could investigate solely the role of PGE_2-induced OX40L expression. The PGE_2-mediated enhanced T cell stimulatory capacity of mature MoDCs could be inhibited by OX40L blockage. As the effect of OX40L blockage was only partially, the elevated levels of CD70 and 4-1BBL expression on PGE_2-matured MoDCs could also contribute to the enhanced T cell stimulation. The expression of co-stimulatory molecules of the TNF superfamily by DCs are important for DC-based immunotherapies, highlighted by a study, in which murine DCs transfected with OX40L showed improved anti-tumor immunity (Danull 2005). OX40L, CD70 and 4-1BBL are essential signals for generation of memory T cells due to their role in maintenance of T cell survival. Vaccination against tumors relies on the

induction of both tumor-specific effectors and memory T cells. As other immunotherapeutic approaches like antibody therapy or adoptive T cell transfer are unlikely to produce T cell memory, the potential of DCs to do so clearly favours them as targets for immunotherapy (Palucka 2007). The PGE_2-induced expression of co-stimulatory molecules of the TNF family on mature MoDCs should improve tumor-specific memory T cell development. The induction of OX40L in particular could provide an additional beneficial feature of PGE_2-matured MoDCs, as signals provided by OX40L negatively regulate Tregs .

Although we established PGE_2 as a factor that in combination with different maturation stimuli induces phenotypic mature MoDCs with the ability to migrate and the enhanced ability to stimulate T cells, this stimulation might however not lead to IL-12 production (Luft 2002, Kalinski 1997), which is important for Th1 differentiation. It has been tried to improve maturation of MoDCs to generate a mature DC phenotype with strong migratory properties and the ability to produce high levels of Th1 cytokines. Addition of IFN-γ to induce IL-12 production by DCs was attempted, but resulted in decreased CCR7 expression and inhibited CCR7-mediated migration (Alder 2006 Vaccine 24:7087-94). Although these results showed no beneficial effect of IFN-γ addition on DC phenotype, they have interesting implications, since IFN-γ which is produced in large amounts in the course of an efficient Th1 response could act as a negative feedback factor for DCs migration into draining lymph nodes.

An alternative maturation cocktail consisting of IFN-α, poly I:C, TNF-α, IL-1ß and IFN-γ has been shown to produce mature MoDCs with the ability to migrate and produce high levels of IL-12 (Mailliard 2004). Although, migratory responses after CCR7 stimulation were attenuated in these cells compared to MoDCs matured by a cytokine cocktail containing PGE_2, migration could still be sufficient. Thus, this alternative maturation cocktail could be used for generation of DCs for vaccination protocols. Clinical trials are necessary to determine anti-tumor inducing efficiency of DCs matured with this new approach. Since we identified PGE_2 as a key factor for induction of OX40L and CD70 on mature MoDCs, the addition of PGE_2 to this new cocktail could achieve even better results. It has to be tested, if PGE_2 would limit IL-12 production under those conditions. The design of a combination of stimuli that results in optimal DC maturation is still a challenge for the future. The use of PGE_2 for DC maturation could be of further advantage, as PGE_2 has been implied in the increase of apoptosis resistance in human DCs by induction of survivin expression (Baratelli 2005). A prolonged survival of tumor antigen-bearing DCs could enhance priming of specific T cells. MoDCs that are generated in the presence of IL-4 do not express endogenous PGE_2 (Chapter 2) due to the inhibitory effect of IL-4 on COX-2 expression. This lack might have positive influence on stimulation of T cells, since PGE_2 has been reported to suppress T cell proliferation (Goodwin 1977, Goodwin 1989).

Early clinical studies established the feasibility and safety of DC vaccination; however, DC-based vaccination trials have not been overwhelmingly successful (Soruri 2005). No evidence was presented so far that DC-based immunotherapy provides superior protective immunity in cancer patients compared to other vaccination strategies. In most studies, only a small fraction of patients show immune responses against the vaccinated antigen, which most often fail to correlate with clinical responses (Gilboa 2007, Palucka 2007). Vaccination of melanoma patients with DC matured with cytokines and PGE_2 showed no improved responses compared to standard dacarbazine chemotherapy (Schadendorf 2006). However, since DC vaccination has been shown to work in principle (Schuler 2003), it is now the task to refine and improve the multiple steps that are involved in the process of DC preparation for vaccination such as the type of DCs and their activation, the type and formulation of antigen, the cell number, frequency and way of injection, or combination with other strategies like cytostatic drugs (Palucka 2007, Gilboa 2007).

Summary

This thesis summarizes a series of studies aiming to characterize multiple effects of the prostanoid prostaglandin E_2 (PGE_2) on dendritic cell function. Monocyte-derived dendritic cells (MoDCs) are most commonly used in dendritic cell (DC)-based vaccination protocols against cancer. In the procedure to generate highly immunogenic DCs that are capable of eliciting effective anti-tumor immune responses DCs are matured by stimulation with inflammatory mediators. Among those, PGE_2 has been described as a modulator of immune cell functions. Several effects of PGE_2 on DC functions, which have implications for the use of DCs in immunotherapeutic approaches, have been identified in this thesis.

The results obtained in this study establish PGE_2 as the key factor to induce a migratory phenotype of mature human DCs. The presence of PGE_2 during maturation of DCs is a general prerequisite for chemotactic responsiveness that is not restricted to migration mediated by one specific receptor. The requirement of PGE_2-provided signals for a migratory phenotype also applies to blood myeloid DC, and is therefore not a unique feature of MoDCs. Endogenous production of PGE_2 by mature MoDCs can not promote migration. In mice, the PGE_2 receptor EP4 has been identified to mediate signals critical for Langerhans cell migration from the periphery into draining lymph nodes (Kabashima 2003). Data obtained in this study clearly demonstrate an important difference between mice and humans in this respect, as in human MoDCs PGE_2 facilitates enhanced migratory responsiveness through the PGE_2 receptors EP2 and EP4, a finding with important implications for proposed approaches for DC therapy improvement involving specific EP receptor targeting.

To determine the mechanism by which PGE_2 induces migratory responsiveness in mature MoDCs a global gene expression analysis was performed with MoDCs matured in the absence or presence of PGE_2. With this approach, Sprouty2 and Spred1 were identified for the first time to be expressed in human DCs. The PGE_2-mediated enhanced migratory phenotype of mature MoDCs could be correlated with PGE_2-induced down-regulation of Sprouty2 and Spred1 expression. Over-expression studies revealed a role for Sprouty2 and Spred1 in chemokine receptor-mediated signaling, which has not been described before.

A member of the regulator of G protein signaling (RGS9) protein family, namely RGS9, was found for the first time to play a role in DC functions. RGS9 mRNA was induced by PGE_2 in mature MoDCs, and experiments *in vivo* using RGS9-deficient mice revealed strong defects in DC migration and induction of a specific immune response.

Although PGE_2-mature MoDCs produce active indoleamine 2,3-dioxygenase, which contributes to inhibition of T cell proliferation due to depletion of tryptophan, T cell stimulatory capacities of PGE_2-mature MoDCs are enhanced. T cells activated by MoDCs matured in the

presence of PGE_2 express elevated levels of tryptophanyl-tRNA-synthetase (TTS), which could protect T cells from tryptophan deprivation by IDO.

MoDCs matured in the presence of PGE_2 possess an enhanced capacity to stimulate allogenic and antigen-specific T cell responses. This ability could by linked to PGE_2-induced expression of OX40L, CD70 and 4-1BBL on mature MoDCs, co-stimulatory molecules promoting survival of T cells and generation of memory T cells.

This thesis provides new insides into the regulation of DCs functions by the lipid mediator PGE_2 representing a link between innate and adaptive immunity. The new parameters described herein might help to further optimize maturation procedures in DC-based immunotherapies. Additionally, the identification of new regulatory mechanisms of chemokine receptor signaling could present interesting targets for intervention of chemokine receptor-mediated processes.

Zusammenfassung

Die vorliegende Arbeit umfasst eine Reihe von Studien zur Charakterisierung der vielfältigen Effekte von Prostaglandin E_2 (PGE_2) auf Funktionen dendritischer Zellen (DCs). DCs, die aus Monozyten differenziert werden können (MoDCs), werden häufig für Vakzinierungsstrategien gegen Krebs verwendet. Um für diesen Zweck DCs mit grossem immunogenen Potential zu generieren, welche in der Lage sind Immunantworten gegen Tumore auszulösen, werden DCs mit inflammatorischen Substanzen stimuliert. Zu diesen Substanzen zählt auch PGE_2, welches modulatorischen Einfluss auf Funktionen von Immunzellen ausübt. In dieser Arbeit wurden verschiedenste Effekte von PGE_2 auf Funktionen humaner dendritischer Zellen identifiziert und charakterisiert. Diese können für die Optimierung von Vakzinierungsstrategien mit DCs hilfreich sein.

Die Ergebnisse dieser Arbeit charakterisieren PGE_2 als Schlüsselfaktor während der Reifung von DCs für die Ausbildung eines migrationsfähigen Phänotyps. Dabei ist die Anwesenheit von PGE_2 während der Reifung für die Wanderung über verschiedene Rezeptoren notwendig. Die Notwendigkeit von PGE_2 für die Ausbildung eines wanderungsfähigen Phänotyps ist keine spezielle Eigenschaft von MoDCs, sondern scheint generelle Gültigkeit für myeloide dendritische Zellen zu besitzen. PGE_2, welches von reifen DCs endogen produziert wird, ist nicht in der Lage Migrationsfähigkeit zu induzieren. Im murinen System konnte gezeigt werden, dass über den PGE_2 Rezeptor EP4 ein kritisches Signal für die Wanderung von Langerhans Zellen aus der Peripherie in sekundäre lymphatische Organe vermittelt wird (Kabashima 2003). Daten der vorliegenden Arbeit zeigen diesbezüglich einen klaren Unterschied zum menschlichen System, da PGE_2-vermittelte Signale, die die Fähigkeit zur Wanderung auslösen, sowohl über EP2 als auch über EP4 übertragen werden konnten. Da die Verwendung von PGE_2 Rezeptoragonisten für die klinische Verwendung vorgeschlagen wurde, ist dieser Befund von therapeutischer Bedeutung.

Um den Mechanismus zu identifizieren, über welchen PGE_2 die Fähigkeit von DCs zur Wanderung auslöst, wurde eine Genexpressionsanalyse mit MoDCs, die in der Anwesenheit oder Abwesenheit von PGE_2 gereift wurden, durchgeführt. Dabei wurden Sprouty2 und Spred1, deren Expression bis dahin in DCs unbekannt war, als Ziel für PGE_2-gesteuerte Genregulation identifiziert. Es konnte gezeigt werden, dass die Expression von Sprouty2 und Spred1 während der Reifung durch die Anwesenheit von PGE_2 stark inhibiert wird; ein Effekt der mit verbesserter Wanderung korrelierbar ist. Mit Hilfe künstlicher Überexpression von Sprouty2 und Spred1 konnte zum ersten Mal eine Beteiligung dieser Proteine an der Regulierung von Chemokinrezeptor-vermittelten Signalen gezeigt werden.

Ebenfalls zu ersten Mal in humanen DCs, konnte RGS9, ein Mitglied der Familie der RGS (regulator of G protein signaling) Proteine, beschrieben werden. Die Expression von RGS9

mRNA wurde durch PGE_2 während der Reifung von MoDCs induziert. *In vivo* Experimente mit RGS9-defizienten Mäusen deuten auf eine Beteiligung von RGS9 in migratorischen Prozessen von DCs hin, da $RGS9^{-/-}$ DCs stark beeinträchtigt waren in angrenzende Lymphknoten zu wandern. Dies ging mit der Induktion einer abgeschwächten antigenspezifischen Immunantwort einher.

Die Anwesenheit von PGE_2 während der Reifung von MoDCs induziert die Produktion des Enzyms Indolamin 2,3-dioxygenase (IDO), welches an der Eindämmung von exzessiver T Zellproliferation durch Depletion von Tryptophan beteiligt ist. Obwohl PGE_2-gereifte MoDCs IDO produzieren, sind sie in der Lage verstärkte T Zellteilung auszulösen. Ein möglicher Schutzmechanismus von T Zellen, die durch PGE_2-gereifte MoDCs stimuliert wurden, konnte ermittelt werden. Solche T Zellen produzieren verstärkt TTS (Trytophanyl-tRNA-Synthetase), welche sie vor Tryptophandepletion schützt.

MoDCs, die in der Gegenwart von PGE_2 gereift wurden, zeigen eine verbesserte Fähigkeit zur Stimulation allogener und antigenspezifischer T Zellproliferation. Diese Fähigkeit konnte der durch PGE_2-induzierten Expression von OX40L, CD70 und 4-1BBL zugeschrieben werden. Diese drei Moleküle gehören zur TNF-Familie und fördern das Überleben von T Zellen und die Generierung von Memory T Zellen.

Diese Arbeit beschreibt neue Erkenntnisse über die Regulation von DC Funktionen durch PGE_2, welches eine Verbingung zwischen angeborener und adaptiver Immunität darstellt. Die Ergebnisse dieser Arbeit können helfen Protokolle zur Krebsvakzinierung mit DCs zu optimieren. Desweiteren, wurde ein neuer regulatorischer Mechanismus für Chemokinrezeptor-vermittelte Signale entdeckt, der ein interessantes Ziel für therapeutische Interventionen in Chemokin-vermittelten Prozessen sein könnte.

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Abgrenzung der Eigenleistung

Soweit im Folgenden nicht anders erwähnt, wurden alle Experimente dieser Arbeit von mir selbst erdacht, geplant, ausgeführt und ausgewertet. Die Manuskripte in Kapitel 2 und 5 wurden in Zusammenarbeit mit meinem Betreuer Dr. Daniel F. Legler verfasst, während alle anderem Kapitel von mir allein verfasst wurden. Die Abbildung 1 in Kapitel 7 wurde von Karin Schäuble graphisch gestaltet.

Alle Experimente in Kapitel 4 wurden unter meiner Anleitung im Rahmen einer Masterarbeit von Stefanie Bürger durchgeführt, wobei alle *in vivo* Arbeiten von mir assistiert wurden. Vorarbeiten zu Kapitel 6 wurden im Rahmen einer Masterarbeit von Christina Uermösi unter meiner Anleitung durchgeführt. Alle dargestellten Experimente in Kapitel 6 wurden jedoch von mir selbst ausgeführt.

Die HPLC-Analysen in Kapitel 5 wurden von Paula Darley und Janosch Klebensberger (Lehrstuhl für mikrobielle Ökologie, Prof. Schink, Universität Konstanz) durchgeführt, wobei die Vorbereitung der Proben sowie die Auswertung der Ergebnisse von mir vorgenommen wurde.

Die Konstrukte für Sprouty2 sowie die Sprouty2-Mutante (Y55A) wurden von Dr. Tarun Patel (Loyola University of Chicago) bzw. Dr. Akihiko Yoshimura (Kyushu University, Japan) zur Verfügung gestellt. Die verwendeten RGS9-defizienten Mäuse stammen von Prof. Johannes Schwarz (Universität Leipzig), und die Zucht der Mäuse wurde von den Mitarbeitern der Tierforschungsanlage der Universität Konstanz übernommen.