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The properties of human CD40-activated B cells as antigen-presenting cells are not affected by PGE_2

ALEXANDER SHIMABUKURO-VORNHAGEN*, ANDREAS DRAUBE*, TANJA LIEBIG, ALEXEY POPOV, ACHIM ROTHE and MICHAEL VON BERGWELT-BAILDON

Cologne Interventional Immunology (CII), Department I of Internal Medicine, University Hospital of Cologne, D-50924 Cologne, Germany

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Abstract. Tumor vaccination represents a promising immunotherapeutic strategy in cancer. However, the inherent ability of many tumors to evade immune responses by suppression of immune cell function represents a major barrier. Prostaglandin E_2 (PGE_2) has been shown to be a critical tumor-derived immunosuppressive factor. It affects a broad range of immune cells including T cells, macrophages and dendritic cells (DCs). CD40-activated B cells are being studied as a potential alternative to DCs as antigen-presenting cells for immunotherapy. So far, it is not known whether PGE_2 affects their antigen presenting capacity. We, therefore, investigated the influence of PGE_2 on the phenotype, migratory potential and antigen-presenting function of CD40-activated human B cells. Here, we demonstrate that the immunostimulatory properties of CD40-activated B cells are not affected by PGE_2. These results support the use of CD40-activated B cells as cellular adjuvants, especially in settings where PGE_2 is present in the tumor microenvironment.

Introduction

Active immunotherapy represents a promising modality for the treatment of malignant diseases. However, due to low clinical response rates cancer vaccination with the use of antigen-presenting cells (APCs) faced substantial skepticism some years ago (1). Meanwhile, growing body of knowledge on cancer immunosurveilance, and loss thereof, led to a refinement of immunotherapeutic strategies (2). Particularly for cancer vaccinations this has been further encouraged by recent progress in this field with several successful trials which even resulted in the first approval for a cellular vaccine by the US FDA (3). Nevertheless, further progress will depend on the ability to circumvent the different tumor escape mechanisms (4-6), specifically tumor-induced immunosuppression which represents one of the major barriers to successful tumor immunotherapy.

Prostaglandin E_2 (PGE_2) belongs to the eicosanoid family of lipid mediators and is a potent immunomodulator. PGE_2 and its receptors play a role in a broad range of physiologic processes and have been implied in a number of pathologic conditions such as inflammatory disease, infections and cancer (7-9). It has diverse and often opposing effects on the immune system. In cancer PGE_2 has been identified as one of the major soluble tumor-derived factors contributing to the immunosuppressive tumor environment (10). Many tumors exhibit increased expression of cyclooxygenase-2 (COX-2) subsequently leading to an increased production of PGE_2. The suppressive effects are mediated by inhibition of the production of pro-inflammatory cytokines, by upregulation of the expression of immunosuppressive cytokines and by inhibiting the function of important immune effector cells such as T cells, natural killer cells and APCs (11-14). Further evidence for the importance of PGE_2 in mediating immunosuppression stems from experiments in which inhibition of COX-2 by non-steroidal anti-inflammatory drugs resulted in an enhanced antitumor immune response (15).

The complex functions of PGE_2 are also reflected in the debate about its use for the maturation of monocyte derived DCs (moDCs). PGE_2 is included in most maturation cocktails as it has been shown to upregulate the expression of CCR7 which is essential for the migration to secondary lymph organs (16,17). On the other hand PGE_2 seems to inhibit the differentiation and immunostimulatory function of moDCs by the upregulation of IDO expression (18,19) and by suppressing DC mediated attraction of naïve T cell (20).

In recent years B cells are increasingly recognized as important APCs capable of inducing antigen-specific $CD4^+$ and $CD8^+$ T cell responses under physiologic and pathologic conditions (21,22). CD40-activated B cells are currently being studied as an alternative type of APC for cellular vaccines (23-26). Most importantly, they can be easily expanded *ex vivo* from peripheral blood of cancer patients (27). However, in

Correspondence to: Dr Michael von Bergwelt-Baildon, Cologne Interventional Immunology (CII), Department I of Internal Medicine, University Hospital of Cologne, Kerpener Str. 62, D-50924 Cologne, Germany
E-mail: michael.bergwelt@uk-koeln.de

*Contributed equally

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contrast to DCs there is little knowledge on the regulation of antigen presentation by B cells. This is also true with regard to the influence of PGE_2. We therefore studied the effects of PGE_2 on key factors for the induction of an immune response by APCs such as the expression of costimulatory molecules, migratory potential to secondary lymphoid organs and finally the induction of T cell activation and proliferation.

Materials and methods

Preparation of human CD40-activated B cells. Human CD40-activated B cells were prepared as described previously (28). Briefly, whole PBMC were cultured on irradiated NIH3T3 cells transfected with CD154 (tCD40L) in the presence of recombinant human interleukin 4 (rhIL-4; 2 ng/ml; R&D Systems, Minneapolis, MN, USA) and clinical-grade cyclosporin A (CsA; $5.5x10^{-7}$ M; Novartis, Basel, Switzerland) in Iscove's modified Dulbecco's medium (IMDM; Invitrogen, Karlsruhe, Germany) supplemented with 10% pooled human serum. The cells were recultured every 3-4 days. After 3 weeks CD40-activated B cells were used for experiments. The CD40-activated B cells were cultured in the presence of PGE_2 (Sigma-Aldrich, St. Louis, MO, USA) or vehicle. Of note, the inhibitory biological activity of PGE_2 was confirmed at different concentrations in T cell proliferation assays of T cells activated by magnetic beads coated with anti-CD3/anti-CD28 monoclonal antibodies as previously described (29,30). After 3 days CD40-B cells were harvested and used for flow cytometric analysis, and functional assays. To obtain antigen-presenting cells free of PGE_2 the cells were washed extensively prior to their use in functional assays.

Flow cytometry. Immunophenotypic analyses were performed using fluorescence-activated cell sorting (FACS). Cells were analyzed for the expression of CD19, CD25, CD80, CD86, HLA-DR (BD Pharmingen, Heidelberg, Germany), CCR7, CXCR4 (R&D Systems) and EP2 and EP4 (Cayman Chemical, Ann Arbor, MI, USA) using a FACSCanto flow cytometer (Becton-Dickinson).

Chemotaxis assay. To assess B cell migration, $5x10^5$ CD40-activated B cells were transferred into the upper chamber of 5-μm pore size transwell plates (Costar, Cambridge, MA, USA). Varying amounts of the chemokines SDF-1α and SLC (R&D Systems) were added to the lower chamber. After 2 h at 37˚C, the number of cells that had migrated into the lower chamber was determined using a hemacytometer.

Allogeneic mixed lymphocyte reaction. $CD4^+$ T cells were obtained from buffy coats by negative selection using Rosette Sep® human $CD4^+$ T cell enrichment cocktail (StemCell Technologies, Vancouver, Canada) according manufacturer's instructions. Prior to allogeneic mixed lymphocyte reaction (MLR) $CD4^+$ T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes, Eugene, OR, USA) according to standard protocols. A total of $1x10^5$ CFSE-labeled $CD4^+$ T cells were co-incubated with allogeneic CD40-activated B cells as stimulators at various B to T-cell ratios ranging from 1:1 to 1:10. After 5-7 days proliferation was assessed by flow cytometry.

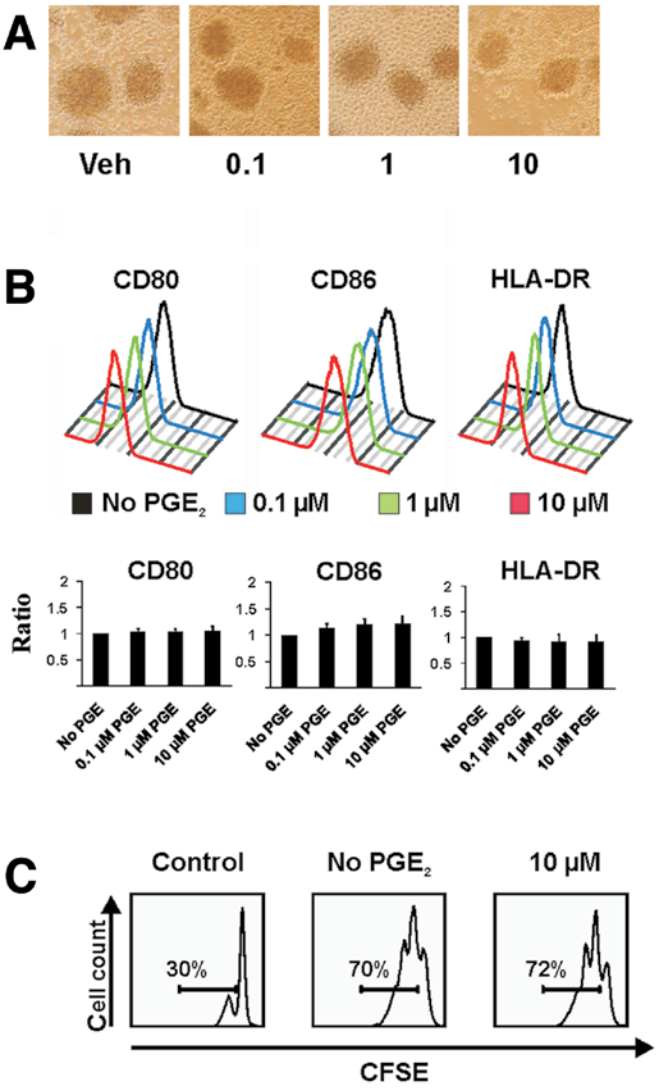

Figure 1. Morphology, proliferation and phenotype of CD40-activated B cells exposed to PGE_2. (A) Morphology of CD40-activated B cells was not changed by the exposure to PGE_2. CD40-activated B cells were cultured in the presence of vehicle (Veh), 0.1, 1 or 10 μM PGE_2 for 3-4 days. (B) Flow cytometry analysis showed that the expression of the costimulatory molecules CD80 and CD86 as well as HLA-DR was not significantly changed by exposure to the indicated concentrations of PGE_2. Representative histograms are shown. The bar charts summarize the data of 8 individual experiments as expression level ratios of PGE_2 treated CD40-activated B cells in comparison to untreated cells as controls. Shown are the means ± standard deviation. (C) Addition of 10 μM PGE_2 did not influence the proliferation of purified CFSE-labeled B cells activated via CD40 by culture on CD40 ligand expressing NIH3T3 cells (tCD40L NIH3T3). B cells cultured in medium containing IL-4 but in the absence of tCD40L NIH3T3 served as control.

Results

Phenotype of PGE_2-treated CD40-activated B cells. First, we assessed the effect of different concentrations of PGE_2 on the phenotype of CD40-activated B cells. Morphology and cell surface expression of costimulatory molecules CD80 and CD86 as well as major histocompatibility complex (MHC) class II of treated cells were compared to untreated CD40-activated B cells. PGE_2-treated cells were of normal morphology and formed round clusters through homotypic adhesion (Fig. 1A). Also, the surface expression of CD80, CD86 and HLA-DR was

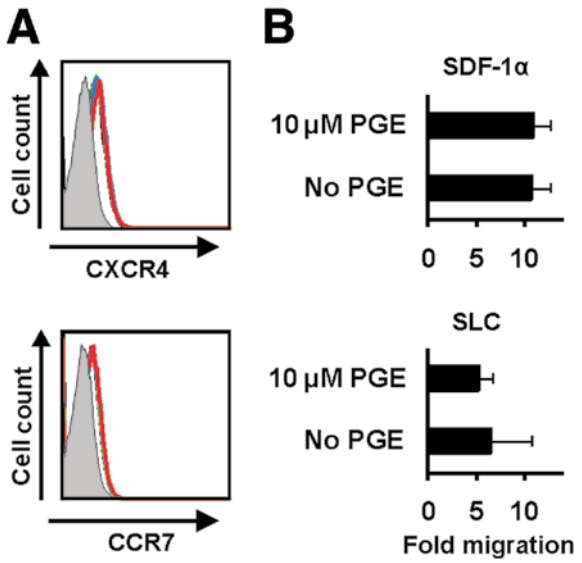

Figure 2. Unchanged CCR7 and CXCR4 chemokine receptor expression and migration towards their ligands. (A) Expression of CCR7 and CXCR4 on CD40-activated B cells cultured in the presence of 0.1 μM (blue lines), 1 μM (green lines), 10 μM (red lines) or no (dashed black lines) PGE_2. The grey-shaded area indicates the isotype controls. (B) The impact of PGE_2 on the migratory capacity of CD40-activated B cells towards secondary lymphoid tissue was studied comparing migration of CD40-activated B cells treated with 10 μM PGE_2 or vehicle towards SLC, the ligand for CCR7 or SDF-1α, the ligand for CXCR4. The bars represent the means of 4 individual experiments ± standard deviation.

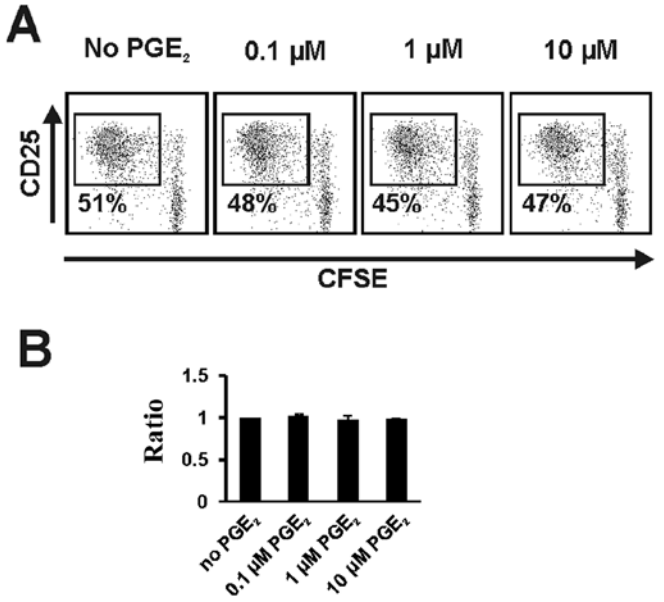

Figure 3. PGE_2 has no influence on T cell activation and proliferation induced by CD40-activated B cells. (A) CFSE-labeled $CD4^+$ T cells were incubated with CD40-activated B cells which were previously cultured for 3-4 days in the presence of the indicated concentration of PGE_2. After 5-6 days no significant changes in the proliferation of $CD4^+$ T cells was seen. Representative FACS plots (x-axis, CFSE; y-axis, CD25). (B) The bar chart displays the proliferation ratio of T cells co-cultured with untreated or PGE_2 treated CD40-activated B cells in the indicated PGE_2 concentrations. The means of 5 individual experiments ± standard deviation.

not significantly affected by the exposure to PGE_2 in a series of experiments (Fig. 1B). Next, we investigated the potential influence of PGE_2 on the proliferation of CD40-activated B cells: the continuous proliferation of CD40-activated B cells was likewise not affected by exposure to PGE_2 (Fig. 1C).

Expression of chemokine receptors CXCR4 and CCR7 as well as migration to their ligands are not affected by PGE_2. The migration of activated APCs to the secondary lymphoid organs is a crucial step in the induction of an immune response. The structure of the secondary lymphoid organs provides the ideal microenvironment for the interaction of antigen-specific T cells with APCs. Mainly, this is driven by chemokines and their receptors. We therefore addressed the expression and function of relevant chemokine receptors which are involved in the migration of APCs to secondary lymph organs. Treatment with PGE_2 did not affect the expression of CXCR4 and CCR7, the receptors for SDF-1α and SLC, respectively (Fig. 2A). We next, assessed the migration of CD40-activated B cells with an *in vitro* migration assay to test whether the function of these chemokine receptors are influenced by PGE_2. As demonstrated in Fig. 2B the migration of CD40-activated B cells to SDF-1α and SLC was not affected by PGE_2.

Immunostimulatory function of CD40-activated B cells with exposure to PGE_2. To examine the effects of PGE_2 on the immunostimulatory function of CD40-activated B cells we performed allogeneic MLRs with purified $CD4^+$ T cells. Changes in activation and proliferation of T cells were assessed

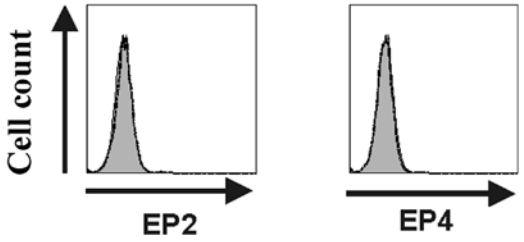

Figure 4. The PGE_2 receptors EP2 and EP4 are not expressed by CD40-activated B cells. The EP2 and EP4 fluorescence intensity of CD40-activated B cells are shown as black lines, the grey-shaded area represents the isotype control.

by tracing expression of CD25 and proliferation of CFSE-labeled T-cells from Day 5 to Day 7. No significant changes in T cell activation and proliferation were detectable when PGE_2-treated CD40-activated B cells were used as stimulators (Fig. 3A). Proliferation difference of T-cells were investigated in a series of experiments and also displayed by the ratio of proliferating T cells after co-culture with CD40-activated B cells untreated or treated with the indicated concentration of PGE_2. We observed no significant changes in T cell proliferation between untreated CD40-activated B cells and CD40-activated B cells exposed to different concentrations of PGE_2. Even at high doses PGE_2 had no effect on the stimulatory capacity of CD40-activated B cells (Fig. 3B).

CD40-activated B cells do not express the PGE_2 receptors EP2 and EP4. It has previously been shown that the immuno-

suppressive effects of PGE_2 are mediated through the EP2 and EP4 receptors. We thus studied the expression of these two receptors in CD40-activated B cells by flow cytometry analyses. Fig. 4 shows representative flow cytometry plots (n=3) demonstrating the absence of EP2 and EP4 expression on the cell surface of CD40-activated B cells. The lack of EP2 and EP4 receptor expression might explain the inherent resistance of CD40-activated B cells to PGE_2-mediated immunosuppression.

Discussion

PGE_2-mediated immunosuppression is a major barrier to the induction of antitumor immune responses and therefore has an important impact on the design of tumor vaccination strategies. One important mechanism by which tumor-derived PGE_2 suppresses immune responses is the induction of APC dysfunction *in vivo* resulting in an inhibition of T cell responses against the tumor. This has been demonstrated for human epithelial cancers such as head and neck and cervical cancer (31,32). Several mechanisms by which PGE_2-induced tolerogenic DCs prevent tumoricidal immune reactions have been discovered: they promote T helper type 2 (Th2) instead of T helper type 1 (Th1) responses and they attract regulatory T cells (33). Beside these *in vivo* effects the use of PGE_2 for DC maturation *in vitro* is currently under debate. It has been included as a major component in most moDC maturation cocktails since it enhances DC maturation and the expression of CCR7 which is crucial for the lymph node homing. However, it has been shown that PGE_2 induces the expression of indoleamine 2,3-dioxygenase (IDO) in moDCs which results in an inhibition of T cells (18,19). Nevertheless, this drawback is still discussed controversially as other authors stated that at least T cell stimulation is not affected by PGE_2 albeit an increased IDO expression (34). Scarce clinical trial data exist which addresses this question. At least, a small DC-based cancer vaccine trial in melanoma raised the concern that use of PGE_2 for the maturation of DCs might be detrimental. Following vaccination an accumulation of IDO-expressing DCs and regulatory T cells was observed at the injection site. All patients had a rapid progressive disease and a short overall survival (35).

We and others have shown that B cells activated *in vitro* by CD40 are potent APCs which could be used for clinical cancer vaccination trials. Main advantages are their easy and apparently unlimited proliferation capacities (26,28). With regard to the growing knowledge on the adverse immunoregulatory effects of PGE_2 on other APCs, especially DCs, it was the aim of this work to investigate the influence of PGE_2 on the phenotype and the functional properties of CD40-activated B cells.

In order to increase their antigen-presenting functions B cells have to be stimulated by inflammatory mediators, such as pathogen associated molecular patterns. Following activation they upregulate the expression of MHC and costimulatory molecules and undergo clonal proliferation. Upon activation B cells like DCs upregulate the expression of several chemokine receptors (e.g., CCR7, CXCR4, CD62L), which enable them to enter secondary lymphoid organs (25). This step is essential to enable the complex interactions between immune cells that are required for the induction of an effective immune response (36). Among these chemokine receptors CCR7 and CXCR4 have been identified to be crucial in controlling the migration of DCs to lymph nodes (37,38).

We did not find any changes in the expression of costimulatory and MHC-II molecules after exposure to PGE_2 at different concentrations. Furthermore, the proliferative capacity of CD40-activated B cells was unaffected. In addition, we could not find either a positive or a negative effect of PGE_2 on the expression of CCR7 and CXCR4 on CD40-activated B cells. In line with these findings, the function of these receptors, the migration of CD40-activated B cells to the two important lymph node homing chemokines SLC (ligand for CCR7) and SDF-1α (ligand for CXCR4) was not altered. CD40-activated B cells generated from healthy individuals and tumor patients have been shown to possess the capacity to stimulate T cells (27,39,40). It is not known though whether these cells are susceptible to tumor-induced immunosuppression. Importantly, we could exclude any inhibitory effect of PGE_2 on T cell activation and proliferation induced by CD40-activated B cells suggesting that CD40-activated B cells would not be affected by tumor-derived PGE2 *in vivo*. PGE_2 exerts its effects through the four PGE_2 receptors EP1-4. At least in DCs the immunosuppressive effects of PGE_2 seem to be exclusively mediated by the receptors EP2 and EP4 (41,42). The finding that EP2 and EP4 are absent on CD40-activated B cells provides a mechanistic explanation for their inherent resistance to the inhibitory effects of PGE_2. These data were further substantiated by gene expression analyses showing no differential EP2 and EP4 gene expression of CD40-activated B cells compared to unstimulated B cells (data not shown). These findings are of special interest as it has been shown both for mouse and human B cells that under defined activation B cells express EP2 and EP4 receptors (43,44). Thus, it has to be addressed by further investigations whether the utilized method to generate CD40-activated B cells precludes the expression of EP2 and EP4.

Taken together, our data demonstrate that the immunostimulatory function of CD40-activated B cells is not affected by PGE_2. These results have important implications for the potentially clinical application of B cells for immunotherapy. The use of B cells as APCs either by targeting antigen to B cells *in vivo* or by *ex vivo* peptide pulsing of activated B cells seems to be a promising strategy especially in settings where PGE_2 might prevent the induction of an immune response.

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