Immunobiology 217 (2012) 1017–1024

Contents lists available at SciVerse ScienceDirect

Immunobiology

journal homepage: www.elsevier.de/imbio

Targeted delivery of CpG ODN to CD32 on human and monkey plasmacytoid dendritic cells augments IFNα secretion

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ARTICLE INFO

Article history:
Received 5 December 2011
Received in revised form 18 January 2012
Accepted 18 January 2012

Keywords:
Allergy
CD32a
CpG ODN
Plasmacytoid dendritic cell
Targeting

ABSTRACT

Atopic diseases are characterized by the presence of Th2 cells. Recent studies, in mice and man, demonstrated that allergen-specific Th2 responses can be shifted to Th0/Th1 responses. Plasmacytoid dendritic cells (pDCs) produce large amounts of type I interferons (IFNs) after stimulation of Toll Like Receptor 9 (TLR9) and are likely to play an important role in the reorientation of these Th2 cells. The expression of CD32a on the cell surface of pDCs makes this cell type attractive for targeted delivery of antigen and TLR agonists to revert Th2 responses. Therefore we sought to determine the efficacy of targeted delivery of CpG-C ODN to CD32a on the ability of human and monkey pDCs to secrete inflammatory cytokines. Here we demonstrate that targeted delivery of 3′-biotinylated CpG-C to CD32a on pDC induced phenotypical maturation as determined by CD80, CD83 and CD86 expression. Furthermore, targeting both monkey and human pDCs strongly augmented the secretion of IFNα compared to the delivery of CpG-C in an untargeted fashion ($p < 0.001$). TLR9 induced activation hampers the ability of human pDCs to internalize CD32a. Therefore we opted for targeted delivery of CpG-ODNs to CD32a, which reduces the risk of undesired side effects of systemic TLR treatment and in addition delivers a superior signal for the activation of pDCs. This approach opens new treatment principles for allergic patients.

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Introduction

Dendritic cells (DCs) constitute a family of antigen presenting cells defined by their morphology, phenotype and their unique capacity to attract and interact with naïve T cells (Steinman 1991). Tissue DCs are generally found as immature cells, which undergo a complex maturation process, and migrate to lymph nodes where they present antigens to T cells, following infection or inflammation. Recent progress in understanding DC activity to produce cytokines has revealed that myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) show different susceptibilities to products derived from pathogens, depending on their toll-like-receptor (TLR) expression profiles (Ito et al. 2002, 2005; Kadowaki et al. 2001; Schreibelt et al. 2010).

PDCs are one of two major subsets of human DCs that circulate in the peripheral blood, and are characterized as $CD4^+CD45RA^+$ IL-$3R\alpha^+$ (CD123) $ILT3^+ILT1^-$ $CD11c^-$ lineage$^-$ cells (Colonna et al. 2004). PDCs can be further characterized by BDCA-2 and BDCA-4 that in humans are exclusively expressed by pDCs in peripheral blood and bone marrow (Dzionek et al. 2002). Upon viral stimulation and subsequent TLR-mediated signaling, human pDCs produce large amounts of type I IFNs (IFNα/β) that stimulate T cell function of the Th1 type and stimulate NK cell cytolytic activity (Romagnani et al. 2005). Type I IFNs also promote differentiation, maturation, and immunostimulatory functions of myeloid DCs (Yoneyama et al. 2005). Moreover, type I IFNs are important for antiviral immunity, but several studies show that they also play a role in bacterial infections, allergy and anti-cancer immunity (Piccioli et al. 2009; Schettini and Mukherjee 2008; Trinchieri 2010). Because of their high IFN production pDCs are considered to have an important role in controlling both innate and adaptive immune responses. In a resting state pDCs might induce unbiased Th, Th2 or regulatory responses whereas their activated equivalents have stimulatory capacities and trigger Th1 responses (Moseman et al. 2004; Sinigaglia et al. 1999; Wenner et al. 1996).

Atopic diseases are characterized by the presence of Th2 cells that secrete interleukin (IL)-4, IL-5 and/or IL-13 but no or only limited amounts of the Th1 cytokine IFNγ (Benson et al. 2001; Grewe et al. 1995; Maggi 1998). However, recent studies

Abbreviations: DC, dendritic cell; IFN, interferon; mDC, myeloid DC; pDC, plasmacytoid DC; TLR, Toll Like Receptor.

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doi:10.1016/j.imbio.2012.01.016

demonstrated that allergen-specific Th2 responses can be shifted, *in vitro* in man and *in vivo* in mice, toward Th0 or even Th1 responses (Farkas et al. 2004; Parronchi et al. 1999; Kline et al. 2002; Fili et al. 2006; Gupta and Agrawal 2010). PDCs, as potential producers of large quantities of type I IFNs, thereby instigating Th1 responses, represent therefore an attractive target for antigen specific treatment of allergic diseases. The potency of activated human pDCs to revert allergy induced Th2 responses into Th1 responses has already been exploited in experimental animal models (Horner and Raz 2002). Previously performed studies have provided pivotal information on how TLR agonists can be used for redirecting Th2 responses. However, these TLR agonists have been delivered to pDCs in a non-targeted fashion. In addition, several recent studies highlight the importance of co-delivery of antigen and TLR agonists for the induction of superior immune responses over the separate delivery of the components (Krishnamachari and Salem 2009). It is known since the early nineties that antigen uptake by DCs can be boosted tremendously by targeting the antigens to FcRs (Sallusto and Lanzavecchia 1994). It is tempting to speculate that also receptor-mediated uptake of TLR agonists and antigens is superior to non-targeted uptake in stimulating pDCs (Yarovinsky et al. 2006). However, not all receptors may have the desired effect. Like in antigen uptake through IgE may lead to deterioration of the disease (Santamaria et al. 1993; Mudde et al. 1995, 1990), whereas IgG specific for an allergen may be able to counteract this effect (Mudde et al. 1996).

There is now ample evidence that pDCs express several receptors that may mediate antigen uptake, *e.g.* DEC-205, BDCA-2 and DCIR, which can be targeted to facilitate antigen delivery, but at the same time hamper the ability of pDCs to secrete type I IFNs (Dzionek et al. 2001; Meyer-Wentrup et al. 2008; Tel et al. 2011). Also other surface receptors expressed by human pDCs are described that negatively influence TLR-induced IFNα secretion upon receptor cross-linking by various mechanisms (Cao et al. 2006; Fuchs et al. 2005). We recently reported that pDCs can internalize soluble exogenous antigen bound to antigen-specific antibodies *via* CD32a, resulting in $CD4^+$ T cell activation without hampering the TLR-induced IFNα secretion (Benitez-Ribas et al. 2006, 2008). Here we describe the potency of targeting CpG ODNs to the Fc receptor CD32a on human and monkey pDCs.

Materials and methods

Monoclonal antibodies

The following mAb were used: CD20V450 (clone L27), CD3FITC (clone SP34), CD16FITC (clone 3G8), CD80PE (clone L307.4), CD86PE (clone IT2.2), anti-IL-12p40/70PE (clone C11.5), anti-TNFαPE (clone Mab11), CD123PerCP-Cy5, CD11cAPC (clone S-HCL3), CD4PE-Cy7 (clone SK3), anti-TNFαPE-Cy7 (clone Mab11), HLA-DRAPC-CY7 (clone L243) all from Becton & Dickinson, CD8FITC (clone DK25, DAKO), CD14PE-TxRed (clone RM052, Beckman Coulter), CD83PE (clone HB15a, Beckman Coulter), CD32biot (clone AT-10, AbD Serotec), CD32FITC (clone IV.3, Stemcell Technologies), mIgG1-PE, mIgG1-APC, anti-CD83-PE, anti-CD80-PE and anti-CD86-APC (all BD Bioscience Pharmingen, San Diego, CA, USA), CD16 and CD64 (both DAKO). For IFNα detection the mAb MMHA2 was used (In Vitrogen). Mean fluorescence intensity and percentage of positive human cells are based upon inclusion of cells in a live cell gate and determined on a FACSCalibur (BD Biosciences, San Jose, CA, USA).

Monkey whole blood stimulation assay

Heparinized blood was obtained from captive-bred, mature (4–5-year old) out-bred Indian origin rhesus macaques (*Macaca mulatta*). This study was critically reviewed and approved and undertaken by the institute's animal ethical committee and performed in accordance with Dutch law and international guidelines for the use of animals in research (BPRC IACUC ID 610). Collected heparin blood was used within 3 h of collection. The assay was performed in 5 ml polypropylene tubes (Becton & Dickinson), to which 200 μl of whole blood was added. Subsequently, a part of the tubes were incubated for 20 min at 4 °C with 5 or 10 μl of biotinylated anti CD32 mAb (clone AT-10, 100 μg/ml) and thereafter washed twice with 1 ml ice-cold PBS. The tubes were then incubated with 100 μl PE-Cy7 labeled streptavidin (1:500 in PBS, Becton & Dickinson) for 20 min at 4 °C. After two washes with PBS, a part of the tubes were incubated with either CpG (M362) or biotinylated CpG (M362) (both from Girundus, USA) for 20 min at 4 °C. Subsequently, a part of the tubes were washed twice with PBS. The stimulation assay was performed by adding to all tubes 800 μl RPMI1640 medium (GIBCO), 15 U/ml heparin, 0.1% FCS (GIBCO), β mercapto-ethanol (50 μM, GIBCO), penicillin (50 U/ml) and streptomycin (50 mg/ml), 10 ng/ml recombinant human IL-3 (Peprotech). The tubes were incubated either without further stimulus or in the presence of CpG (M362) at 37 °C for different time intervals as described in 'Results' section. Golgiplug (1:1000, Becton & Dickinson) was added either directly or after 1 or 2 h of incubation, to prevent protein trafficking.

Monkey FACS analysis

After incubation the tubes were centrifuged for 10 min at 500 × *g* and supernatant was harvested from the tubes in which no Golgiplug had been added. Subsequently, the cells were washed with cold PBS. After discarding the supernatant the cells were incubated for 20 min at 4 °C with a mixture of mAb for surface staining, consisting of: CD20V450, CD3FITC, CD8FITC, CD16FITC, CD14PE-TxRed, CD123PerCP-Cy5, CD11cAPC, HLA-DRAPC-CY7, supplemented with CD83PE or in some experiments with CD80PE or CD86PE. Subsequently, cells were washed once with 1 ml cold PBS and then 2 ml lysing solution (Becton & Dickinson) was added to all tubes followed by incubation at room temperature for 10 min, then centrifugation for 10 min at 500 × *g*. The supernatant was aspirated and the cells were either re-suspended in 1 ml PBS with 2% paraformaldehyde or incubated with an anti-IFNα PE labeled complex or 5 μl anti-IL-12PE or 4 μl anti-TNFαPE diluted in Becton & Dickinson perm/wash solution for 30 min at 4 °C in the dark. After washing with 1 ml of perm/wash solution, 1 ml PBS with 2% paraformaldehyde was added and all samples were stored overnight at 4 °C. For detection of IFNα in rhesus macaques only an unlabeled mAb (MMHA2) was available. The mAb was labeled with PE in house using the Zenon labeling technology (Zenon mouse IgG1 kit, Molecular Probes). With this method the unlabeled mAb is first incubated with PE labeled Fab fragments of an antibody directed against mouse IgG1 Fc (reagent A) and then incubated with non-specific IgG, which complexes unbound Fab fragments (reagent B). The anti-IFNα complex was prepared by diluting 1 mg of mAb MMHA2 (stock 0.5 mg/ml) in 20 μl PBS and adding 5 μl of compound A. After 5 min incubation at room temperature, 5 μl of compound B was added and the mixture was incubated for another 5 min. Then the volume was adjusted to 50 μl with PBS and diluted in 1 ml of perm/wash solution. For staining of IFNα 100 ml of this mixture was added per tube. Flow cytometry was performed on a LSRII or FACSaria machine using Diva software (Becton & Dickinson).

Monkey ELISA

For determination of IFNα a Verikine Cynomolgus/rhesus IFNα serum ELISA kit (Pestka Biomedical Laboratories, Inc., Piscataway,

NJ, USA). Assays were performed according to the manufacturer's instructions.

Purification of human pDCs

Buffy coats were obtained from healthy volunteers after informed consent and according to institutional guidelines and pDCs were purified by positive isolation using anti-BDCA-4-conjugated magnetic microbeads (Miltenyi Biotec) and cultured in X-VIVO-15 medium (Cambrex) supplemented with 2% human serum (Sanquin, Nijmegen, The Netherlands). PDC purity was routinely up to 95%, as assessed by double staining BDCA-2/CD123 (Miltenyi Biotec) (Fig. 1A) performed on a FACSCalibur using CellQuest Pro software (Becton & Dickinson).

Stimulation of human PBMCs and purified pDCs

Freshly isolated human pDCs were cultured as indicated with 10 ng/ml rhIL-3 (Cellgenix, Freiburg, Germany) or activated in the presence of ODN-CpG-C (M362, Axxora), ODN-CpG-C-3′-biotin or ODN-CpG-C-5′-biotin (both M362, Biosearch Technologies).

PBMCs and freshly isolated pDCs were incubated with biotinylated anti-CD32 (10 μg/ml, clone AT-10, AbD Serotec,) in PBA (PBS containing 5% BSA) on ice for 30 min and washed twice with PBA, followed by an incubation with 10 μg/ml streptavidin-Alexa647 in PBA on ice for 30 min and two times washing with PBA. Subsequently, pDCs were incubated on ice for 15 min with either PBA, 5 μg/ml CpG-C or 5 μg/ml CpG-C-3′-biotin. Unbound CpG was washed away three times with PBA and pDCs were cultured overnight in X-VIVO-15 medium, supplemented with 2% human serum. After overnight stimulation phenotypical maturation was analyzed by flow cytometry and supernatants were collected from PBMC and pDC cultures, and IFNα production was analyzed with murine monoclonal capture and HRP-conjugated anti-IFNα antibodies and IL-6, IL-8 and TNFα was analyzed with a human Multiplex kit (all BenderMed System) according to the manufacturer's instructions.

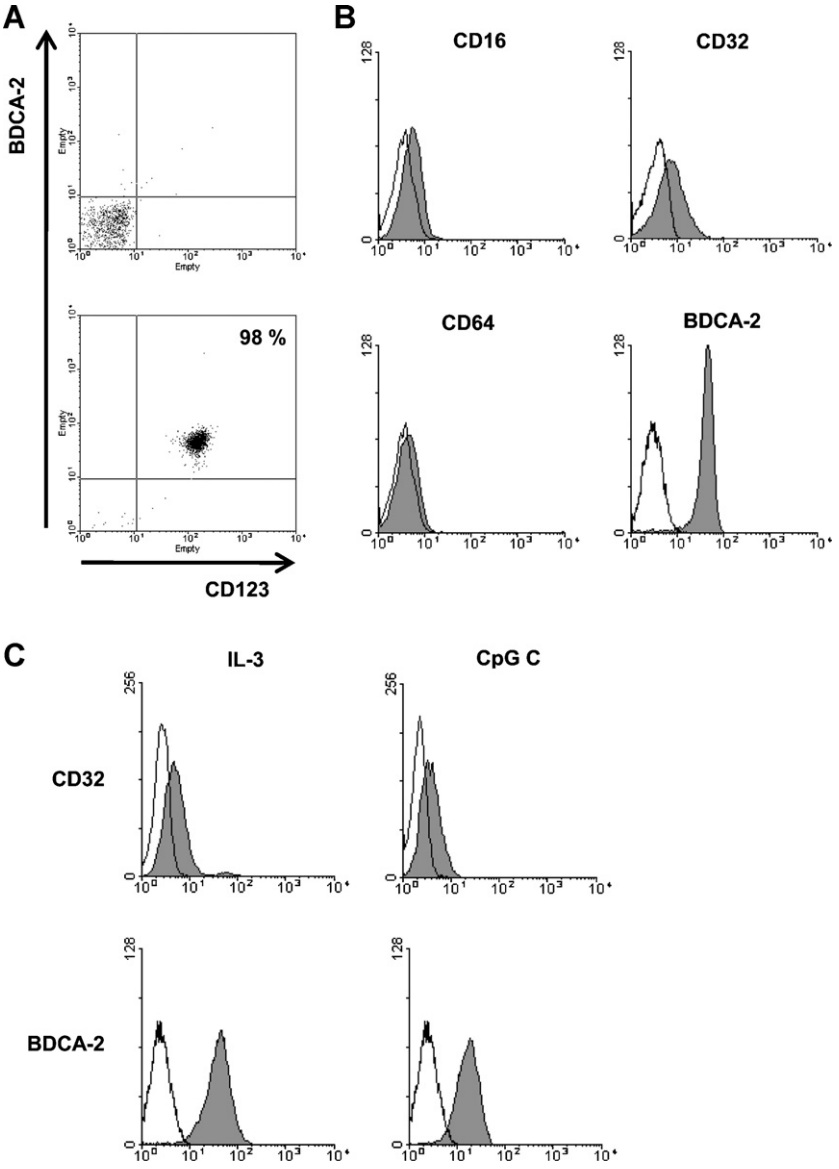

Fig. 1. Fc receptor expression on human pDCs. (A) Forward scatter (FSC)–Sideward scatter (SSC) plot of cell population following MACS isolation and staining of pDCs with CD123-FITC and BDCA-2-PE. MACS isolation yields a population of >95% pure pDCs. (B) Freshly isolated pDCs express CD32a and BDCA-2 but minimally CD16 and CD64 (gray filled histograms) compared to isotype controls (black lined histograms). (C) IL-3 cultured or CpG-C activated pDCs downregulate BDCA-2 receptor expression while CD32a expression remains unaffected (gray filled histograms) compared to isotype controls (black lined histograms). Data shown are from 1 representative experiment out of at three performed.

Statistics

All results are shown as the mean ± SEM. Statistical differences as determined by (paired) Student's *t*-test or by one-way ANOVA followed by Tukey's multiple comparison test as indicated.

Results

Fc receptor expression and regulation on human pDCs

To investigate the expression of antigen uptake receptors, pDCs were isolated from blood of healthy donors using anti-BDCA-4 coated beads and magnetic separation (Fig. 1A). In accordance with the literature (Dzionek et al. 2001), freshly isolated human pDCs expressed the pDC-specific CLR BDCA-2 and low levels of CD32, and expressed only minimal levels of CD16 and CD64 (Fig. 1B). Given that endocytic receptors are differentially regulated during pDC activation, we investigated CD32a expression after activation. In accordance with previous findings we could demonstrate that BDCA-2 expression is decreased after CpG-C induced activation (Fig. 1C) while CD32a expression remains unaffected.

Functionality of CpG-C after 3′-biotinylation remains unaffected

Next we determined whether biotinylation of CpG-C was feasible without hampering its stimulatory function. For the generation of targeting constructs we used 3′- and 5′-biotinylated CpG-C ODNs. As a positive control we used unbiotinylated CpG-C. Both the 3′- and 5′-biotinylated CpG-C were capable to induce a full phenotypical maturation based on the surface expression of CD80, CD83 and CD86 (Fig. 2A). Unexpectedly, biotinylation of CpG-C on the 5′-end hampered the ability of this ODN to induce IFNα secretion by pDCs (Fig. 2B), while 3′-biotinylation of CpG-C left the integrity unaltered resulting in high secretion of IFNα after stimulation (Fig. 2B). In a dose finding experiment we could demonstrate that 5 μg/ml 3′-biotin-CpG-C was most effective for the generation of fully mature pDCs (Fig. 2A and B).

TLR9 induced activation hampers CD32a receptor internalization on human pDCs

The dogma that mature DCs are incapable to endocytose and present encountered antigens was recently challenged by several groups (Tel et al. 2011; Drutman and Trombetta 2010; Platt et al. 2010). Therefore, we studied the capacity of freshly isolated and CpG activated and matured pDCs to internalize their CD32a receptor. Upon triggering surface receptor molecules, freshly isolated pDCs internalized BDCA-2 (data not shown) and CD32a as determined by analyzing surface expression levels (Fig. 3A–C). Although pDCs still expressed CD32a after CpG-C induced activation they were incapable to internalize CD32a upon receptor triggering (Fig. 3A–C).

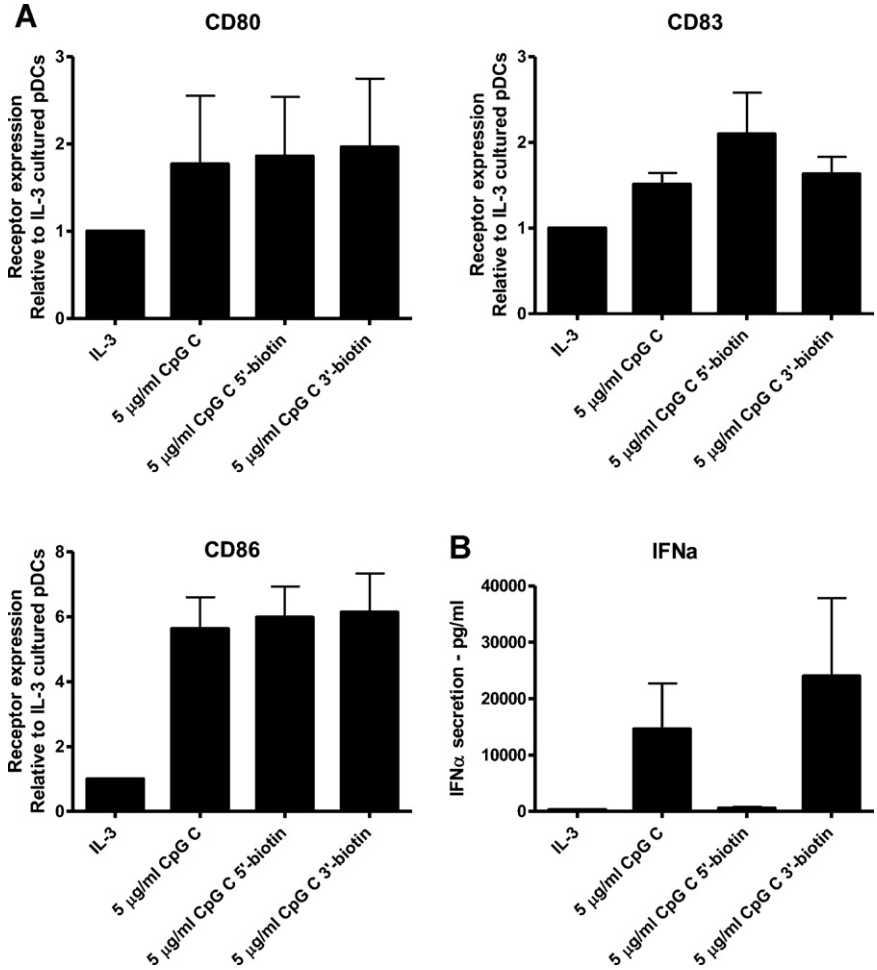

Fig. 2. Functionality of CpG-C remains unaffected after 3′-biotinylation. Freshly isolated pDCs were cultured or activated overnight with either IL-3, CpG-C, 3′-biotin-CpG-C or 5′-biotin-CpG-C. (A) CpG-C induced activation resulted in upregulation of surface receptor expression of CD80, CD83 and CD86 compared to IL-3 cultured pDCs. The graphs show the receptor expression (MFI) relative to IL-3 cultured pDCs. (B) Supernatants of pDC cultures following incubation were analyzed for the presence IFNα. The graphs show the mean ± SEM of three independent experiments performed with different donors.

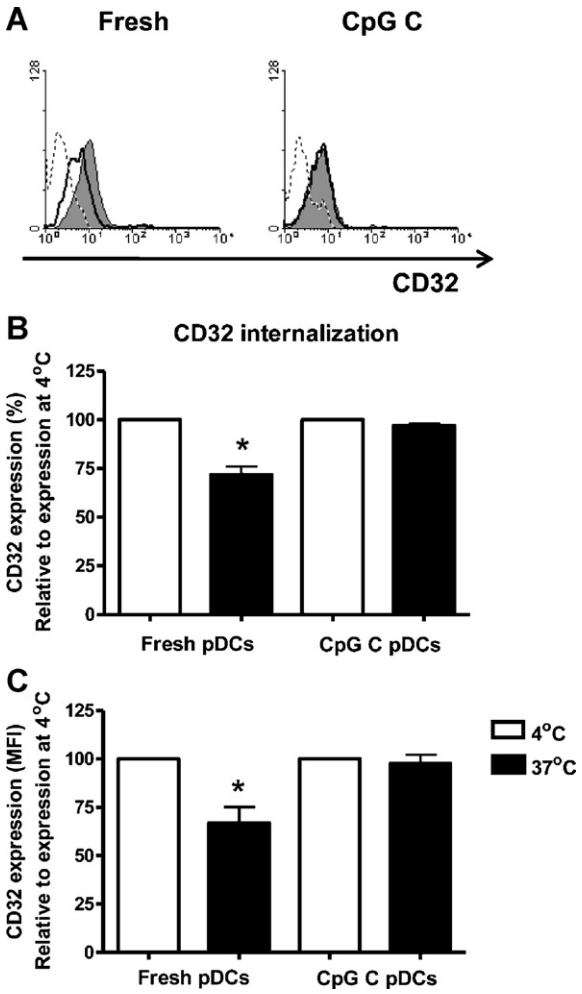

Fig. 3. Freshly isolated but not TLR-activated human pDCs internalize CD32a upon triggering with specific antibodies. (A) CD32a surface expression levels at 4 °C (gray filled histograms) and after internalization at 37 °C (black lined histograms) compared to isotype control (dotted lined histograms) on freshly isolated and CpG-C activated pDCs. Results shown are representative data of one experiment out of 3 performed. (B) CD32a receptor expression (black bars) depicted as surface expression levels (%) following internalization at 37 °C for 30 min relative to surface expression following incubation at 4 °C (white bars). (C) CD32a receptor expression (black bars) depicted as surface expression levels (MFI) following internalization at 37 °C for 30 min relative to surface expression following incubation at 4 °C (white bars). The graphs show the mean ± SEM of three independent experiments performed with different donors. Significant differences as determined by paired Student's *t*-test (*$p < 0.05$).

Targeted delivery of CpG-C to CD32a on human pDCs enhances secretion of inflammatory cytokines

In agreement with previous studies using human pDCs, it was observed that without stimulation there was a loss of pDCs after overnight culture (data not shown). Subsequent experiments showed that the addition of IL-3 could preserve these cells, with minimal induction of activation markers (Fig. 4A, first bar in graph). The maturation status of pDCs was studied by determining CD83 expression levels. Furthermore, expression of CD80 and CD86 was analyzed to determine the potential capacity to provide co-stimulation along with antigen presentation. To study the efficacy of CpG-C delivery we incubated pDCs with biotinylated αCD32 monoclonal antibody and streptavidin followed by a short incubation with either biotinylated (targeted) or unbiotinylated (non-targeted) CpG-C at 4 °C. This strategy ensured that solely bound CpG-C internalized upon subsequent incubation at 37 °C. Interestingly, we could demonstrate that both targeted and non-targeted delivery of CpG-C was able to induce a mature phenotype as demonstrated by upregulated expression of CD80, CD83 and CD86 compared to IL-3 treated pDCs (Fig. 4A). These data reveal that CpG-C despite rigorous washing, binds or sticks to the plasma membrane of human pDCs in sufficient amounts to induce activation (data not shown). Moreover, short incubation with targeted and non-targeted delivery of CpG-C was able to induce the same phenotypical maturation as pDCs activated overnight at 37 °C with 3′-biotin-CpG-C (Fig. 4A). The receptor for the binding of CpG-C to the cell surface is not identified although candidates have been suggested in the literature (Gursel et al. 2006; Hu et al. 2003).

Thereafter, we determined the effect of targeting CpG-C to CD32a on the ability to secrete inflammatory cytokines. As expected, untargeted pDCs cultured in IL-3 did not secret any IFNα (Fig. 4B and C) nor IL-6, IL-8 and TNFα (Fig. 4C). However, when CpG-C was targeted to CD32a we observed a 4 fold increase in the IFNα secretion as compared to pDCs that were stimulated with CpG-C in an untargeted fashion (Fig. 4B and C). Unexpectedly, we also demonstrated that targeted delivery of CpG-C was superior in inducing IFNα secretion as compared to pDCs that were activated with the optimal concentration of 3′-biotin-CpG-C overnight (Fig. 4B). Furthermore, targeted delivery of CpG-C also resulted in significantly enhanced secretion of IL-6, IL-8 and TNFα by human pDCs (Fig. 4C). Since human pDCs do not uniquely express CD32a and TLR9, we investigated whether targeting pDCs in whole PBMC cultures also enhanced inflammatory cytokine secretion. In accordance with the findings in purified pDCs, targeted delivery of CpG-C to CD32a in PBMCs also significantly enhanced the secretion of IFNα (Fig. 4D) and slightly enhanced IL-8 and TNFα secretion (Fig. 4D). In contrast to purified pDCs, IL-6 production could not be enhanced by targeted delivery of CpG-C (Fig. 4D).

Targeted delivery of CpG-C to CD32a on monkey pDCs enhances secretion of inflammatory cytokines

The goal of the monkey study was to develop conditions to test our targeting modality in a non human primate animal model and to compare the result to those obtained from similar experiments with human cells. Because of the low number of pDCs in peripheral blood of rhesus macaques, 2.71 ± 1.57 pDCs/μl blood, and the small volume of blood that can be ethically drawn from these animals, experiments on purified pDCs were not possible. Instead, a whole blood assay was developed to determine cytoplasmatic cytokine production in monkey pDCs (Fig. 5A) and the results obtained were confirmed using whole blood cytokine production measured by ELISA. After incubation, cells were first stained for several surface markers that allowed discrimination of different antigen presenting cell subsets including B-cells, monocytes, pDCs and mDCs. As shown in Fig. 5A, a Mhc-DR positive, lineage (CD3, CD8, CD16) negative subset could be discriminated, which contained $CD20^+$ B-cells, $CD14^+$ monocytes and a subset of CD20, CD14 double negative cells. The latter contained a $CD123^+/CD11c^-$ pDC subset and a $CD123^-/CD11c^+$ mDC subset. After surface staining, the cells were permeabilized and subsequently stained for the presence of intracellular IFNα and TNFα. As expected, targeted delivery of CpG-C did not induce IFNα or TNFα production by mDCs, B-cells or monocytes (data not shown). This confirms the close similarity between the immune system of monkeys and humans. However, targeted delivery of CpG-C significantly enhanced IFNα production, whereas the production of TNFα showed only a trend as determined by intracellular FACS (Fig. 5B). Like with human cells, targeted delivery of CpG-C to PBMC also induced a significant increase in IFNα as measured by ELISA ($p < 0.001$) (Fig. 5C).

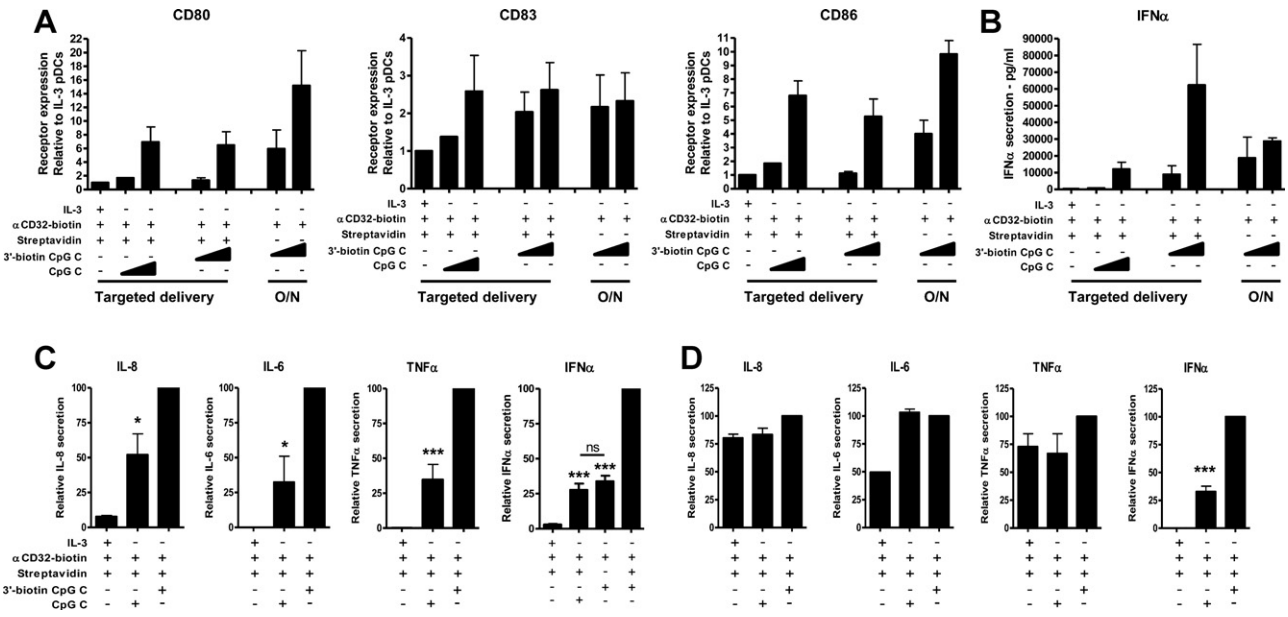

Fig. 4. Targeted delivery of CpG-C to CD32a on human pDCs enhances secretion of inflammatory cytokines. Freshly isolated pDCs (A–C) or total PBMCs (D) were incubated with (1) biotinylated αCD32 followed by streptavidin-Alexa647 and subsequently with 3′-biotin-CpG-C, (2) biotinylated αCD32 followed by streptavidin-Alexa647 and subsequently with CpG-C at 4 °C. After short incubation unbound CpG-C was washed away and cells were cultured overnight at 37 °C. (3) PDC were incubated with biotinylated αCD32 and subsequently activated overnight with 3′-biotin-CpG-C without washing. (A) Phenotypical maturation of differently activated pDCs determined by the expression of CD80, CD83 and CD86. The graphs show the receptor expression (MFI) relative to IL-3 cultured pDCs incubated with αCD32. (B) Graphs show the secretion of IFNα after overnight culture. (A and B) ▂▃▅ ; 0.5 or 5 μg/ml of either CpG-C or 3′-biotin-CpG-C as indicated. The graphs show the cytokine secretion relative to IL-3 cultured pDCs incubated with biotinylated αCD32 (C) and PBMCs (D) under different targeting conditions. The graphs show the mean ± SEM of at least three independent experiments performed with different donors. Significant differences as determined by one-way ANOVA followed by Tukey's multiple comparison test (*$p < 0.05$; ***$p < 0.001$).

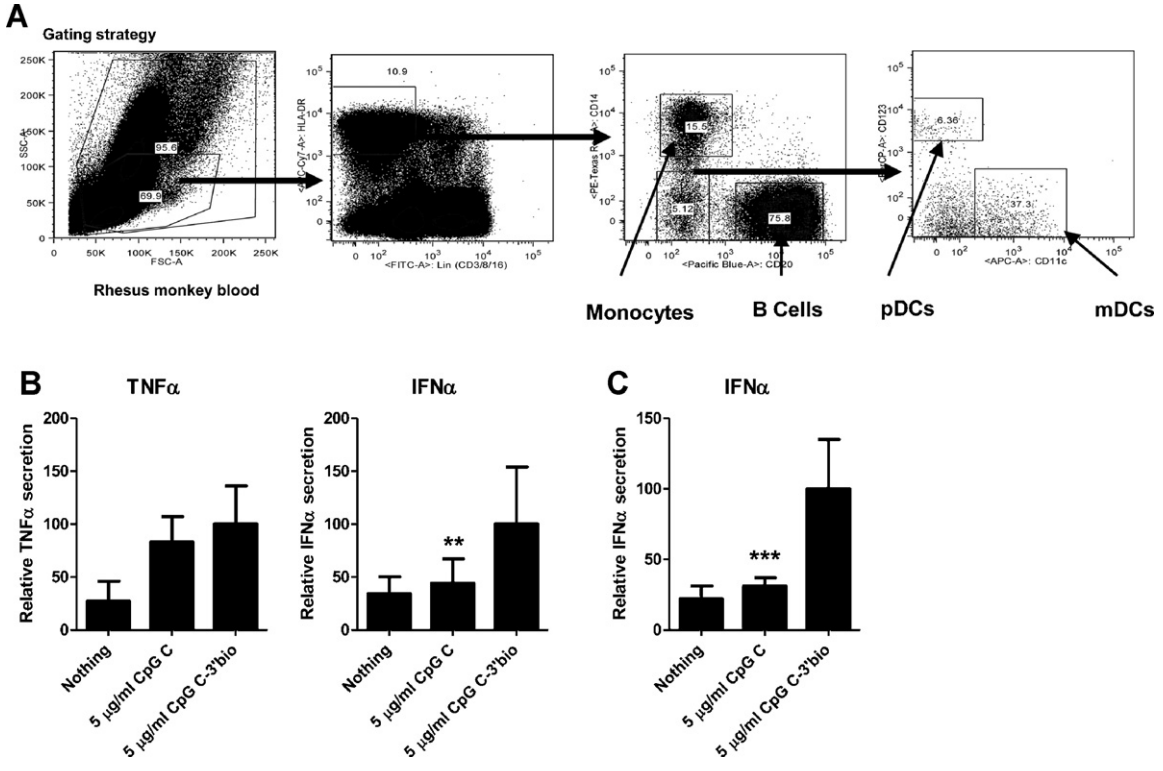

Fig. 5. Targeted delivery of CpG-C to CD32a on monkey pDCs enhances secretion of inflammatory cytokines. A whole blood assay was setup to test the ability of our targeting modality to specifically target pDCs in a non-human primate model. (A) Gating strategy for detection of intracellular cytokine production in monkey pDCs. (B) Graphs show the effect of the different targeting strategies on the relative production of IFNα and TNFα after overnight culture. (C) After overnight incubation of the differently targeted monkey pDC supernatant was harvested and tested for the presence of IFNα. The graphs show the mean ± SEM of at least five independent experiments performed with different animals. Significant differences as determined by paired Student's *t*-test (**$p < 0.01$; ***$p < 0.001$).

Discussion

Atopic diseases are characterized by the presence of activated Th2 cells (Benson et al. 2001; Grewe et al. 1995; Maggi 1998). However, recent studies demonstrated that allergen-specific Th2 responses can be skewed, *in vitro* in man and *in vivo* in mice, to Th0 or even Th1 responses (Farkas et al. 2004; Parronchi et al. 1999; Kline et al. 2002; Fili et al. 2006). PDCs, as potential producers of large quantities of type I IFNs thereby instigating Th1 responses, represent therefore a potential target for specific treatment of allergic diseases. The potency of activated pDCs to revert allergy induced Th2 responses into Th1 responses has already been exploited in experimental animal models (Horner and Raz 2002). Until now, studies were performed wherein CpG and allergen were applied separately and at random to the immune system. Some investigators have explored, in mice, the potential of linking the allergen directly to danger signals that activate DC *via* TLR. In a clinical trial this same concept was exploited to reduce the symptoms of ragweed allergic individuals. Unfortunately, a multicenter placebo-controlled trial (DARTT) involving over 700 patients with ragweed allergy was discontinued because only minimal ragweed-specific disease was observed in the overall study population and no significant efficacy could be assessed. On the other hand the ragweed allergen has been conjugated to 5′-end of the CpG, which leads to a decreased stimulation potential of the CpG (Putta et al. 2010 and our Fig. 2). However, to date neither in preclinical animal models nor in the multicenter trial, pDCs were specifically harnessed for massive amounts of type I IFN secretion. In the current study we demonstrate that targeted delivery of CpG-C to CD32a on both human and monkey pDCs augments the secretion of IFNα. Anticipating future targeting modalities that facilitate the targeted delivery of CpG-C and antigen simultaneously through CD32, we showed that rhesus macaques have a strong immunological homology with humans and can therefore be used as a preclinical animal model for this strategy.

Unmethylated CpG ODNs that target TLR9 were demonstrated to prevent and reverse airway allergy in experimental animal models (Horner and Raz 2002). Although in mice TLR9 is expressed by many cell types, including B cells and several subsets of DCs and monocytes/macrophages, under normal conditions TLR9 expression in man is restricted to B cells, granulocytes and pDCs (Krieg 2003). PDCs constitutively express the interferon regulatory factor-7 (IRF-7), but this is not the mere reason that only pDCs, and not other TLR9 expressing cells, efficiently produce type I IFNs. Moreover, the levels of IRF-7 are often even higher in other cells than the expression of IRF-7 in pDCs (Asselin-Paturel and Trinchieri 2005). Notwithstanding, the contribution of other immune cells in type I IFN secretion in response to TLR9 agonists is marginal. Honda et al. demonstrated that the capacity of pDCs to induce IRF-7 activation differed from other TLR9 expressing cells (Honda et al. 2005). In pDCs, the TLR9-associated MyD88–IRF-7 complex was retained for a longer period of time in the endosomal compartment after treatment with CpG ODNs, whereas in other cell types, the complex rapidly translocated to lysosomes (Honda et al. 2005). Taken together, pDCs are the main cell to produce large amounts of type I IFN after TLR9 ligation.

Both Farkas et al. and Schroeder et al. demonstrated the dichotomy of pDCs in controlling immune responses by the expression of TLR9 and the IgE receptor (Farkas et al. 2004; Schroeder et al. 2005). Their studies unambiguously showed that TLR9-induced activation resulted in complete downregulation of the IgE receptor which correlated with a selective suppression of allergen-induced $CD4^+$ T cell proliferation (Farkas et al. 2004; Schroeder et al. 2005). These findings illustrate that properly activated pDCs have the potential to revert allergic Th2 responses.

Although advantageous immune responses were observed after TLR agonist administration in mice and man, often systemic or high dose administration of TLR agonist also resulted in multiple undesired side effects and toxicity at the same time. These reports emphasize that both the specific induction of the immune response as well as the reduction of undesired side effects would greatly benefit from targeted delivery of TLR-ligands (Tacken et al. 2011). There are already reports demonstrating an improved and safe strategy by covalently linking CpG-ODN to the allergen Amb a 1 in order to revert the allergen-specific Th2 response in a non-targeted fashion (Simons et al. 2004; Tulic et al. 2004). Various studies have demonstrated that endocytic receptors, apart from DEC-205, are downregulated after pDC activation (Tel et al. 2011). Furthermore, triggering of these endocytic receptors on pDCs in a steady state impairs the ability of pDCs to secrete type I IFNs (Dzionek et al. 2001; Meyer-Wentrup et al. 2008; Tel et al. 2011). Here we describe that although CD32a receptor expression remains unaltered upon activation, receptor internalization is hampered. Therefore, we propose that targeted delivery of antigens and TLR agonists should be contemporaneous for the generation of a superior immune response.

The finding that conjugation of moieties to the 5′-end of CpG motifs abrogates their immunostimulatory potential was previously demonstrated by 2 different groups (Putta et al. 2010; Kandimalla et al. 2002). Interestingly, we observed that although the IFNα secretion was hampered, conjugation of biotin at the 5′-end left the ability of CpG-C to induce phenotypical maturation unaffected. However, since the induction of IFNα is a key target for manipulation of immune responses, targeting modalities exploiting this feature should be designed by conjugation of moieties at the 3′-end of CpG ODNs. In the previously discontinued multicenter trial TOLAMBA™ (Dynavax) was used, which consisted of an immunostimulatory CpG sequence linked at the 5′-end with the ragweed allergen Amb-1. The impaired IFNα production might partially explain the low success rate of previous studies.

Concluding remark

Administration of TLR agonists benefits the reversion of allergen-specific Th2 responses in unbiased Th0/Th1 responses. Our results envision that specific targeting of pDCs, as main producers of type I IFN, is an attractive approach that can be exploited to revert the Th2 responses. Moreover, these findings pave the way to improve the efficacy of currently used strategies by using targeting modalities wherein antigen and TLR agonist are delivered simultaneously.

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