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Research Paper

CXCL14 Acts as a Specific Carrier of CpG DNA into Dendritic Cells and Activates Toll-like Receptor 9-mediated Adaptive Immunity

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ABSTRACT

CXCL14 is a primordial chemokine that plays multiple roles in tumor suppression, autoimmune arthritis, and obesity-associated insulin resistance. However, the underlying molecular mechanisms are unclear. Here, we show that CXCL14 transports various types of CpG oligodeoxynucleotide (ODN) into the endosomes and lysosomes of bone marrow-derived dendritic cells (DCs), thereby activating Toll-like receptor 9 (TLR9). A combination of CpG ODN (ODN2395) plus CXCL14 induced robust production of IL-12 p40 by wild-type, but not Tlr9-knockout, DCs. Consistent with this, ODN2395-mediated activation of DCs was significantly attenuated in Cxcl14-knockout mice. CXCL14 bound CpG ODN with high affinity at pH 7.5, but not at pH 6.0, thereby enabling efficient delivery of CpG ODN to TLR9 in the endosome/lysosome. Furthermore, the CXCL14-CpG ODN complex specifically bound to high affinity CXCL14 receptors on DCs. Thus, CXCL14 serves as a specific carrier of CpG DNA to sensitize TLR9-mediated immunosurveillance.

1. Introduction

Innate immune responses are initiated by microbe-derived products known as pathogen-associated molecular patterns (PAMPs) (Blasius and Beutler, 2010; Kawai and Akira, 2010). PAMPs activate pattern recognition receptors (PPRs) expressed on or in dendritic cells (DCs), thereby triggering inflammatory reactions, interferon responses, and DC maturation; these are followed by activation of the adaptive immune response that completely eliminates the pathogen (Blasius and Beutler, 2010; Kawai and Akira, 2010). Among the known PPRs, Toll-like receptor 9 (TLR9) recognizes unmethylated cytosine-phosphate guanine (CpG)-containing DNA (CpG DNA) present in bacterial DNAs; this type of DNA is less abundant in mammalian genomic DNA (Hemmi et al., 2000; Ishii and Akira, 2006). Thus, unmethylated CpG DNA acts as a sensor for TLR9-mediated immunosurveillance (Krug et al., 2004a,b; Lund et al., 2003; Tabeta et al., 2004).

Basic and clinical research studies have used synthetic CpG oligodeoxynucleotide (CpG ODN) as a ligand for TLR9 (Krieg, 2006; Ishii and Akira, 2006). Agonistic CpG ODN molecules fall into four main categories, A, B, C, and P, based on their structure and function. A-class CpG ODNs possess poly-G and palindromic sequences that have strong interferon-α-inducing activity in plasmacytoid DCs (pDCs). B-class CpG ODNs do not contain palindromic sequences and predominantly act on B cells. C-class CpG ODNs have the properties of both A-class and B-class molecules, and include palindromic sequences at the 3′-end. P-class CpG ODNs have two copies of a palindromic sequence and are more potent than C-class molecules with respect to induction of cytokine production.

Since TLR9 is localized within the endosomes and lysosomes of DCs, CpG DNA must be internalized prior to binding. CpG ODNs bind to DEC205 and mannose receptors to trigger TLR9-dependent responses (Lahoud et al., 2012; Caminschi et al., 2013; Moseman et al., 2013). Granulin, HMGB1, LL-37, and β-defensin also facilitate incorporation of CpG ODNs (Park et al., 2011; Ivanov et al., 2007; Hurtado and Peh, 2010; Tewary et al., 2013). Some of these secreted factors directly bind to CpG ODNs (Park et al., 2011; Ivanov et al., 2007; Tewary et al., 2013); however, it is not clear how CpG ODNs are transported into DCs to activate the TLR9 signaling.

CXCL14 is a non-ELR (glutamic acid-leucine-arginine) chemokine with multiple immunological functions (Lu et al., 2016; Hara and Tanegashima, 2012). At very high concentrations, CXCL14 acts as a

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chemoattractant for DCs, activated macrophages, and NK cells (Hara and Tanegashima, 2012). Consistent with this, CXCL14 promotes NK cell-mediated suppression of transplanted melanoma cell metastasis in a CXCL14 transgenic mouse line (Hata et al., 2015). CXCL14 transgenic mice with collagen-induced arthritis show augmented Th1 responses (Chen et al., 2010). Conversely, Cxcl14-knockout (KO) mice show weaker inflammatory responses when they become obese (Nara et al., 2007). Although these results clearly indicate that CXCL14 is involved in Th1 immune responses, the underlying molecular mechanisms require clarification.

Since CXCL14 is abundantly present in mucosal tissues (the Human Protein Atlas: http://www.proteinatlas.org/ENSG00000145824-CXCL14/tissue) and displays anti-microbial activity, we speculated that CXCL14 might be involved in innate immunity. Here, we demonstrate that CXCL14 specifically binds CpG DNAs, delivers them to the endosomal/lysosomal compartments in DCs via receptor internalization, and efficiently activates TLR9-mediated cytokine production and DC maturation.

2. Materials and Methods

2.1. Mice and Cell Preparation

C57BL/6N mice were obtained from Nihon SLC (Hamamatsu, Japan). As previously described (Tanegashima et al., 2010), *Cxcl14*$^{-/-}$ male and Cxcl14$^{+/-}$ female mice with a C57BL/6 N background were crossed to produce Cxcl14$^{+/-}$ and *Cxcl14*$^{-/-}$ mice. LysM-Cre knock-in mice (Clausen et al., 1999) were obtained from RIKEN (RBRC02302), and CD11c-Cre (stock number 007567) and Cxcr4$^{F/F}$ (stock number 008767) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Tlr9-KO mice (Hemmi et al., 2000) were obtained from Oriental Bioservice, Inc. (Kyoto, Japan).

BMDCs were prepared from 6-week-old C57BL/6N, Tlr9-KO, and Cxcr4-CKO mice by culturing bone marrow cells in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA), mouse granulocyte-macrophage colony stimulating factor (10 ng/ml; Peprotech, Rocky Hill, NJ), and mouse IL-4 (10 ng/ml; Peprotech) for 10 days. Splenocytes were prepared from spleen removed from 6 to 8 week-old C57BL/6N mice. Red blood cells were lysed in RBC lysis buffer (140 mM NH_4Cl, 17 mM Tris-HCl, pH 7.6). All mice were housed in a pathogen-free animal facility under a 12 h light/dark cycle. All experimental procedures were pre-approved by the ethical committee of Tokyo Metropolitan Institute of Medical Science.

2.2. Reagents

Endotoxin-free ODN2395 was purchased from InvivoGen (San Diego, CA). ODN2395mut (phosphorothioester modified sequence), 5′Cy3-ODN2395, 5′Cy3-ODN2395(p), 5′Cy3-ODN2395mut(p), ODN1826, ODN1826(p), 5′Cy3-ODN1826, 5′Cy3-ODN1826(p), ODN1585(p), 5′Cy3-ODN1585(p), ODN-D-SL03, 5′Cy3-ODN-D-SL03, ODN21798, 5′Cy3-ODN21798, ODN1668, ODN1668(p), 5′Cy3-ODN1668, 5′Cy3-ODN1668(p), and 5′Cy3-D35 were synthesized by Eurofins Genomics (Tokyo, Japan). D35 was purchased from Gene design (Osaka, Japan). CXCL14, CXCL14-K-biotin (referred to as CXCL14-bio in this study), and CXCL14-Alexa488 were chemically synthesized (Tsuji et al., 2015) based on the human CXCL14 sequence (Supplementary methods and Fig. S1). Synthetic CXCL14 was as active as recombinant CXCL14 in terms of the inhibition of CXCL12-mediated cell migration. The endotoxin level of the synthetic CXCL14 preparation was <0.1 EU/mg, as measured by a LAL Quantitation Kit (Thermo Fisher Scientific). LPS, Poly-U, and Poly-IC were purchased from Sigma-Aldrich (St. Louis, MO).

2.3. Antibodies

The following antibodies/conjugates were purchased: Allophycocyanin (APC)-conjugated anti-CD3ε (TONBO, Cat# 20–0032, RRID:AB_2621538, Burlingame, CA), fluorescein isothiocyanate (FITC)-conjugated anti-CD8α (BioLegend, Cat# 100706, RRID:AB_312745, San Diego, CA), APC-rat anti-CD45R/B220 (BioLegend, Cat# 103212, RRID:AB_312997), Brilliant Violet (BV) 421-conjugated anti-CD11c (BioLegend, Cat# 117329 RRID:AB_10897814), AlexaFluor488-anti-CD11b (BioLegend), FITC-anti-CD19 (BioLegend, Cat# 152404 RRID:AB_2629813), FITC-anti-CD3ε (BioLegend, Cat# 100203 RRID:AB_312660), APC-anti-CD184 (CXCR4; BioLegend, Cat# 146508 RRID:AB_2562785), phycoerythrin (PE)-conjugated anti-MHC class II (BD Biosciences, San Jose, CA), PE-anti-CD86 (BioLegend, Cat# 105106 RRID:AB_313159), mouse anti-EEA1 (eBioscience, San Diego, CA), Alexa488-labeled goat anti-mouse IgG, and FITC-anti-LAMP1 (BioLegend, Cat# 121606 RRID:AB_572007).

2.4. FACS Analysis

BMDCs were stained with a BV421-labeled anti-CD11c antibody to confirm DC differentiation. Splenic cells were stained to identify CD8$^+$ cDCs (BV421-CD11c$^+$/FITC-CD8$^+$/APC-CD3ε$^-$/APC-CD45R/B220$^-$), CD11b$^+$ cDCs (BV421-CD11c$^+$/Alexa488-CD11b$^+$/APC-CD3ε$^-$/APC-CD45R/B220$^-$), and pDCs (BV421-CD11c$^+$/FITC-CD19$^-$/FITC-CD3ε$^-$/APC-CD45R/B220$^+$). Cells were analyzed using a LSR Fortessa X-20 cytometer (BD Biosciences).

2.5. FACS and Microscopic Analyses of Incorporated CpG ODNs

BMDCs were plated onto 24 well plates (10^5 cells/well; Corning, Corning, NY) and incubated for 1 h at 37 °C with Cy3-ODNs in the presence or absence of CXCL14 or CXCL12 in RPMI-1640 medium containing 20 mM Hepes-NaOH, pH 7.5, and 0.1% BSA (Sigma, fatty acid free). Cells were then trypsinized, stained with BV421-labeled anti-CD11c, and analyzed by FACS.

For confocal microscopy analysis, BMDCs were plated onto 35 mm glass plates (IWAKI, Tokyo, Japan) and incubated for 1 h at 37 °C with CXCL14-Alexa488, Cy3-ODN2395, or Cy3-ODN2395 + CXCL14 in RPMI-1640/20 mM Hepes-NaOH pH 7.5/0.1% BSA. Cells were then fixed with 4% paraformaldehyde/PBS for 1 h, permeabilized in 0.04% Saponin/PBS (Wako, Osaka, Japan), and stained with anti-EEA1 or anti-LAMP1 antibodies. Fluorescent images were analyzed using a Leica TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany).

2.6. Enzyme-Linked Immunoassay (ELISA)

BMDCs were plated onto 24-well plates (10^5 cells/well) (Corning) and incubated in RPMI-1640/20 mM Hepes-NaOH pH 7.5/0.1% BSA for 6 h at 37 °C with CpG ODNs in the presence or absence of CXCL14 or CXCL12. Culture supernatants were then tested using ELISA MAX™ Kits (BioLegend).

2.7. In Vitro Binding Assay

CXCL14-bio (10 pmol) was coupled to streptavidin-agarose (Sigma-Aldrich) and incubated with Cy3-ODN [100 nM in 100 μl of binding buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% BSA)] for 1 h at 4 °C. Precipitates were then washed and eluted in SDS sample buffer at 70 °C for 10 min. Cy3-ODN was separated by TBE-Urea-SDS polyacrylamide gel electrophoresis, and Cy3 fluorescence was measured in a LAS-3000 (Fuji Film, Tokyo, Japan). CXCL14-bio was blotted onto a PVDF membrane and incubated with peroxidase-conjugated streptavidin (GE Healthcare, Pittsburgh, PA). Chemiluminescence detection was performed using the ECL Plus detection reagent (GE Healthcare), and signals were measured in a LAS-3000.

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