Dendritic Cells Are Decreased in Blood and Accumulated in Granuloma in Tuberculosis

Kazutaka Uehira,* Ryuichi Amakawa,** Tomoki Ito,* Kenichirou Tajima,* Shinsuke Naitoh,* Yoshio Ozaki,* Toshiki Shimizu,* Kazuyuki Yamaguchi,* Yoshiko Uemura,† Hiroyuki Kitajima,‡ Seibun Yonezu,§ and Shirou Fukuhara*

*First Department of Internal Medicine and †Surgical Pathology, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi, Osaka 570-8506, Japan; ‡Department of Hematology, Kishiwada City Hospital, 2 Gakuwara-cho, Kishiwada, Osaka 596-0822, Japan; and §Department of Internal Medicine, Hyogo Prefectural Amagasaki Hospital, 1-1-1 Daimotsu-cho, Amagasaki, Hyogo 660-0828, Japan

Immunity against tuberculosis consists of innate and adaptive immune responses. In this study, we investigated the dynamics of dendritic cells (DC), which are known to elicit a variety of immune responses, in patients with tuberculosis. CD11c+ peripheral blood DC were decreased in patients with tuberculosis. Immunohistochemical analyses demonstrated that a number of fascin+, CD11c+, HLA-DR+ DC were infiltrating the lymphocyte areas of the tuberculous granulomas (tubercles). Immunohistochemical analyses also demonstrated that interferon-γ-producing Th1 cells were increased in the tubercles of the patients, indicating the presence of Th1 polarization at least in the context of inflammatory tissues. In vitro coculture of autologous naïve T cells with CD11c+ or CD11c– DC pretreated with Bacillus Calmette Guérin augmented the production of Th1 cells. These findings suggested that the trafficking of DC from the peripheral blood into the tubercles causes a dominant Th1 balance and thus plays an essential role in the immunity against tuberculosis.

Key Words: tuberculosis; dendritic cell; Th balance; BCG; interferon-γ.

INTRODUCTION

Mycobacterium tuberculosis (M. tb), the causative microbe of tuberculosis, is responsible for 8 million new cases and almost 3 million deaths per year in the world (1). Protective immunity against M. tb infection consists of both innate and adaptive immune responses.

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†To whom correspondence and reprint requests should be addressed at the First Department of Internal Medicine, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi, Osaka 570-8506, Japan. Fax: 81-6-6994-8344. E-mail: amakawa@takii.kmu.ac.jp.

Innate immunity to tuberculosis primarily depends on macrophages, while adaptive immunity is mediated by antigen-specific effector cells, especially T cells (2, 3). Although many studies demonstrated the importance of a Th1 response in the protective immunity against tuberculosis, the mechanism of skewing toward a Th1 balance in patients with tuberculosis remains largely unknown.

Dendritic cells (DC) are potent antigen-presenting cells and are the unique cell type that has the capacity to prime naïve T cells. Recent progress in DC biology has highlighted the integral roles of this cell type in eliciting and regulating the Th balance. Monocyte-associated DC (Mo-DC), functionally termed DC1, primarily drive naïve Th cells into IFN-γ-producing Th1 cells, while plasmacytoid DC, named DC2, basically induce IL-4-producing Th2 cells (4). In addition, accumulating evidence has recently demonstrated that various cytokines affect the DC subtypes and modulate their original Th1- or Th2-inducing capacity (5). Furthermore, it has also been demonstrated that the individual DC subtypes display different functions depending on their activation states and antigens (6, 7). Thus, the DC system exhibits lineage heterogeneity and functional plasticity, residing at the central part of the immune system as the major commander of immune responses. This, together with the evidence that DC are involved in various kinds of infections, strongly suggests that DC also play important roles in immunity to tuberculosis. However, the precise nature of involvement of DC in tuberculosis is currently unknown, especially in vivo, although there are several documented studies which have examined the in vitro effects of mycobacterial antigens on DC (8–10).

We recently demonstrated that human peripheral blood DC (PBDC) are categorized into three fractions based on the differential expression of CD1a and CD11c (fraction 1, CD1a+/CD11c–; fraction 2, CD1a+/CD11c+; fraction 3, CD1a+/CD11c–) (11). Fractions 1 and 2 DC are associated with Mo-DC, while fraction 3
DC corresponds to precursors of plasmacytoid DC or interferon-α-producing cells (IPC) (12). These fractions of DC are in immature stages and thus are regarded as being en route to tissues of various organs. In the healthy situation, the PBDC appear to be the main source of steady-state "sentinel" DC in tissues. On the other hand, a line of evidence indicates that under some disease conditions, recruitment of PBDC is facilitated from PB to affected tissues presumably as a critical response to "danger" signals. Indeed, we recently found that in primary Sjögren's syndrome fraction 1 DC were decreased due to selective trafficking from PB to salivary gland, implicating the essential contribution of DC in the pathophysiology of Sjögren's syndrome (13). Thus, PBDC provide an essential viewpoint and serve as a unique scientific tool in dissecting the nature of various disease conditions. In this study, we demonstrate that in patients with tuberculosis active and selective trafficking of fractions 1 and 2 DC occurred from PB to tuberculous tissues (tubercles), causing a dominant Th1 polarization.

MATERIALS AND METHODS

Patients

The subjects enrolled in this study consisted of 42 patients with tuberculosis. Twenty-four patients had pulmonary tuberculosis and 4 patients had pleural tuberculosis, while 6 and 2 patients were suffering from tuberculous lymphadenitis and subcutaneous tuberculosis, respectively. The remaining 6 patients had pleural tuberculosis or tuberculous lymphadenitis in addition to pulmonary tuberculosis. The patients were diagnosed as having tuberculosis in our or in affiliated hospitals. Diagnosis of tuberculosis was made based on the clinical and radiological findings and was confirmed by identification of M. tb by the polymerase chain reaction (Cobas Amplicor M. tuberculosis; Roche Diagnostics KK, Tokyo, Japan) method; acid-fast stains of sputum, gastric juice, or samples obtained by transbronchial lung biopsy; and/or histological examination of biopsied samples of affected tissues. All patients satisfied the definition of the 1990 edition of Diagnostic Standards and Classification of Tuberculosis, published by the American Lung Association. Twenty-one of the 42 patients were subjected to analysis of PBDC, PB T cells. The control group for this study consisted of 15 gender- and age-matched healthy volunteers. None of the control subjects had any diseases and none of the patients had received any antituberculosis therapy before this study. Furthermore, we performed a histological examination of biopsied samples (tubercles) in the remaining 21 patients. The tissues biopsied from those patients included lung, pleura, subcutaneous tissues, and lymph nodes. The biopsied samples were subjected to hematoxylin–eosin (H–E) staining and immunohistochemical staining using various monoclonal antibodies. Since the tissue biopsies were performed as diagnostic procedures for all patients, none had experienced antituberculosis treatment at the time of the biopsies. All patients gave their informed consent for this study.

Media and Reagents

The following medium was used for the cell culture throughout this study: phosphate-buffered saline supplemented with 10% FBS (fetal bovine serum) and 5 mM EDTA.

Purification and Analysis of PBDC

Peripheral blood mononuclear cells (PBMC) from normal donors and patients with tuberculosis were prepared by Lymphoprep (Nycomed, Oslo, Norway) density-gradient centrifugation of heparinized venous blood. PBMC were incubated with anti-CD3, anti-CD14, and anti-CD19 monoclonal antibodies (mAbs) (PharMingen, San Diego, CA) for 30 min on ice, and cells binding these mAbs were removed using sheep anti-mouse IgG-coated magnetic beads (M-450; Dynal, Oslo, Norway). Using this method, the percentage of PBDC (less than 1% of total PBMC) was increased to 2–5% (DC-enriched population).

Three fractions of PBDC were identified. (i) CD1a+/CD11c+ (fraction 1) PBDC: The PBDC-enriched population was stained with FITC-labeled anti-CD1a mAbs (Biosource, Camarillo, CA), a mixture of PE-labeled mAbs against lineage markers (CD3, CD14, CD16, and CD19, Exalpa, Boston, MA; CD15, Coulter, Hialeah, FL; CD56, Becton–Dickinson, San Jose, CA), and PC5-conjugated anti-HLA-DR mAbs, and the CD1a+DR+/lin− population was identified as CD1a+/CD11c+ PBDC. Note that we confirmed that the identified CD1a+/DR+/lin− PBDC are CD11c+ by four-color staining. (ii) CD1a+/CD11c− (fraction 2) and CD1a+/CD11c− (fraction 3) PBDC: The PBDC-enriched population was stained with FITC-labeled mAbs against lineage markers (CD3, CD14, and CD16, Exalpa; CD15, CD19, and CD56, Becton–Dickinson) and CD1a, PE-labeled anti-CD11c (Becton–Dickinson) and PC5-conjugated anti-HLA-DR mAbs. CD1a+/lin−/CD11c+/DR− PBDC and CD1a+/lin+/CD11c+/DR− PBDC were identified as fraction 2 and fraction 3 PBDC, respectively. Before staining, the cells were incubated with polyclonal mouse immunoglobulin (Cappel, Aurora, OH) to block nonspecific binding of labeled mAbs.

The absolute numbers (per milliliter) of DC were calculated by multiplying the percentage of the lin−/DR− population among the DC-enriched population by
the count (per milliliter) of the DC-enriched population. The absolute number (per milliliter) of each fraction of DC was calculated by multiplying the percentage of each fraction within the lin⁻/DR⁺ population by the total number of the lin⁻/DR⁺ population.

Histopathological and Immunohistochemical Analyses of Tubercles

Formalin-fixed, paraffin-embedded sections of biopsied specimens were stained with H-E. We examined either paraffin-embedded or frozen sections for immunohistochemical staining using various mAbs. The frozen specimens were embedded in OCT compound and were stored at −80°C until sectioning. The immunohistochemical analyses of paraffin-embedded sections was performed on the 21 patients using mAbs against CD1a (Novocastra, Newcastle, UK), CD4, CD8, HLA-DR (Nichirei, Tokyo, Japan), and fascin (DAKO, Carpinteria, CA) and the DAKO EnVision kit. Immunostaining of frozen sections of four cases with tuberculous lymphadenitis was carried out using mAbs against IFN-α (Genzyme, Cambridge, MA), IFN-γ, IL-4, IL-10 (R&D, Minneapolis, MN), and CD11c (Ancell, Bayport, MN).

Autologous T Cell Response Induced by Mycobacterium bovis Bacillus Calmette and Gueïrin (BCG)-Stimulated CD11c⁺ DC (Fractions 1 + 2) and CD11c⁻ DC (Fraction 3)

CD4⁺/CD45RA⁺ naive T cells were obtained from normal volunteers by negative depletion using a CD4⁺ T cell isolation kit (Miltenyi Biotec, CA) and positive selection using CD45RA microbeads (Miltenyi). The purity of the acquired cells was demonstrated to be >93% by reanalysis using anti-CD45RA mAb (Immunotech) and CD4 mAb (Immunotech). Sorting procedure of CD11c⁺ DC and CD11c⁻ DC was as follows: after staining of the PBDC-enriched population with a mixture of FITC-labeled mAbs against lineage markers (CD3, CD14, and CD16, Exalpha; CD15, CD19, and CD56, Becton–Dickinson), PE-labeled anti-CD11c mAbs (Becton–Dickinson), and PC5-conjugated anti-HLA-DR mAbs, the CD11c⁺/lin⁻/HLA-DR⁺ and the CD11c⁻/lin⁺/HLA-DR⁺ populations were identified as fractions 1 + 2 and fraction 3 DC using EPICS ALTRA flow cytometer (Coulter). CD11c⁺ or CD11c⁻ DC were cocultured with BCG (Calbiochem, La Jolla, CA) at a multiplicity of infection of 2 to 5. After overnight culture at 37°C, DC were harvested and centrifuged at 800 rpm for 8 min. Autologous CD4⁺/CD45RA⁺ naive T cells (5 × 10⁶) were cultured with harvested DC (1 × 10⁶) in a 96-well plate in 200 µl of RPMI 1640 medium containing heat-inactivated 10% FBS, 2 mM l-glutamine, 100 units/ml penicillin, and 100 ng/ml streptomycin. After 7 days culture, the T cells were washed and subsequently restimulated with PMA (40 ng/ml) and ionomycin (2 µg/ml) for 8 h. Brefeldin A (5 µg/ml) was added to the cultures for the last 4 h, and then cells were treated according to the intracellular cytokine staining protocol described above.

Statistical Analysis

The Mann–Whitney U test was used for statistical analysis with a StatView statistical program (Abacus Concepts, Berkeley, CA). Differences were considered significant when tied P values were less than 0.05.

RESULTS

Absolute Counts of PBDC and Each Fraction of DC

We compared the absolute count of total PBDC and each fraction of DC in tuberculosis patients with those in age- and gender-matched control subjects (Fig. 1). The absolute count of total PBDC was significantly decreased in TB patients (mean 9711/ml, range 2414–27,819/ml) compared with normal controls (mean 17,132/ml, range 8313–32,708/ml) (P = 0.001). This decrease was shown to be predominantly due to the decrease in fraction 1 (mean 4106/ml, range 352–10,998/ml) and fraction 2 DC (mean 648/ml, range 52–1303/ml) in patients (controls: fraction 1, mean 9236/ml, range 4300–18,496/ml; fraction 2, mean 1639/ml, range 637–3264/ml) (P < 0.0005 and P < 0.0001, respectively) (Fig. 1). Furthermore, the absolute count of fraction 3 DC in patients (mean 4957/ml, range 1589–15,527/ml) was slightly decreased, although there was no significant difference observed between patients and control subjects (mean 6258/ml, range 3061–10,948/ml) (P = 0.057).

Immunohistochemical Analyses of Tubercles

The H-E staining of tubercles from the 21 patients showed a histological architecture typical of tuberculosis, in which epithelioid granuloma with caseous necrosis and Langhans' giant cells accompanied with infiltration of numerous mononuclear cells were detected (Fig. 2A). Immunohistochemical analyses of tubercles were performed on the paraffin-embedded tissue sections from the same 21 patients and on the frozen sections from 4 patients with tuberculous lymphadenitis and consistent results were obtained. It was demonstrated that many fascin⁺ cells were scattered among numerous fascin⁻ mononuclear cells in the lymphocyte area around epithelioid cells (Fig. 2B). These fascin⁺ cells possessed many dendritic projections and were shown to be HLA-DR⁺ in sequential sections (Fig.
Furthermore, there were no cells detected which were positive for EBNA-2 and LMP-1 in any cases, excluding the possibility that the fascin/H11001 cells were EBV-infected B cells (data not shown). The majority of the fascin/H11001/HLA-DR/H11001 cells appeared to be CD11c/H11001 based on the relative frequency of fascin/H11001 and CD11c/H11001 cells, both of which exhibited the dendritic cell morphology (Fig. 2D). However, the fascin/H11001/HLA-DR/H11001 cells were shown to be mostly negative for CD1a in the serial cutting preparations (Fig. 2E). On the other hand, a small number of IFN-γ/H9251-positive cells were also found to exist in the lymphocyte area (Fig. 2F). Numerous CD4 single-positive and CD8 single-positive cells were shown to have infiltrated throughout the lymphocyte area at similar frequencies (Figs. 2G and 2H). A number of INF-γ-producing cells were also found to have infiltrated the whole lymphocyte area (Fig. 2I), while significantly fewer numbers of IL-4-producing and IL-10-producing cells were found to be present in the same area (Figs. 2J and 2K). Based on the relative frequency of each cell type, the majority of CD4/H11001 and CD8/H11001 cells seemed to produce IFN-γ.

**DISCUSSION**

In this study, patients with pulmonary and pleural tuberculosis showed a significant decrease in the number of total PBDC, compared with normal individuals. The decrease was demonstrated to be due to the selective decrease in fraction 1 and fraction 2 PBDC. It is suggested that the decrease might have been caused by preferential and continuous trafficking of these fractions of DC into tuberculous lesions (tubercles) from circulating blood, because numerous fascin/H11001/HLA-DR/H11001 cells were found to be infiltrated in the tubercles of all cases examined (21/21). Fascin is an actin-bundling protein and its expression in blood cells is restricted to Epstein-Barr virus-infected B cells, mature dendritic cells, and Reed-Sternberg cells of Hodgkin’s disease (14, 15). Immunohistochemical analysis excluded the possibility that the fascin− cells were EBV-infected B cells (data not shown). This, together with the finding that the fascin− cells showed many dendritic projections, indicated that these cells were DC. The infiltrated DC were shown to be positive for CD11c but mostly negative for CD1a, raising a possibility that the infiltrated DC were fraction 2 DC, of which the phenotype is CD1a/CD11c+.

**Differentiation of Naive T Cells into Th1 Cells Driven by CD11c DC**

We next examined whether CD11c+ or CD11c− DC had the capacity to induce Th1 or Th2 cells in the presence of BCG. CD11c+ or CD11c− PBDC from normal individuals were pretreated with or without BCG for 18 h, and after intensive washing they were subjected to coculture with autologous naive CD4+ T cells. After 7 days culture, the percentages of IFN-γ- or IL-4-producing CD4+ T cells were determined by the intracellular cytokine staining method. Both CD11c+ and CD11c− DC pretreated with BCG were demonstrated to facilitate the induction of IFN-γ-producing T cells, compared with those untreated with BCG (Fig. 3). The numbers of single producers of IL-4 and double producers of IFN-γ/IL-4 were substantially decreased when naive CD4+ T cells were cocultured with autologous CD11c− DC precultured with BCG (Fig. 3).
well be that the majority of the infiltrated CD1a⁺/CD11c⁺ DC were derived from fraction 1 DC in PB. This presumption apparently contrasts with the expression profile of fraction 1 DC in PB, which were CD1a⁻. This discrepancy might be explained by the down-regulation of CD1a⁺ in fraction 1 DC during recruitment. Indeed, Langerhans cells, which were shown to be induced from fraction 1 DC (11), down-regulate CD1a upon maturation (16). Furthermore, it was reported that down-regulation of CD1a molecules occurred in monocyte-derived DC by infection with M. tb (17). Recruitment of blood DC into bacteria-induced granuloma and subsequent induction of specific T cell response have recently been reported in mice (18). The current study findings also suggested the involvement of DC in the formation of granuloma and in the induction of Th1 balance in tuberculosis. Indeed, cytokine analysis demonstrated the presence of many IFN-γ-producing CD4⁺ T cells in pulmonary tubercles (Fig. 2) and peripheral blood (data not shown), indicating the existence of a generalized predominant Th1 balance in patients with tuberculosis. This was at least partly in accordance with many previous findings which sug-

**FIG. 2.** Histological analyses of tuberculous tissues. (A) H-E staining of tuberculous tissue showing Langhans’ giant cells typical of tuberculosis. (B–K) Immunohistochemical analyses of tuberculous tissues. (B) Fascin, (C) HLA-DR, (D) CD11c, (E) CD1a, (F) IFN-γ, (G) CD4, (H) CD8, (I) IFN-γ, (J) IL-4, (K) IL-10.
gested the relevance of a Th1 balance with the pathophysiology of tuberculosis (19–22). In light of the intrinsic property of DC, the Th1 polarization in the patients might presumably be induced by multistep processes in which DC play important roles. The processes may include recruitment of DC from blood to tubercles, uptake of M. tb by the recruited DC, trafficking of the DC into regional lymph nodes, antigen presentation by the DC to naive CD4+ T cells, and subsequent induction of Th1 cells. The Th1 cells generated in lymph nodes may presumably reach tuberculous lesions through blood vessels and participate in the inflammatory reactions there. On the other hand, it is also possible that mobilized DC and effector T cells interacted with each other in the inflammatory tubercle, resulting in the expansion of the T cells.

We also found many IFN-γ-producing CD8+ T cells (Tc1 cells) in tubercles. The Tc1 cells might be generated through interaction with Th1 cells or directly by DC. CD8+ T cells may activate macrophages to eliminate the intracellular M. tb by secreting IFN-γ (3) and/or directly kill the intracellular bacteria presumably through perforin/granzyme pathways (23).

We consistently detected a small number of IL-4-producing T cells as well as IL-10-producing T cells in the tissues of tuberculous lymphadenitis. Although the biological significance of infiltration of these types of T cells is currently unknown, it might be the reflection of
a feedback mechanism that quenches the overload of the Th1 balance, because both IL-4 and IL-10 are known to inhibit IFN-γ production.

Immunohistochemical analyses also demonstrated that a small number of IFN-α strongly positive cells were present in the tissues of tuberculous lymphoadenitis. The IFN-α-positive cells appeared to be derived from fraction 3 DC in PB, because fraction 3 DC are known to be the major IPC (12) and the number of fraction 3 DC were slightly decreased in the peripheral blood of the patients. The present study demonstrated that the coculture of autologous naive CD4+ T cells with CD11c+ DC (fraction 3) pretreated with BCG induced IFN-γ-producing Th1 cells, but not Th2 cells, suggesting the cooperative role of CD11c+ DC with CD11c+ DC in inducing a Th1 balance in M. tb infection. In this context, it is tempting to note that CD11c+ DC induce IFN-γ-producing Th1 cells upon viral infection (6, 12).

In conclusion, we have suggested a hierarchical mechanism of adaptive immune responses in tuberculosis, in which DC may play important roles in determining the Th balance, although we did not examine the innate part of the immune responses in tuberculosis. In addition, this may be the first report that described the possible recruitment of naturally circulating blood DC into the granuloma in patients with tuberculosis. The findings obtained in this study will clearly provide a better understanding of the nature of tuberculosis and thus may serve as a platform on

**FIG. 3.** In vitro differentiation of autologous naive T cells into Th1 cells driven by fraction 1+2 DC (CD11c+ DC) and fraction 3 DC (CD11c- DC). FACS analyses of in vitro coculture of CD11c+ DC (A) or CD11c- DC (B) with autologous naive CD4+ T cells. The DC were pretreated with or without BCG for 18 h and then were cultured with naive CD4+ T cells for 7 days. Double stainings with anti-IL-4 mAbs and anti-IFN-γ mAbs were performed.
which new basic and therapeutic approaches will be conducted for this still worldwide prevalent disease.

REFERENCES


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