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Dendritic cell-derived TNF- α is responsible for development of IL-10-producing CD4⁺ T cells

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Abstract

Immature dendritic cells (DCs) appear to be involved in peripheral immune tolerance via induction of IL-10-producing CD4⁺ T cells. We examined the role of TNF- α in generation of the IL-10-producing CD4⁺ T cells by immature DCs. Immature bone marrow-derived DCs from wild type (WT) or TNF- α ^{-/-} mice were cocultured with CD4⁺ T cells from OVA specific TCR transgenic mice (OT-II) in the presence of OVA₃₂₃₋₃₃₉ peptide. The WT DCs efficiently induced the antigen-specific IL-10-producing CD4⁺ T cells, while the ability of the TNF- α ^{-/-} DCs to induce these CD4⁺ T cells was considerably depressed. Addition of exogenous TNF- α recovered the impaired ability of the TNF- α ^{-/-} DCs to induce IL-10-producing T cells. However, no difference in this ability was observed between TNF- α ^{-/-} and WT DCs after their maturation by LPS. Thus, TNF- α appears to be critical for the generation of IL-10-producing CD4⁺ T cells during the antigen presentation by immature DCs.

Keywords

Dendritic cells, CD4⁺ T cells, TNF- α , IL-10, Antigen presentation

1. Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells which are primarily responsible for the initiation and regulation of immune responses against various antigens [1-3]. DCs exhibit a unique ability to activate naive T cells. The DC ability for the antigen presentation to T cells depends on their maturation stage. Immature DCs are present in almost all tissues as sentinels of the immune system. When encountering pathogens, immature DCs recognize the pathogen-derived components via pattern recognition receptors such as Toll-like receptors (TLRs) and differentiate to mature DCs [4]. The mature DCs highly express major histocompatibility complex (MHC) and co-stimulatory molecules on their surface and potentially activate the antigen specific CD4⁺ T cells to eliminate the pathogens.

DCs also play a role in the maintenance of peripheral tolerance to self-antigens in the steady state. Actually, immature DCs induce T cell anergy or IL-10-producing regulatory T cells in vitro and in vivo [5-8]. Thus, it has been considered that interaction of T cells with immature DCs cause immune tolerance, while the interaction with mature DCs generates T cell immunity. However, the precise mechanism underlying the immature DC-mediated induction of IL-10 producing T cells remains unclear.

Tumor necrosis factor (TNF)- α is a major inflammatory cytokine and promotes various inflammatory responses. However, it has been reported that TNF- α -pretreated DCs ameliorate experimental autoimmune encephalomyelitis [9]. It seems that TNF- α exhibits not only proinflammatory functions but also displays immunoregulatory properties.

In the present study, we examined the role of DC-produced TNF- α in the development of IL-10-producing CD4⁺ T cells in vitro using bone marrow-derived DCs (BMDCs) and ovalbumin (OVA)-specific T-cell receptor (TCR) transgenic T cells (OT-II transgenic T cells). We demonstrate herein that the DC-derived TNF- α is responsible for the development of the IL-10-producing CD4⁺ T cells by immature DCs.

2. Materials and Methods

2.1. Mice

Wild type (WT) C57BL/6 mice were purchased from Japan SLC Inc (Hamamatsu, Japan). TNF- α knock out (TNF- $\alpha^{-/-}$) mice and OT-II TCR (OVA₃₂₃₋₃₃₉ peptide specific) transgenic mice on a C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in a specific pathogen-free condition of our animal facility at Hokkaido University. All experiments were approved by regulations of Hokkaido University Animal Care and Use Committee.

2.2. Flow cytometry

FITC-conjugated anti-mouse CD86 mAb (GL1), FITC-conjugated anti-mouse IFN- γ mAb (XF G1.2), PE-conjugated anti-mouse CD40 mAb (3/23), biotin-conjugated anti-mouse I-A^b, PerCPTM-conjugated anti-mouse CD4 (RM4-5), and streptavidin-PerCPTM were purchased from BD Pharmingen (San Diego, CA). PE-conjugated anti-mouse IL-10 mAb (JES5-16E3) and allophycocyanin-conjugated anti-mouse IL-4 mAb (11B11) were obtained from BioLegend Inc. (San Diego, CA). Cells were stained using FITC-, PE-, PerCPTM-, allophycocyanin-, or biotin-conjugated mAbs and streptavidin-PerCPTM. The fluorescence intensity of the cells was analyzed by flow cytometry on EPICS XL (Beckman Coulter Inc., Miami, FL) or FACSCanto II (BD Biosciences, San Jose, CA).

2.3. DC culture

Murine BMDCs were generated by a well established method as previously described [10-12]. Bone marrow cells were prepared from femur and tibial bone marrow of WT or TNF- $\alpha^{-/-}$ mice. After lysis of erythrocytes, MHC class II-, CD45R (B220)-, CD4- and CD8-positive cells were removed by killing with mAbs (1E4, RA3-6B2, GK1.5 and 53-6.7) and rabbit complement.

The cells were extensively washed to remove mAbs, complement, and cell debris. The cells were cultured in 5% FCS RPMI-1640 containing 20 ng/ml GM-CSF (BMDC medium) at a density of 1×10^6 cells/ml/well (24-well plate). On day 2, the medium was gently exchanged to fresh medium. On day 4, non-adherent granulocytes were removed without dislodging clusters of developing DCs, and fresh medium was added. On day 6, free-floating and loosely adherent cells were collected and were used as BMDCs ($>95\%$ CD11c⁺ B220⁻). Unstimulated BMDCs were used as immature DCs. BMDCs cultured with 1 μ g/ml LPS for 24 h were used as mature DCs.

2.4. T cell differentiation in the presence of WT or TNF- α ^{-/-} DCs

CD4⁺ T cells were isolated from spleens and LNs of OT-II TCR transgenic mice. After lysis of erythrocytes, CD4⁺ T cells were selected using anti-CD4 (L3T4) MicroBeads and a MACS column (Miltenyi Biotec, Auburn, CA). Purity of the CD4⁺ T cells (CD4⁺ TCR β ⁺) was $>95\%$. The purified OT-II CD4⁺ T cells (5×10^4 cells) were cocultured with WT or TNF- α ^{-/-} DCs (1×10^4 cells) in the presence of 0.1 μ M OVA₃₂₃₋₃₃₉ peptide in 200 μ l RPMI-1640 containing 10% FCS and 50 μ M 2-ME for 5 days using a 96-well round-bottom plate. In some experiment, 100 ng/ml TNF- α was added to the culture. The culture supernatants were subjected to quantification of cytokines by ELISA using OptEIA Set (BD Biosciences). For intracellular cytokine staining, the cells were harvested and restimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of GolgiPlug (BD Biosciences) for 5 h. The intracellular staining of IL-4 and IL-10 was performed using the Cytotfix/Cytoperm Kit (BD Biosciences). Proportion of IL-4 and IL-10 positive cells in the CD4⁺ T cells were determined by flow cytometry.

3. Results

3.1. Cell Surface expressions of maturation markers on $TNF-\alpha^{-/-}$ DCs

In the present study, we used BMDCs (>95% $CD11c^{+}$) from WT and $TNF-\alpha^{-/-}$ mice to stimulate antigen-specific $CD4^{+}$ T cells (OT-II). These BMDCs were positive for CD11b and negative for CD8 and B220 (data not shown), a pattern typical of conventional DCs. We first examined the cell surface expression of maturation markers, CD86, CD40 and $I-A^b$, on these DCs (Fig. 1). Both types of DCs from WT and $TNF-\alpha^{-/-}$ mice showed immature phenotype, low expression of CD86 and CD40 and moderate expression of $I-A^b$. No significant difference was detected in CD86 and $I-A^b$ expression between WT and $TNF-\alpha^{-/-}$ DCs. CD40 expression on $TNF-\alpha^{-/-}$ DCs was slightly but significantly lower than that of WT DCs. However, this change was negligible compared to that induced by treatment with LPS (data not shown) [13].

3.2. IL-10 production in the culture of antigen specific $CD4^{+}$ T cells with WT or $TNF-\alpha^{-/-}$ DCs

It has been reported that immature DCs induced IL-10-producing $CD4^{+}$ T cells [4-6]. To explore the role of DC-derived $TNF-\alpha$ to induce the antigen-specific IL-10-producing $CD4^{+}$ T cells, WT or $TNF-\alpha^{-/-}$ immature DCs were cocultured with OT-II $CD4^{+}$ T cells in the presence of OVA₃₂₃₋₃₃₉ peptide (0.1 μ M) for 5 days, and cytokine levels in the culture supernatant were quantitated using ELISA (Fig. 2). Significant IL-10 and IL-4 production was observed in the T cell culture with WT DCs in the presence of OVA₃₂₃₋₃₃₉ peptide (Fig. 2A, B). Interestingly, $TNF-\alpha^{-/-}$ DCs with OVA₃₂₃₋₃₃₉ peptide induced considerably lower level of IL-10 production in the culture with $CD4^{+}$ T cells than WT DCs (Fig. 1A). In contrast, no significant differences in IL-4 production were detected between WT and $TNF-\alpha^{-/-}$ DCs (Fig. 1B). Negligible production of IL-10 or IL-4 was detected in the absence of OVA₃₂₃₋₃₃₉ peptide (Fig. 1A, B). Either type of DCs with OVA₃₂₃₋₃₃₉ peptide failed to induce substantial level of IFN- γ in the

culture with CD4⁺ T cells (data not shown).

We also analyzed TNF- α level in the culture of OT-II CD4⁺ T cells with WT or TNF- α ^{-/-} immature-DCs (Fig. 2C). TNF- α was detected in the culture of CD4⁺ T cells and WT DCs in the absence of OVA₃₂₃₋₃₃₉ peptide. The TNF- α level was markedly increased by addition of the antigen. No TNF- α production was detected in the culture with TNF- α ^{-/-} DCs in the absence of the antigen. However, addition of the antigen resulted in significant TNF- α production in the culture of CD4⁺ T cells and TNF- α ^{-/-} DCs.

3.3. Development of IL-10-producing CD4⁺ T cells with WT or TNF- α ^{-/-} DCs

We next performed intracellular cytokine staining of OT-II CD4⁺ T cells after the antigen presentation by WT or TNF- α ^{-/-} immature DCs for 5 days (Fig. 3A upper, B). Considerable proportions of the OT-II CD4⁺ T cells were IL-10 and/or IL-4 positive after the antigen stimulation with WT DCs. In contrast, the proportions of IL-10⁺IL-4⁻ and IL-10⁺IL-4⁺ T cells in the culture with TNF- α ^{-/-} DCs were significantly lower than those with WT DCs. Thus, the ability of DCs to induce IL-10-producing CD4⁺ T cells was attenuated by the TNF- α deficiency in DCs. Nevertheless, the proportion of IL-4 single positive T cells was slightly increased by the TNF- α deficiency in DCs.

We also examined whether addition of exogenous TNF- α during the antigen presentation by TNF- α ^{-/-} DCs recovered their impaired ability to develop IL-10-producing CD4⁺ T cells (Fig. 3A lower, B). Exogenous TNF- α significantly increased the proportions of IL-10⁺IL-4⁻ and IL-10⁺IL-4⁺ T cells in the culture with TNF- α ^{-/-} DCs but not WT DCs. Thus, the TNF- α supply could recover the impaired ability of TNF- α ^{-/-} DCs to induce IL-10-producing CD4⁺ T cells. On the other hand, the TNF- α addition showed no significant effects on the proportion of IL-4 single positive cells in the culture with WT or TNF- α ^{-/-} DCs.

We next examined the role of TNF- α in ability of mature DCs to induce IL-10 producing CD4⁺ T cells. WT or TNF- α ^{-/-} DCs were cultured with LPS for 24 h and used as mature DCs. Both types of DCs showed mature phenotype, high level expressions of CD86, CD40 and I-A^b. No significant difference in the expression level of these molecules was detected between WT and TNF- α ^{-/-} DCs (data not shown). The WT or TNF- α ^{-/-} mature-DCs were cocultured with OT-II CD4⁺ T cells for 5 days in the presence of OVA₃₂₃₋₃₃₉ peptide, and the proportion of IL-10 producing CD4⁺ T cells was determined (Fig. 4A, B). The proportion of IL-10 producing CD4⁺ T cells after the antigen presentation with WT mature-DCs was lower than that with WT immature-DCs compared Fig. 4 with Fig. 3. The TNF- α deficiency of mature DCs exerted no significant influence in the proportion of IL-10⁺ and/or IL-4⁺ T cells after the antigen presentation. Exogenous TNF- α addition showed no significant effects on the proportion of IL-10 producing CD4⁺ T cells in the culture with WT or TNF- α ^{-/-} DCs (data not shown).

4. Discussion

Increasing evidence indicates that DCs play pivotal roles not only in T cell immunity but also immune homeostasis [14, 15]. Immunogenicity of DCs appears to be dependent on their maturational stage. It is generally considered that immature DCs are tolerogenic and involved in peripheral immune tolerance to self antigens in the steady state, while mature DCs are immunogenic and initiate immune responses against harmful foreign antigens in the state of infection. Actually, it has been reported that immature, but not mature, DCs induce T cell anergy and IL-10 producing regulatory T cells in vitro and in vivo [5-8]. The tolerogenic properties of immature DCs may be attributed to the absence or low level expression of costimulatory molecules. However, the precise mechanism underlying induction of the IL-10 producing CD4⁺ T cells by immature DCs are poorly understood.

Our present study also demonstrated that immature DCs induced development of

antigen-specific IL-10-producing CD4⁺ T cells. We then examined the role of DC-derived TNF- α in the induction of IL-10-producing CD4⁺ T cells. The ability of immature DC to induce IL-10-producing CD4⁺ T cells was significantly attenuated by their deficiency of TNF- α . It should be noted that the addition of exogenous TNF- α recovered the impaired ability of TNF- α ^{-/-} DCs to induce IL-10-producing CD4⁺ T cells. Thus, we conclude that TNF- α is a critical factor for the generation of IL-10-producing CD4⁺ T cells during the antigen presentation by immature DCs.

TNF- α promotes inflammatory responses in infected sites and contributes to elimination of foreign pathogens. However, several studies have indicated that TNF- α also exhibits immune regulatory nature. It has been reported that TNF- α -pretreated DCs prevent experimental autoimmune encephalomyelitis [9]. In tumor immunity, TNF- α induces apoptosis of tumor-infiltrating T cells and may blunt the immune surveillance against tumor cells within the tumor site [16]. Thus, we consider that TNF- α not only promotes inflammatory responses but also regulates immune responses. In the present study, we found a novel role of DC-derived TNF- α in the immature DCs-mediated development of IL-10 producing CD4⁺ T cells, which appear to be involved in the immune regulations.

The ability of DCs to induce IL-10-producing CD4⁺ T cells was decreased after the maturation by LPS. TNF- α deficiency of the mature DCs did not affect their ability to induce IL-10-producing CD4⁺ T cells. Thus, the role of TNF- α in the DC ability to induce IL-10-producing CD4⁺ T cells appeared to be restricted in the immature state. It seems to us that TNF- α promotes the development of IL-10 producing CD4⁺ T cells for the immune homeostasis in the steady state. After the continuous infection in which DCs are matured, TNF- α appears to contribute no longer to the development of the IL-10 producing CD4⁺ T cells.

At least three distinct types of regulatory T cells have been identified; T regulatory (Treg) cells, T helper type 3 (Th3) cells and T regulatory type 1 (Tr1) cells [17]. Tr1 cells mainly produce IL-10 and thereby suppress inflammatory responses [17, 18]. It has been suggested that IL-10-producing T cells induced by immature DCs are Tr1-like cells [7]. Tr1 cells are Foxp3 negative different from Treg cells. We could not detect Foxp3 expression in the IL-10 producing CD4⁺ T cells induced by immature DCs in the present study (data not shown). Thus, it seems that these IL-10 producing CD4⁺ T cells are Tr1-like cells as was shown in the previous study [7].

We described a novel immune regulation system involving TNF- α and immature DCs for the development of IL-10-producing CD4⁺ T cells. As the IL-10 producing T cells play a critical role in immune homeostasis, elucidation of the precise mechanism underlying this regulation system may lead to the development of new treatments for various immune disorders.

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References

- [1] R.M. Steinman, The dendritic cell system and its role in immunogenicity, *Annu. Rev. Immunol.* 9 (1991) 271–296.
- [2] D.N.J. Hart, Dendritic cells: unique leukocyte populations which control the primary immune response, *Blood* 90 (1997) 3245–3287.
- [3] J. Banchereau, R.M. Steinman, Dendritic cells and the control of immunity, *Nature* 392 (1998) 245–251.
- [4] S. Akira, K. Takeda, T. Kaisho, Toll-like receptors: critical proteins linking innate and acquired immunity, *Nat. Immunol.* 2 (2001) 675–680.
- [5] M. B. Lutz, N.A. Kukutsch, M. Menges, S. Rübner, G. Schuler, Culture of bone marrow cells in GM-CSF plus high doses of lipopolysaccharide generates exclusively immature dendritic cells which induce allo antigen-specific CD4 T cell anergy in vitro, *Eur. J. Immunol.* 30 (2000) 1048–1052.
- [6] M. B. Lutz, R. M. Suri, M. Niimi, A. L. J. Ogilvie, N. A. Kukutsch, S. Rübner, G. Schuler, J. M. Austyn, Immature dendritic cells generated with low doses of GMCSF in the absence of IL-4 are maturation-resistant and prolong allograft survival in vivo. *Eur. J. Immunol.* 30 (2000) 1813–1822.
- [7] H. Jonuleit, E. Schmitt, G. Schuler, J. Knop, A. H. Enk, Induction of interleukin 10-producing, non proliferating CD4⁺ T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J. Exp. Med.* 192 (2000) 1213–1222.
- [8] M. V. Dhodapkar, R. M. Steinman, J. Krasovsky, C. Munz, N. Bhardwaj, Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J. Exp. Med.* 193 (2001) 233–238.
- [9] M. Menges, S. Rössner, C. Voigtländer, H. Schindler, N. A. Kukutsch, C. Bogdan, K.

- Erb, G. Schuler, M. B. Lutz, Repetitive injections of dendritic cells matured with tumor necrosis factor alpha induce antigen-specific protection of mice from autoimmunity. *J. Exp. Med.* 195 (2002) 15–21.
- [10] K. Inaba, M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, R.M. Steinman, Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor, *J. Exp. Med.* 176 (1992) 1693–1702.
- [11] S. Yamazaki, A.J. Bonito, R. Spisek, M. Dhodapkar, K. Inaba, R.M. Steinman, Dendritic cells are specialized accessory cells along with TGF- for the differentiation of Foxp3⁺ CD4⁺ regulatory T cells from peripheral Foxp3 precursors, *Blood* 110 (2007) 4293–4302.
- [12] S. Yamazaki, M. Patel, A. Harper, A. Bonito, H. Fukuyama, M. Pack, K.V. Tarbell, M. Talmor, J.V. Ravetch, K. Inaba, R.M. Steinman, Effective expansion of alloantigen-specific Foxp3⁺ CD25⁺ CD4⁺ regulatory T cells by dendritic cells during the mixed leukocyte reaction, *Proc. Natl. Acad. Sci. USA* 103 (2006) 2758–2763.
- [13] N. Hirata, Y. Yanagawa, T. Ebihara, T. Seya, S. Uematsu, S. Akira, F. Hayashi, K. Iwabuchi, K. Onoé, Selective synergy in anti-inflammatory cytokine production upon cooperated signaling via TLR4 and TLR2 in murine conventional dendritic cells, *Mol. Immunol.* 45 (2008) 2734–2742.
- [14] Y. Belkaid, G. Oldenhove, Tuning microenvironments: induction of regulatory T cells by dendritic cells. *Immunity* 29 (2008) 362–371.
- [15] J. L. Coombes, F. Powrie, Dendritic cells in intestinal immune regulation. *Nat Rev Immunol.* 8 (2008) 435–446.
- [16] J. B. Mumm, M. Olt, Cytokine-based transformation of immune surveillance into tumor-promoting inflammation. *Oncogene* 27 (2008) 5913–5919.
- [17] E. Bettelli, M. Oukka, V. K. Kuchroo, T_H-17 cells in the circle of immunity and

autoimmunity. *Nat. Immunol.* 8 (2007) 345-350.

[18] A. Awasthi, Y. Carrier, J. P. Peron, E. Bettelli, M. Kamanaka, R. A. Flavell, V. K.

Kuchroo, M. Oukka, H. L. Weiner, A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells, *Nat. Immunol.* 8 (2007) 1380-1389.

Figure legends

Fig. 1. Expression of surface molecules on WT and TNF- $\alpha^{-/-}$ DCs. BMDCs were generated from WT or TNF- $\alpha^{-/-}$ mice. Expressions of CD86, CD40 and I-A^b were analyzed by flow cytometry. (A) Representative histogram of each molecule on DCs. (B) Each column represents the mean $\pm$ SE of three independent experiments. MFI, mean fluorescence intensity. Statistical significance was calculated by Student's *t*-test (*: < 0.05).

Fig. 2. Cytokine production in the culture of CD4⁺ T cells and WT or TNF- $\alpha^{-/-}$ DCs. Purified OT-II CD4⁺ T cells were cocultured with WT or TNF- $\alpha^{-/-}$ DCs in the absence or presence of OVA₃₂₃₋₃₃₉ peptide (OVA - or +). After 5 days, the culture supernatants were subjected to quantification of IL-10 (A), IL-4 (B), and TNF- α (C) by ELISA. Each column represents the mean $\pm$ SE of four independent experiments. Statistical significance was calculated by Student's *t*-test (**: < 0.01).

Fig. 3. The effect of TNF- α deficiency on DC ability to induce IL-10-producing CD4⁺ T cells. Purified OT-II CD4⁺ T cells and WT or TNF- $\alpha^{-/-}$ DCs were cocultured with OVA₃₂₃₋₃₃₉ peptide for 5 days in the absence (medium) or presence (TNF- α +) of exogenous TNF- α . The cells were restimulated with PMA and ionomycin for 5 h and the proportions of IL-4 and IL-10 positive cells in the CD4⁺ T cells were determined by flow cytometry. (A) Dot plots of IL-4 and IL-10 positive cells in CD4⁺ T cells. Data are representative of four independent experiments. (B) The proportion of IL-4 and IL-10 positive cells in CD4⁺ T cells. Each column represents the mean $\pm$ SE of four independent experiments. Statistical significance was calculated by Student's *t*-test (*: < 0.05).

Fig. 4. The ability of mature DCs to induce IL-10-producing CD4⁺ T cells. WT or TNF- α ^{-/-} DCs were stimulated with LPS for 24 h and used as mature DCs. Purified OT-II CD4⁺ T cells were cocultured with each type of mature DCs in the presence of OVA₃₂₃₋₃₃₉ peptide for 5 days. The cells were restimulated with PMA and ionomycin for 5 h and the proportions of IL-4 and IL-10 positive cells in the CD4⁺ T cells were determined by flow cytometry. (A) Dot plots of IL-4 and IL-10 positive cells in CD4⁺ T cells. Data are representative of four independent experiments. (B) The proportion of IL-4 and IL-10 positive cells in CD4⁺ T cells. Each column represents the mean $\pm$ SE of four independent experiments.

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