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Murine Th9 cells promote the survival of myeloid dendritic cells in cancer immunotherapy

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Abstract

Dendritic cells (DCs) are professional antigen-presenting cells to initiate immune responses, and DC survival time is important for affecting the strength of T-cell responses. Interleukin (IL)-9-producing T-helper (Th)-9 cells play an important role in anti-tumor immunity. However, it is unclear how Th9 cells communicate with DCs. In this study, we investigated whether murine Th9 cells affected the survival of myeloid DCs. DCs derived from C57BL/6 mice bone marrow were cocultured with Th9 cells from OT-II mice using Transwell, and the survival of DCs was examined. DCs cocultured with Th9 cells had longer survival and fewer apoptotic cells than DCs cultured alone in vitro. In melanoma B16-OVA tumor-bearing mice, DCs conditioned by Th9 cells lived longer and induced stronger anti-tumor response than control DCs did in vivo. Mechanistic studies revealed that IL-3 but not IL-9 secreted by Th9 cells was responsible for the prolonged survival of DCs. IL-3 upregulated the expression of antiapoptotic protein Bcl-xL and activated p38, ERK and STAT5 signaling pathways in DCs. Taken together, our data provide the first evidence that Th9 cells can promote the survival of DCs through IL-3, and will be helpful for designing Th9 cell immunotherapy and more effective DC vaccine for human cancers.

Keywords

Th9 cells; Dendritic Cells; survival; IL-3; cancer immunotherapy

Introduction

Dendritic cells (DCs) are the most potent antigen presenting cells to induce primary immune responses by effectively priming naïve T cells. DCs reside in tissues as immature cells, capture and process antigen, and then mature in response to inflammatory cytokines. Mature DCs are characterized by upregulated expression of MHC class I and II and costimulatory molecules. DCs migrate to the draining lymph node, where antigen-bearing DCs activate

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CD4⁺ or CD8⁺ T cells to initiate immune responses. DCs are messengers between the innate and adaptive immunities as well as important regulator of adaptive immunity in cancer [1].

DCs are diverse cell populations which include at least five major subsets: plasmacytoid DCs; CD11b⁺ DCs; CD8⁺ DCs; Langerhans cells; and the monocyte-derived DCs [2]. Depending on different DC subsets and maturation states, the survival of DCs in vivo varies from 1 day to several weeks [3,4]. After presenting antigen, mature DCs undergo rapid apoptosis. Prolonged survival of DCs in secondary lymphoid tissues may affect quantity and quality of immune responses including T cell priming and the strength of the immune responses both in inflammatory diseases and cancer [3]. The fate of DCs is controlled by components of innate and adaptive immune systems. These include pathogen-derived molecules such as CpG as ligand for Toll-like receptors, inflammatory cytokines, and interaction with T cells through CD40 ligand or tumor necrosis factor (TNF)-related activation-induced cytokine [5-9].

Recently, T-helper (Th)-9 cells are identified as a specific subset of CD4⁺ T cells and distinct from other Th cells by predominant producing interleukin (IL)-9. Th9 cells can be induced by culturing naïve CD4⁺ T cells with transforming growth factor (TGF)- β and IL-4 in vitro and its development requires transcription factors including PU.1, IRF4, and STAT6 [10-13]. The inflammatory function of Th9 cells has been described in allergic inflammation and autoimmune diseases [10,12]. Most recently, our lab and Kupper's group revealed a new role for IL-9 and Th9 cells in the antitumor immunity [14,15]. Adoptive transfer of Th9 cells decreased tumor development in melanoma-bearing mice by the induction of CC chemokine ligand 20, and recruitment of CD8⁺ T cells, DCs and mast cells [14,15]. In other studies, Th9 cells seem to regulate the immune responses as negative or positive factors in inflammatory diseases [16,17]. However, it is unclear whether Th9 cells can regulate the survival and function of DCs during anti-tumor immune responses.

In present study, we investigated and showed that Th9 cells could regulate the survival of DCs via secreted cytokine(s). Mechanistic studies were performed by using cytokine array to analyze the molecule(s) that mediated the regulatory effect on DCs. Finally, we injected Th9-conditioned DCs into melanoma tumor-bearing mice to investigate the in vivo survival and anti-tumor ability of DCs. We found that the survival of DCs was promoted by coculture with Th9 cells and IL-3 produced from Th9 cells was responsible for the prolonged survival of DCs. We also showed that in vitro Th9-conditioned DCs had enhanced survival and increased anti-tumor ability in vivo in melanoma-bearing mice. Thus, our study identifies that Th9 cells promote the survival and function of mature DCs, and sheds light on the immunoregulatory function of Th9 cells. These results may be helpful to develop new strategy to improve the cellular immunotherapy in human cancers.

Materials and Methods

Mice and cell lines

Male C57BL/6, OT-I (C57BL/6-Tg(Tcr α Tcr β)1100Mjb/J) and OT-II (C57BL/6-Tg(Tcr α Tcr β)425Cbn/J) mice were purchased from National Cancer Institute and the

Jackson laboratory respectively. All mice were maintained in a specific pathogen-free barrier facility at the University of Texas MD Anderson Cancer Center.

B16-OVA melanoma cell line (ATCC) was cultured in complete Iscove's modified Dulbecco's medium (Gibco) supplemented with 10% heat-FBS (Hyclone), 2 mM glutamine (Invitrogen), and 100 U/mL penicillin-streptomycin (Invitrogen). All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center.

Generation of murine DCs and differentiation of Th9 cells

DCs were generated from mouse bone marrow (BM) with 10 ng/mL murine Granulocyte-macrophage colony-stimulating factor (GM-CSF, R&D systems) according to protocol described previously [18]. On day 7 of culture, non-adherent and loosely adherent cells were collected as the immature DCs and were matured with TNF- α (10 ng/mL; R&D systems), IL-1 β (10 ng/mL; R&D systems), and GM-CSF (10 ng/mL). After 24 hours, mature DCs were collected for coculture with Th9 cells.

Generation of ovalbumin (OVA)₃₂₃₋₃₃₉ peptide-specific Th9 cells was performed as described previously [14]. Naïve CD4⁺CD62L⁺ T cells isolated from spleens and lymph nodes of OT-II mice were cultured with irradiated splenocytes depleted of T cells using CD90.2 microbeads (Miltenyi Biotech) from C57BL/6 mice with TGF- β (1 ng/mL; R&D Systems), IL-4 (10 ng/mL; R&D Systems), and anti-interferon (IFN)- γ antibody (Ab) (10 μ g/mL; eBioscience) in the presence of OVA₃₂₃₋₃₃₉ peptide (5 μ g/mL; New England Peptide). After 3-day culture, T cells were harvested for coculture with DCs as OVA-specific Th9 cells.

Coculture with Th9 cells and mature DCs

Th9 cells and DCs were cocultured using Transwell system in complete RPMI-1640 (Gibco) with 10 % FBS. DCs were plated on 24-well plates with 2×10^5 cells in 1000 μ l per well. Th9 cells were added into the top chamber of the Transwell (pore size 0.4 μ m; Costar) at a final concentration of 2×10^5 cells in 200 μ l medium. Control DCs were cultured using the same condition, but in the absence of Th9. Transwell coculture system permitted the two cell populations to grow together in the same well, but prevented direct cell-to-cell contact. After 3 or 6 day coculture, the number of living DCs was counted by trypan blue (Invitrogen) and DCs were stained with FITC-Annexin V (Invitrogen) and propidium iodide (PI; Sigma-Aldrich) for measuring apoptotic cells. In some experiments, mature DCs were stimulated by IL-3 (10 ng/mL, R&D Systems) with inhibitors for ERK (U0126, 10 μ M, Cell Signaling), STAT5 (sc-355979, 50 μ M, Santa Cruz), or p38 (SB 202190, 2.5 μ M, EMD Millipore).

Detection of cytokine expression and production

Cytokine array analysis was used for testing profile of cytokines produced from DCs alone, Th9 alone, and DCs cocultured with Th9 cells. The mouse cytokine array I (RayBiotech, Inc.) was performed with supernatants from DCs alone, Th9 alone, and DCs cocultured with Th9 cells according to the manufacturer's instructions. The membranes were exposed to X-

ray film and proteins quantified by densitometric analysis using the ImageJ software (GE Healthcare).

Secreted cytokines IL-3, IL-9, and IL-17 in supernatants from DCs alone, Th9 alone, and DCs cocultured with Th9 cells for 3 days were measured using ELISA according to the manufacturer's instructions (e-Bioscience).

Real-time PCR was performed to detect relative cytokine mRNA expression in DCs cocultured with or without Th9 cells by using Power Syber Green PCR Master mix (Applied Biosystems). The data were normalized to a GAPDH reference. Each of the primer pairs used for these analyses was listed in **Supplementary Table 1**.

Western blot analysis

DCs cocultured with or without Th9 cells were harvested and lysed with lysis buffer containing protease inhibitor. Proteins were separated on a 12% polyacrylamide gel, and blotted onto nitrocellulose membranes. Mouse monoclonal Ab against Akt was obtained from Cell Signaling, and Ab against phosphorylated (p)-Ark was obtained from Santa Cruz. Rabbit polyclonal Abs against p38 MAPK, STAT5, cleaved (c)-Caspase-3, Bcl-xL, and Bax were obtained from Cell Signaling. Rabbit monoclonal Abs against p-p38, ERK, p-ERK, and p-STAT5 were obtained from Cell Signaling. Rabbit anti- β -actin polyclonal Ab was obtained from Sigma-Aldrich. After blocking with 5% nonfat milk protein/PBS, membranes were incubated with primary Abs and visualized with horseradish peroxidase-conjugated secondary Abs (Bio-rad) and an ECL substrate detection system (Amersham Pharmacia).

In vitro functional test of Th9-conditioned DCs

After 3-day coculture with Th9 cells, DCs were harvested and used to stimulate OVA-specific T cells in vitro. Naïve CD4⁺ T cells from OT-II mice, and naïve CD8⁺ T cells from OT-I mice were isolated using the naïve T cell kits (Stem Cell Technologies). After labeling with 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen), CD4⁺ T cells or CD8⁺ T cells were stimulated by DCs in the presence of OVA₃₂₃₋₃₃₉ peptide or OVA₂₅₇₋₂₆₄ peptide (0.5 μ g/mL, New England Peptide), respectively. CFSE dilution of T cells was detected by flow cytometry after 3-day stimulation. Some activated CD8⁺ T cells were fixed, permeabilized and stained with anti-Granzyme B Ab (eBioscience). The expression of Granzyme B was measured with flow cytometry. Data were analyzed by FlowJo software (Tree Star).

Tumor model and DC treatment in vivo

B16-OVA melanoma cells (2×10^5 cells) were s.c. injected into mice. Three days later, DCs were s.c. injected on the opposite site of mice. DCs cultured with or without Th9 cells for 3 days were pulsed with OVA₂₅₇₋₂₆₄ peptide (5 μ g/mL, New England Peptide), class I (Kb)-restricted peptide epitope of peptide and then were labeled with CFSE by incubation at 37 for 5 minutes [14]. After thorough washing, the labeled DCs (5×10^5 cells) were s.c. injected on opposite site of the B16-OVA bearing mice. Three days after injection of CFSE-labeled DCs, mice were sacrificed and draining lymph nodes, non-draining lymph nodes, and spleens were harvested for analyzing the population of CFSE-labeled DCs by flow

cytometer. Total lymph node cells or spleen cells were gated and the CFSE labeling was analyzed.

To examine the in vivo anti-tumor effect of Th9-conditioned DCs, tumor-bearing mice (n=8/group) were treated with DCs (5×10^5 , s.c. injection) on day 3 and day 10 after B16-OVA inoculation (4×10^5 cells, s.c.). Control DCs or Th9-conditioned DCs were pulsed with OVA₂₅₇₋₂₆₄ peptide, class I (Kb)-restricted peptide epitope, before s.c. injection. Tumor growth was monitored. In some experiments, spleen cells from DC-treated mice on day 24 after tumor injection were isolated and stimulated by irradiated B16-OVA cells in vitro for 3 days. Then T cells were restimulated by phorbol myristate acetate (PMA, 50 ng/mL; Sigma-Aldrich), ionomycin (250 ng/mL; Sigma-Aldrich) and GolgiPlug (1 μ L/mL; BD Bioscience) for 6 hour. Intracellular expression of IFN- γ in T cells was detected by flow cytometry.

Statistical analysis

Statistical analysis was performed using the Prism 4 software (GraphPad software, San Diego, CA). The results are shown as the mean $\pm$ SEM. Statistical analysis was performed using a Student's *t*-test, one-way ANOVA or two-way ANOVA.

Results

Th9 cells promote the survival of DCs

To investigate whether Th9 cells affected the survival of DCs, we cocultured mature DCs with Th9 cells using Transwell system. OVA₃₂₃₋₃₃₉ peptide-specific Th9 cells were generated in vitro, which were characterized with about 25% IL-9- and 10% IL-10- expressing CD4+ T cells, with low expression of IFN- γ , IL-4, and IL-17 [14]. For coculture with DCs and Th9 cells, Transwell was used to separate DCs and Th9 cells. After 1, 3 and 6 day coculture, DCs cocultured with Th9 cells maintained higher numbers than DCs cultured alone ($P < 0.001$; **Fig. 1a**). The apoptosis of DCs was also tested 6 days after coculture. Significantly decreased apoptosis of DCs cocultured with Th9 cells was found ($P < 0.001$; **Fig. 1b-c**). More cleaved caspase 3 was detected in DCs alone than that in DCs cocultured with Th9 cells for 2 days (**Fig. 1d**). As DCs and Th9 cells were separated by Transwell during the coculture, these results indicated that Th9 cells can prolong the survival of mature DCs through soluble molecules.

Next we investigated how long the interaction between DCs and Th9 cells was required for promoting the survival of DCs. We cocultured DCs and Th9 cells for different days (from 1-day coculture to 6-day coculture). After the coculture, Th9 cells in Transwells were removed and DCs were cultured alone until day 6. Control DCs were DCs cultured alone without Th9 for 6 days. A positive effect of Th9 in supporting the survival of DCs was already observed in a 2-day coculture ($P < 0.05$) whereas stronger protection was seen with prolonged (3-6 day) cocultures ($P < 0.001$; **Fig. 1e**). These results suggested that 3-day coculture interaction is enough to maximally prolong the survival of DCs.

We also tested whether coculture with Th9 cells regulated the expression of cytokines in DCs with real-time PCR. The mRNA expression of *IL-1 β* , *IL-4*, *IL-6*, and *monocyte chemotactic protein-1* was increased in DCs cocultured with Th9 cells ($P < 0.05$ to $P < 0.01$,

compared with DCs alone), whereas mRNA expression of *IL-10*, *IL-12*, and *TNF- α* was decreased ($P < 0.05$ to $P < 0.01$, compared with DCs alone). The mRNA expression of *TGF- β* and *IFN- γ* was similar between DCs cultured alone and DCs cocultured with Th9 cells (Supplementary Fig. 1).

IL-3 from Th9 cells is responsible for the prolonged survival of DCs

To identify which soluble factor(s) were responsible for the survival of DCs, we compared the cytokine profile in medium of 3-day coculture of DCs and Th9 cells using cytokine array (Fig. 2a). As compared with medium from DCs alone and from Th9 cells alone, medium from coculture of DCs and Th9 cells contained higher levels of IL-3 and IL-9 ($P < 0.001$; Fig. 2b). The level of IL-6 was similar in culture media from DCs alone and DCs cocultured with Th9 cells. The production of IFN- γ , IL-2, and IL-10 was barely detected. ELISA results confirmed the increased secretion of IL-3, IL-9, and IL-17 in coculture medium of DCs and Th9 cells. Culture medium from coculture of DCs and Th9 cells contained increased levels of IL-3 and IL-9 compared with DCs alone and Th9 cells alone ($P < 0.001$; Fig. 2c). IL-17 secretion was also increased during the coculture of DCs and Th9 cells, but the concentration was quite low as compared with that of IL-3 and IL-9 (Fig. 2c).

Next we examined which cytokine(s) were involved in the survival of DCs by adding neutralizing Abs to the coculture of DCs and Th9 cells. Anti-IL-3 Ab inhibited the enhanced survival of DCs by Th9 cells, whereas anti-IL-9 had no effect (Fig. 3a). When recombinant IL-3, IL-9 or IL-17 were added, alone or in a combination, into DC cultures, only IL-3 was able to increase the number of living DCs and no synergic effect could be seen with the combination of IL-9 or IL-17 (Fig. 3b). We also tested the effect of IL-3 on the apoptosis of DCs. DCs treated with IL-3 showed decreased apoptosis, and the antiapoptotic effect of IL-3 was similar to coculture with Th9 cells. Anti-IL-3 Ab could block the anti-apoptotic effect of Th9 cells on DCs (Fig. 3c and 3d). These results suggested that IL-3 regulates the lifespan and apoptosis of DCs. To investigate whether IL-3 affected the survival of Th9 cells, IL-3 or anti-IL-3 Ab were added to Th9 cell culture. Results showed that IL-3 and anti-IL-3 Ab did not affect the survival of Th9 cells (Supplementary Fig. 2).

Kinetics of IL-3 expression by Th9 cells

To identify the cell origin of IL-3, we measured the mRNA expression of IL-3 in DCs and Th9 cells from the Transwell coculture. The expression of *Il-3* mRNA in Th9 cells was higher than that in DCs, and peaked on day 1 after the coculture ($P < 0.01$; Fig. 4a). We also examined the protein level of IL-3 in coculture medium of DCs and Th9 cells by ELISA. The concentration of IL-3 was significantly higher in coculture medium of DCs and Th9 cells than that from DCs or Th9 cells alone ($P < 0.001$; Fig. 4b). These data indicated that the interaction between DCs and Th9 cells enhance IL-3 production from Th9 cells.

Signaling pathways induced by IL-3 in DCs

To investigate the effect of IL-3 on DCs, we first examined the expression of IL-3 receptor on DCs. Both immature DCs and mature DCs expressed IL-3 receptor, while increased IL-3 receptor expression was detected on DCs cocultured with Th9 cells for 3 days (Fig. 5a).

These results indicated that Th9 cells upregulate the expression of IL-3 receptor on BM derived DCs, which may enhance the response of DCs to IL-3.

Binding of IL-3 to its receptor leads to the stimulation of multiple signaling transduction pathways including Raf/MEK/ERK, PI3K/Akt, and JAK/STAT [19,20]. To determine which signaling pathways were involved in the survival of DCs induced by IL-3, DCs were analyzed by western blotting with specific Abs 1 and 2 days after adding IL-3 into DC cultures. As shown in **Fig. 5b**, compared with DCs alone, increased levels of phosphorylated (p) p38 and pERK were observed in DCs stimulated by IL-3 for 2 days, whereas pAkt was not changed. Expression of pSTAT5 was detectable in DCs with IL-3 stimulation for 1 and 2 days. The cleaved caspase 3 was detected in DCs alone but not in IL-3 stimulated DCs for 2 days. An increased Bcl-xL and a decreased Bax were observed in DCs after 2-day IL-3 stimulation. To identify which signal pathway was essential for the protective effect of IL-3 on DC survival, we used inhibitors for p38, ERK and STAT5 in DC culture. We found that ERK inhibition blocked the protective effect of IL-3 on DC survival (**Supplementary Fig. 3**). These results indicated that IL-3-induced prolonged survival of DCs is mediated through various survival signal pathways, and ERK is the most important one.

Function of Th9-conditioned DCs in B16-OVA-bearing mice

To examine the function of Th9-conditioned DCs in vitro, we tested the ability of DCs to stimulate OVA-specific CD4⁺ and CD8⁺ T cells from OT-II and OT-I mice, respectively. We found that Th9-conditioned DCs had similar capacity to stimulate the proliferation of T cells (**Fig. 6a**). The expression of Granzyme B was similar in CD8⁺ T cells stimulated by Th9-conditioned DCs and control DCs, too (**Fig. 6b**). These results indicate that Th9-conditioned DCs function well in vitro to simulate T cells.

To examine whether DCs cocultured with Th9 cells survived longer in vivo, we used the melanoma B16-OVA s.c. injected mice. Three days after B16-OVA injection, control DCs or DCs conditioned by Th9 cells for 3 days in vitro were labeled with CFSE and then s.c. injected into B16-OVA-bearing mice. Three days after DC injection, mice were sacrificed, and CFSE-labeled DCs were identified and enumerated in the draining lymph nodes, non-draining lymph nodes, and spleens by flow cytometry. The percentage of CFSE-labeled DCs in mice injected with DCs conditioned by Th9 cells was significantly higher in the draining lymph nodes and non-draining lymph nodes but not in the spleens than in mice injected with DCs cultured alone ($P < 0.001$; **Supplementary Fig. 4a and 4b**). To investigate the anti-tumor function of DCs cocultured with Th9 cells in vivo, OVA₂₅₇₋₂₆₄ peptide-pulsed control DCs or Th9-conditioned DCs were used to immunize mice bearing B16-OVA tumors. We measured IFN- γ expression in CD4⁺ and CD8⁺ T cells from spleens of the tumor-bearing mice treated with control DCs or Th9-conditioned DCs. T cells were restimulated by irradiated B16-OVA cells ex vivo. Results showed that T cells from mice treated with Th9-conditioned DCs expressed more IFN- γ (**Fig. 6c**). Tumor growth curve showed that Th-9 conditioned DCs had stronger anti-tumor effect than control DCs (**Fig. 6d**). These results indicate that DCs conditioned by Th9 cells can induce stronger anti-tumor responses in melanoma-bearing mice in vivo.

Discussion

In this study, we found that Th9 cells could enhance the survival of mature BM derived DCs through IL-3 secreted by Th9 cells. Expression of IL-3 receptor on DCs was upregulated during coculture with Th9 cells, which resulted in prolonged survival of DCs by upregulation of Bcl-xL, ERK and STAT5 dependent pathways. DCs cocultured with Th9 cells survived longer than control DCs did and had stronger anti-tumor effect in melanoma-bearing mice in vivo.

Th9 subset is a less-well investigated Th population as compared with other subsets such as Th1, Th2, Th17 and Treg. The anti-tumor effect of Th9 was identified recently, which suggests that Th9 cells are an important component of the immune system. However, the interaction between Th9 cells and other immune cells is unclear. Here we investigated the regulatory effect of Th9 cells on DCs using in vitro Transwell coculture system. We found that coculture of Th9 cells and DCs could improve the survival of DCs in vitro and in vivo. IL-9 is well known for its ability to promote the survival of diverse cell types including T cells, mast cells, eosinophils, as well as neurons, tumor cells and epithelial cells [21]. Because Th9 cells mainly produce IL-9 and one of the main functions of IL-9 is to mediate antiapoptotic effects in target cells, we expected that IL-9 could regulate the survival of DCs. However, adding exogenous IL-9 or neutralizing IL-9 had no effect on the survival of DCs. By using cytokine array, we found that IL-3 secretion was elevated after Th9-DC coculture, and neutralizing IL-3 could abrogate the improved survival of DCs. Furthermore, real-time PCR measurement confirmed that Th9 cells contributed to IL-3 expression in the coculture. These findings enriched our knowledge in the cytokine profile of Th9 cells, and in the interaction between Th9 cells and DCs.

IL-3 is a pleiotropic cytokine that can stimulate the development of a broad range of hematopoietic cell lineages from BM stem cells, including mast cells, basophils, neutrophils, eosinophils, macrophages, erythrocytes, megakaryocytes and DCs [22,23]. Human monocytes cultured in the presence of IL-3 could differentiate into DCs that had suppressed IL-12 secretion and favored Th2-cell responses [24]. On the other hand, IL-3 together with IFN- β induced differentiation of monocytes to DCs. Although these DCs secrete low levels of IL-12 in response to LPS, they could trigger vigorous T-cell immune responses including production of high levels of IFN- γ and increased proliferation [25]. The expression of IL-3 receptor α -chain has been regarded as one of distinctive markers for human plasmacytoid DCs ex vivo and IL-3 is required for pDC survival and maturation as well as development [26,27]. In this study, we found that IL-3 also promotes the survival of mature myeloid DCs derived from murine BM, indicating that IL-3 is a very important regulatory molecule for DCs.

As shown in **Fig. 5**, mature DCs expressed low levels of IL-3 receptor, but DCs cocultured with Th9 cells had increased expression of the receptor, although we do not know which factor(s) in the coculture are responsible for upregulating the expression of IL-3 receptor on DCs. We also investigated the signaling transduction pathways after treatment of IL-3 in DCs. Hou and Van Parijs reported that Toll-like receptors and T-cell costimulatory molecules activate DC survival pathway that is dependent on Bcl-xL [28]. STAT5 regulates

the expression of Bcl-xL, but not Bcl-2 or Bax in hematopoietic cells in response to IL-3 [29]. Our results showed that pSTAT5 was upregulated in DCs treated with IL-3 and it was correlated with the upregulation of Bcl-xL, indicating that IL-3 suppresses apoptosis of DC by upregulating the expression or phosphorylation of the Bcl-2 gene family members. The cleaved caspase 3 appeared in DCs cultured for 2 day, but not in DCs cocultured with Th9 cells on the same time. The fact that DC apoptosis was protected by caspase inhibitor was noted by Chen et. al [30]. Our data indicated that after stimulation of IL-3, prolongation of DC lifespan was attributed to the survival signal pathways such as Bcl-xL, p38, ERK, and STAT5 signaling pathways. These different signaling pathways appear to participate in DC survival depending on the stimulatory factors.

Recently, we reported that Th9 cells exert anti-tumor immunity by recruiting effector cells as well as DCs into tumor site [14]. Although DCs cocultured with Th9 cells expressed different cytokine profile compared with control DCs (**Supplementary Fig. 1**), our in vivo results showed that Th9-conditioned DCs stimulated stronger anti-tumor response in melanoma-bearing mice. These data indicated that the anti-tumor function of Th9 cells may act through recruiting DCs [14] as well as protecting DCs from apoptosis.

In conclusion, our study reports for the first time that Th9 cells promote the survival of myeloid DCs through IL-3, and Th9 conditioning can improve the anti-tumor effect of DCs. This finding not only sheds new light on the function of Th9 cells and provides new evidence for the application of Th9 cells in cancer treatment, but also will be helpful to improve the strategy of DC vaccine in tumor immunotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Ab	Antibody
BM	Bone marrow
CFSE	5(6)-carboxyfluorescein diacetate succinimidyl ester
DC	Dendritic cells
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IFN	Interferon
IL	Interleukin
OVA	Ovalbumin

PMA	Phorbol myristate acetate
Th	T-helper
TGF	Transforming growth factor
TNF	Tumor necrosis factor

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Précis

Th9 cells improve the survival and anti-tumor function of dendritic cells, so facilitating their development may enhance dendritic cell-based cancer vaccines.

Fig. 1. Th9 cells prolong the survival of DCs in vitro

a Survival of DCs was enhanced by coculture with Th9 cells. Mature DCs were cultured alone or cocultured with Th9 cells in Transwell (0.4 m pore size) for 6 days. The number of living DCs was counted on day 1, day 3, and day 6 after the coculture using trypan blue. **b** Apoptosis of DCs was inhibited by coculture with Th9 cells. Apoptosis of DCs during the culture was analyzed with Annexin V-PI staining on day 6 of the coculture. **c** Data of apoptotic DCs were summarized. **d** Activation of caspase 3 in DCs during the coculture. Control DCs and Th9-conditioned DCs were harvested for Western blot analysis to detect the cleaved caspase 3. **e** Survival of DCs maintained after removal of Th9 cells. DCs were cocultured with Th9 cells for different days (1 to 6 days). After DC-Th9 coculture, Th9 cells in Transwells were removed and DCs were continued to culture until day 6. Control DCs were DCs cultured without Th9 cells for 6 days. Cell number was counted by trypan blue on day 6. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with DCs cultured alone.

Fig. 2. DC-Th9 coculture alters cytokine profiles

a Cytokine profiles were tested in supernatants from DCs cultured alone, DC-Th9 coculture and Th9 cells cultured alone. DCs cultured alone and Th9 cells cultured alone were used as controls. Supernatants were taken from 3-day culture of DCs cultured alone, Th9 cells cultured alone, and Transwell coculture of DCs and Th9 cells for cytokine array analysis. **b** Data of the cytokine secretion were quantified. The relative levels of secreted cytokines were quantified as area (pixel number) by densitometry using ImageJ software. **c** Secretion of IL-3, IL-9 and IL-17 was measured in cell culture supernatants using ELISA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with DCs cultured alone.

Fig. 3. Th9 cells enhance the survival of DCs via IL-3

a Anti-IL-3 Ab prevented the prolonged survival of DCs during the DC-Th9 coculture. Neutralizing Abs against IL-3, IL-9 or IL-17 were added to the coculture of DC and Th9 cells. After 6 days of culture, the number of DCs was counted by trypan blue. **b** IL-3 promoted DC survival. Recombinant IL-3 (10ng/ml), IL-9 (10ng/ml) or IL-17 (10ng/ml), alone or in a combination, were added to DC culture. Coculture of DC and Th9 cells was served as positive controls. Six days later, the number of DCs was counted by trypan blue. **c** IL-3 inhibited apoptosis of DCs. IL-3 or anti-IL-3 Ab was added to the culture of DCs alone and to DC-Th9 coculture, respectively. Six days later, apoptotic DCs were measured by Annexin V-FITC assay. **d** Data of apoptosis were summarized from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with DCs cultured alone.

Fig. 4. Th9 cells increase IL-3 secretion after coculture with DCs

a Kinetics of relative *IL-3* mRNA expression were tested in DCs and Th9 cells. DCs and Th9 cells were harvested separately on indicated days during DC-Th9 Transwell coculture. Real-time PCR was performed to examine *IL-3* mRNA expression. **b** IL-3 secretion was enhanced by coculture of DC and Th9 cells. Culture media from DCs alone, Th9 cells alone, and coculture of DCs and Th9 cells were collected on indicated days, and IL-3 protein was measured by ELISA. Representative results from 2 independent experiments are shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 5. IL-3 activates survival signaling in DCs

a Expression of IL-3 receptor was increased on DCs by coculture of DC and Th9 cells. Different DC samples were harvested and stained with anti-IL-3 receptor Ab and analyzed by flow cytometry. These samples included immature DCs (iDC), mature DCs (mDC), mature DCs cultured alone for another 3 days (DC), and mature DCs cocultured with Th9 cells (DC+Th9) for 3 days. **b** Survival signaling pathways in DCs was induced by IL-3. One and 2 days after DCs cultured with or without IL-3, cells were harvested and lysate was prepared. Western blot was performed with Abs against p38, phosphorylated (p) p38, ERK, pERK, Akt, pAkt, STAT5, pSTAT5, cleaved (c)-caspase 3, Bcl-xL, Bax and β-Actin. Representative results from 2 independent experiments are shown.

Fig. 6. Th9-conditioned DCs possess improved anti-tumor function in B16-OVA-bearing mice
a Proliferation of OT-II CD4⁺ T cells and OT-I CD8⁺ T cells stimulated by control DCs or Th9-conditioned DCs. Naïve T cells were isolated from OT-II or OT-I mice, labeled with CFSE and stimulated by DCs at the DC:T ratio of 1:5. CFSE dilution was measured after 3-day culture. **b** Expression of Granzyme B in CD8⁺ T cells stimulated by control DCs or Th9-conditioned DCs. **c** Expression of IFN-γ in T cells from DC-treated tumor-bearing mice. Spleen cells from B16-OVA bearing mice treated with DCs were restimulated by irradiated B16-OVA ex vivo for 3 days. Intracellular expression of IFN-γ was detected by flow cytometry. **d** Inhibition of tumor growth by DCs cocultured with Th9 cells. OVA₂₅₇₋₂₆₄ peptide pulsed DCs (5×10^5) were s.c. injected to B16-OVA bearing mice on day 3 and day 10 after tumor inoculation. n=8/group. Representative results of two independent experiments were shown. DC(Th9), DCs conditioned by Th9 in vitro; DC, control DCs.