Role of Interleukin-23-Dependent Antifungal Immune Responses in Dendritic Cell-Vaccinated Mice

Mingquan Zheng,1 Rekha R. Rapaka,2 Amy C. Yu,1 Judd E. Shellito,3 and Jay K. Kolls1*

1LSU Health Sciences Center, Department of Genetics, New Orleans, Louisiana; 2Children’s Hospital of Pittsburgh, Pittsburgh, Pennsylvania; and LSUHSC Section of Pulmonary/Critical Care and LSUHSC Gene Therapy Program, New Orleans, Louisiana

Received 31 March 2011/Returned for modification 15 April 2011/Accepted 24 June 2011

CD40 ligand (CD40L) transduction of antigen-pulsed dendritic cells (DCs) can result in antigen-specific humoral immune responses even in CD4+ T-cell-depleted settings. Here, we show that CD40L transduction of DCs results in the induction of interleukin-12p40 (IL-12p40), IL-12p70, and IL-23. Using DCs that were deficient in IL-12p40, IL-12p35, or IL-23p19, we show that these molecules are dispensable for primary IgG1 responses to Pneumocystis, but IgG2c was dependent on IL-12p40 and IL-23p19 expression in DCs and were not affected by the lack of IL-12p35. To confirm that this defect in recall was due to IL-23, transduction of IL-12p40−/−DCs with a recombinant adenovirus expressing functional IL-23 restored recall responses in DC-vaccinated CD4-deficient mice. These data show that DC-produced IL-23 is critical for vaccine-induced antigen-specific IgG2c and recall antibody responses in the setting of CD4+ T-cell depletion.

Pneumocystis (PC) is an opportunistic pathogen of clinical importance since the onset of the AIDS epidemic (14, 19). Chemoprophylaxis of HIV-infected patients at high clinical risk for PC pneumonia and the introduction of highly active antiretroviral therapy (HAART) have contributed to a reduced incidence of PC pneumonia (14). Despite this, PC pneumonia remains a common opportunistic pneumonia and the most common life-threatening infectious complication in HIV-infected patients (14). Although it has been well recognized that CD4+ T cells are critical for host defense in both human, nonhuman primate, and rodent models of PC infection (3, 9, 22), the role of B cells in protection against PC has received increased recognition (11, 12).

B-cell-deficient mice are permissive to chronic PC infection, and recent data suggest that B cells serve critical roles as both antigen-presenting cells and effector cells by producing PC-specific antibody (11). However, dendritic cells (DCs) pulsed with PC antigen have been shown also to elicit protective antibody responses in both CD4-repleted and CD4-depleted mice, and adoptive transfer of B cells or serum suggested that the protection can also be conferred by anti-PC IgG (29). However, in the context of CD4-depleted mice, protection was observed only when the DCs were engineered to express CD40L, suggesting that CD40-CD40L interactions were critical for B-cell class switching and antibody production in CD4-depleted mice (29). Furthermore, in support of a role of B cells in these responses, DNA-based vaccination with kexin, an immunodominant antigen of PC, along with CD40L as a genetic adjuvant induced strong anti-PC IgG responses and protection against PC in CD4+ T-cell-deficient mice (28). Again, this protection could be transferred by B cells or IgG, suggesting that anti-PC antibody was critical for this response.

Here, we show that transduction of murine bone marrow-derived dendritic cells with an adenovirus encoding murine CD40L potently induces interleukin-12p70 (IL-12p70), IL-12p40, and IL-23, a heterodimer of IL-23p19 and IL-12p40. IL-12p70 is critical in regulating Th1 responses, whereas IL-12p40 has been shown to regulate DC migration to draining lymph nodes (11). IL-23p19 has been shown to regulate antigen-specific antibody responses to T-cell-dependent antigens (7). In order to investigate the specific roles of these molecules in regulating anti-PC humoral immune responses, after the CD40L-modified DC-based vaccination, we used DCs from wild-type (WT) or IL-12p40−/−, IL-12p35−/−, or IL-23p19-deficient mice transduced with AdCD40L and pulsed with PC antigen as DC-based vaccines in CD4-depleted C57BL/6 mice. Mice vaccinated with WT, IL-12p40−/−, IL-12p35−/−, or IL-23p19−/−DCs were all able to mount similar levels of anti-PC IgG after 2 rounds of vaccination. However, only WT mice and IL-12p35−/− mice were capable of mounting antigen-specific recall responses to PC antigen, whereas recall responses were absent in IL-12p40−/− and IL-23p19−/− mice. In order to determine the contributions of IL-23 in these responses, we investigated whether we could rescue recall responses of IL-12p40−/− DC-vaccinated mice by transducing these DCs with an adenovirus encoding IL-23 in addition to CD40L. Providing exogenous IL-23 rescued antigen-specific recall responses in IL-12p40−/− DC-vaccinated mice, demonstrating that IL-23 is critical for antigen-specific recall responses to PC antigens. These data suggest that IL-23 is critical for the generation of PC-specific antigen recall responses by B cells and that IL-23 may be a potential adjuvant for PC-based vaccination strategies in CD4-deficient environments.
MATERIALS AND METHODS

Adenovirus vectors. AdCD40L is an E1-E3 replication-deficient recombinant adenovirus type 5 (Ad5)-based vector containing and expressing the full-length murine CD40L cDNA under the cytomegalovirus immediate-early promoter (29). The control AdLuc vector is identical to this but encodes firefly luciferase as previously described (29). AdIL-23 encodes IL-23p19 and IL-12/23p40, separated by an internal ribosomal entry site, as previously described (30). Viruses were propagated in 293 cells and purified as previously described (29).

Dendritic cells. Bone marrow-derived DCs were obtained from hematopoietic progenitors from the femurs and tibiae of 6- to 8-week-old male WT, IL-12p40−/−, IL-12p35−/−, and IL-23p19−/− mice from a C57BL/6 background and grown in complete RPMI 1640 medium (10% fetal bovine serum [FBS], 2 mM l-glutamine, 100 mg/ml streptomycin, and 100 units/ml penicillin) supplemented with 100 units/ml recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) and 20 ng/ml recombinant mouse IL-4 (both from R&D Systems Inc., Minneapolis, MN). DC preparations were harvested on day 6, and over 90% were positive for class II major histocompatibility complex (MHC) class II I-A (I-A^d) and CD11c, with less than 1% of the cells positive for CD4, CD8, CD19, or DX-5, an NK cell marker (BD Pharmingen, San Jose, CA). We have previously shown that a multiplicity of infection (MOI) of 100 was found to result in over 90% of the DCs being transduced, so all experiments were carried out with this MOI (29). Prior to use in vivo, transduced DCs were assayed for adenovirus vectors (AdCD40L, AdLuc, or AdIL-23 at an MOI of 100). To assess DC activation, culture medium was collected after 24 h, and the level of mouse IL-12p70, IL-12p40, or IL-23 in the culture medium was determined. IL-12p40 and IL-12p70 were measured by Bio-Plex (Bio-Rad, Hercules, CA), and IL-23 was measured by enzyme-linked immunosorbent assay (ELISA) (eBioscience).

Vaccination protocol. Male 6- to 8-week-old C57BL/6 mice were depleted of CD4 cells as previously described (28, 29) by the administration of 0.3 mg GK1.5, a depleting anti-CD4 monoclonal antibody (4). This dose of GK1.5 results in over 97% depletion of CD4+ T cells in the spleen and lung, as measured by staining with anti-CD4+ (BD Pharmingen), an anti-CD4 antibody that is not blocked by GK1.5. GK1.5 was then administered weekly to maintain CD4 depletion. Three days after CD4 depletion, subgroups of mice were vaccinated as previously described (29) with 5 × 105 PC-pulsed DCs from WT, IL-12p40−/−, IL-12p35−/−, or IL-23p19−/− mice in 100 ml phosphate-buffered saline (PBS) injected intravenously. We have previously shown that CD4-depleted mice that received PBS, DCs only, DCs pulsed with PC, or DCs transduced with AdLuc pulsed or unpulsed with PC have minimal increases in anti-PC antibody titers (29). Based on this, the experimental group consisted of AdCD40L-transduced DCs, and the control group consisted of AdLuc-transduced DCs pulsed with PC. DCs were administered at zero and 3 weeks, and anti-PC IgG1 and IgG2c levels were measured by ELISA at weeks 2 and 5. At week 12, anti-PC recall responses were assessed by administering 100 μg of PC antigen intraperitoneally (i.p.), and anti-PC IgG1 and IgG2c levels were determined 1 week later by ELISA.

PC antigen preparation and anti-PC ELISA. PC antigen was generated as previously described (16). For vaccine preparation, day 6 DCs were transduced with adenovirus vectors or mock transduced followed by pulsing, with or without PC antigen, for 4 h at 37°C at a ratio of 1 μg protein to 106 DCs. Anti-PC IgG1 and IgG2c titers were measured by ELISA as previously described (28).

Opsonization killing assay. To assess whether samples containing anti-PC antibody by ELISA contained opsonic activity against PC, we used an in vitro killing assay that detects both nonopsonic and opsonic macrophage-mediated killing of PC, as previously described (24, 28). Briefly, alveolar macrophages were obtained from male C57BL/6 mice by bronchoalveolar lavage. Cell preparations were greater than 98% enriched macrophages. Macrophages (1 × 106/ml) in a volume of 100 μl were cocultured with 100 μl PC (1 × 106 cysts/ml) for 16 h at 37°C, 5% CO2. Controls for 100% viability included PC incubated with medium alone. The contents of each well were collected and pelleted, and total DNA was isolated from the cell pellets using TRizol reagent (Invitrogen Corp.). PC viability was analyzed using real-time PCR measurement of rRNA copy number and quantified by employing a standard curve of known copy number of PC rRNA, as previously described (29). For opsonization studies, PC was incubated with 10 μl of serum from control or vaccinated mice prior to incubation with macrophages.

Adoptive transfer studies. For adoptive transfer studies, 6- to 8-week-old male B6 SCID mice received 200 μl of naive or immune serum (from mice previously immunized with PC-pulsed DCs). Twenty-four hours later, all mice were challenged with 2 × 108 PC cysts. Mice were sacrificed at 2 weeks for intensity of PC infection by real-time PCR.

RESULTS

IL-12 family production induced by AdCD40L transduction. We have previously shown that transduction of bone marrow-derived DCs transduced with AdCD40L and pulsed with PC elicits PC-specific IgG1 and IgG2c responses in CD4-depleted mice (29). To determine if AdCD40L transduction resulted in the production of IL-12 family members, we transduced bone marrow-derived DCs with AdCD40L or AdLuc at a dose of MOI (we have previously shown results in >90% of the cells expressing CD40L in the AdCD40L group). Transduction with AdCD40L induced significant amounts of IL-12p40, IL-12p70, and IL-23 (Fig. 1). All these levels increased significantly compared to those observed with AdLuc-transduced DCs. The level of IL-12p40 exceeded the levels of IL-12p70 and IL-23, suggesting the formation of free IL-12p40 or IL-12p40 homodimer in addition to IL-12p70 and IL-23.

Requirement of IL-12 family members in primary IgG responses to PC vaccination. CD4-depleted mice were administered two rounds of DC vaccination with DCs from WT, IL-12p40−/−, IL-12p35−/−, or IL-23p19−/− mice. All DC preparations had equivalent baseline I-A, CD80, and CD86 expression after adenovirus transduction (data not shown). For these studies, we focused on the generation of IgG1 and IgG2c, as we have previously shown that the opsonic activity of DC-PC-vaccinated mice is mediated by FcγRIII, which mediates opsonic phagocytosis by these IgG isotypes. PC-pulsed WT DCs transduced with AdCD40L elicited significant anti-PC IgG1 titers measured at 5 weeks (Fig. 2A) compared to DCs transduced with a control adenovirus encoding luciferase (AdLuc). CD4-depleted mice vaccinated with AdCD40L-modified DCs from IL-12p40−/−, IL-12p35−/−, or IL-23p19−/− mice also had significant increases in anti-PC IgG1 at 5 weeks. Moreover, these levels were not significantly different than those of CD4-depleted mice vaccinated with WT DCs, suggesting that these immune responses to PC are independent of IL-12p70, IL-12p40, or IL-23. In contrast to IgG1, however,
the IgG2c response to AdCD40L/DC vaccination was dependent on IL-12p40 and IL-23 (Fig. 2B), as CD4-depleted mice vaccinated with DCs from IL-12p40 or IL-23 knockout (KO) mice had significantly lower IgG2c responses in serum than mice that received WT or IL-12p35−/− DCs (Fig. 2B).

**Requirement of IL-23 for recall IgG responses to PC vaccination.** We next assessed antigen-specific recall responses in DC-vaccinated CD4 depleted by administering PC antigen in vivo and assessing postrecall titers in serum 1 week after antigen boosting. CD4-depleted mice that received CD40L-modified WT dendritic cells showed significant increases in anti-PC IgG1 (Fig. 3A) and IgG2c (Fig. 3B) after antigen boosting consistent with an anamnestic humoral immune response. CD4-deficient mice that received DCs transduced with the AdLuc control vector did not have demonstrable recall responses as previously published (29) (data not shown). Recall IgG1 and IgG2c responses were retained in CD4-depleted mice vaccinated with CD40L-modified IL-12p35−/−-deficient mouse DCs despite 97% depletion of CD4+ T cells in the spleen (data not shown). However, both IgG1 and IgG2c recall responses were significantly attenuated in mice vaccinated with IL-12p40−/− or IL-23−/− DCs, strongly suggesting that AdCD40L transduction of DCs requires DC production of IL-23 to elicit functional recall responses in vivo (Fig. 3A and B). We have previously shown that vaccine-induced protection is correlated with the ability of anti-PC antibody to mediate opsonic phagocytosis in vitro (25). Based on this, we next examined the requirements of DC-derived IL-12 or IL-23 in this response. Serum from antigen-boosted mice demonstrated significant opsonic activity in a macrophage-mediated killing assay of PC (Fig. 3C). We have previously shown that this enhanced in vitro killing is mediated by Fcy receptors (25, 28). In contrast to mice vaccinated with WT DCs or IL-12p35−/− DCs, serum from boosted animals that were vaccinated with either IL-12p40−/− or IL-23p19−/− DCs failed to enhance macrophage-mediated killing of PC in vitro (Fig. 3C). We next performed adoptive transfers of this serum to SCID mice followed by PC challenge. Similar to the in vitro activity, adoptive transfer of serum from CD4-depleted mice vaccinated with AdCD40L-transduced WT DCs conferred a significant reduction in PC lung burden compared to that of naïve serum (Fig. 3D). Mice vaccinated with IL-12p35−/− DCs also showed protection compared to naïve serum. However, serum from either IL-12p40−/− or IL-23p19−/− DCs failed to augment PC clearance upon adoptive transfer to SCID mice (Fig. 3D).

In order to determine the strict requirement of IL-23 in this response, we performed two rounds of vaccination in CD4-depleted mice with WT or IL-12p40−/− DCs that were transduced with AdCD40L and then vehicle exposed and additionally transduced with AdLuc or with AdIL-23 to reconstitute IL-23 production in the DCs. Using WT AdCD40L-transduced DCs that were treated with vehicle or AdLuc elicited efficient recall responses to antigen after two rounds of DC-based vaccination (Fig. 4A). Transduction with AdIL-23 significantly enhanced both anti-PC IgG1 and IgG2c responses (Fig. 4A and B). AdLuc- or vehicle-exposed IL-12p40−/− DCs (that were transduced with AdCD40L) elicited poor recall responses, but the additional transduction of these DCs with AdIL-23 resulted in robust recall responses for both anti-PC IgG1 and IgG2c (Fig. 4A and B). These data were confirmed also by Western blotting. Serum from mice vaccinated with WT DCs and then boosted with PC antigen reacted with multiple bands, with dominant bands at 100 kDa and 55 kDa (Fig. 4C). In contrast, mice vaccinated with IL-12p40−/− AdCD40L-transduced DCs exposed to the vehicle or transduced with AdLuc displayed weak reactivity to the same antigens. Serum from mice that were vaccinated with two rounds of DC vaccination and with 12p40−/− DCs that were transduced by AdIL-23 and pulsed with PC antigen followed by antigen boosting reacted robustly with the 100-kDa and 55-kDa antigens in addition to multiple other bands analogous to WT DCs (Fig. 4C).

**DISCUSSION**

Ghiardi and colleagues have shown that mice with a genetic deletion of IL-23p19 have impaired IgG isotype responses to T-dependent but not T-independent antigens (7). These investigators observed normal primary IgG responses in IL-23p19−/− mice, similar to the data described herein, but responses after antigen boosting were significantly attenuated. As IL-23 is largely made by antigen-presenting cells, the authors speculated whether IL-23 was required for the generation of CD4+ memory cells (7). Our study significantly extends this study in several key areas. First, we demonstrated that it is DC-derived IL-23 that is required for recall responses to Pneumocystis antigen and that IL-23 is also required for CD40L-activated DCs to elicit antigen-specific recall responses. Moreover, we showed that IL-23 could serve as an adjuvant to achieve enhanced humoral immune responses in the setting of CD4 deficiency. These findings may aid in developing vaccines for opportunistic infections in individuals with compromised CD4+ T-cell immunity. Although we observed ≥97% CD4+
T-cell depletion in the spleen throughout our protocol, we cannot exclude some remnant of CD4+ helper function that results in DC-based IgG class switching in vivo. However, even if this were the case, this response requires IL-23. Additionally, we focused on anti-PC IgG1 and IgG2c, given their association with Th1 and Th2 immunity, but we realize that other isotypes, including IgM (20) and IgG3 (6), are induced during PC infection and may play important roles in vivo. Having stated that, however, the in vitro opsonic activity of serum after DC vaccination is FcγR dependent, suggesting that IgG3 is not responsible for this activity (21).

Neither primary IgG1 nor IgG2c nor recall responses by these isotypes were dependent on IL-12p35, suggesting that IL-23 is required for efficient IgG2c responses in this model. IL-23 has been shown to be a critical survival factor for ThIL-17 cells (1, 27), and IL-17A−/− mice have impaired humoral immunity to vaccines as well (15). However, the effect of IL-23 in this model is likely independent of classical ThIL-17 cells, as the effect was preserved in mice that were depleted of over 97% of their CD4+ T cells with GK1.5. This, of course, does not exclude a role for other IL-17-producing cells, such as γδ T cells, which could make IL-17 in an IL-23-dependent fashion (10, 13, 23, 26). IL-23 can also regulate GM-CSF production, which could also act as an adjuvant in the DC vaccine model (1). In addition to the observed activity in regulating humoral responses, other groups have also shown that IL-23 potently also regulates Th1 response in Mycobacterium infection (8, 15) and regulates cytotoxic T lymphocyte (CTL) activity in tumor vaccine models (17). Both these responses are dependent on enhancing Th1 immunity, and thus our data are the first data to our knowledge to demonstrate CD4-independent effects of IL-23. Both IL-23R (18) and IL-12βR1 (5) have been reported to be expressed on B cells as well as DCs (2), and thus it will be important to investigate if IL-23 has direct effects on DCs or B-cell proliferation or class switching in this model.
In these series of experiments, DC vaccination with IL-12p40−/− DCs still resulted in priming, as there was reactivity to the immunodominant antigens of PC by both ELISA and Western blotting. However, recall responses were absent in these vaccinated mice. Transduction of IL-12p40−/− DCs with AdIL-23 restored robust recall responses. These data suggest that neither IL-12 nor IL-23 is required for priming and that IL-23 likely regulates expansion of the recall response. As the enhanced recall response was observed in CD4-depleted mice, these data suggest that IL-23 may be an excellent adjuvant for vaccine-induced humoral immunity in patients with T-cell deficiency.

ACKNOWLEDGMENTS

We acknowledge support from PHS grants P50HL084932 and 5R01HL061271 and thank Nico Ghilardi at Genentech for providing the IL-23p19−/− mice.

REFERENCES

16. Numasaki, M., et al. 2005. IL-17 enhances the net angiogenic activity and in

Editor: G. S. Deepe, Jr.