The CD40–CD40L axis and IFN-γ play critical roles in Langhans giant cell formation

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Abstract

The presence of Langhans giant cells (LGCs) is one of the signatures of systemic granulomatous disorders such as tuberculosis and sarcoidosis. However, the pathophysiological mechanism leading to LGC formation, especially the contribution of the T cells abundantly found in granulomas, has not been fully elucidated. To examine the role of T cells in LGC formation, a new in vitro method for the induction of LGCs was developed by co-culturing human monocytes with autologous T cells in the presence of concanavalin A (ConA). This system required close contact between monocytes and T cells, and CD4+ T cells were more potent than CD8+ T cells in inducing LGC formation. Antibody inhibition revealed that a CD40–CD40 ligand (CD40L) interaction and IFN-γ were essential for LGC formation, and the combination of exogenous soluble CD40L (sCD40L) and IFN-γ efficiently replaced the role of T cells. Dendritic cell-specific transmembrane protein (DC-STAMP), a known fusion-related molecule in monocytes, was up-regulated during LGC formation. Moreover, knock-down of DC-STAMP by siRNA inhibited LGC formation, revealing that DC-STAMP was directly involved in LGC formation. Taken together, these results demonstrate that T cells played a pivotal role in a new in vitro LGC formation system, in which DC-STAMP was involved, and occurred via a molecular mechanism that involved CD40–CD40L interaction and IFN-γ secretion.

Keywords: concanavalin A, granuloma, multinucleated giant cell

Introduction

Granuloma formation is a specialized inflammatory response observed in infections by certain pathogens such as Mycobacterium and Cryptococcus (1, 2). Granulomas are also found in immunodeficient conditions such as chronic granulomatous disease (CGD) (3). In addition, this response is a pathological hallmark of non-infectious idiopathic inflammatory disorders including systemic sarcoidosis and Blau syndrome/early-onset sarcoidosis (BS/EOS) (4–7). Granulomas consist of various cell types including macrophages, T cells, plasma cells and epithelioid cells, but the presence of multinucleated giant cells (MGCs) is the cardinal feature of granulomatous inflammation (1, 4). Granuloma formation is generally considered a host defense mechanism against persistent irritants or chronic infection and occurs as a consequence of the failure of the host to eliminate invading pathogens. When the irritants are large and indigestible, MGCs are formed by the fusion of monocyte-macrophage lineage cells (1, 4, 8). However, the pathophysiological mechanism of MGC development and associated granuloma formation is not well understood.

MGCs are morphologically classified into Langhans giant cells (LGCs), which show a circular peripheral arrangement of nuclei, and foreign body giant cells (FBGs), which show irregular scattering of nuclei (9). LGCs are characteristic of systemic granulomatous disorders such as tuberculosis, sarcoidosis and BS/EOS, whereas FBGs are observed as a consequence of a reaction against foreign bodies such as an implant. To explore the mechanisms of MGC formation, particularly LGC formation in systemic granulomatous disease, several in vitro systems of human monocyte culture have been developed. These involve the use of stimuli such as phorbol myristate acetate (10–12) and lectins such as concanavalin A (ConA) to induce LGCs (13, 14). However, a considerable degree of concurrent FBG formation is observed in systems that employ ConA.

Cytokines are also used to induce MGCs. For example, IFN-γ (15–19), IL-3 (15, 18, 19) and granulocyte macrophage colony-stimulating factor (15) are frequently used to induce LGCs, while IL-4 is known to induce FBGs (15, 20–23). Notably, IFN-γ has been regarded as a crucial factor...
The CD40–CD40 ligand (CD40L) axis is required for LGC formation because LGC formation is inhibited by antibodies against IFN-γ (24). However, the roles of other factors and cell types in LGC development have not been elucidated.

T cells are one of the main components of granulomas. Therefore, the present study focused on T cells and investigated their role in the development of LGCs. A novel system was developed in which LGC-dominant MGCs could be generated from freshly isolated monocytes co-cultured with autologous T cells in the presence of ConA, and this system was used to evaluate the role of the CD40–CD40 ligand (CD40L) interaction and IFN-γ in the formation of LGCs. Moreover, the hypothesis that the stimulation of monocytes with exogenous soluble CD40L (sCD40L), recombinant human IFN-γ (rhIFN-γ) and ConA would be sufficient to induce monocyte fusion into LGCs in the absence of T cells was investigated. The findings of this study revealed a pivotal role for T cells in LGC formation and suggest a new pathophysiological mechanism contributing to LGC formation in granulomatous disorders.

Methods

Reagents and antibodies

Blocking antibodies against human CD40 (clone 82102; antagonistic antibody), CD40L, IFN-γ, IL-12 and isotype control IgG and rhIL-12 were purchased from R&D systems (Minneapolis, MN, USA). ConA, LPS and PMA were purchased from Sigma Chemical Co. (St Louis, MO, USA). sCD40L was kindly provided by Dr Ashish Jain (NIAID/NIH, Bethesda, MD, USA) and also purchased from Adipogen (Incheon, Korea). rhIFN-γ was purchased from PeproTech (Rocky Hill, NJ, USA). Inhibitory reagents JSH-23, BMS-345541, U0126, MD, USA) and also purchased from Adipogen (Incheon, Korea). rhIFN-γ was kindly provided by Dr Ashish Jain (NIAID/NIH, Bethesda, MD, USA) and also purchased from Adipogen (Incheon, Korea). rhIFN-γ was kindly provided by Dr Ashish Jain (NIAID/NIH, Bethesda, MD, USA) and also purchased from Adipogen (Incheon, Korea). rhIFN-γ was kindly provided by Dr Ashish Jain (NIAID/NIH, Bethesda, MD, USA) and also purchased from Adipogen (Incheon, Korea).

Differentiation of MGCs from peripheral CD14+ monocytes

Blood was collected from healthy volunteer donors after informed consent was obtained in accordance with the guidelines of the Institutional Review Board of Kyoto University. PBMCs were isolated from whole blood using the Lymphoprep system (Axis-Shield PoC, Oslo, Norway). PBMCs were incubated with anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and CD14+ cells were isolated using a AutoMACS benchtop magnetic cell sorter according to the manufacturer’s protocol (Miltenyi Biotec). Similarly, CD3+, CD4+ and CD8+ cells were collected in a similar fashion, using CD3, CD4 and CD8 beads (Miltenyi Biotec), respectively. The purity of the isolated cells was >95%, as demonstrated by flow cytometry using a FACS Calibur system (BD Biosciences, Franklin Lakes, NJ, USA). In some experiments, CD3+ T cells were first depleted from PBMCs, and CD14+ monocytes were isolated from the negative fraction to obtain highly purified monocytes. (Contaminating T cells comprised <0.5% of the total cell population.)

The isolated CD14+ monocytes were re-suspended at a density of 100 000 cells per well in 48-well plates in RPMI-1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 μM β-mercaptoethanol, 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin. Cells were then cultured for 72 h with various supplemental reagents as described in Results. At the end of the culture period, cells were treated with Giemsa stain to detect nuclei. MGCs were defined as cells with more than three nuclei per cell, according to definitions established by previous studies (9–24).

The stained plates were examined under an Olympus IX70 microscope (Olympus, Tokyo, Japan) using a ×20 or ×10 objective lens with a ×10 eyepiece, and the number of nuclei and MGCs in the representative area was counted. The fusion index was calculated according to the following formula: fusion index = (number of nuclei within MGCs)/(total number of nuclei counted). More than 300 nuclei were counted for each experiment. Images were acquired with an Axio Cam camera (Carl Zeiss, Germany).

Enzyme-linked immunosorbent assay

The concentration of IFN-γ in the culture supernatants was measured by enzyme immunoassay using an OptEIA human IFN-γ ELISA set (BD Biosciences) according to the manufacturer’s instructions.

Immunohistochemistry

Immunohistochemical staining was performed using an indirect immunofluorescent technique. Briefly, after fixation with 4% paraformaldehyde, the cells were incubated with a mouse antibody directed against-human CD3 (BD Biosciences), followed by incubation with an Alexa488-labeled goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA). Cells were then stained with DAPI (Sigma) and visualized with an Olympus laser microscope IX70. Merged reconstruction images were created using Axio Vision SP4 software (Carl Zeiss).

Reverse transcription–PCR

RNA was extracted directly from culture plates using the RNeasy mini kit (Qiagen, Hilden, Germany). After DNase treatment, cDNA was obtained by reverse transcription using the Omniscript RT system (Qiagen). Reverse transcription (RT)–PCR was performed using Takara LA Taq (Takara, Shiga, Japan) with the following primer pairs: 5'-GATTGGTGTCAGCACTTTTGT-3' and 5'-CCTCCCAAGTGAAGTGATTG-3' for CD40L; 5'-GGGAGATTACAGTGTCCTCAGGG-3' and 5'-GGGAGGACAACACCTCTGTG-3' for dedicator of cytokinesis cell-specific transmembrane protein (DC-STAMP); 5'-GGCAGAGAACCAGGTGAATG-3' and 5'-CTCTGGTGGAATCTCTCTG-3' for triggering receptor expressed on myeloid cells 2 (TREM-2); 5'-TGGAACCTGTCCTCAGGATG-3' and 5'-ATCACGACCTCCAAAAAGG-3' for signal regulatory protein (SIRP) α; 5'-CGGAGATCGATTGAAATAAAC-3' and 5'-GAGGTTCGTGCTGTGTCG-3' for CD44; 5'-CAAGGACATCCTCTCGAG-3' and 5'-TCTGGTCAGGGATGTAAGC-3' for matrix metalloproteinase 9 (MMP-9) and 5'-AGTTGGAAGTGGAGGTCAAC-3' and 5'-ACCTGGTGCTCAGGTagGCC-3' for glyceraldehyde 3-phosphate dehydrogenase.
siRNA transfection

Pre-designed siRNAs against DC-STAMP or control siRNAs (Applied Biosystems, Carlsbad, CA, USA) were transfected into newly isolated CD14+ cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturers’ instructions. Additional transfections were performed after 12 and 24 h of culture.

Western blotting

Antibodies against phospho-(p)-ERK1/2, ERK1/2, p-JNK, JNK, p-p38, p38, p-p65 and p65 were purchased from Cell Signaling Technology (Danvers, MA, USA). Isolated CD14+ cells were incubated without stimulation for 2 h, followed by stimulation with ConA, rhIFN-γ, sCD40L and LPS for 1 h.

The harvested cells were lysed with RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 1 mM orthovanadate) containing a protease inhibitor cocktail (Nacalai, Kyoto, Japan). Equal amounts of total protein were resolved on SDS–polyacrylamide gels and transferred onto Immobilon PVDF membranes (Millipore, Billerica, MA, USA). The membranes were treated with blocking buffer (10 mM Tris–HCl, 150 mM NaCl, 0.1% tween-20, 0.05% NaN3 and 1% FBS) and then incubated overnight with primary antibody. The membranes were then incubated for 1 h with an HRP-conjugated goat anti-rabbit secondary IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and developed with ECL Plus Western blotting detection kit (GE Healthcare, Uppsala, Sweden).
**Results**

**Co-culturing human monocytes with autologous T cells induces LGC formation**

LGCs in granulomas are a specific indication of systemic granulomatous disorders. The observation that T cells are one of the main constituents of granulomas led to the hypothesis that T cells may play a key role in LGC formation. To understand the pathophysiology underlying LGC formation, a new *in vitro* LGC formation system was developed using human monocytes and T cells. When freshly isolated peripheral blood monocytes were co-cultured with autologous T cells under ConA stimulation, LGCs formed within 3 days (Fig. 1A, left), while very few FGCs were detected (Fig. 1A, right). LGC formation, as measured by the fusion index, as well as the number of LGCs per monocyte and the number of nuclei per LGC, increased along with the number of co-cultured T cells, peaking at a monocyte:T cell ratio of 1:1 (Fig. 1B–D).

**IFN-γ is required for LGC formation in the co-culture of human monocytes and autologous T cells**

The cytokine IFN-γ, which is reported to be critical in LGC formation (15–19, 24), was detected in the supernatants of the ConA-stimulated co-cultures (data not shown). The addition of a neutralizing antibody against IFN-γ to the co-culture system resulted in a dose-dependent inhibition of LGC formation (Fig. 2A). Furthermore, exogenous addition of rhIFN-γ accelerated LGC formation in a dose-dependent manner (Fig. 2B). These results show that IFN-γ is important in LGC formation in this co-culture system of human monocytes and autologous T cells.

Close contact between monocytes and T cells is required for LGC formation

ConA-stimulated T cells secrete several cytokines and up-regulate the expression of surface molecules that can stimulate nearby cells. Notably, differentiated LGCs were surrounded by CD3+ T cells (Fig. 3A), suggesting that direct contact between monocytes and T cells also contributed to LGC formation. The separation of T cells and monocytes by a semipermeable membrane reduced LGC formation (Fig. 3B).

We next co-cultured monocytes with either CD4+ or CD8+ T cells and assessed whether these two T cell subtypes differed in their ability to induce LGC formation. At every T cell-to-monocyte ratio tested, CD4+ T cells induced LGCs more efficiently than an equivalent number of CD8+ T cells (Fig. 3C). Since IFN-γ was required for LGC formation, the IFN-γ concentration was measured in the supernatants of the various culture conditions. The co-cultures with CD4+ T cells contained more IFN-γ than cultures with CD8+ T cells (Fig. 3D). However, the exogenous addition of rhIFN-γ to co-cultures with CD8+ T cells did not enhance LGC formation to the levels observed with CD4+ T cells (Fig. 3E). These results showed that factors other than IFN-γ, potentially surface molecules preferentially expressed on CD4+ T cells, are required for the efficient formation of LGCs in this system.

**The CD40–CD40L axis is necessary for LGC formation**

A literature search for differentially expressed surface molecules between CD4+ and CD8+ T cells in the expression profiles of activated human T cells (25) identified tumor necrosis factor superfamily 5 (TNFSF5 or CD40L) as a candidate molecule affecting LGC formation. The preferential expression of CD40L by CD4+ cells was confirmed in our co-culture system (Fig. 4A). To evaluate whether CD40L was indeed important in LGC formation, antibodies that disrupt the CD40–CD40L interaction were added to the co-culture. Blocking antibodies against CD40 and CD40L inhibited LGC formation in a dose-dependent manner (Fig. 4B). Furthermore, exogenous addition of sCD40L to the co-culture system enhanced LGC formation in a dose-dependent manner (Fig. 4C).

The importance of IFN-γ and CD40-CD40L axis in LGC formation suggested that IL-12 might contribute to LGC formation in this co-culture system since IL-12 is produced by monocytes in response to CD40 ligation and causes T cells to produce IFN-γ (26). The blocking antibody against IL-12 failed to inhibit LGC formation (Fig. 4D), although the exogenous addition of rhIL-12 to the co-culture system enhanced LGC formation (Fig. 4E). CD40 ligation also enhanced LGC formation in the presence of anti-IL-12 antibodies (Fig. 4D). Interruption of the CD40–CD40L interaction as well as the neutralization of IFN-γ abrogated LGC formation even in the presence of exogenous IL-12 (Fig. 4E). These data together indicate that the mechanisms through which CD40/CD40L and IFN-γ enhance LGC formation under the co-culture conditions employed in this study are mostly IL-12 dependent, although IL-12 may enhance the LGC formation process when it is present in a high concentration.
**T cells induce LGC formation by providing IFN-γ and CD40 stimulation**

To more specifically evaluate the role of T cells in inducing LGCs, monocytes were cultured with either rhIFN-γ or sCD40L in the presence of ConA without T cells. The addition of rhIFN-γ alone did not induce LGC formation from ConA-stimulated monocytes (Fig. 5A), suggesting that T-cell factors besides or in addition to IFN-γ were required to induce the formation of LGCs. Stimulation with exogenous sCD40L did induce monocytes to differentiate into LGCs (Fig. 5B), indicating the necessity for an interaction between T cell-derived CD40L and monocyte-expressed CD40. Despite the fact that rhIFN-γ alone had no effect on LGC formation from ConA-stimulated monocytes in the absence of T cells, IFN-γ was required for LGC formation when monocytes were co-cultured with autologous T cells under ConA stimulation (Fig. 2A). Furthermore, a blocking antibody against IFN-γ abrogated LGC formation by monocytes cultured with sCD40L and ConA (Fig. 5C), indicating that the LGC formation induced by stimulating cultured monocytes with exogenous sCD40L and ConA required IFN-γ.

Because monocytes do not produce large amounts of IFN-γ, it was possible that contaminating T cells were the source of IFN-γ when monocytes were stimulated with exogenous sCD40L and ConA. Indeed, when highly purified CD14+ cells were stimulated with exogenous sCD40L and ConA, significantly less LGC formation was observed than in cultures using crude monocytes (Fig. 5D). In addition, supplementing the culture medium of highly purified monocytes with rhIFN-γ as well as exogenous sCD40L and ConA enhanced LGC formation to the degree observed with CD4+ T cells. The culture condition was the same as Fig. 3(C and D), and the ratio of monocytes to T cells was 1:0.2. Values represent the mean fusion index and error bars indicate the standard mean of the error (n = 3 independent co-cultures).

**Fig. 3.** Direct contact between monocytes and T cells is required for LGC formation. (A) Immunohistochemical staining of LGCs. Co-cultured cells were stained with an antibody against CD3 (green), and nuclei were stained with DAPI (blue). (B) A semipermeable membrane between the monocytes and T cells (1:1 ratio) stimulated with ConA (2.5 µg ml⁻¹) abrogated LGC formation. Nuclei were distributed in a circular pattern in the absence of the semipermeable membrane (upper left), whereas this distribution was disturbed in the presence of the membrane (upper right). The mean fusion index of the cultures are shown, and error bars indicate the standard mean of the error (n = 3 independent co-cultures) (bottom). (C) CD4+ T cells induced LGCs more efficiently than CD8+ T cells. 'Monocyte' refers to the number of monocytes × 10⁶, and 'T cell' refers to the number of T cells × 10⁶. Values represent the mean fusion index calculated without distinguishing between monocytes and T cells, and error bars indicate the standard mean of the error of the indicated ratio of monocytes to T cells stimulated with ConA (5 µg ml⁻¹) (n = 3 independent co-cultures). (D) CD4+ T cells produced more IFN-γ than CD8+ T cells. The data show the results of triplicate ELISAs for IFN-γ concentration in the supernatants of the experiment shown in Fig. 3(C). Error bars indicate the standard mean of the error of three independent cultures. (E) Addition of rhIFN-γ to the co-culture of monocytes with CD8+ T cells did not enhance LGC formation to the degree observed with CD4+ T cells. The culture condition was the same as Fig. 3(C and D), and the ratio of monocytes to T cells was 1:0.2. Values represent the mean fusion index and error bars indicate the standard mean of the error (n = 3 independent co-cultures).
Taken together, these results demonstrate that T cells play a pivotal role by providing IFN-γ and CD40 stimulation to monocytes, but that a direct effect of ConA is also required for the induction of LGCs from human monocytes.

**DC-STAMP is involved in LGC formation**

Recently, it has been reported that several fusion-related molecules are involved in MGC formation, particularly in osteoclast differentiation and FGC formation (27–31). However, the involvement of these molecules in LGC formation has not been established. The mRNA expression of well-known fusion-related genes was therefore examined by RT-PCR. Notably, DC-STAMP, which is required for osteoclast formation and FGC formation, was up-regulated in monocytes stimulated with sCD40L, rhIFN-γ and ConA, whereas the expression of TREM-2, DOCK180, SIRPα, CD44, CD9, CD81 and MMP-9 was observed in the monocytes cultured with media alone (Fig. 6A).

To further explore the involvement of DC-STAMP in the LGC formation, siRNA-mediated knock-down of DC-STAMP was performed. Down-regulation of DC-STAMP mRNA was confirmed by RT-PCR (Fig. 6B). Transfection of monocytes with siRNAs against DC-STAMP decreased LGC formation in comparison with monocytes transfected with control siRNA, confirming the involvement of DC-STAMP in LGC formation by human monocytes stimulated with exogenous sCD40L, IFN-γ and ConA (Fig. 6C).

Inhibitors against nuclear factor (NF)-κB and mitogen-activated protein (MAP) kinases were used to delineate the signal transduction pathways involved in DC-STAMP up-regulation since CD40 stimulation induces the activation of these molecules (32–34). First, the activation of NF-κB and the MAP kinases (ERK kinase, JNK kinase, p38 kinase) during LGC formation was confirmed (Fig. 6D). The NF-κB inhibitors JSH-23 and BMS-345541, the ERK kinase inhibitor U0126, JNK kinase inhibitor II and the p38 kinase inhibitor SB-203580 reduced DC-STAMP mRNA expression and inhibited LGC formation (Fig. 6E and F). These data suggest that DC-STAMP up-regulation occurred through activation of NF-κB and MAP kinases and implied that this up-regulation is critical for LGC formation from monocytes stimulated with sCD40L, IFN-γ and ConA.

### Discussion

Granulomas are generally formed as a consequence of the failure of the host to eliminate a specific pathogen such as *Mycobacterium* (1) or *Cryptococcus* (2). They also occur when the host is immunodeficient, as in the case of CGD (3). Non-infectious granulomas cause clinical symptoms in diseases such as systemic sarcoidosis and BS/EOS. In systemic sarcoidosis, idiopathic granuloma formation in the bronchus or pleura causes respiratory disorders (4) and in BS/EOS, granuloma formation in the uvea or synovium causes loss of vision or joint contractures, respectively.
These granulomatous diseases are pathologically characterized by the presence of LGCs, which are considered to be closely related with the formation of granulomas. However, the pathophysiological mechanisms of LGC and granuloma formation are not well understood.

Based on the fact that granulomas usually contain LGCs and are surrounded by T cells (4, 35), a novel culture system was established whereby human monocytes were co-cultured with autologous T cells that were activated by ConA. This system resulted in efficient formation of LGCs. Although many systems that employ cytokines such as IFN-γ have been reported (15–19, 24), this is the first demonstration that a co-culture of monocytes and autologous T cells induces LGC-dominant MGC differentiation. Using this novel co-culture system, it was demonstrated that T cells play a pivotal role in LGC formation by providing IFN-γ and CD40 stimulation.

Since CD40 ligation on monocytes stimulates IL-12 production leading to IFN-γ production from activated T cells (26), it was possible that LGC formation did not require downstream signaling of CD40 in monocytes but required IFN-γ subsequently secreted from nearby T cells. However, LGC formation was not observed in monocytes stimulated with rhIFN-γ and ConA, and further addition of exogenous sCD40L was required to induce LGC formation (Fig. 5A and D). In addition, the contribution of IL-12 to LGC formation in the co-culture system of monocytes and T cells was minimal (Fig. 4D and E). Thus, CD40 signaling in monocytes contributes to LGC formation by a mechanism other than the induction of IL-12 production.

Although the molecular mechanism of macrophage fusion has been studied extensively in relation to osteoclast and FGC formation, little is known about the basis of LGC formation (27–31). In the present study, an in vitro human LGC formation system was established and the molecular mechanisms underlying the formation of LGCs were investigated.
Among the genes reported to be involved in macrophage fusion, DC-STAMP was involved in LGC formation in this co-culture system, similar to its involvement in osteoclast (36–39) and FGC formation (40). DC-STAMP was up-regulated by stimulating monocytes with exogenous sCD40L (300 ng ml⁻¹), rhIFN-γ (100 U ml⁻¹) and ConA (5 μg ml⁻¹). Additional transfections were performed at 12 and 24 h of the culture. (C) LGC formation was reduced by siRNA against DC-STAMP. Highly purified monocytes transfected with either control or DC-STAMP siRNA were cultured with exogenous sCD40L, rhIFN-γ or ConA as above. Values represent the mean fusion index, and error bars indicate the standard mean of the error (n = 3 independent cultures). *P < 0.01 by Student’s t-test. (D) NF-κB and MAP kinase signaling were involved in LGC formation in this system. Immunoblotting of whole cell lysates stimulated as indicated (LPS, 1 μg ml⁻¹; PMA, 4 μg ml⁻¹). (E) DC-STAMP up-regulation during LGC formation was abrogated in the presence of inhibitors of NF-κB or MAP kinases. RT-PCR analysis of DC-STAMP expression in the culture is shown. Each inhibitor was added 1 h before stimulation with ConA, sCD40L and rhIFN-γ (JSH-23, 20 μM; BMS-345541, 5 μM; U0126, 10 μM and JNK inhibitor II, 40 μM; SB203580, 10 μM). (F) LGC formation from highly purified monocytes stimulated with ConA, sCD40L and rhIFN-γ was prevented by the addition of inhibitors of NF-κB or the MAP kinases. (JSH-23, 10–20 μM; BMS-345541, 2.5–5 μM; U0126, 5–10 μM and JNK inhibitor II, 20–40 μM; SB203580, 5–10 μM).
RANKL does not appear to be involved in LGC formation in the present system, as a neutralizing antibody against RANKL failed to abrogate LGC formation (data not shown). These data imply that the differentiation of monocytes into LGCs versus osteoclasts occurs through different pathways.

In chronic inflammatory lesions of various etiologies, chemokines such as monocyte chemoattractant protein-1 produced by macrophages or vascular endothelial cells induce the chemotaxis of monocytes and T cells (45–48). The present study suggests that the interaction between monocytes and activated T cells caused LGC formation through pathways involving a CD40L and IFN-γ, both of which are provided by activated T cells. A potential scheme for LGC formation based on this data is shown in Fig. 7. ConA was a requisite for LGC formation in our system and acted directly on monocytes (Fig. 5D), and furthermore, the addition of methyl-α-D-mannopyranoside (Sigma), a ConA neutralizing agent, completely abrogated LGC formation (Supplementary Figure 1A is available at International Immunology Online). However, methyl-α-D-mannopyranoside did not inhibit the DC-STAMP up-regulation, the activation of NF-κB or the activation of MAP kinases (Supplementary Figure 1B and C is available at International Immunology Online). Although we speculate that ConA stimulation of monocytes yielded an activated phenotype, further study is required to delineate the mechanism through which ConA affects LGCs.

The present LGC formation system showed that the CD40-CD40L axis plays a critical role in LGC differentiation and might be a potential therapeutic target for pharmacologic treatment of granulomatous diseases. Although lethal thromboembolic events were initially reported for the humanized anti-CD40L antibody BG9588 (49), the humanized anti-CD40 monoclonal antibody dacetuzumab was well tolerated in a phase I study of patients with non-Hodgkin’s lymphoma (50). The use of anti-CD40-CD40L axis agents for the treatment of diseases such as sarcoidosis and CGD colitis seems contradictory because these diseases are known to involve infectious agents and disrupting the CD40-CD40L axis weakens host defenses. However, these diseases are currently treated with corticosteroids, which also have both anti-inflammatory and immunosuppressive effects, which control the tissue damage caused by the granulomatous lesions. Thus, the treatment of granulomatous diseases with anti-CD40-CD40L axis agents as a targeted therapy could be a viable approach.

In conclusion, the present study demonstrates that the CD40–CD40L interaction as well as IFN-γ production was necessary for LGC formation in a new in vitro LGC formation system using human monocytes and autologous T cells. T cells were shown to initiate signaling to monocytes via CD40- and IFN-γ-mediated pathways, and DC-STAMP was involved in the fusion of monocytes into LGCs. These findings provide new insights into the molecular mechanism of LGC formation and have the potential to contribute to the establishment of novel therapeutics against corticosteroid-resistant or -dependent granulomatous diseases.

Supplementary data

Supplementary data are available at International Immunology Online.
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