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Cutting Edge: Antibody-Mediated TLR7-Dependent Recognition of Viral RNA

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TLR7 recognizes the genome of ssRNA viruses such as Coxsackievirus B. Because TLR7 is expressed in intracellular compartments, viral RNA must be internalized before its recognition by TLR7. In this study, we define plasmacytoid dendritic cells (pDC) as peripheral blood mononuclear immune cells that respond to Coxsackievirus. pDC activation by Coxsackievirus B requires the presence of specific antiviral Abs. We show that Fc receptors mediate the recognition of virus-Ab complexes and that TLR7 is required for human and murine pDC production of cytokines. These data define a pathway by which intracellular TLR7 senses viral RNA and indicate a role for TLRs in association with Abs in sustaining virus-specific responses. The Journal of Immunology, 2007, 178: 3363–3367.

Plasmacytoid dendritic cells (pDC) are highly specialized cells that are important during viral infection, where they are recruited and can produce IFN-α (1). TLRs are crucial mediators in innate immune responses to pathogens. Of at least 11 TLR family members, TLR7 and TLR9 are expressed in pDC. These endosomal pattern-recognition receptors recognize viral genomic ssRNA and dsDNA, respectively, and engagement leads to the production of cytokines including IFN-α (3–5). IFN-α may be protective in the host response to many viruses, but proinflammatory cytokines activated by TLRs have the potential to adversely affect the host.

Igs play an essential role in the host defense against pathogens. However, they can also contribute to autoimmune diseases like systemic lupus erythematosus, in which high levels of Abs against nuclear Ags are present. Immune complexes (IC) comprised of either small nuclear ribonucleoprotein particles or chromatin can stimulate pDC to produce high amounts of IFN-α via TLR7 or TLR9, a phenomenon mediated by FcR (6–9). IC may contribute to ssRNA viral diseases, including “atypical” measles, dengue hemorrhagic fever, and enhanced respiratory syncytial virus disease. Prior immune sensitization to such viruses can lead to overwhelming cytokine production and influx of inflammatory cells, and IC deposition has been implicated as a major disease-causing factor (10–14). Furthermore, recent studies have shown that IFN-α induction by certain viruses is enhanced in the presence of virus-specific Ig and is mediated by FcR (15, 16).

We define a role for virus-specific Ig activation of TLR7 by Coxsackievirus B (CVB), a nonenveloped, ssRNA picornavirus of the enterovirus group. CVB is commonly associated with subclinical human disease but can cause acute or chronic myocarditis. Myocardial damage may result from either cytopathic effects of CVB or from immune-mediated destruction (reviewed in Ref. 17). In our studies, we generated murine bone marrow-derived DC (BMDC) and found that they produce IFN-α and IL-12 p40 in response to CVB serotype 3 (CVB3) only in the presence of anti-CVB Abs. Human pDC also produce IFN-α in response to CVB3 only in conjunction with Ig. We demonstrate that both TLR7 and FcR participate in the virus-induced production of IFN-α by both murine and human pDC and that the presence of virus-specific Ab is essential.

Materials and Methods

Reagents

CVB3 strain Nancy was prepared as described previously (18) and for some experiments was UV-inactivated with 1 J/cm² UVA light for 15 min. R-848 was a gift from D. Golenbock (University of Massachusetts, Worcester, MA). CpG 2336 was purchased from Coley Pharmaceuticals. Human AB serum was a gift from D. Golenbock (University of Massachusetts, Worcester, MA). CpG 2336 was purchased from Coley Pharmaceuticals. Human AB serum was obtained from Sigma-Aldrich and Sandoglobulin intravenous immune globulin (IVIG) from Novartis. Anti-CVB3 mAb and IgG1 were purchased from Chemicon International, anti-CD64 and IgG2a from eBioscience, anti-CD32 from BD Pharmingen. Immunoregulatory DNA sequence (IRS) 661 was synthesized on a phosphorothioate backbone by MWG Biotech.

Mice

FcγRII−/− (B6.129P2-FcγRII−/−) mice and FcγRII−/− (B6.129-FcγRII−/−) mice, each backcrossed 10 generations to C57BL/6, were obtained from The Jackson Laboratory.
Jackson Laboratory, Fc receptor common γ-chain-deficient (B6.129P2-FcεR1tm1; backcrossed 12 generations to C57BL/6) mice were obtained from Taconic Farms. MyD88–/– (backcrossed 12 generations to C57BL/6) and TLR7–/– mice (backcrossed 7 generations to C57BL/6) were a gift from S. Akira (Osaka University, Osaka, Japan). Serum was obtained from whole blood of wild-type (WT) mice 12 days after infection with CVB3 (5000 PFU i.p.) or from uninfected control mice.

Cell culture

Human PBMC and pDC were isolated as previously described (19). Mouse bone marrow cells were cultured for 7 days with 10 ng/ml mouse fms-related tyrosine kinase 3 ligand (R&D Systems), resulting in a population of BMDC with 30–40% pDC by FACS staining. For some experiments, DC were sorted on a FACS Aria (BD Biosciences) to isolate pDC (CD11c+CD11b+/CD45R+). Mouse pDC purity was ≥98%.

Cytokines

Cell culture supernatants were collected at 18 h. ELISA was used to quantify human IFN-α (Bender MedSystems), murine IL-12 p40 (BD Biosciences), and murine IFN-α (PBL Biomedical Laboratories). Luminex testing was performed at the Baylor Luminex National Institute of Allergy and Infectious Diseases Core facility (Dallas, TX) for fibrolast growth factor basic, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, IFN-γ-inducible protein 10, KC, MCP-1, MIG, MIP-1α, TNF-α, and vascular endothelial growth factor.

Results and Discussion

Murine pDC activation by CVB3 requires specific anti-Coxsackievirus Ab

We examined Ab-mediated responses to CVB3 by murine BMDC. Incubation of bone marrow cells with fms-related tyrosine kinase 3 ligand results in a population of cells rich in pDC which secrete IL-12, IFN-α, and other cytokines in response to TLR7 and TLR9 ligands, including ssRNA viruses (3, 5, 20–22). We found that CVB3 did not induce IL-12 p40 production in BMDC unless opsonized with ≥1 µg/ml mAb specifically targeting CVB3. This combination led to vast production of IL-12 p40 (Fig. 1). At these concentrations, mAb neutralized CVB3, causing a decrease in viral plaque formation in HeLa cells by nearly 100-fold (data not shown). To confirm that in vivo infection leads to generation of specific antiviral Abs, we infected a mouse with CVB3 i.p. and collected serum 12 days later. Opsonization of CVB3 with this serum induced a strong IL-12 p40 response from DC, whereas opsonization with serum from a CVB3-naïve, uninfected mouse did not. Under these conditions, UV-treated virus did not induce cytokines; either viral replication is required for DC activation or UV treatment alters the structure of viral RNA such that it can no longer engage with TLR7.

Since BMDC are a mixed population, we used FACS to isolate a pure population of pDC and found that pDC produced cytokines in response to virus only in the presence of specific Abs. Using Luminex multiplex testing, we found several cytokines to be released by pDC, including IL-6, IL-12 p40, IFN-γ-inducible protein 10, and MIP-1α (data not shown). All other tested cytokines were negative (see Materials and Methods).

Murine pDC activation by CVB3 IC requires expression of FcR common γ-chain

We then asked whether opsonized CVB3 triggers cytokine responses by engagement with FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16), or FcγRIV (reviewed in Ref. 23). We used BMDC from FcγRI chain−/−, FcγRII−/−, and FcγRIII−/− mice to establish the role of FcR in the recognition of opsonized CVB3. Cells from FcγRI chain−/− mice lack the γ-chain subunit of FcR, which is essential for cell surface expression of FcγRI, FcγRIII, and FcγRIV. FcγRI chain−/− DC did not produce any IL-12 p40 or IFN-α in response to CVB3 opsonized with anti-CVB3 mAb (Fig. 2a), whereas DC from FcγRII−/− and FcγRIII−/− mice produced comparable amounts of cytokine to WT DC (Fig. 2b). Identical FcR requirements were observed with virus opsonized with serum from a CVB3-infected mouse (data not shown). Thus, either FcγRI or FcγRIV is required for cytokine induction by opsonized CVB3 in murine cells. This requirement is distinct from that of chromatin IC, which utilize murine FcγRIII receptors for TLR9-mediated DC production of IL-12 p70 and TNF-α (8).

Murine DC activation by opsonized CVB3 requires TLR7 and MyD88

CVB, specifically its ssRNA genome, has been shown to interact with TLR7 and contribute to the production of inflammatory cytokines (24). We determined that TLR7 and the adapter molecule MyD88 are required for murine cytokine responses to opsonized CVB3 by using DC from mice deficient in these molecules. Induction of IL-12 p40 and IFN-α was absent from the cells from knockout mice (Fig. 2c); therefore, FcR-mediated delivery of viral RNA to intracellular TLR7 is critical for cytokine responses by pDC.

pDC are the primary responders among human PBMC to opsonized CVB3

Human PBMC demonstrate Ab-enhanced production of IFN-α in response to CVB (25). We examined IFN-α production in human PBMC in response to opsonized CVB3, testing three sources of Ig: autologous serum, serum from human male blood type AB (human AB serum), and pooled IVIG. Opsonization was performed by incubating virus with an equal volume of serum or IVIG for 15 min at 37°C before addition to cells. Each enabled dose-dependent production of IFN-α in response to live, but not UV-irradiated opsonized CVB3 (Fig.
Unopsonized virus did not lead to any IFN-α production. Of note, in three independent experiments using PBMC from different adult donors, virus opsonized with autologous serum induced IFN-α. Nearly all adults are seropositive for CVB3, accounting for the presence of specific anti-CVB IgG in serum and IVIG (26).

Given that pDC are strong IFN-α secretors, we suspected that pDC were responsible for the majority of the IFN-α produced by PBMC in response to CVB3. To generate a population of PBMC depleted of pDC, we added anti-CD304 MicroBeads to cells and passed them through a MACS Separator (Miltenyi Biotec). The resulting population of PBMC was largely devoid of pDC. When compared with the total PBMC population, pDC-depleted PBMC produced negligible IFN-α in response to opsonized CVB3 (Fig. 3b). pDC-depleted PBMC had equivalent IL-6 production following stimulation with the TLR2 ligand Pam3CSK4 (100 ng/ml) in comparison to total PBMC (16.1 ± 1.8 ng/ml vs 11.6 ± 2.7 ng/ml IL-6); therefore, altered viability of the pDC-depleted PBMC was not a contributing factor. Of positively selected pDC, high amounts of IFN-α were produced in response to opsonized CVB3 at a multiplicity of infection (MOI) of 30 (42.7 ± 8.8 ng/ml), whereas CVB3 alone did not induce IFN-α.

Human pDC activation by CVB3 is mediated by CD32 and TLR7

Recent studies investigating mechanisms of TLR activation have shown that CD32a (FcγRIIa) is important for the activation of human pDC by IC involving small nuclear ribonucleoprotein particle or systemic lupus erythematosus-DNA (6, 7, 9). To establish the role for FcR in human pDC recognition of opsonized CVB3, we incubated pDC in the presence of neutralizing Abs against CD16 (FcγRIII), CD32a (FcγRIIa), or CD64 (FcγRI) for 30 min before addition of stimuli. We found IFN-α responses to be mediated by CD32a, but not CD16 or CD64; results from a representative donor are shown in Fig. 4a.

In experiments using multiple donors, anti-CD32a mAb (0.5 μg/ml) blocked virus-induced IFN-α production to 54% compared with isotype control (n = 5, p < 0.01 by Student’s t test). Induction of IFN-α by R-848 was unaffected by the presence of neutralizing Ab (data not shown). Mice are naturally deficient in the CD32a isoform and only express the inhibitory FcγRIIb (38, 39).

FIGURE 3. IFN-α production by human PBMC in response to CVB3 requires opsonization of virus and is predominantly from the pDC population. A, CVB3 (MOI, 100, 10, or 1) was opsonized with autologous serum, human IVIG, or human AB serum. For some conditions, CVB3 was UV-irradiated before opsonization. Results are representative of three independent experiments with different human donors. B, PBMC and PBMC depleted of pDC were stimulated with CVB3 opsonized with autologous serum. Controls included R-848 (20 μM) and CpG (10 μg/ml).
cytokines could be associated with adverse outcomes. In a model of CVB3-induced myocarditis, MyD88-deficient mice had improved survival, decreased cardiac inflammation, and decreased levels of inflammatory cytokines in comparison to WT mice (29). Further in vivo studies will define the role for FcR and TLR7 in the presence of virus-specific Ab and may identify mechanisms by which immune sensitization to virus can cause disease.

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Disclosures

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