Molecular Immunology 81 (2017) 52–58

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

CD14 is not involved in the uptake of synthetic CpG oligonucleotides

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ARTICLE INFO

Article history:
Received 2 October 2016
Received in revised form
14 November 2016
Accepted 18 November 2016
Available online 12 December 2016

Keywords:
Dendritic cells
CD14
CpG
DNA receptor
Pathogen pattern recognition receptor

ABSTRACT

We have previously shown that DEC205, a surface receptor expressed at high levels on $CD8^+$DC, is able to capture synthetic CpG oligonucleotides (ODN) and is required for optimal responsiveness. However, even in the absence of DEC205, $CD8^+$DC are able to respond to CpG ODN, albeit suboptimally. This suggested that additional receptors might contribute to the uptake of CpG ODN. CD14 represented an ideal candidate as it is expressed by DC and has been shown to bind and facilitate the uptake of CpG ODN. However, when CD14-deficient ($CD14^{-/-}$) mice and normal B6 mice were injected with CpG ODN, $CD8^+$DC were equivalently activated as assessed by the upregulation of the co-stimulatory molecules CD40 and CD80. Furthermore, the level of serum IL-6 and IL-12 produced in response to CpG ODN was comparable in $CD14^{-/-}$ and B6 mice. Importantly, mice deficient in both DEC205 and CD14 had comparable responses to mice lacking DEC205 alone, both in terms of cytokine production and DC activation, arguing that CD14 did not contribute to responses to CpG ODN. For CD14 to act as an uptake receptor for CpG ODN, it must first capture CpG ODN. To this end we assessed the capacity of cell surface CD14 to bind CpG ODN. Although we unequivocally confirmed that CD14 is required for the binding of its known ligand LPS, CD14 was not required for binding or responses to A-, B-, and C- Class CpG ODN. Our studies dispute the claim that CD14 is involved in CpG ODN capture.

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1. Introduction

The immune system utilizes an array of receptors to recognize pathogen associated molecular patterns (PAMP) (Kumar et al., 2011). Nucleic acids of pathogen origin are a clear indicator of invasion and an intricate network of innate sensors specialize in detecting such microbial DNA and RNA (Roers et al., 2016). Compared to mammalian DNA, bacterial and viral DNA has a high frequency of non-methylated cytosine-guanosine (CpG) motifs, that are readily detected by endosomal Toll-like receptor 9 (TLR9) and result in the initiation of pro-inflammatory responses (Kumar et al., 2011; Roers et al., 2016; Wagner, 2008). Synthetic oligodeoxynucleotide (ODN) can also activate TLR9 and mimic the immunostimulatory properties of bacterial DNA, and consequently, are attractive vaccine adjuvants. Extensive research has focused on maximizing the therapeutic potential of CpG ODN. Phosphorothioation of the ODN backbone imparts nuclease resistance thereby extending in vivo pharmacokinetics, and specific stimulatory motifs have been identified (Krieg, 2006, 2012). There are three major types of immuno-stimulatory ODN, (A-, B-, and C- Class) varying in their base pair composition, extent of phosphorothioation and biological activity (Krieg, 2012). Given that the stimulatory properties of CpG ODN require TLR9 (Vollmer et al., 2004), and that TLR9 largely resides in intracellular compartments (Kawai and Akira, 2010), it is unclear how extracellular CpG ODN gain access to this compartment. Various receptors have been implicated in the uptake of DNA, including the receptor for advanced glycation end-products (RAGE) (Sirois et al., 2013), the mannose receptor 1 (Moseman et al., 2013), KIR3DL2 on NK cells (Sivori et al., 2010), CXCL16 (Gursel et al., 2006) on plasmacytoid dendritic cells (pDC) and CD14 on macrophages and dendritic cells (DC) (Baumann et al., 2010). We have previously shown that DEC205

Abbreviations: EDTA, ethylenediamine tetra-acetic acid disodium salt; PE, phycoerythrin; SA, streptavidin; PBS, phosphate buffered saline; i.v., intravenous; LPS, lipopolysaccharide.

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http://dx.doi.org/10.1016/j.molimm.2016.11.015
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facilitates the uptake of B-Class ODN and is required for optimal cytokine production and DC activation (Lahoud et al., 2012). In the mouse, DEC205 is expressed at high levels on the cross-presenting $CD8^+$DC and accordingly, was required by this DC subset for optimal activation in response to CpG ODN. B cells also express DEC205, albeit at moderate levels, yet still rely on this receptor for optimal responses to CpG. However, even in the absence of DEC205, DC are partially activated in response to B-Class ODN, suggesting that other receptors could contribute to the uptake of these ODN. Of particular interest was CD14, a glycosylphosphatidylinositol-anchored, membrane-associated protein shown to be involved in the uptake of CpG ODN and required for optimal responses by bone marrow derived DC (BMDC) and macrophages (BMDM) (Baumann et al., 2010). CpG ODN was shown to bind CD14 by a competitive ELISA, where lipopolysaccharide (LPS), a well-established ligand of CD14, could block the CpG ODN-CD14 interaction. Furthermore, CD14 on the surface of peritoneal macrophages was also shown to contribute to the capture of CpG ODN. Since CD14 appeared to contribute to the response of BMDC to CpG ODN, we hypothesized that this receptor might facilitate the uptake of CpG in $DEC205^{-/-}$ DC, thereby explaining the remaining stimulatory effects of CpG ODN. Despite expression of CD14 by $CD8^+$DC, however, detailed *in vitro* and *in vivo* analyses failed to substantiate a role for CD14 in the capture or responsiveness to CpG ODN.

2. Materials and methods

2.1. Mice

C57BL/6J wehi (B6), $DEC\text{-}205^{-/-}$ (B6.129P-$Ly75^{tm1Mnz}$/J) and $CD14^{-/-}$ mice (Moore et al., 2000) were bred under specific pathogen free conditions at the Walter and Eliza Hall Institute (WEHI) or at the Burnet Medical Research Institute. Gender and age-matched mice were used and handled according to the guidelines of the National Health and Medical Research Council of Australia. Experimental procedures were approved by the Animal Ethics Committee, Melbourne Health Research Directorate.

2.2. CpG ODN

The following fully phosphorothioated 3'biotinylated ODN were purchased from Geneworks (Adelaide): 1668 B-Class CpG ODN: 5′TCC ATG ACG TTC CTG ATG CT; 1826 CpG ODN: 5′ TCC ATG ACG TTC CTG ACG TT; 2006 CpG ODN: 5′ TCG TCG TTT TGT CGT TTT GTC GTT; 2395 C-Class CpG ODN 5′ TCG TCG TTT TCG GCG CGC GCC G and the A-class ODN 2216, which was only phosophorothioated at the sites indicated by asterisk: 5′G*G*GGGACGATCGTC*G*G*G*G*G*G. For in vitro and in vivo experiments, 1668 CpG was also synthesized without the 3'biotin tag or with a 5′ Cy3-tag.

2.3. Isolation of DC and peritoneal macrophages

DC were isolated as described previously (Caminschi et al., 2007; Vremec et al., 2000). Briefly, spleens were chopped, digested with collagenase and DNAse at room temperature, and treated with EDTA. Low-density cells were obtained by density centrifugation (1.077 g/cm^3 Nycodenz) and non-DC-lineage cells were then depleted. Depletion of non-DC was achieved by labeling these cells with mAb (KT3-1.1, anti-CD3; T24/31.7, anti-Thy1; TER119, anti-erythrocytes; 1D3 anti-CD19; 1A8 anti-Ly6G) then removing them using anti-rat Ig magnetic beads (Biomag beads; QIAGEN). Peritoneal macrophages were obtained by flushing the peritoneum with media (2.5% FCS, RPMI-1640).

2.4. Antibodies and fluorescent staining

The following fluorochrome-conjugated mAb were used against mouse Ag: CD11c (N418-PeCy7, −PerCP5.5), CD4 (GK1.5-Pe.Cy7), CD8 (YTS169.4-APC), CD317 (120-G8-FITC), CD40 (FGK45.5-PE), CD80 (16-10.A1-PE; eBioscience), CD86 (PO3.1-PE; eBioscience), MHC class II (M5/114-PE), CD45R (B220-Pacific Blue; BD Bioscience), CD11b (M1/70-APC), F4/80-FITC, CD14-biotin (eBioscience), DEC205-biotin and isotype control IgG2a-biotin (eBioscience). Fc-mediated binding was blocked by pre-incubating cells (10 min 4 °C) with rat Ig and anti-FcR mAb (2.4G2). Biotin was detected using streptavidin (SA) conjugated to PE. Propidium iodide (PI) (0.5 μg/ml) was added to the final cell wash and PI positive dead cells were excluded from analysis. $CD8^+$DC ($CD11c^+CD8^+CD45R^-$ or $CD11c^+CD8^+CD317^-$), $CD8^-$DC ($CD11c^+CD8^-CD45R^-$ or $CD11c^+CD8^-CD317^-$,) pDC ($CD11c^{int}CD45R^+$ or $CD11c^{int}CD317^+$) and peritoneal macrophages ($CD11b^+F4/80^+$) were analyzed as indicated in the Figure legends. Flow cytometry analysis was performed on a FACSCalibur or LSR II (Becton Dickinson, San Jose, CA).

2.5. Cytokine assay

In vivo production of cytokines was measured using Mouse Th1/Th2/Th17/TH22 13plex Kit Flow cytomix (eBioscience) according to manufacturer's guidelines. Serum IL-12 was quantitated by ELISA. Plates (Costar, Broadway, Cambridge, UK) were coated overnight at 4 °C with 0.5–1 μg/ml of anti-IL-12p70 mAb (R29A5) in PBS. Unbound protein was washed away (PBS, 0.05% Tween-20), and plates were blocked for 2 h at room temperature (0.3% BSA/PBS). Serum samples or serial dilutions of recombinant IL-12 (R&D Systems) were incubated at 4 °C overnight. Bound IL-12 was detected using biotinylated anti-IL-12p40 mAb (C17.8) and SA-HRP, then visualized using ABTS.

2.6. CpG ODN binding assays

Peritoneal cells were incubated with either biotinylated LPS (InvivoGen), biotinylated ODN (1668, 1826, 2006, 2216, 2395) or 1668-CpG-Cy3 for 30 min at 4 °C in 2% FCS/BSS-EDTA (BSS-EDTA: 150 mM NaCl, 3.75 mM KCl, 5 mM EDTA), or with 1668-CpG-Cy3 for 1 h at 37 °C in complete media (modified RPMI-1640 medium containing 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin, 10^{-4} M B-2-mercaptoethanol). Cells were then washed in 2% FCS/BSS-EDTA and counterstained with CD11b (with or without SA-PE) before analysis by flow cytometry.

2.7. Statistical analysis

Unpaired *t*-test was performed on the data. The significance of differences is listed as follows: not significant (n/s), $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Analysis was performed in Prism (GraphPad Sofware, Inc).

3. Results

We have previously shown that DEC205 binds synthetic CpG oligonucleotides, facilitates uptake, and is required for optimal responses (Lahoud et al., 2012). However, $DEC205^{-/-}$ DC are able to respond to CpG ODN, albeit sub-optimally suggesting that other receptors may be involved (Lahoud et al., 2012). CD14 was recently shown to bind CpG ODN and act as an uptake receptor (Baumann et al., 2010). To determine whether CD14 could explain the stimulatory activity of CpG in $DEC205^{-/-}$ mice, we compared the expression pattern of DEC205 and CD14 on DC (Fig. 1). DEC205 is expressed at high levels on $CD8^+$DC, at low levels on $CD8^-$DC, and is

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