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CpG oligodeoxynucleotides enhance FcγRI-mediated cross presentation by dendritic cells

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Abstract

Dendritic cells (DC) can trigger naive CD8$^+$ T cell responses by their capacity to cross-present exogenous antigens via the major histocompatibility complex class I pathway. The myeloid class I IgG receptor, FcγRI (CD64), is expressed on DC, and *in vivo* targeting of antigens to FcγRI induces strong humoral and cellular immune responses. We studied the capacity of human FcγRI (hFcγRI) to facilitate DC-mediated cross presentation and T cell activation, and assessed the effect of CpG oligodeoxynucleotides on this process. We generated hFcγRI expressing immature DC from hFcγRI transgenic and immature DC from non-transgenic mice. Antigens were targeted to Fcγ receptors as ovalbumin immune complexes, or selectively to hFcγRI via ovalbumin–CD64 mAb fusion proteins. Co-incubation of immature DC with CpG ODN led to markedly increased MHC class I presentation of FcγR-targeted antigens. When OVA was selectively targeted to hFcγRI, few differences were observed between Tg and NTg DC. However, upon co-incubation with CpG ODN, hFcγRI-triggered cross presentation was enhanced. These results document the capacity of hFcγRI on DC to trigger cross presentation via MHC class I upon co-culture with CpG ODN.

Introduction

Dendritic cells are professional antigen-presenting cells, with a unique capacity to induce primary immune responses. Tissue resident immature DC exert high endocytic and phagocytic activities that are mitigated upon maturation in favor of an enhanced capacity to present antigens (1). Dendritic cell-mediated antigen presentation initiates specific immune responses involving both CD4$^+$ and CD8$^+$ T cell activation. In general, exogenous antigens are presented on MHC class II molecules, and endogenous antigens via the MHC class I pathway. However, cross presentation by DC of exogenous antigens on class I molecules can represent a potent pathway to elicit primary CD8$^+$ T cell responses (2,3). Cross presentation is a rather inefficient process, as fluid phase internalization only results in class I restricted presentation at high concentrations of antigen (4,5). FcγR-mediated uptake of complexed antigens can markedly enhance the efficiency of cross presentation (6).

The human receptor with high affinity for IgG, hFcγRI (CD64), is exclusively expressed on cells of the myeloid lineage including monocytes, macrophages, granulocytes (upon cytokine induction) and DC (7). hFcγRI is unique among leukocyte FcγR, because of its structure, limited cell distribution and function. This receptor has three Ig-like extracellular domains that bind monomeric IgG with high affinity, in contrast to the other FcγR. hFcγRI on antigen-presenting cells can facilitate antigen-specific CD4$^+$ T cell responses by antigen-presenting cells, and effectively triggers anti-tumor vaccine responses (8–11). Because of these features and the fact that hFcγRI-targeted antigens co-localize with MHC class I molecules in subcellular organelles (12), we

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postulated hFcγRI to be a candidate for facilitation of DC cross presentation.

Bacterial DNA has direct and indirect immunostimulatory effects on immune cells due to the presence of unmethylated CpG dinucleotide motifs that bind to Toll-like receptor 9 (TLR9), which is a pattern recognition receptor expressed by a subset of cells including DC. These unmethylated CpG dinucleotides are present in the expected frequency in bacterial DNA but methylated and suppressed in vertebrate DNA (13,14). Synthetic immunostimulatory oligodeoxynucleotides containing CpG motifs (CpG ODN) can interact with TLR9, activate immune cells, and induce the production of various cytokines (15). In addition, CpG ODN exhibit the capacity to induce growth, activation and maturation of DC (16–18).

To study the capacity of hFcγRI to facilitate DC-mediated cross presentation of targeted exogenous antigen, we used bone marrow-derived DC (BMDC) from unique hFcγRI transgenic mice (8). Ovalbumin (OVA) was targeted to FcγRI by OVA-IgGαOVA immune complexes, by an OVA–CD64 antibody fusion protein, or by an OVA–CD64 chemically linked protein. These latter two constructs selectively target OVA to an epitope on hFcγRI, located outside the IgG-binding domain (19). Our results document hFcγRI to efficiently facilitate DC-mediated cross presentation, upon incubation of DC with CpG ODN.

Methods

Mice

Human FcγRI Tg animals (8) crossed with C57Bl6 (F1), or Balb/c mice (F12) were bred and maintained at the Transgenic Mouse Facility of the Central Animal Laboratory, Utrecht University. C57Bl/6 and Balb/c mice were obtained from Harlan (Horst, The Netherlands). 8–12-week-old human CD64-expressing animals were used in the experiments, as well as their NTg littermates. The Utrecht University animal ethics committee approved all experiments.

Cell lines

The RF33 cell line, expressing a TCR recognizing the H-2^b-restricted OVA epitope SIINFEKL (20), and the OVA-specific D011.10 cell line, that recognizes the OVA peptide in an Ia^d restricted way (21), were cultured in RPMI 1640 medium (Gibco BRL, Life Technologies, Paisley, UK), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Fetalclone I, Hyclone, Logan, UT), 50 IU/ml penicillin (Gibco BRL) and 50 μg/ml streptomycin (Gibco BRL). The interleukin 2 (IL-2) dependent CTLL-2 cell line (22) was propagated in RPMI 1640 medium, with 10% FBS, 50 IU/ml penicillin, 50 μg/ml streptomycin and 100 U/ml IL-2 (Immunokine, Boehringer Ingelheim, Alkmaar, The Netherlands).

Antibodies

CD80 (clone 1G10), CD86 (clone GL1), CD11b FITC (clone M1/70), CD11c biotin (clone HL3), CD32/16 PE (clone 2.4G2) and Gr-1 PE (clone RB6-8C5) were obtained from PharMingen (BD Biosciences, BD PharMingen, San Diego, CA). CD64 PE and SA-PE were purchased from Becton Dickinson (BD Biosciences, San Jose, CA). F4/80 biotin (clone Cl: A3-1) was obtained from Serotec (Oxford, UK). CD40 PE (clone 3.23) was purchased from Immunotech (Marseille, France), and $F(ab')_2$ fragments of mouse anti-rat IgG (H+L) were purchased from Jackson Immunoresearch (West Grace, PA). NLDC-145 (23) and M5/114 anti-class II (24) were kindly provided by Dr Georg Kraal (Vrije Universiteit, Amsterdam, The Netherlands).

Antigens

The immunodominant peptide of OVA, SIINFEKL (OVA 257–264), was obtained from Isogen (Maarssen, The Netherlands). Ovalbumin complexes were generated by incubation of 40 μg/ml chicken egg OVA (Sigma, St Louis, MO) with 80 μg/ml rabbit IgG anti-OVA (Sigma) for 20 min at 37°C. 22×OVA conjugates were prepared using *N*-succinimidyl *S*-acetylthioactetate (SATA) (Pierce, Rockford, IL) and SPDP (Pierce) as chemical cross-linkers (25). 22-OVA fusion protein was generated by standard methods. Briefly, the VH and VL encoding regions of H22 (19) were used to generate the 22sFv vector [kindly provided by Dr Joel Goldstein (Medarex, Annandale, NH)]. cDNA encoding the OVA sequence was inserted into the 22sFv vector, producing a 22sFv and OVA gene fusion (22-OVA). 22-OVA recombinant baculovirus was generated using the BD Baculogold Transfection kit as recommended by the manufacturer. *Trichopluscia Ni* (Hi-5) insect cells were infected with high titre baculovirus encoding 22-OVA at a multiplicity of infection of 10. After 4 days, supernatant was collected, concentrated and purified. Purified protein constructs were run out on 6% acrylamide gels and stained with Coomassie brilliant blue to test for purity. All protein constructs were tested for lipopolysaccharide (LPS) contamination by the Limulus Amebocyte Lysate QCL-1000 assay kit (BioWhittaker, Walkersville, MD).

CpG ODN

Synthetic ODN were provided by Coley Pharmaceutical Corporation (Wellesly, MA). CpG ODN 1826 with the following sequence was used: TCCATGACGTTCCTGACGTT. In addition, CpG ODN were tested and proved to contain $<$12.5 ng/mg of lipopolysaccharides levels by Limulus Amebocyte Lysate QCL-1000 assay kit.

Generation of DC

Bone-marrow derived DC (BMDC) were obtained as described by Inaba *et al.* (26). Briefly, bone marrow was flushed from mouse femurs, erythrocytes were lysed and cells were grown at 1×10^6/ml in filtered $RPMI^+$ (RPMI 1640 medium with 10% FBS, 50 IU/ml penicillin and 50 μg/ml streptomycin) in the presence of either 10 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF; Immunex, Seattle, WA) or 10 ng/ml GM-CSF + 50 ng/ml tumor necrosis factor alpha (TNF-α; Hycult, Uden, The Netherlands). Non-adherent cells were replated on day 1, and non-adherent cells were removed on days 2 and 4 from the cultures, with concomitant refreshment of culture media. Non-adherent and loosely adherent DC were harvested on days 7, 8 or 9.

Flow cytometric analyses

Day 7 DC (DC7) (1×10^5), day 8 DC cultured for 24 h with 10 μg/ml CpG ODN, and DC7 which were cultured for 48 h with 1 μg/ml LPS (Sigma), were blocked with 5% heat-inactivated mouse serum for 30 min at room temperature (RT). Cells were washed with FACS buffer [phosphate-buffered saline (PBS), 0.1% azide, 1% bovine serum albumin (BSA)] and incubated with different mAb for 20 min at RT. Cells were washed again and, if required, incubated with secondary antibodies. Cells were analyzed by flow cytometry using a FACSCalibur (BD Biosciences). Unstained cells, isotype controls and secondary (fluorochrome-labeled) antibodies were used as negative controls.

MHC class II antigen presentation

DC7 (1×10^5 cells) were washed twice in RPMI$^+$ and resuspended in 100 μl RPMI$^+$/well. DC were incubated with OVA (300 ng/ml) and various concentrations of OVA-IgGαOVA complexes, and 1×10^5 D011.10 T cells for 24 h at 37°C. Excess OVA (0.4 mg/ml) was used as a positive control. The presence of IL-2 released by D011.10 cells was determined by culturing 5×10^3 IL-2 dependent CTLL-2 cells with supernatants. After overnight incubation, 1 μCi of [^{3}H]thymidine (Amersham, Buckinghamshire, UK) was added to each well, and cells were harvested 24 h later onto glass fibre filters (Wallac, Turku, Finland) for liquid scintillation counting.

MHC class I antigen presentation

DC7 (1×10^5) were washed twice in RPMI$^+$ medium and resuspended in 150 μl RPMI$^+$/well. DC were incubated with OVA alone (100 ng/ml), OVA-IgGαOVA complexes, 22-OVA or 22×OVA, either with or without 10 μg/ml CpG ODN 1826 for 24 h at 37°C. The SIINFEKL peptide (0.29 mg/ml) served as a positive control. OVA-IgGαOVA complexes were generated by incubating DC with 100 ng/ml of OVA and varying concentrations of rabbit anti-OVA. After 24 h, cells were washed once with RPMI$^-$ medium (RPMI 1640 medium only) and fixed using 1.5% paraformaldehyde for 20 min at RT. Cells were washed once in RPMI$^-$ medium and quenched with 50 mM NH_4Cl for 60 min at RT. This was followed by washing trice in RPMI$^-$ medium and resuspension in 100 μl RPMI$^+$/well. RF33 cells, 1×10^5, were added in a volume of 50 μl, and incubated for 36 h at 37°C. One hundred microliters of supernatant were harvested from each well. The presence of IL-2 released by RF33 cells in culture supernatants was determined as above.

Statistical analysis

Differences between groups were analyzed by one-way ANOVA. A P-value <0.05 was considered significant.

Results

Effect of culture conditions on DC phenotype and expression of hFcγRI

To study hFcγRI on dendritic cells as target molecule for vaccine development, we assessed bone marrow-derived DC. To obtain DC with hFcγRI expression, we cultured bone marrow from hFcγRI-transgenic mice for 7 days in the presence of GM-CSF or GM-CSF/TNFα. The well-described GM-CSF/IL-4 culture method was not used, as IL-4 downregulates hFcγRI expression on myeloid cells (8,27). BMDC cultured for 7 days (DC7) with GM-CSF showed a clear immature DC phenotype, indicated by expression of CD11c, DEC-205, high expression of Fcγ receptors and low expression levels of costimulatory and MHC molecules (Fig. 1). DC cultured with GM-CSF/TNFα demonstrated a higher expression of CD86 and MHCII, representing a more mature phenotype (data not shown). In addition, DC7 expressed F4/80, and no B or T cell markers (data not shown). Expression of GR-1 was consistently found on DC upon 7 days of culture (Fig. 1), which was down modulated upon full maturation (data not shown). The presence of the

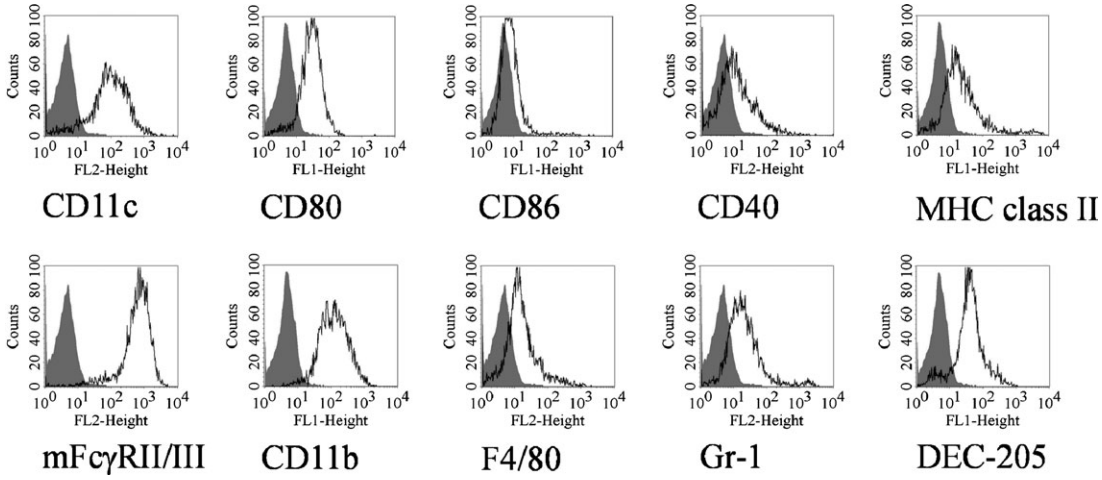

Fig. 1. Effect of culture conditions on DC7 cell surface marker expression. Human FcγRI Tg DC were cultured for 7 days in the presence of GM-CSF. Expression of different markers was analyzed by flow cytometry. Controls are depicted as shaded histograms, staining with monoclonals as open histograms. Percentages of CD11c+ cells vary from culture to culture, with the minimum of CD11c+ cells being >60%. One representative experiment out of four is shown.

hFcγRI did not cause any change in marker expression, as NTg and Tg DC exhibited similar expression patterns for all markers tested (data not shown).

Human FcγRI is expressed on cells of the myeloid lineage, including monocytes, macrophages and dendritic cells. To document that hFcγRI was present on dendritic cells in our cultures, we triple-stained for CD11b, CD11c and hFcγRI. Both GM-CSF (Fig. 2) and GM-CSF/TNFα (data not shown) $CD11b^+CD11c^+$ DC7 expressed hFcγRI, albeit hFcγRI expression levels were lower on GM-CSF/TNFα DC7. This is consistent with earlier observations that DC maturation coincides with down regulation of hFcγRI expression (28).

MHC class II antigen presentation is equivalent in NTg and hFcγRI Tg DC

Next, we measured MHC class II dependent antigen presentation by DC derived from NTg and Tg DC, to exclude differences in functionality of Tg DC. DC were incubated with excess OVA (400 μg/ml), to assess its capacity to internalize antigen via fluid phase antigen uptake, and subsequent processing. To study FcγR-mediated uptake and processing, DC were incubated with 300 ng/ml OVA-IgGαOVA immune complexes. As read out we used OVA-specific MHC class II restricted D011.10 T cells. hFcγRI Tg and NTg DC7 exhibited similar capacity to pinocytoze and process fluid phase OVA (Fig. 3). OVA-immune complexes triggered T cell proliferation at 100-fold lower concentrations, confirming that receptor-mediated endocytosis and processing is more efficient. Importantly, hFcγRI Tg and NTg DC were similar in their ability to activate class II restricted T cells (Fig. 3). This demonstrated class II antigen presentation, and subsequent T cell activation, not to be influenced by the transgenic hFcγRI.

Effect of CpG ODN on DC cell surface marker expression

CpG ODN can exhibit direct effects on DC differentiation and maturation, and enhance cross presentation of MHC class I restricted peptides and fluid phase-internalized antigens (17). Before studying the effect of CpG ODN on FcγR-triggered antigen presentation, we analyzed the effect of CpG ODN on cell surface marker expression of GM-CSF and GM-CSF/TNFα immature DC. DC7 were co-cultured with CpG ODN for 24 h, and marker expression levels examined. CpG ODN clearly activated DC7, as reflected by upregulation of the level of co-stimulatory and MHC class II molecules, and down regulation of FcR, such as human FcγRI, and mouse FcγRII/FcγRIII (Fig. 4).

CpG ODN enhance cross presentation of FcγR-targeted immune complexes

In order to study the effect of CpG ODN on FcγR-triggered cross presentation, we established whether the presence of

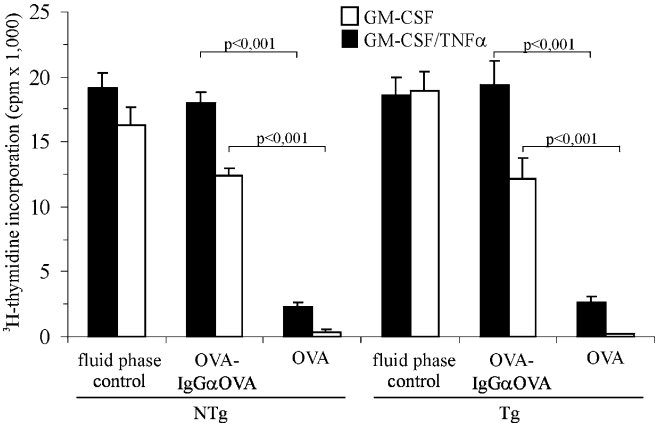

Fig. 3. MHC class II antigen-presenting capacity of DC. Human FcγRI Tg and NTg DC, cultured for 7 days in the presence of either GM-CSF or GM-CSF/TNFα, were incubated with excess OVA (400 μg/ml) as fluid phase control, with OVA-IgGαOVA immune complexes (300 ng/ml), or with OVA (300 ng/ml), and OVA-specific MHC class II restricted DO11.10 T cells for 24 h at 37°C. IL-2 production by T cells was quantified by CTLL-2 proliferation assays. Data represent means of duplicate determinations in one representative experiment out of four, in which the specific signal minus background is depicted. Statistic significant differences in antigen presentation of immune complexes vs ovalbumin by NTg or Tg DC are depicted in the figure.

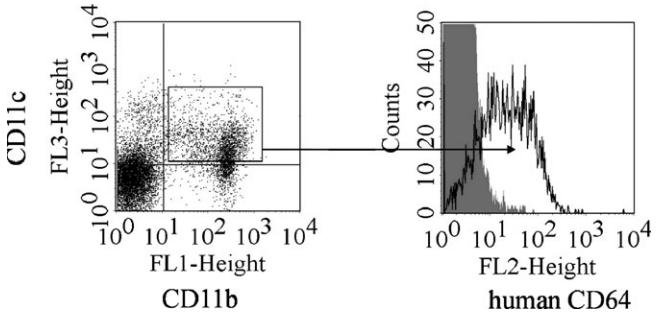

Fig. 2. Expression of hFcγRI on dendritic cells. Human hFcγRI Tg and NTg DC were cultured for 7 days in the presence of GM-CSF and GM-CSF/TNFα. Triple staining for CD11b, CD11c and hFcγRI was performed and analyzed by flow cytometry. Expression of hFcγRI by the CD11b/CD11c double positive cell population is depicted, and one representative experiment out of four is shown.

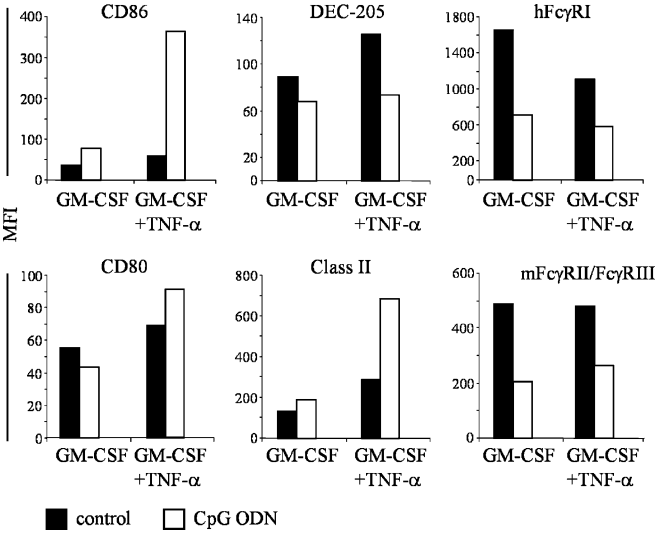

Fig. 4. Effect of CpG ODN on DC cell surface marker expression. Human FcγRI Tg DC were cultured for 7 days in the presence of GM-CSF or GM-CSF/TNFα. Subsequently, CpG ODN (10 μg/ml) were added for 24 h. Cell surface expression of different markers was analyzed by flow cytometry. Data are representative of three independent experiments, yielding essentially identical results.

hFcγRI affected the ability of DC7 to activate class I restricted T cells. DC7 from hFcγRI Tg and NTg mice, cultured with GM-CSF and GM-CSF/TNFα, were incubated with the class I restricted OVA peptide SIINFEKL with or without CpG ODN. After 24 h, DC were fixed and MHC class I restricted OVA-specific RF33 T cells were added for 36 h. In the absence of CpG ODN, both types of DC showed similar ability to activate class I restricted T cells. Additional stimulation with CpG ODN further enhanced DC ability to activate class I restricted T cells (Fig. 5A).

Subsequently, hFcγRI Tg and NTg DC were incubated with OVA immune complexes at varying concentrations. Immune complex-triggered cross presentation was higher in hFcγRI Tg than in NTg DC (Fig. 5B), reaching a statistically significant difference in GM-CSF/TNFα DC, indicative of an effect mediated by the transgenic hFcγRI. Stimulation with CpG ODN further increased cross presentation (Fig. 5B). CpG ODN clearly had an additive effect on hFcγRI-mediated cross presentation, as antigen presentation in the Tg + CpG group was enhanced to a higher extend than antigen presentation by DC of the NTg + CpG group.

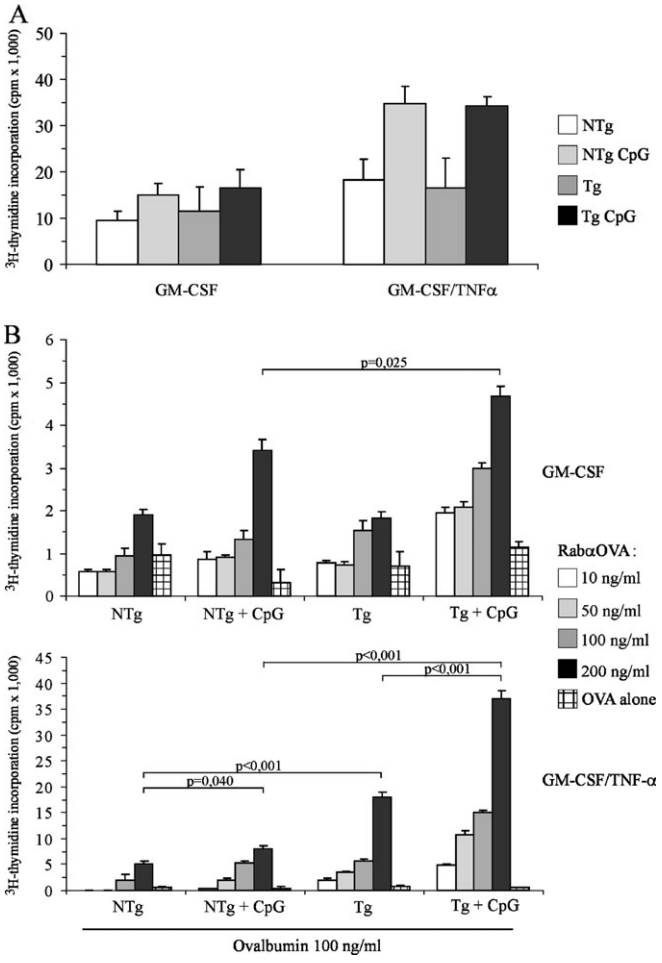

Fig. 5. Effect of CpG ODN on MHC class I presentation of FcγR-targeted immune complexes. Human FcγRI Tg and NTg DC were cultured for 7 days in the presence of GM-CSF or GM-CSF/TNFα. DC were then incubated with either 124 μg/ml SIINFEKL (A), immune complexes or OVA alone (B), in the presence or absence of 10 μg/ml CpG ODN for 24 h at 37°C. Cells were fixed, washed and incubated with MHC class I restricted OVA-specific RF33 T cells for 24 h at 37°C. IL-2 released by T cells was determined by CTLL-2 proliferation assays. One representative experiment out of three, in which the specific signal minus background is depicted, is shown. Statistical differences are depicted in the figure (determined for a rabαOVA concentration of 200 ng/ml).

CpG ODN enhance cross presentation of human FcγRI-targeted antigens

The capacity of hFcγRI to induce DC-mediated cross presentation of OVA and the effect of CpG ODN stimulation on this process was examined next. To target OVA to hFcγRI, two approaches were followed. Firstly, an scFv fragment of mAb H22 to hFcγRI was genetically linked to OVA (22-OVA). As this construct only contains 22 scFv, it is not expected to cause massive receptor crosslinking (10,29). Secondly, OVA was chemically cross-linked to whole IgG of monoclonal antibody H22 (22×OVA), which results in a molecule targeting OVA to hFcγRI that cross-links the receptor (19). DC7 from hFcγRI Tg and NTg mice, cultured with GM-CSF or GM-CSF/TNFα, were incubated with either 22-OVA, or 22×OVA, in the presence or absence of CpG ODN for 24 h. DC were fixed, and subsequently incubated with MHC class I restricted OVA-specific RF33 T cells for 36 h. In the absence of CpG ODN, very limited hFcγRI-triggered cross presentation of OVA by DC was detected (Fig. 6). When CpG ODN were added, an increase in hFcγRI-mediated antigen presentation was observed, as reflected by higher antigen presentation of CpG ODN-stimulated hFcγRI Tg DC7, compared to NTg DC7 (Fig. 6). This difference reached statistical significance using a concentration of 1 μg/ml for 22-OVA, and 5 μg/ml for 22×OVA. Ovalbumin alone used at concentrations of either 1 or 5 μg/ml was applied as control, inducing an average [^{3}H]thymidine incorporation of 500 c.p.m. Ovalbumin co-incubated with CpG reached an average incorporation of 1000 c.p.m. The fact that both 22-OVA and 22×OVA triggered cross presentation indicated cross-linking of hFcγRI not to be crucial for this process.

Discussion

To study the role of hFcγRI as a target molecule on DC, we used a unique and well-defined FcγRI Tg mouse model (8,10). We cultured bone marrow from hFcγRI transgenic mice with GM-CSF or GM-CSF/TNFα, to generate BMDC which express human FcγRI in addition to mouse FcγR. Consistent with earlier work (1,26,30), these protocols yielded immature DC.

In this study, we focused on the effect of CpG ODN on FcγR-mediated cross presentation. Our results confirmed that CpG ODN enhance expression of costimulatory molecules, induce down modulation of FcγR, and augment MHC class I restricted peptide presentation. Importantly, FcγR-mediated cross presentation of OVA immune complexes was up-regulated 2- to 4-fold upon CpG ODN activation, despite the fact that CpG ODN down regulated FcγR expression. This is consistent with earlier data in the literature, documenting CpG ODN to act as adjuvants for MHC class I restricted epitopes (18), and CpG ODN treatment of peptide- or protein-pulsed DC to enhance activation of class I restricted T cells (31,32). However, these data show for the first time that CpG activation of DC enhances FcγR-mediated antigen presentation.

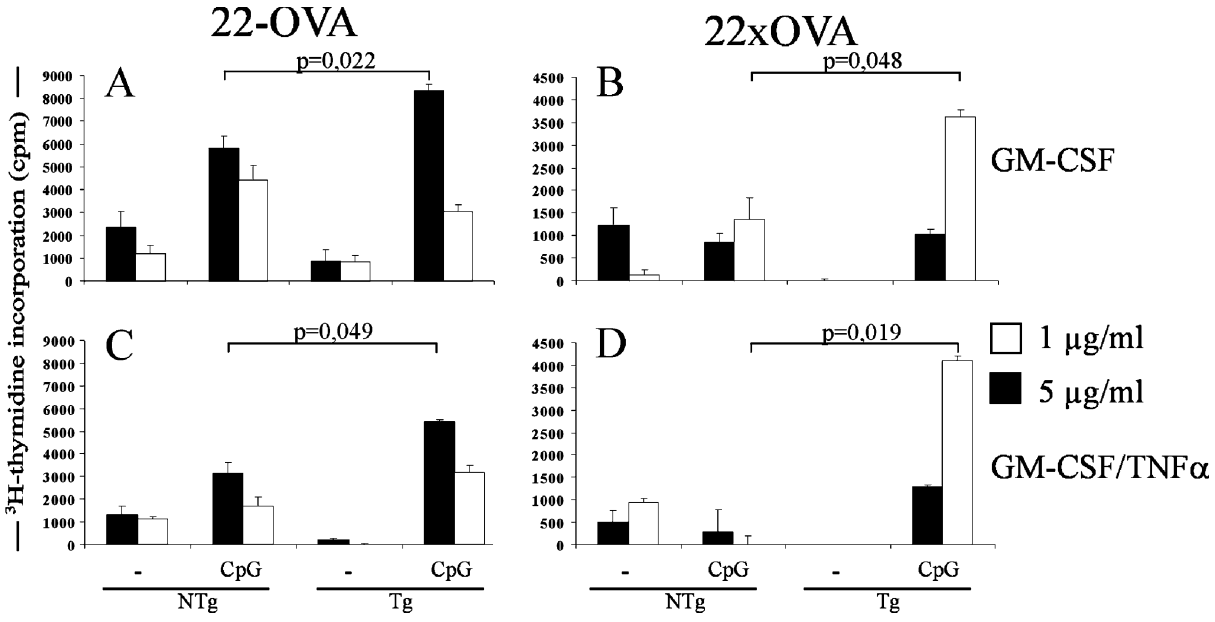

Fig. 6. Effect of CpG ODN on hFcγRI-mediated cross presentation. Human FcγRI Tg and NTg DC were cultured for 7 days in the presence of either GM-CSF (A and B) or GM-CSF/TNFα (C and D), and were then incubated with 1 or 5 μg/ml 22-OVA (genetically engineered) (A and C) or 22×OVA (chemically cross-linked) (B and D), either with or without CpG ODN, for 36 h at 37°C. Cells were fixed, washed and incubated with MHC class I restricted OVA-specific RF33 T cells for 24 h at 37°C. IL-2 released by T cells was determined by CTLL-2 proliferation assays. One representative experiment out of three, in which the specific signal minus background is depicted, is shown. Statistical differences are depicted in the figure.

hFcγRI is the high affinity receptor for monomeric IgG, and is, therefore, likely occupied with serum IgG *in vivo* (33). To study potential *in vivo* dendritic cell based strategies, we targeted hFcγRI outside its IgG ligand-binding domain using monoclonal antibody H22. By using genetically linked 22-OVA, or chemically-linked 22×OVA, we selectively targeted OVA to hFcγRI, without interference of murine FcγR. Human FcγRI targeting on Tg DC resulted in no cross presentation. Incubation of DC with hFcγRI-targeted antigens and CpG ODN clearly induced cross presentation of antigen-derived peptides on MHC class I molecules, both when OVA was targeted via 22-OVA and 22×OVA. Binding of 22×OVA to Tg DC induces crosslinking, whereas 22-OVA is not likely to induce massive crosslinking and this difference in type of activation of the receptor might lead to different intracellular trafficking and presentation of OVA. 22-OVA antigen uptake by NTg DC was apparently influenced by CpG, in that more 22-OVA was non-specifically (without interacting with the human FcγRI) taken up and presented upon CpG co-incubation. Differences observed in NTg DC between 22-OVA and 22×OVA might be attributable to the size of the molecule, as 22-OVA is smaller than 22×OVA and more efficient non-FcR-mediated uptake might be envisaged. Data in this paper show that antigen presentation by the human high affinity receptor for IgG on DC can be enhanced by CpG activation.

Our data may underlie the well-documented anti-tumor and vaccine responses seen upon targeting hFcγRI in hFcγRI-transgenic mouse models (11,34,35). Consistent with the present data, FcγRI-targeted antigens can co-localize with MHC class I molecules in human monocytic U937 cells (12), and were presented on MHC class I in the human THP-1 myeloid cell line (36).

The mechanism by which hFcγRI directs antigens to MHC class I processing pathways has not been elucidated. Cross presentation upon targeting antigens to FcR by liposomes (37), or by immune complexes (6,38), was shown to require proteosomal degradation and a functional TAP. This suggests an escape of antigens from phagosome to cytosol to be involved in FcγRI-mediated cross presentation. In addition, recent publications indicate an ER–phagosome fusion compartment to be an important cell organelle for cross presentation (39–41). This represents an additional route for loading of exogenous antigen-derived peptide on MHC class I molecules, which might be of importance for FcγRI-mediated cross presentation. Human FcγRI exists as a hetero-oligomeric receptor complex, with a ligand binding α-chain and a promiscuous FcR γ-chain homodimer (7,33). We previously documented hFcγRI to be unique within the leukocyte FcγR family, in that the α-chain exhibits the capacity to route endocytozed antigens to intracellular compartments positive for MHC class II (10). Whether the FcγRI α-chain is also involved in routing antigens to the MHC class I cross presenting pathway is currently under active investigation.

Dendritic cells express different subsets of Toll-like receptors, which enables them to react to diverse pathogens. TLR9 has been recognized as a receptor for CpG DNA (42). Several studies indicate that CpG are internalized and bind to TLR9, located within the endosome (43–45). Binding of TLR9 triggers cell signaling pathways including the mitogen activated protein kinases (MAPKs) and NF-κB (46). In DC this results in maturation, Th1-driven cytokine production and prolonged

survival (14,16,17). In the present study we demonstrate that CpG ODN can increase cross presentation of antigens selectively targeted to hFcγRI. Increased receptor-mediated uptake of antigens, improved hFcγRI-triggered cross presentation by transiently augmenting antigen processing, increased expression of co-stimulatory molecules or enhanced half-life of peptide–MHC complexes are putative mechanisms underlying the influence of CpG on antigen presentation (47).

In mice, TLR9 is expressed in both myeloid and plasmacytoid DC (pDC), whereas in humans expression is only apparent in pDC (48,49). In humans, hFcγRI is only expressed on myeloid DC, and whether combining CpG ODN and hFcγRI as target molecule on myeloid DC leads to efficient vaccination in humans remains to be shown. CpG-triggered IFN-α production by pDC, and subsequent activation of (hFcγRI-expressing) myeloid DC may represent an attractive concept for immunotherapy (50,51).

Due to the fact that DC represent the most potent initiators of immune responses (1,52), and are capable of funneling exogenous antigens into the MHC class I restricted antigen presentation pathway (5,53), they may represent tools for the development of future therapeutic concepts. Carefully designed combination strategies of CpG ODN and hFcγRI-targeting, which have shown promising results in murine models (54), may have potential to further improve DC-based vaccines.

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Abbreviations

BMDC	bone marrow-derived dendritic cell
CpG ODN	CpG oligodeoxynucleotides
DC	dendritic cell
FBS	fetal bovine serum
FcγR	receptor for Fc domain of IgG
GM-CSF	granulocyte macrophage colony stimulating factor
hFcγRI	human FcγRI
MAPK	mitogen activated protein kinase
NTg	non-transgenic
OVA	ovalbumin
pDC	plasmacytoid DC
RPE	r-phycoerythrin
Tg	transgenic
TLR9	Toll-like receptor 9

References

1 Cella, M., Sallusto, F. and Lanzavecchia, A. 1997. Origin, maturation and antigen presenting function of dendritic cells. *Curr. Opin. Immunol.* 9:10.
2 Rock, K. L. 1996. A new foreign policy: MHC class I molecules monitor the outside world. *Immunol. Today* 17:131.
3 Brossart, P. and Bevan, M. J. 1997. Presentation of exogenous protein antigens on major histocompatibility complex class I molecules by dendritic cells: pathway of presentation and regulation by cytokines. *Blood* 90:1594.
4 Rock, K. L., Gamble, S. and Rothstein, L. 1990. Presentation of exogenous antigen with class I major histocompatibility complex molecules. *Science* 249:918.
5 Mitchell, D. A., Nair, S. K. and Gilboa, E. 1998. Dendritic cell/macrophage precursors capture exogenous antigen for MHC class I presentation by dendritic cells. *Eur. J. Immunol.* 28:1923.
6 Regnault, A., Lankar, D., Lacabanne, V., Rodriguez, A., Thery, C., Rescigno, M., Saito, T., Verbeek, S., Bonnerot, C., Ricciardi-Castagnoli, P. and Amigorena, S. 1999. Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. *J. Exp. Med.* 189:371.
7 van Vugt, M. J. and van de Winkel, J. G. J. 2000. Fc receptors. Nature Publishing Group, London, UK.
8 Heijnen, I. A., van Vugt, M. J., Fanger, N. A., Graziano, R. F., de Wit, T. P., Hofhuis, F. M., Guyre, P. M., Capel, P. J., Verbeek, J. S. and van de Winkel, J. G. 1996. Antigen targeting to myeloid-specific human Fc gamma RI/CD64 triggers enhanced antibody responses in transgenic mice. *J. Clin. Invest.* 97:331.
9 Guyre, P. M., Graziano, R. F., Goldstein, J., Wallace, P. K., Morganelli, P. M., Wardwell, K. and Howell, A. L. 1997. Increased potency of Fc-receptor-targeted antigens. *Cancer Immunol. Immunother.* 45:146.
10 van Vugt, M. J., Kleijmeer, M. J., Keler, T., Zeelenberg, I., van Dijk, M. A., Leusen, J. H., Geuze, H. J. and van de Winkel, J. G. 1999. The FcgammaRIa (CD64) ligand binding chain triggers major histocompatibility complex class II antigen presentation independently of its associated FcR gamma-chain. *Blood* 94:808.
11 Keler, T., Guyre, P. M., Vitale, L. A., Sundarapandiyan, K., van De Winkel, J. G., Deo, Y. M. and Graziano, R. F. 2000. Targeting weak antigens to CD64 elicits potent humoral responses in human CD64 transgenic mice. *J. Immunol.* 165:6738.
12 Guyre, C. A., Barreda, M. E., Swink, S. L. and Fanger, M. W. 2001. Colocalization of Fc gamma RI-targeted antigen with class I MHC: implications for antigen processing. *J. Immunol.* 166:2469.
13 Bird, A. P. 1986. CpG-rich islands and the function of DNA methylation. *Nature* 321:209.
14 Krieg, A. M., Yi, A. K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., Koretzky, G. A. and Klinman, D. M. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374:546.
15 Liu, H. M., Newbrough, S. E., Bhatia, S. K., Dahle, C. E., Krieg, A. M. and Weiner, G. J. 1998. Immunostimulatory CpG oligodeoxynucleotides enhance the immune response to vaccine strategies involving granulocyte-macrophage colony-stimulating factor. *Blood* 92:3730.
16 Sparwasser, T., Koch, E. S., Vabulas, R. M., Heeg, K., Lipford, G. B., Ellwart, J. W. and Wagner, H. 1998. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur. J. Immunol.* 28:2045.
17 Hartmann, G., Weiner, G. J. and Krieg, A. M. 1999. CpG DNA: a potent signal for growth, activation and maturation of human dendritic cells. *Proc. Natl Acad. Sci. USA* 96:9305.
18 Vabulas, R. M., Pircher, H., Lipford, G. B., Hacker, H. and Wagner, H. 2000. CpG-DNA activates *in vivo* T cell epitope presenting dendritic cells to trigger protective antiviral cytotoxic T cell responses. *J. Immunol.* 164:2372.
19 Graziano, R. F., Tempest, P. R., White, P., Keler, T., Deo, Y., Ghebremariam, H., Coleman, K., Pfefferkorn, L. C., Fanger, M. W. and Guyre, P. M. 1995. Construction and characterization of a humanized anti-gamma-Ig receptor type I (Fc gamma RI) monoclonal antibody. *J. Immunol.* 155:4996.
20 Grant, E. P. and Rock, K. L. 1992. MHC class I-restricted presentation of exogenous antigen by thymic antigen-presenting cells *in vitro* and *in vivo*. *J. Immunol.* 148:13.
21 Shimonkevitz, R., Kappler, J., Marrack, P. and Grey, H. 1983. Antigen recognition by H-2-restricted T cells. I. Cell-free antigen processing. *J. Exp. Med.* 158:303.
22 Gillis, S., Ferm, M. M., Ou, W. and Smith, K. A. 1978. T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* 120:2027.

23 Kraal, G., Breel, M., Janse, M. and Bruin, G. 1986. Langerhans' cells, veiled cells and interdigitating cells in the mouse recognized by a monoclonal antibody. *J. Exp. Med.* 163:981.
24 Bhattacharya, A., Dorf, M. E. and Springer, T. A. 1981. A shared alloantigenic determinant on Ia antigens encoded by the I-A and I-E subregions: evidence for I region gene duplication. *J. Immunol.* 127:2488.
25 Graziano, R. F., Somasunduram, C. and Goldstein, J. 1998. The production of bispecific antibodies, p. 1. Springer-Verlag, New York, NY.
26 Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S. and Steinman, R. M. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176:1693.
27 te Velde, A. A., de Waal Malefijt, R., Huijbens, R. J., de Vries, J. E. and Figdor, C. G. 1992. IL-10 stimulates monocyte Fc gamma R surface expression and cytotoxic activity. Distinct regulation of antibody-dependent cellular cytotoxicity by IFN-gamma, IL-4 and IL-10. *J. Immunol.* 149:4048.
28 Fanger, N. A., Voigtlaender, D., Liu, C., Swink, S., Wardwell, K., Fisher, J., Graziano, R. F., Pfefferkorn, L. C. and Guyre, P. M. 1997. Characterization of expression, cytokine regulation and effector function of the high affinity IgG receptor Fc gamma RI (CD64) expressed on human blood dendritic cells. *J. Immunol.* 158:3090.
29 Guyre, C. A., Keler, T., Swink, S. L., Vitale, L. A., Graziano, R. F. and Fanger, M. W. 2001. Receptor modulation by Fc gamma RI-specific fusion proteins is dependent on receptor number and modified by IgG. *J. Immunol.* 167:6303.
30 Chen, B., Shi, Y., Smith, J. D., Choi, D., Geiger, J. D. and Mule, J. J. 1998. The role of tumor necrosis factor alpha in modulating the quantity of peripheral blood-derived, cytokine-driven human dendritic cells and its role in enhancing the quality of dendritic cell function in presenting soluble antigens to CD4+ T cells *in vitro*. *Blood* 91:4652.
31 Warren, T. L., Bhatia, S. K., Acosta, A. M., Dahle, C. E., Ratliff, T. L., Krieg, A. M. and Weiner, G. J. 2000. APC stimulated by CpG oligodeoxynucleotide enhance activation of MHC class I-restricted T cells. *J. Immunol.* 165:6244.
32 Datta, S. K., Redecke, V., Prilliman, K. R., Takabayashi, K., Corr, M., Tallant, T., DiDonato, J., Dziarski, R., Akira, S., Schoenberger, S. P. and Raz, E. 2003. A subset of Toll-like receptor ligands induces cross-presentation by bone marrow-derived dendritic cells. *J. Immunol.* 170:4102.
33 van de Winkel, J. G. and Capel, P. J. 1993. Human IgG Fc receptor heterogeneity: molecular aspects and clinical implications. *Immunol. Today* 14:215.
34 Honeychurch, J., Tutt, A. L., Valerius, T., Heijnen, I. A., Van De Winkel, J. G. and Glennie, M. J. 2000. Therapeutic efficacy of FcgammaRI/CD64-directed bispecific antibodies in B-cell lymphoma. *Blood* 96:3544.
35 van Spriel, A. B., van den Herik-Oudijk, I. E. and van de Winkel, J. G. 2001. Neutrophil Fc gamma RI as target for immunotherapy of invasive candidiasis. *J. Immunol.* 166:7019.
36 Wallace, P. K., Tsang, K. Y., Goldstein, J., Correale, P., Jarry, T. M., Schlom, J., Guyre, P. M., Ernstoff, M. S. and Fanger, M. W. 2001. Exogenous antigen targeted to FcgammaRI on myeloid cells is presented in association with MHC class I. *J. Immunol. Methods* 248:183.
37 Machy, P., Serre, K. and Leserman, L. 2000. Class I-restricted presentation of exogenous antigen acquired by Fcgamma receptor-mediated endocytosis is regulated in dendritic cells. *Eur. J. Immunol.* 30:848.
38 Rodriguez, A., Regnault, A., Kleijmeer, M., Ricciardi-Castagnoli, P. and Amigorena, S. 1999. Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells. *Nat. Cell Biol.* 1:362.
39 Ackerman, A. L., Kyritsis, C., Tampe, R. and Cresswell, P. 2003. Early phagosomes in dendritic cells form a cellular compartment sufficient for cross presentation of exogenous antigens. *Proc. Natl Acad. Sci. USA* 100:12889.
40 Guermonprez, P., Saveanu, L., Kleijmeer, M., Davoust, J., Van Endert, P. and Amigorena, S. 2003. ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. *Nature* 425:397.
41 Houde, M., Bertholet, S., Gagnon, E., Brunet, S., Goyette, G., Laplante, A., Princiotta, M. F., Thibault, P., Sacks, D. and Desjardins, M. 2003. Phagosomes are competent organelles for antigen cross-presentation. *Nature* 425:402.
42 Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K. and Akira, S. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408:740.
43 Wagner, H. 1999. Bacterial CpG DNA activates immune cells to signal infectious danger. *Adv. Immunol.* 73:329.
44 Wagner, H. 2001. Toll meets bacterial CpG-DNA. *Immunity* 14:499.
45 Takeda, K., Kaisho, T. and Akira, S. 2003. Toll-like receptors. *Annu. Rev. Immunol.* 21:335.
46 Krieg, A. M. 2002. CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* 20:709.
47 Askew, D., Chu, R. S., Krieg, A. M. and Harding, C. V. 2000. CpG DNA induces maturation of dendritic cells with distinct effects on nascent and recycling MHC-II antigen-processing mechanisms. *J. Immunol.* 165:6889.
48 Kadowaki, N., Ho, S., Antonenko, S., Malefyt, R. W., Kastelein, R. A., Bazan, F. and Liu, Y. J. 2001. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J. Exp. Med.* 194:863.
49 Krug, A., Towarowski, A., Britsch, S., Rothenfusser, S., Hornung, V., Bals, R., Giese, T., Engelmann, H., Endres, S., Krieg, A. M. and Hartmann, G. 2001. Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. *Eur. J. Immunol.* 31:3026.
50 Palucka, A. K., Banchereau, J., Blanco, P. and Pascual, V. 2002. The interplay of dendritic cell subsets in systemic lupus erythematosus. *Immunol. Cell Biol.* 80:484.
51 Gursel, M., Verthelyi, D. and Klinman, D. M. 2002. CpG oligodeoxynucleotides induce human monocytes to mature into functional dendritic cells. *Eur. J. Immunol.* 32:2617.
52 Banchereau, J. and Steinman, R. M. 1998. Dendritic cells and the control of immunity. *Nature* 392:245.
53 Bachmann, M. F., Lutz, M. B., Layton, G. T., Harris, S. J., Fehr, T., Rescigno, M. and Ricciardi-Castagnoli, P. 1996. Dendritic cells process exogenous viral proteins and virus-like particles for class I presentation to CD8+ cytotoxic T lymphocytes. *Eur. J. Immunol.* 26:2595.
54 van Ojik, H. H., Bevaart, L., Dahle, C. E., Bakker, A., Jansen, M. J., van Vugt, M. J., van de Winkel, J. G. and Weiner, G. J. 2003. CpG-A and B oligodeoxynucleotides enhance the efficacy of antibody therapy by activating different effector cell populations. *Cancer Res.* 63:5595.