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Osteopontin Is Not Required for the Development of Th1 Responses and Viral Immunity

Brian Abel,* Stefan Freigang,‡ Martin F. Bachmann,† Ursula Boschert,§ and Manfred Kopf*‡

Osteopontin (OPN) has been defined as a key cytokine promoting the release of IL-12 and hence inducing the development of protective cell-mediated immunity to viruses and intracellular pathogens. To further characterize the role of OPN in antiviral immunity, OPN-deficient (OPN−/−) mice were analyzed after infection with influenza virus and vaccinia virus. Surprisingly, we found that viral clearance, lung inflammation, and recruitment of effector T cells to the lung were unaffected in OPN−/− mice after influenza infection. Furthermore, effector status of T cells was normal as demonstrated by normal IFN-γ production and CTL lytic activity. Moreover, activation and Th1 differentiation of naive TCR transgenic CD4+ T cells by dendritic cells and cognate Ag was normal in the absence of OPN in vitro. Contrary to a previous report, we found that OPN−/− mice mounted a normal immune response to Listeria monocytogenes. In conclusion, OPN is dispensable for antiviral immune responses against influenza virus and vaccinia virus. The Journal of Immunology, 2005, 175: 6006–6013.

To effectively protect a mammalian host from colonization by intracellular pathogens, the immune system has evolved an adaptive response characterized by the generation of CD4 Th1 cells and CTLs. These Th1 cells are required for the induction of Ig class switching to generate Abs, and provide help for CTLs, which engage infected cells and lyse them. To trigger a rapid and effective type-1 cell-mediated immune response, the cytokine milieu should be dominated by the production of the Th1-inducing cytokine, IL-12. The extracellular matrix protein osteopontin (OPN), as also known as Eta-1 (early T lymphocyte activation-1), has been reported to induce a strong IL-12 response from macrophages via interactions with the α,β, integrin and to inhibit the anti-inflammatory cytokine IL-10 through CD44 engagement (1). Ashkar et al. (1) described an impairment in the ability of OPN-deficient mice to mount a robust immune response against HSV type 1 (HSV-1) and the intracellular pathogen, Listeria monocytogenes. OPN−/− mice have also been reported to have attenuated host resistance against Mycobacterium bovis bacillus Calmette-Guérin (2) but, unexpectedly, normal host defense against Borrelia burgdorferi and the concurrent development of arthritis (3). Murine deficiency in OPN has been demonstrated to ameliorate the development of several autoimmune diseases including experimental autoimmune encephalomyelitis (EAE) (4, 5), anti-type II collagen Ab-induced arthritis (CAIA) (6), and collag

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3 Abbreviations used in this paper: OPN, osteopontin; EAE, experimental autoimmune encephalomyelitis; CAIA, anti-type II collagen Ab-induced arthritis; CIA, collagen-induced arthritis; DC, dendritic cell; LCMV, lymphocytic choriomeningitis virus; sm2, smarta2; tg, transgenic; BHI, brain heart infusion; B/AL, bronchoalveolar lavage.

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amplified by specific PCR for OPN exons 6–7. A hypoxanthine phosphoribosyltransferase PCR was performed to ensure the integrity of the cDNAs. Primers used for amplification of OPN transcripts were as follows: Ex6for, 5′-AATGCTGCTTACAGCTGGAT-3′, and Ex7rev, 5′-TAGGGTCTAAGGCTAGTCTG-3′, which yields a 410-bp cDNA fragment. The hypoxanthine phosphoribosyltransferase reaction was used as a control for the quality of the cDNA. PCR were performed as described by Liaw et al. (10).

**Viruses, bacteria, and mouse infections**

Infectious stocks of the PR8 strain of influenza virus were grown and purified, and viral titers were determined on MDCK cells as described (12). Vaccinia virus WR-G2 (VV-G2) expressing the glycoprotein of LCMV was provided by M. Bachmann (13), and titers were determined using the plaque assay on BSC40 cells (13). L. monocytogenes (wild-type strain L10403S) was grown from frozen stocks in brain heart infusion (BHI) medium. Titers of viable bacteria in the inoculum were determined by plating serial dilutions on BHI agar plates. Mice were infected intranasally with 50 PFU of influenza PR8 virus (50 μl; administrations; two times), or i.p. with 2 × 10⁶ PFU of VV-G2. For Listeria infections, mice were injected i.v. with 8000 CFU.

**Cell lines and peptides**

EL-4 thymoma cells (H2b) and MC57 fibroblasts were used as targets for the in vitro Cr release assays of influenza virus and VV-G2 virus infections, respectively. MDCK cells and BSC-40 cells were used to determine the titer of influenza virus and VV-G2 virus, respectively. All cells were grown in IMDM (BioWhittaker) containing 5% FCS and supplemented with 2-ME and l-glutamine. LCMV glycoprotein-derived peptide gp61 (sequence GLNGPDIYKGVYQQSKVFD; aa 61–80) was synthesized by a solid-phase method and purchased from Eurogentec. Leptospira bacteria (PN68 [ASNNMDAM]; 366–374; H2-Kb-restricted) was derived from the influenza virus nucleoprotein of the 1968 strain A/HK/8/68. The Listeria peptid (VAYGRQVYL; aa 296–304; H2-Kb-restricted) was derived from the listeriolysin (O) protein of L. monocytogenes (14).

**Collection and analysis of bronchoalveolar lavage (BAL) cells**

At days indicated, influenza-infected mice were killed by CO2 inhalation, a tracheal cannula was inserted, and BAL was performed with four 0.3-ml aliquots of PBS. The total number of leukocytes was determined using a Coulter Counter (IC Instruments). BAL cell suspensions were spun down, the supernatant was harvested for the measurement of Ab levels, and cells were resuspended for in vitro culturing or ex vivo FACS analysis. BAL cells (5 × 10⁷) were isolated and spun onto glass slides in PBS with 20% BSA by centrifugation (600 × g; 10 min). Cells were differentially stained with May-Grünewald-Giemsa staining: After fixation with methanol (96%; 2.5 min) cells were stained with undiluted May-Grünewald solution (Fluka) for 3 min. Thereafter, the staining was performed in a 50% May-Grünewald/Giemsa staining: After fixation with methanol (96%; 2.5 min) and 3 min. Thereafter, the staining was performed in a 50% May-Grünewald/Giemsa staining: After fixation with methanol (96%; 2.5 min) and then May-Grünewald solution for an additional 3 min. In a last step, cells were stained in 7% Giemsa solution (Fluka) for 10–12 min. Slides were rinsed with tap water and air dried overnight. Dried cells were embedded within Eukit solution under glass coverslips. The percentages of eosinophils, lymphocytes, macrophages, and neutrophils were determined by counting 200 cells/BAL sample.

**Tetramer staining and intracellular cytokine staining**

An aliquot of BAL cells was incubated at 37°C for 30 min with soluble PE-conjugated tetramers comprised of biotinylated murine class I monomers (H2-D^d), human β2-microglobulin, and influenza virus peptide NP68 (ASNNMDAM) or LCMV peptide gp33 (KAVYNFATC). Allophycocyanin-conjugated anti-CD8 mAbs were added subsequently and incubated on ice for 15 min. An aliquot of BAL cells from influenza-infected mice were stimulated for 6 h with 5 μg/ml of influenza virus (1 × 10⁸ PFU/ml) as described. Splenocytes from VV-G2-infected mice were stimulated for 6 h with VV-G2 (1 × 10⁶ PFU) or PMA/ionomycin for 4 h. Splenocytes from Listeria-infected mice were stimulated for 6 h with heat-killed Listeria (1 × 10⁶ CFU/ml) or Listeria-derived H2-Kb-restricted peptide (10⁻⁷ M). Brefeldin A was added for the last 2 h to retain cytokines in the cytoplasm. Cells were stained with FITC-labeled anti-CD4 and PE-labeled anti-CD8 for 15 min, washed, and fixed with 2% paraformaldehyde. Subsequently, cells were permeabilized using 0.5% saponin and stained intracellularly with allophycocyanin-labeled anti-IFN-γ. Cells were washed and analyzed by flow cytometry (FACScan; BD Biosciences) using CellQuest software.

**Cytolytic activity of CTLs**

Secondary CTL responses of influenza virus-infected mice were measured in a conventional ⁵¹Cr release assay essentially as described (13) using EL-4 cells pulsed with peptide NP68 (20 ng/ml; 90 min at 37°C) as target cells and in vitro expanded peribronchial cells as effectors. Peribronchial cells (5 × 10⁶) were stimulated for 5 days with NP68 peptide (20 ng/ml) in 2 ml of IMDM supplemented with 10% FCS. Primary ex vivo CTL responses of vaccinia virus-infected mice were performed using VV-G2-infected MC57 fibroblasts (multiplicity of infection, 5:1) as targets and splenocytes as effectors (6 days after infection). Limiting-dilution analysis was performed as described. Unlabelled target cells were used as controls. Radioactivity in cell culture supernatants was measured in a Wallac Microbeta Trilux counter (PerkinElmer).

**Measurement of influenza virus-specific Abs**

Microtiter plates were coated overnight at 4°C with UV-inactivated influenza virus (1 × 10⁶ PFU/well). Subsequent steps were performed at room temperature and followed by three washes with PBS. Coated plates were blocked with BSA (1% w/v) for 2 h. Sera and BAL fluid were serially diluted in PBS containing 0.1% BSA, and incubated at 4°C overnight. Alkaline phosphatase-labeled goat anti-mouse IgM and IgG Abs (Southern Biotechnology Associates) were added for 2 h followed by the addition of p-nitrophenyl phosphate as substrate. Plates were read at 405 nm. Values indicate serum and BAL dilutions at half-maximum OD₅₀. End-point sera titers were also comparable between groups of mice (data not shown).

**Determination of viral and bacterial titers**

To determine influenza viral titers in the lungs, samples were collected on various days after infection, homogenized (Polytron PT 1300 D), and serially diluted with MDCK cells as described (12). For the determination of vaccinia viral titers, ovaries were collected 6 days after infection, and the plaque assay was performed on BSC-40 cells as previously described. Listeria bacterial titers were assessed in livers and spleens of mice infected 5 days previously. Briefly, homogenates were serially diluted 10-fold, plated on BHI agar plates, and incubated for 24–36 h at 37°C before counting the colonies.

**Isolation of splenic DCs and naive CD4⁺ T cells**

Primary DCs were isolated from spleens of wild-type C57BL/6 and OPN⁻/⁻ mice as previously described (15). Spleens were minced and incubated with 1 mg/ml collagenase D twice for 30 min at 37°C in a water bath. Resulting cell suspensions were washed and stained with CD11c-conjugated MACs beads (Miltenyi Biotec), and DCs were sorted using positive magnetic sorting. Purity of CD11c⁺ MHC class II⁺ cells was usually 80–90%. Naive C626L⁺CD4⁺ T cells were isolated from spleens of OPN⁻/⁻ × sm2 and OPN⁻/⁻ × sm2 tg mice using positive magnetic sorting (CD4⁺ conjugated MACs beads; Miltenyi Biotec). Purity of C626L⁺CD4⁺ T cells was usually 90–95%.

**In vitro differentiation of naive CD4⁺ T cells**

Splenic DCs (1 × 10⁶/well) were cocultured with LCMV gp61-specific naive CD4⁺ T cells (5 × 10⁶/well) from sm2 tg mice (11) in the presence of 100 nM gp61 peptide. IMDM supplemented with 5% FCS, Pen/Strep, and glutamine was used for all cell cultures (cell culture reagents were from Cambrex Bio Science). After 3 days, CD4⁺ T cells were restimulated with DC (1 × 10⁶/well) and 100 nM gp61 peptide. After a further 2 days, CD4⁺ T cells were analyzed for the expression of intracellular cytokines.

**rOPN protein production and purification**

Amplification of the mouse opn cDNA was performed using standard PCR techniques in an Eppendorf MasterCycler. The constructs were cloned into expression vectors using the Gateway cloning system (Invitrogen Life Technologies). Protein expression was performed in Spodoptera frugiperda (Sf9) insect cells (Invitrogen Life Technologies) grown in suspension in SF900 II (Invitrogen Life Technologies) using baculovirus expression vectors as described (16). For medium-scale cultures, cells were grown in 3-L culture flasks (Corning) on an orbital shaker rotating at 170 rpm, at 27°C. Cells were infected at a density of 1.5 × 10⁶ cells/ml at a multiplicity of infection of 5.0. Culture supernatant was harvested 48 h postinfection and concentrated by ultrafiltration on a 10-kDa membrane (Millipore). Sf9 insect cell-conditioned culture supernatant was filtered and applied to a column packed with 50 ml of Ni-NTA-agarose resin (Qiagen) according to the manufacturer’s specifications. Bound proteins were eluted with 250 mM imidazole, and fractions containing the mouse OPN protein
were pooled, concentrated, and further chromatographed by size exclusion on a Superdex-75 column (Amersham Biosciences) equilibrated in PBS. Fractions containing the protein were used for analysis in bioassays.

For bacterial protein expression, murine OPN-A was subcloned into the pGEX-4T3-LETD-ASSAM vector. After transfection in Escherichia coli DH5α, fermentation was conducted in a Biolatitte stirred reactor (continuously stirred tank reactor) with 5 liters working volume. At a culture density of OD600, sterile isopropyl β-D-thiogalactoside was injected to a final concentration of 1 mM. After 45 min of induction, the culture is harvested by centrifugation, and the cell pellet is kept at −20°C until purification. Murine OPN-A-6His has been purified by GST affinity and protein was cleaved by Casp8. After recovery from the digested material on a Ni-NTA affinity column, the protein was chromatographed by size exclusion on a Superdex-75 column (Amersham Biosciences), equilibrated in PBS, and kept at −80°C until use.

**Oli-neu proliferation assays**

Oli-neu cells were grown under insulin-deprived conditions for 24 h in poly-L-lysine-coated flasks and then seeded into BioCoat poly-L-lysine-precoated 96-well plates (356516; BD Biosciences) at a concentration of 4 × 10^5 cells in 100 μl of medium per well. Incubation with rOPN either in the presence or absence of 10 μM cyclo-Arg-Gly-Asp (RGD) and cyclo-Arg-Ala-Asp (RAD) peptides (Peptides International) was performed for 48 h. rOPN was denatured via boiling for 1 h as a negative control. Insulin-like growth factor-1 (R3-IGF-1) was used as a positive control. Proliferation was measured by adding 10 μl of Alamar Blue (BioSource International), a fluorometric/colorimetric redox growth indicator that reflects metabolic activity, to the cultures and incubating at 37°C and 10% CO2 for 3 h after the 48-h treatment. Seventy microliters of the medium was transferred from each sample well to 96-well flat-bottom black polystyrene culture plates (Corning). Fluorescence was measured at a 530- to 560-nm emission wavelength on a Wallac Victor2 multilabel counter (PerkinElmer Instruments).

**Collection of peritoneal macrophages and in vitro macrophage assays**

For assays with resident peritoneal macrophages, peritoneal lavage was performed with 10 ml of 1% BSA/PBS. For assays with thioglycolate-induced macrophages, cells were harvested from mice 5 days after a 0.5 ml peritoneal injection of 5% Brewers thioglycolate solution. A total of 2 × 10^5 total cells was seeded in 96-well flat-bottom plates, and nonadherents were removed 2 h later. Cells were left unstimulated in medium, or were stimulated with LPS alone (100 or 300 ng/ml) or with IFN-γ (100 U/ml), OPN (R&D Systems; non-IG-secreting, nonlight chain-synthesizing myeloma-derived, or baculovirus-produced, 200 or 1000 ng/ml) alone or with LPS (300 ng/ml), or Cpg (100 μM) and IFN-γ (100 U/ml). LPS from *E. coli* 0111:B4 was purchased from Sigma-Aldrich, and phosphorothioate CpG, RITTCATAGCTCCTGATGCT (synthesized by Microsynth). Supernatants were collected after 24 or 48 h, and sandwich ELISAs were performed to quantify IL-10 and IL-12p40.

**Results**

**OPN**−/− mice have an unimpaired recruitment of inflammatory cells to the lung after influenza infection. C57BL/6 mice (○) and OPN−/− mice (□) were infected intranasally with 50 PFU of influenza virus PR-8 (n = 4/group), and BAL was performed on days 7, 10, and 13. A and B, Total BAL cells were counted (A) and surface stained with anti-βTCR-FITC, anti-CD8-PE, and anti-CD4-allophycocyanin (BD Pharmingen) before flow cytometry analysis (BD Biosciences FACSCalibur) (B). Results are means ± SD and are representative of three independent experiments (n = 4 mice/group). C, Cytospins were prepared from BAL cells, stained with May-Grünwald/Giemsa, and morphologically differentiated under the microscope. Two hundred total BAL cells were counted per sample, and frequencies of respective cells were calculated accordingly. Results are mean ± SD and are representative of two independent experiments (n = 4 mice/group).

**Specific T cell responses and influenza virus clearance are not affected in the absence of OPN**

To quantify the frequency of specific CTLs generated after the infection, tetramer staining was performed on BAL cells harvested 13 days after infection. These data revealed that there was no significant difference between the frequency of influenza virus NP68-specific CD8 T cells in wild-type and OPN−/− mice (Fig. 2A). Moreover, BAL cells restimulated with UV-inactivated influenza for 6 h and intracellularly stained for IFN-γ, revealed comparable frequencies of IFN-γ-producing CD4 and CD8 effector T cells (Fig. 2B). Although frequencies of NP68-specific CD8 T cells were comparable in wild-type and OPN−/− mice, we performed a secondary killer assay to assess the lytic activity of CD8 T cells. Total peribronchial lymph node cells were expanded in vitro for 5 days with NP68 peptide and used as effectors in a cytotoxic killer assay. As shown in Fig. 2C, there was a comparable lysis of NP68 peptide-loaded target cells between the two groups of mice. The
restimulation of peribronchial lymph node cells with titrating amounts of UV-irradiated influenza virus revealed that there is no difference in the expansion of specific lymphocytes (Fig. 2D). Moreover, the clearance of influenza virus was assessed in lungs from wild-type and OPN−/− mice taken 7 and 11 days after infection. As shown in Fig. 2C, there were comparable titers of influenza virus at day 7, and both groups of mice cleared the virus by day 11.

Ab responses to influenza virus are independent of OPN

To analyze the anti-influenza B cell response, we measured specific Ab levels in serum and BAL fluid by ELISA during the course of the influenza infection. As shown in Fig. 3A, wild-type and OPN−/− mice have comparable titers of the Th cell-independent IgM in serum and BAL fluid at days 9 and 15. Furthermore, Th cell-dependent IgG titers are similar in the serum and BAL at days 9 and 15 after infection.

OPN is not required for the control of vaccinia virus and the generation of antiviral T cell responses

OPN has been suggested to be required for the induction of IL-12 and the inhibition of anti-inflammatory IL-10 responses (1). We have shown previously that clearance of vaccinia virus is mediated by IL-12 and inhibited by IL-10 (17). Therefore, we addressed the role of OPN in the immune response to vaccinia virus, a cytopathic DNA virus. Ovaries were removed from infected mice and analyzed in a viral plaque assay. As shown in Fig. 4A, there is no significant difference in vaccinia titers between wild-type and OPN−/− mice 6 days after infection with 2 × 10⁶ PFU of VV-G2. Furthermore, an ex vivo killer assay using vaccinia virus-infected fibroblasts as targets, demonstrated no difference in the lytic activity between the two strains of mice (Fig. 4B). Splenocytes restimulated with either PMA/ionomycin (Fig. 4C, left panel) or UV-inactivated wild-type vaccinia virus (right panel), demonstrated a comparable frequency of IFN-γ-producing CD4 and CD8 effector T cells (Fig. 4C). Additionally, expansion of CTLs in vivo, as demonstrated by the frequency of gp33-specific CD8 T cells to an infection with VV-G2, revealed no defect in OPN−/− mice (Fig. 4D). Thus, control of vaccinia virus and antiviral T cell responses are unimpaired in OPN−/− mice.

OPN-deficient mice have an unimpaired immune response to L. monocytogenes infection

It was reported previously that OPN−/− mice have an impaired immune response to infection with the intracellular pathogen, L.
monocytes (1). Because we demonstrate absolutely normal immune responses to influenza virus and vaccinia virus, we wanted to reassess the role mediated by OPN during Listeria infection. Surprisingly, the wild-type and OPN−/− mice appear to control the bacterial burden after 5 days in the spleen (Fig. 5A) and liver (data not shown) comparably. The experiment was repeated with the OPN−/− mice generated by Rittling et al. (31) and used in the original paper (1), but now backcrossed onto C57BL/6 background for 10 generations. These OPN−/− mice had indistinguishable bacterial titers from the wild-type mice (data not shown). Moreover, in contrast to the aforementioned study, stimulation of splenocytes with either H2-Kb peptide derived from Listeria (Fig. 5B) or heat-killed Listeria (data not shown) resulted in unimpaired frequencies of IFN-γ-producing CD4 and CD8 T cells, and IFN-γ production (data not shown).

The physiological role of some genes may be overlooked due to in vivo redundancy. Because OPN has been reported to promote the production of IL-12 from macrophages and monocytes (1, 19), and might induce the polarization of naive CD4 T cells to Th1 IFN-γ-producing cells, we wanted to address the role of OPN in the in vitro differentiation of naive CD4 T cells.

![FIGURE 3](https://example.com/figure3.png)

**FIGURE 3.** Normal Ab responses in OPN−/− mice after influenza infection. Groups of C57BL/6 mice (○) and OPN−/− mice (□) were infected intranasally with 50 PFU of influenza virus. A, Anti-influenza IgM was measured at days 9 and 15 after infection in the serum (left panel) and BAL fluid (right panel). B, Anti-influenza IgG was measured at days 9 and 15 after infection in the serum (left panel) and BAL fluid (right panel). ELISAs were performed on plates coated with UV-inactivated influenza virus. Results are representative of three independent experiments (n = 6 mice/group for day 9; n = 3 mice/group for day 15).

![FIGURE 4](https://example.com/figure4.png)

**FIGURE 4.** OPN is not required for the control of vaccinia virus and the generation of antiviral T cell responses. Groups of C57BL/6 mice and OPN−/− mice were infected i.v. with 2 × 10⁶ PFU of VV-G2 and sacrificed on day 6 for analysis. A, Vaccinia virus titers in ovaries were determined by plaque assay on BSC40 cells. Results are representative of two independent experiments (n = 6 mice/group). B, CTL lytic activity was assessed in a primary killer assay using splenocytes from C57BL/6 (○) and OPN−/− (□) VV-G2-infected mice as effectors, and VV-G2 infected 35Cr-labeled MC57 fibroblasts as targets. No peptide 35Cr-labeled MC57 fibroblasts were used to demonstrate the specificity of the killing (△, C57BL/6 mice; □, OPN−/− mice). Results are representative of two independent experiments (n = 4 mice/group). C, The frequency of splenic IFN-γ−producing CD4 and CD8 T cells were analyzed in C57BL/6 (○) and OPN−/− mice (□) after restimulation with either PMA/ionomycin (left panel) for 4 h or with VV-WR (right panel) for 6 h. Results are representative of two independent experiments (n = 5 mice/group). D, The frequency of specific CTLs was assessed in C57BL/6 (○) and OPN−/− mice (□) by staining splenocytes with gp33-loaded MHC class I tetramer-PE and anti-CD8-allophycocyanin before FACS analysis. Results are representative of two independent experiments (n = 5 mice/group).

![FIGURE 5](https://example.com/figure5.png)

**FIGURE 5.** OPN is not important for the control of L. monocytogenes. Groups of C57BL/6 and OPN−/− mice were infected i.v. with 8000 CFU of L. monocytogenes and sacrificed 5 days later for analysis. A, Spleens from C57BL/6 and OPN−/− mice were homogenized and plated serially on BHI agar to determine bacterial load. Results are representative of two independent experiments (n = 6 mice/group). B, Splenocytes from C57BL/6 (○) and OPN−/− (□) were restimulated with the H2-Kb-restricted Listeria-derived peptide LLO296 for 6 h and subsequently stained with CD8 on the surface, and intracellularly for IFN-γ.
analyze this, we crossed mouse strains containing a transgene for a class II-restricted TCR specific for LCMV gp61 (sm2 tg mice) with OPN<sup>−/−</sup> mice. Naive CD4 T cells isolated from TCR transgenic OPN<sup>−/−</sup> and OPN<sup>+/+</sup> mice were cultured with primary DCs purified from wild-type and OPN<sup>−/−</sup> mice and 100 nM gp61 peptide for 3 days. The CD4 T cells were restimulated with respective DCs and 100 nM gp61 peptide for a further 2 days before analysis for the intracellular expression of IFN-γ and IL-4 by FACS analysis. Results are representative of three independent experiments.

No effect of OPN on Th1 cell differentiation

We have shown previously that the strength of TCR signaling determines Th subset development with high Ag concentrations resulting in Th1 differentiation and low Ag concentrations inducing Th2 differentiation in cocultures of DCs and naive CD4<sup>+</sup> T cells (20, 21). To determine whether OPN derived from DCs or CD4<sup>+</sup> T cells mediates a role in the induction of Th1 differentiation of naive CD4<sup>+</sup> T cells, four-way cocultures were set up with BL/6 or OPN<sup>−/−</sup> DCs cocultured with TCR transgenic CD4<sup>+</sup> T cells specific for an I-A<sup>b</sup> restricted peptide (gp61) of the glycoprotein from LCMV from either BL/6 × sm2 mice or OPN<sup>−/−</sup> × sm2 mice. As shown in Fig. 6, stimulation of the coculture with 100 nM gp13 resulted in a comparable frequency of IFN-γ-producing sm2 CD4<sup>+</sup> T cells. Furthermore, addition of rOPN to the cultures did not influence Th1 differentiation (data not shown). Thus, OPN is not required for differentiation of naive CD4<sup>+</sup> T cells in vitro.

No effect of rOPN on macrophage IL-12p40 production or inhibition of IL-10 induced by LPS

Because we observed no effect of endogenous OPN on the induction of IFN-γ-producing CD4<sup>+</sup> T cells by Ag-presenting DCs, we wanted to assess whether exogenously added rOPN affects in vitro macrophage cultures as published previously (1). We produced murine rOPN both in insect cells and <i>E. coli</i> using baculovirus and bacterial expression systems. As shown in Fig. 7A, either LPS and IFN-γ, or CpG and IFN-γ, induce IL-12p40 from resident macrophages; however, murine rOPN derived from insect cells or LPS alone was unable to induce the release of IL-12p40. Furthermore, thiglycolate-induced peritoneal macrophages, which are partially activated in vivo, release IL-12p40 in response to LPS stimulation alone, but rOPN does not induce IL-12p40 (Fig. 7C). Moreover, resident peritoneal macrophage cultures produced IL-10 in response to stimulation with LPS alone, LPS and IFN-γ, CpG and rOPN (Fig. 7D). The addition of rOPN to the cultures did not influence IL-12p40 production or reduce LPS-induced IL-10 from macrophages, but it does induce proliferation of Oli-neu cells. A and B, Resident peritoneal macrophages were stimulated with LPS alone (300 ng/ml), LPS (300 ng/ml) and IFN-γ (100 U/ml), or LPS (300 ng/ml) and rOPN (1 μg/ml) as indicated; supernatants were harvested 48 h later; and IL-12p40 (A) or IL-10 (C) were measured in the supernatant by ELISA. Thiglycolate (TG)-induced macrophages were stimulated with LPS (100 ng/ml), rOPN (200 ng/ml), or LPS (100 ng/ml) and rOPN (200 ng/ml) for 48 h, supernatants were harvested 48 h later, and IL-12p40 was measured in the supernatant by ELISA. Results are representative of two independent experiments (n = 2 mice/group). D, Insulin-deprived Oli-neu cells were either left untreated, stimulated with R3-IGF-1, or incubated with the indicated concentrations of baculovirus- or <i>E. coli</i>-produced rOPN for 48 h. Proliferation was measured 3 h after the addition of Alamar Blue by reading the relative fluorescence at 530–560 nm. Results are representative of two independent experiments.

IFN-γ; however, no inhibition of LPS-induced IL-10 was observed when rOPN was added (Fig. 7B). To ensure that the OPN was bioactive, a proliferation assay was performed with an oligodendrocyte precursor-like cell line (Oli-neu), which has been shown to proliferate in response to OPN (22). As shown in Fig. 7D, the addition of baculovirus-produced OPN leads to a dose-dependent proliferation response in Oli-neu cells, indicating that the protein is bioactive. Interestingly, rOPN derived from <i>E. coli</i> and murine rOPN purchased from R&D Systems demonstrated no effect on either the proliferation of Oli-neu cells or IL-12 production by macrophages. Therefore, contrary to previously published data, we find that rOPN does not directly induce the release of IL-12p40 from macrophages, nor does it inhibit the LPS-induced IL-10 response.

Discussion

In this report, we focus on the role of OPN in the development of antiviral immune responses. Mice deficient in OPN were demonstrated to have an impaired type-1 cell-mediated immune response, resulting in impaired immunity against HSV and the intracellular pathogen <i>L. monocytogenes</i> (1). To gain further insight into the role of OPN in the development of antiviral responses, we initially performed infections with influenza virus PR8 strain. Surprisingly, OPN<sup>−/−</sup> mice mounted an unimpaired immune response against influenza virus as demonstrated by comparable clearance of the virus from the lungs. OPN has been reported to act as a chemoattractant factor for many cell types (18, 23–26), but we found that...
recruitment of inflammatory cells into the lung after influenza infection was comparable for wild-type and OPN−/− mice. Furthermore, OPN has been reported to promote a Th1 response via the induction of macrophage-, monocyte-, or DC-derived IL-12 (1, 19, 27); however, we found no impairment in the frequency of IFN-γ-producing CD4 and CD8 T cells or the amount of IFN-γ produced after ex vivo restimulation of BAL cells. These findings are supported by a study by Nau et al. (2), in which M. bovis bacillus Calmette-Guérin-infected OPN−/− mice had a defect neither in cellular recruitment nor in the IFN-γ response to Ag restimulation. Moreover, the OPN−/− mice in these experiments even displayed an exaggerated recruitment of cells into the peritoneum and enhanced granuloma formation (2). Murine Lyme arthritis has been demonstrated to be regulated by IL-12 and IL-10 (28–30), cytokines apparently affected by OPN (1); however, Potter et al. (3) reported that OPN has no influence on the development of the disease. Indeed, the OPN−/− mice on a mixed background displayed more severe arthritis than wild-type littermates, which was suggested to be a consequence of closely linked genes. Furthermore, OPN−/− macrophages and splenocytes produced similar quantities of IL-12 and IL-10 upon stimulation with the Pam3Cys-spleen, and mounted a normal IFN-γ response. Furthermore, we found no impairment in the frequency of IFN-γ-producing CD4 and CD8 T cells or the amount of IFN-γ produced after ex vivo restimulation of BAL cells. These findings are supported by a study by Nau et al. (2), in which M. bovis bacillus Calmette-Guérin-infected OPN−/− mice had a defect neither in cellular recruitment nor in the IFN-γ response to Ag restimulation. Moreover, the OPN−/− mice in these experiments even displayed an exaggerated recruitment of cells into the peritoneum and enhanced granuloma formation (2). Murine Lyme arthritis has been demonstrated to be regulated by IL-12 and IL-10 (28–30), cytokines apparently affected by OPN (1); however, Potter et al. (3) reported that OPN has no influence on the development of the disease. Indeed, the OPN−/− mice on a mixed background displayed more severe arthritis than wild-type littermates, which was suggested to be a consequence of closely linked genes. Furthermore, OPN−/− macrophages and splenocytes produced similar quantities of IL-12 and IL-10 upon stimulation with the Pam3Cys-modified lipoprotein from B. burgdorferi, Ospa (3).

We demonstrated that OPN−/− mice mount a normal protective immune response to vaccinia virus, which has been shown to be dependent on IL-12 production, but inhibited by IL-10 (17). OPN−/− and wild-type mice developed similar frequencies of CTLs which were shown to be functionally active in a primary killer assay. In agreement with our influenza findings, OPN−/− mice had no defect in the generation of IFN-γ-producing T cells or IFN-γ levels after vaccinia infection. Because immunity to both influenza and vaccinia infection was shown to be normal, we decided to reassess the role of OPN in the protective response to infection with L. monocytogenes. Unexpectedly, OPN−/− and wild-type mice had comparable listerial titers in the liver and spleen, and mounted a normal IFN-γ response. Furthermore, we obtained the OPN−/− mice generated by Ritting (31), but now backcrossed onto C57BL/6 for 10 generations, and infected them with Listeria. These OPN−/− mice also mounted a comparable protective response to Listeria, and bacterial titers were not significantly different in the spleen and liver. OPN has been reported to exacerbate disease in several autoimmune models, i.e., EAE (4, 5), CAIA (6), and CIA (7); however, contradictory findings have recently been published (8, 9). Blom et al. (9) reported that using fully backcrossed OPN−/− mice, OPN had no influence on EA, CAIA, or CIA, and concluded that the dramatic phenotypes published are due to one or several polymorphic genes linked to the OPN locus. Blom further states that many closely linked polymorphic genes derived from the 129 strain and linked to the OPN locus could influence the inflammatory response or resistance to pathogens, including the chemokines Cxcl-1, Cxcl-2, Cxcl-5, Cxcl-9, Cxcl-10, Cxcl-15, and the neuronal nitric oxide synthase, Nos1 (9). Several studies have addressed the effect of allelic polymorphism of OPN in the development of autoimmune and host defense with varying outcomes (32–39). Importantly, the C57BL/6 mice used in our study and the C57BL6 × 129SV mice used in previous studies express the same OPN allele, namely Eta-1, which excludes this additional factor from the interpretation (40, 41). Finally, using the sm2 coculture system, we observed that OPN derived from either CD4 T cells or DCs is not required for the differentiation of naïve CD4 T cells to Th1 IFN-γ producers. Moreover, contrary to previously published findings (1), we demonstrated that exogenous rOPN produced in baculovirus or E. coli is unable to induce the production of IL-12p40 or to inhibit LPS-induced IL-10 from either resident peritoneal- or thioglycollate-induced macrophages.

These data are in agreement with an investigation by Potter et al. (3), in which rOPN mediated no effect on the production of IL-12p40 or IL-10 from bone marrow macrophages or splenocytes. A proliferation assay with the oligodendrocyte precursor-like cell line, Oli-neu, demonstrated that our baculovirus-produced rOPN is bioactive, but interestingly, the E. coli-produced rOPN had no effect on expansion of the Oli-neu cells. This might indicate that glycosylation of OPN is necessary for activity of the protein; however, Ashkar et al. (1) reported that an E. coli-derived OPN preparation was shown to induce IL-12 from macrophages. OPN has been shown to be posttranslationally regulated by several factors including thrombin cleavage, phosphorylation, trans-glutamination, and glycosylation (42–44), processes that modulate the bioactivity of OPN. Of note, Konno et al. (45) reported recently, after testing several mouse and human rOPN preparations, that endotoxin contamination leads to many of the effects previously attributed to OPN in vitro. Thus, interpretation of in vitro results may be confounded by the presence of LPS, which becomes all the more relevant because high concentrations of OPN are usually required to mediate an effect. In conclusion, we find that OPN is dispensable for the development of antiviral and antilisterial immunity, and these findings suggest that the role of OPN should be reconsidered in the development of immune responses.

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Disclosures

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References
