IL-4 deficiency does not impair the ability of dendritic cells to initiate CD4\(^+\) and CD8\(^+\) T cell responses \textit{in vivo}

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Abstract

Several reports have described a role of IL-4 in dendritic cell function. We have examined the number and phenotype of dendritic cells from C57Bl/6 wild-type and IL-4\(^{-/-}\) mice, and compared their ability to induce T cell immune responses \textit{in vivo} and \textit{in vitro}. We observed that the number of dendritic cells in the spleens and lymph nodes of IL-4\(^{-/-}\) mice is comparable to the number found in wild-type mice. In addition, the expression of maturation markers such as MHC II, CD40, CD80 and CD86, and of differentiation markers such as CD4, CD8 and CD11b, was also comparable in the two populations. Splenic wild-type and IL-4\(^{-/-}\) dendritic cells were both able to present antigen to T cell receptor transgenic CD4\(^+\) or CD8\(^+\) T cells in culture. When pulsed with antigen \textit{in vitro} and then injected subcutaneously into C57BL/6 host mice, both populations of dendritic cells were able to induce the division of T cell receptor transgenic CD4\(^+\) or CD8\(^+\) T cells \textit{in vivo}. This was the case regardless of whether the antigen used in these experiments was a low or a high affinity T cell receptor ligand. Similarly, both populations of dendritic cells were able to activate antigen-specific cytotoxic T cell responses and initiate tumor-protective immune responses \textit{in vivo}. We conclude that IL-4\(^{-/-}\) and wild-type dendritic cells have a comparable ability to initiate T cell immune responses when in an IL-4-sufficient environment.

Introduction

The cytokine IL-4 has multiple effects on immune responses, of which the most notable are the ability to direct CD4\(^+\) T cells development towards ‘type 2’ cells which secrete IL-4, IL-5 and IL-13, and the ability to induce the switching of B cells to IgE secretion (1,2). Thus, IL-4 has been principally regarded as a key factor in the initiation of type 2 immune responses.

In addition to the effects described, other effects of IL-4 have also been observed which extend beyond its recognized role in T helper 2 (Th2) immune responses. Somewhat paradoxically, IL-4 appears necessary for the development of cytotoxic T lymphocyte (CTL) responses and type 1 Th immune responses. For example, the presence of IL-4 promotes the development of Th1 and CTL responses to tumors (3,4) and can increase resistance to \textit{Leishmania} infection if administered early during infection (5). Furthermore, IL-4 is also critical for the activation of allogeneic T cells \textit{in vitro}, and for the rapid rejection of histoincompatible skin grafts \textit{in vivo} (6). These effects of IL-4 on type 1 responses appear to be mediated through antigen-presenting cells and an increased secretion of IL-12. IL-4 has been demonstrated to directly increase IL-12 secretion by dendritic cells (DC) \textit{in vitro} (7) and \textit{in vivo} (5). Increased IL-12 in turn allows the differentiation of CD4\(^+\) T cells into IFN-\(\gamma\)-secreting Th1 cells (8), the activation of CD8\(^+\) T cells into potent effector cells (9) and prevents CD8\(^+\) T cell anergy (10). In all these systems, IL-4 appears to be mainly derived from CD4\(^+\) and CD8\(^+\) T cells (4,11), but secretion of IL-4 from other cell types has also been observed (12). Altogether, these observations suggest a critical function of IL-4, not only in Th2 immune responses, but in most T cell mediated immune responses as well.

In contrast to some of the observations above, other authors have reported that IL-4 can have an inhibitory effect on CD8\(^+\) T cells and immune responses (13). Infection with a recombinant vaccinia virus induced stronger CD8\(^+\) T cell activation in IL-4\(^{-/-}\) mice compared to WT mice (14). In addition, IL-4 has been shown to modulate expression of costimulatory molecules on DC (15), which correlated with a strongly decreased incidence of diabetes in transgenic mice where IL-4 secretion had been targeted to the pancreas using the rat insulin promoter.
IL-4-deficient dendritic cells

The antigen-presenting function of DC therefore appears to be regulated by IL-4 through its effects on expression of costimulatory molecules and production of IL-12. It is possible that defects in the activity of DC may also contribute to the multiple defects in immune responses described above, which affected both CD4+ and CD8+ T cell responses. However, no reports to date have directly addressed whether the presence of IL-4 during DC development affects the phenotype or function of DC. To address this point, we compared the ability of DC from WT and IL-4−/− mice to activate T cell responses in vitro and in vivo, and find that this appears completely normal.

Methods

Mice

C57BL/6 mice were obtained from Jackson Laboratories and were maintained at the Biomedical Research Unit of the Wellington School of Medicine. IL-4−/− mice were originally obtained from Dr Manfred Kopf (16). The ‘line 318’ mouse strain, transgenic for a TCR specific for H-2D\textsuperscript{b} + fragment 33–41 of the lymphocytic choriomeningitis virus (LCMV) glycoprotein (LCMV\textsubscript{33-41}) was kindly provided by Dr H. Pircher (Institute of Medical Microbiology, University of Freiburg, Germany). OT-II mice, transgenic for a TCR specific for I-Ab + OVA, were kindly provided by Dr Bill Heath (WEHI, Melbourne, Australia). All experimental protocols were approved by the Wellington School of Medicine Animal Ethics Committee and performed according to Institutional guidelines.

In vitro culture media and reagents

Unless otherwise stated, all cultures were maintained in complete medium comprising Iscove’s modified Dulbecco’s medium with 2 mM glutamine, 1% penicillin–streptomycin, 5 × 10\textsuperscript{-5} M 2-mercapto-ethanol and 5% fetal bovine serum (FBS) (all Invitrogen, Auckland, NZ). Chicken ovalbumin protein (OVA\textsubscript{257-264}) was from Sigma (St Louis, MO, USA). The synthetic peptides OVA\textsubscript{323-339} (KISQAVHAAHAEINEAG), OVA\textsubscript{257-264} (SIINFEKL), LCMV-GP\textsubscript{33-41} (KAVYNFATM) and A4Y (KAVAN-FATM) were from Chiron Mimotopes, Clayton, Australia.

DC preparation

Spleens were harvested and incubated for 30 min at 37°C in collagenase type II (Invitrogen, Auckland, NZ) and DNase (Sigma, St Louis, MO, or Roche Diagnostics NZ Ltd, Auckland, NZ). Digestion was stopped by adding FBS supplemented with 30 mM EDTA; cells were recovered by centrifugation and resuspended at 2 × 10\textsuperscript{7} cells/ml in phosphate-buffered saline (PBS) at room temperature. Unbound dye was quenched by incubation for 2 h at 37°C.

T cell proliferation in vitro

Purified CD4+ T cells were prepared from lymph node cell suspensions by depleting CD8+ and B cells using 2,4-dinitrophenylated and Dynabeads conjugated to streptavidin, and Dynabeads conjugated to rat anti-mouse IgG (Dynal, Victoria, Australia). Purified CD8+ T cells were prepared by depleting lymph node cell suspensions of CD4+ T cells and B cells using GK1.5–biotin and magnetic depletion with Dynabeads. Thymidine incorporation was measured by culturing 1–2 × 10\textsuperscript{5} CD4+ T cells from OT-II mice, or CD8+ T cells from line 318 mice, with various numbers of antigen-loaded DC in 96-well culture plates in complete medium. Cells were pulsed with 1 μCi/well of [\textsuperscript{3}H]thymidine for the final 8 h of a 48 h culture, and then harvested for liquid scintillation counting.

Carboxy-fluorescein diacetate succinimidyld ester (CFSE) labeling

Single cell suspensions were prepared from spleen and lymph nodes by teasing through nylon gauze, and erythrocytes were lysed in 0.14 M NH\textsubscript{4}Cl and 17 mM Tris–HCl. Cells were washed and resuspended at 2 × 10\textsuperscript{7} cells/ml in phosphate-buffered saline (PBS) at room temperature. An equal volume of 2.5 μM CFSE (Molecular Probes, Eugene, OR) in PBS was added to the cell suspension, immediately vortexed, and incubated for 8 min at room temperature. Unbound dye was quenched by the addition of an equal volume of FBS and then washed three times with ice-cold medium containing 5% FBS.

T cell division in vivo

CFSE-labeled lymph node and spleen cell suspensions from OT-II mice or line 318 mice, containing a total of 0.7 × 10\textsuperscript{6} V\textsubscript{a2}+\textsuperscript{V\textbeta5.1}+ transgenic T cells (OT-II) or 1 × 10\textsuperscript{6} V\textsubscript{a2}+\textsuperscript{V\textbeta8}+ transgenic T cells (line 318), were injected intravenously (i.v.) into C57Bl/6 mice in a total volume of 0.3 ml. One day later, the mice were immunized with 1 × 10\textsuperscript{5} antigen-loaded DC subcutaneously (s.c.) into the anterior forelimb. Control mice received DC only. Cells from the draining axillary and brachial lymph nodes were harvested 72 h after immunization.

FACS analysis

Anti-FcR\textgamma\textsubscript{II} (2.4G2), anti-MHC Class II (3JP), anti-CD4 (GK1.5), anti-CD11c (N418) and anti-CD86 (GL-1) antibodies were affinity purified from culture supernatants of the relevant B cell hybridomas using protein G–Sepharose (Pharmacia Biotech, Uppsala, Sweden) and conjugated to biotin, FITC or
APC. Anti-Vx2–PE, anti-Vβ5.1–Bt, anti-Vx2–PE, anti-CD54–PE, anti-CD80–Bt, anti-CD40–PE, anti-CD11b–PE and SA–PerCP were all obtained from Pharmingen (San Diego, CA). Cells were stained in PBS containing 2% FCS and 0.01% sodium azide as described (17) and analyzed on a FACSSort (Becton Dickinson, Mountain View, CA). Necrotic cells were identified by FSC/SSC profile or PI staining as indicated, and excluded from analysis.

**In vivo cytotoxicity assays**

Cytotoxicity was assessed on fluorescence-labeled syngeneic spleen cell populations administered by i.v. injection into groups of immunized mice (n = 4–5) and a non-immunized control group (n = 3). The target cell preparation contained equal proportions of two differently labeled populations: a control population labeled with 10 μM chloromethylbenzoylarnino-tetramethylrhodamine (CM-TMR, Molecular Probes, Eugene, OR), and a population labeled with 1.25 μM CFSE and loaded with 10 μM LCMV-GP33-41 or OVA257-264 peptide. Cytotoxicity was assessed 20 h after target cell administration by FACS analysis of lymph node tissue preparations from inguinal and axillary nodes, and is presented as percent killing calculated using the formula [1 – (targets with peptide/targets without peptide)] × 100. Any variability in proportion of cells in the different target populations was assessed in the non-immunized control group, and experimental groups were adjusted accordingly.

**Tumor protection assays**

Groups of C57BL/6 mice (n = 5) were immunized by s.c. injection into the flank with 10² or 10³ DC loaded with 10 μM LCMV33-41 peptide. Control animals received DC only. All animals were challenged with 10⁶ LL-LCMV tumor cells injected s.c. into the opposite flank. LL-LCMV is a derivative of the Lewis lung carcinoma LLTC (C57BL/6, H-2b) which has been modified to express a minigene encoding LCMV 33–41 of the Lewis lung carcinoma LLTC (C57BL/6, H-2b) which has been modified to express a minigene encoding LCMV 33–41 of the Lewis lung carcinoma LLTC (C57BL/6, H-2b).

**Results**

**Numbers and phenotype of DC in IL-4−/− mice**

The percentages of DC in the secondary lymphoid organs of WT and IL-4−/− mice were examined. Spleens and lymph nodes were harvested from WT and IL-4−/− mice, gently digested in collagenase and DNase, and immediately stained with antibodies specific for a number of DC surface markers. Figure 1 shows representative stainings of these cell populations. As shown in the top row of Fig. 1, spleen cell suspensions from WT and IL-4−/− mice contained comparable proportions of CD11chigh cells. On average, spleens from WT mice contained 2.39 ± 0.48% CD11chigh cells (mean ± SD of three samples), while spleens from IL-4−/− mice contained 2.10 ± 0.14% CD11chigh cells. Lymph node suspensions from WT and IL-4−/− mice also contained similar proportions of DC11chigh cells, with 1.39 ± 0.12% CD11chigh cells in WT mice, and 1.25 ± 0.29% in IL-4−/− mice.

DC comprise a heterogeneous population of cells expressing different surface markers (18). The distribution of DC into different subpopulations was compared in WT and IL-4−/− mice. As shown in Fig. 1, the CD11chigh population from the spleens and lymph nodes of WT and IL-4−/− mice comprised comparable proportions of cells expressing the DC subpopulation markers CD11b, CD8 and CD4. The expression of other markers associated with DC function, MHC II, CD40, CD80 and CD54, were also compared using density-gradient enriched spleen DC populations. As shown in Fig. 2, each of the above markers was expressed at a comparable level on splenic DC from WT or IL-4−/− mice. Because the spleens and lymph nodes of WT and IL-4−/− mice comprised similar numbers of cells, we conclude that IL-4 deficiency did not cause a detectable defect in the development and differentiation of spleen or lymph node DC.

**IL-4−/− DC can stimulate T cell responses in vitro and in vivo**

To establish whether the ability of DC to initiate immune responses was compromised by development in an IL-4-deficient environment, we assessed the ability of WT and IL-4−/− splenic DC to initiate immune responses in vitro and in vivo. Splenic DC were isolated by positive selection using anti-CD11c antibodies and magnetic beads, incubated with the relevant antigens for 2 h, and then cultured for 2 days with specific CD4+ or CD8+ cells purified from the lymph nodes of TCR transgenic mice. The WT and IL-4−/− DC populations used in these studies were of comparable purity in each experiment, and comprised similar proportions of CD8+ and CD4+ cells (data not shown). Antigen-specific CD4+ T cells were obtained from OT-II mice, which carry a TCR specific for OVA+I-A^b, while antigen-specific CD8+ T cells were from line 318, which carry a TCR specific for LCMV-GP33-41+D^b.

To induce the proliferation of CD4+ OT-II cells, DC were incubated in the presence of the minimal OVA epitope OVA323-339, or in the presence of whole OVA protein (OVAp), for 2 h before the assay. As shown in Fig. 3, at every cell concentration tested, DC from WT or IL-4−/− mice induced similar proliferation of OT-II T cells. This was the case regardless of whether OVAp or the minimal OVA epitope OVA323-339 were used as antigens. Figure 3 shows a similar experiment carried out to compare the proliferation of CD8+ T cells to antigen presented on WT or IL-4−/− DC. In this case, DC were incubated in the presence of the synthetic peptide LCMV-GP33-41, which is a high affinity agonist for the line 318 transgenic TCR, or the substituted peptide A4Y, which is a weak agonist. Although T cell proliferation to the weak agonist A4Y was much lower than to the agonist LCMV-GP33-41, undistinguishable thymidine incorporation was observed when using WT or IL-4−/− DC. Together these results indicate that the two populations of DC express comparable levels of MHC and costimulatory molecules, and have comparable abilities to process antigen (in the case of OVAp).

Induction of in vivo immune responses may have more stringent requirements than induction of in vitro proliferation, as both APC and T cells are required to migrate to the appropriate site where interaction can occur. To evaluate the
T cell response in vivo we monitored cell division of antigen-specific T cells labeled with CFSE, where CFSE fluorescence can be observed to halve at each consecutive cell division. TCR transgenic T cells were injected i.v. on day 0, and antigen-loaded DC were administered s.c. on the following day. T cell division was evaluated in the draining lymph nodes at 72 h after DC administration, as this time was found to be optimal in pilot experiments. As shown in Fig. 4, CD4 + OT-II cells underwent a comparable number of divisions regardless of whether antigen was presented in the context of a WT or an IL-4 Δ/Δ DC. Similarly, CD8 + line 318 T cells divided a similar number of times after immunization with antigen presented on WT or IL-4 Δ/Δ DC.

An additional approach to evaluate T cell responses by WT and IL-4 Δ/Δ DC

T cell division as evaluated by CFSE staining is a sensitive measure of early T cell responses to antigen recognition, but does not necessarily reflect the generation of effector cells. In order to establish whether early T cell division had been followed by generation of effector T cells, we evaluated the development of cytotoxic activity in C57Bl/6 mice that had been immunized s.c. with WT or IL-4 Δ/Δ DC that had been coated with LCMV-GP33-41 or OVA257-264 peptide. Unlike the experiments in the previous paragraph, these experiments...
were carried out in C57Bl/6 mice that had received no adoptive transfer of TCR transgenic T cells, thus reflecting the generation of CTL from a naive repertoire. To measure cytotoxic activity, 1 week after immunization, mice were injected i.v. with target spleen cells that had been labeled with CFSE and loaded with LCMV-GP 33–41 or OVA 257–264 peptide, or labeled with CMTMR and loaded with no peptide. The differential survival of these target spleen cells in vivo was evaluated 48 h after target cell transfer. Target cell recoveries were evaluated in immunized mice and compared to recoveries in naive C57Bl/6 mice to minimize confounding effects of the dye label on target survival. As shown in Fig. 5, cytotoxic activity was readily demonstrated in immunized mice. No difference was apparent in the LCMV-GP33–41-specific CTL activity induced by WT or IL-4−/− DC immunization, even when limiting numbers of DC were used. In addition, IL-4−/− DC were also able to induce OVA257–264-specific CTL, although the CTL activity was somewhat lower than in mice immunized with WT DC. Therefore, WT and IL-4−/− DC were both able to induce CTL differentiation in vivo.

As a further measure of the ability of DC to activate effector T cell responses, we also evaluated their capacity to induce tumor-protective immunity. We therefore immunized C57Bl/6 mice with LCMV-GP33–41 peptide-loaded DC prepared from WT or IL-4−/− mice, and evaluated their ability to resist a challenge with LCMV-GP33–41-expressing tumor cells.

Fig. 2. Surface phenotype of splenic DC from WT or IL-4−/− mice: DC were obtained from WT or IL-4−/− mouse spleens by collagenase and DNase digestion, and enriched on a density gradient before analysis by flow cytometry. DC were identified on the basis of positive CD11c staining using the HL-3 or N418 mAbs as in Fig. 1, and counter stained with a second antibody as indicated. Dead cells were excluded from analysis. Thick lines: WT DC; thin lines: IL-4−/− DC; shaded histograms: no staining.

Fig. 3. WT and IL-4−/− DC can prime T cell proliferation in vitro: DC were prepared from WT or IL-4−/− spleens by collagenase digestion and positive selection using anti-CD11c biotin and Streptavidin magnetic beads. The selected populations comprised 75% CD11chigh cells for the WT DC population and 82% for the IL-4−/− DC population. Both populations were loaded in vitro with the indicated antigens by co-incubation for 2 h at 37°C, and their ability to induce T cell proliferation was measured in a 3 day thymidine incorporation assay using purified T cells from the indicated TCR transgenic mouse strains. The number of CD11chigh cells/well is indicated. Data show mean ± SD of triplicate wells.
administered 1 week later. As shown in Fig. 6(A), these freshly prepared DC induced only weak or undetectable anti-tumor immune responses. In the attempt to induce more powerful anti-tumor immune responses, we used DC that had been activated by overnight adherence to plastic. This method of purification also yielded similar numbers of WT and IL-4−/− DC, which had similar surface phenotypes. As shown in Fig. 6(B), both DC populations could induce tumor protective immunity in recipient mice. When lower numbers of DC were used to immunize mice, no effect on tumor growth could be detected (data not shown). We conclude that both WT and IL-4−/− DC are able to induce the activation of effector T cells with comparable tumor-protective activity.

Conclusions

Several reports have described a role for IL-4 in CD4+ T cell responses, CTL generation and DC function. IL-4 appears necessary for the early production of IL-12 by DC (7). Lack of IL-4 production causes altered resistance to infection with the protozoan parasite Leishmania major (5) and defective CTL generation after vaccination with a recombinant protein (12). T cells have been identified as the source of IL-4 in some of these experiments (4), but other cell types, including monocyctic cells, have also been implicated (12). Although DC are critical components of the immune response, and effects of IL-4 on their function have been observed (7,15), the properties of DC in IL-4−/− mice have not been directly addressed.

In this report we compare the ability of WT and IL-4−/− DC to induce the early activation of CD4+ and CD8+ T cells in vitro and in vivo, and initiate tumor-specific CD8+ effector T cells in vivo. In most experiments we used DC that were freshly isolated from murine spleen, and had not been activated by deliberate exposure to infectious stimuli or adherence to plastic. DC were loaded with synthetic peptide antigen and injected into host mice where their ability to induce specific immune responses

**Fig. 4.** WT and IL-4−/− DC can prime T cell proliferation in vivo: C57Bl/6 mice received an adoptive transfer of CFSE-labeled TCR transgenic T cells on day 0, and 1 day later were immunized with 10^6 CD11chigh cells from either WT or IL-4−/− mice, which had been prepared and loaded with antigen as indicated in the legend to Fig. 3. The results are representative of three independent experiments using populations that comprised 37–38% WT and 42% IL-4−/− CD11chigh cells. T cell division was assessed in the draining lymph nodes 72 h after DC injection. Bars indicate the number of cells at each division ± SD.

**Fig. 5.** WT and IL-4−/− DC can initiate cytotoxic T cell responses in vivo: C57Bl/6 mice were immunized with the indicated numbers of CD11chigh cells from either WT or IL-4−/− mice, which had been prepared and pulsed with antigen as described in the legend to Fig. 2. For the response to LCMV-GP33–41, the DC populations used comprised 53.5% CD11chigh cells for the WT DC population and 58% for the IL-4−/− DC population. For the response to OVA257–264, the percentages of CD11chigh cells were 46% and 40% for WT and IL-4−/−, respectively. Seven days after immunization, the cytotoxic activity in recipient mice was evaluated by co-injecting CFSE-labeled and CMTMR-labeled spleen target cells that had been pulsed with the relevant peptide antigens or left untreated, respectively. The preferential elimination of peptide-loaded targets was assessed 24 h later by flow cytometry of lymph node cell suspension, and is expressed as percent killing ± SEM, according to the formula described in the Methods.
mediated in large part by secretion of IFN-\(\gamma\). In our model, protective immunity to tumor challenge is activated by overnight culture. We have previously shown that tumor responses required the use of DC that had been induced by the two types of DC were also similar, although anti-proliferation. In addition, CTL activity and anti-tumor activity were elicited regardless of whether WT or IL-4 \(-/-\) DC were used in our experiments, we conclude that sufficient amounts of IL-12 were likely to be present to allow full CD8\(^+\) T cell activation. We therefore conclude that the presence of IL-4 during the final stages of DC maturation is sufficient for the full antigen presenting function of these cells.

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### Abbreviations
- CFSE: carboxy-fluorescein diacetate succinimidyl ester
- CMTMR: chloromethyl-benzoylamino-tetramethylrhodamine
- DC: dendritic cell
- i.v.: intravenous
- LCMV: lymphocytic choriomeningitis virus
- OVA: chicken ovalbumin
- OVAp: OVA protein
- s.c.: sub-cutaneous
- WT: wild type

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