Investigation for regression factors in trehalose 6, 6'-dimycolate(TDM)-induced granulomatous lesions.

(トレハロースジミコレート(TDM)誘発性肉芽腫病変の退縮因子の検索)

Yusuke Sakai

坂井　祐介

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General Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB), is still one of the most important infectious diseases in the world, and is responsible for about 1.6 million deaths annually. Although most of antituberculosis drugs had been found in the period between 1950 and 1970 and such regimens made TB a curable disease [1], broad strains of antibiotics-resistant MTB have emerged in recent years [2-4] and they made TB therapeutics more difficult and complicated [5-7]. These multidrug-resistant (MDR)- MTBs or extensively drug-resistant (XDR)-MTBs threaten not only developing countries [8, 9] but also industrialized countries [10] where TB had once been controlled by use of antibiotics. Such global outbreak of MDR- and XDR- MTBs is a result of genetic mutations and unnatural selection in the bacteria promoted by an indiscriminate use of antibiotics. Thus, in the future, drugs that modify host's immune response to combat MTBs should be developed, because such immuno-potentiaters have few risks to promote the selection and distribution of bacteriocide-resistant MTBs. For this purpose, accumulation of the knowledge about immune response against MTB will be essential.

In addition to the emergence of drug resistant-MTBs, some drawbacks of Bacille Calmett-Guerin(BCG) vaccine, a vaccine used in all of the world, have been reported. First, because live bacterial vaccination is required for effective immunization of cellular immunity that is an indispensable arm against MTB, the vaccine cannot be injected to HIV-positive or immunodeficient patients, one of the most sensitive populations to MTB [11]. Second, BCG vaccination cannot prevent the infection of all strains of MTB. For example, MTB Beijing strain can escape from BCG-induced immunity and, currently, distributes worldwide [12]. Thus, the necessity for new and better anti-tuberculosis vaccine was advocated. To design and evaluate a new vaccine, more detailed information about the immunity against TB are indispensable.

I investigated, in this thesis, granulomatous lesions induced by the injection of trehalose 6, 6'-dimycolate (TDM) that is one of the cell wall components of MTB. Because the granuloma formation is one of the most characteristic immunopathology in MTB- or *Mycobacterium leprae*-infected patients, the comprehension of immunological mechanism is thought to be important for understanding anti-mycobacterial immunity. The nomenclature "granuloma" indicates the lesion consisting of activated and MTB-infected macrophages, called "epithelioid cells" or "foam cells", surrounded by lymphocytes and fibrosis cuffing. MTB is known to parasitize in these macrophages [13-16]. The structure of the lesion is considered to be beneficial for the host because it avoids dissemination of the mycobacteria and destructive inflammatory processes [14]. However, some negative aspects of granuloma formation have been also reported. Macrophages in the lesion cannot kill MTB in their phagosome because MTB have an ability to inhibit phagosome-lysosome fusion [17-19], phagosomal acidification [17, 20, 21] and maturation of phagosome [17, 22, 23]. Beside, macrophages even provide a nutrient-rich environment for the mycobacteria [24]. Antibiotics become less effective when MTBs are in a macrophage, because MTBs undergoes the dormancy state, which is a metabolistically inactivated state and thus resistant to antibiotics [25, 26]. Finally, granulomatous response is physically more destructive than normal exudative inflammations. Also due to these unfavorable effects, understanding how these granulomatous lesions are formed, maintained, and resolved, may aid treatment for the disease.

In this thesis, I induced granulomatous lesions by injecting TDM into mice. Mycobacterial cell wall mainly consists of glycolipids called mycolic acid, and TDM is the major component and constitutes 60% of the cell wall [26-29]. TDM possesses trehalose and branched-chain fatty acid called. The mycolic acid is characteristic

substance of mycobacteria and mainly exists as an esterified form with other cell wall components such as trehalose, wax D and arabinose. TDM is widely used by researchers who investigate on pathology of tuberculosis granuloma because it has granulomagenic property. Injection of the glycolipid into experimental animals induces granuloma formation and cytokine expression mimiking those induced by MTB infection [30-32], while the injection of MTB that lacks TDM to mice results in fewer granulomatous lesions and less intense delayed-type hypersensitivity than mice infected with native MTB [33]. TDM are known to stimulate TLR-2 and TLR-4 and to promote CD-1-dependent antigen presentation [34, 35], and thus to activate an adaptive immune response. Although TDM has such immuno-stimulatory properties, TDM also has the functions that suppress the intracellular processing of the bacterial antigens, for example, TDM inhibits the maturation of phagolysosomes, intracellular trafficking of phospholipid vesicles and acidification of phagolysosome in macrophages [36-38]. Perhaps by this reason, MTB that lacks TDM cannot survive in macrophages [39, 40]. Therefore, TDM is thought to be one of the most important substances determining the virulence of the mycobacteria. The TDM-induced granulomatous lesion develops and regresses more rapidly than the live bacteria-induced model, because TDM does not show long time persistence nor proliferate in macrophages. Therefore, the TDM model seems to suit for understanding throughout the course of the MTB granulomatous lesions.

Cytokines and chemokines are the most important intercellular mediators in inflammation. These molecules activate cells of the immune system and attract them to an appropriate site of inflammation. Therefore, these molecules are thought to control the persistence, intensity, cell population of the inflammation and finally the systemic response to the immunogenic stimuli.

Roles of some cytokines in TB-granuloma development are also well characterized [41-43]. Especially, tumor necrosis factor (TNF)-alpha, interleukin (IL)-1beta, interferon (IFN)-gamma and IL-6 are considered to be crucial molecules for the development of TB lesions.

TNF-alpha and IL-1beta are the representative pro-inflammatory cytokines and are secreted mainly by macrophages but also by a broad spectrum of cells [44, 45]. Either TNF-alpha-, TNFR1-, IL-1- or IL-1R-deficient mice failed to form granulomatous lesions due to poor activation of the immune system, and, as a result, succumbed to MTB infection [44, 46-52]. Therefore, TNF-alpha and IL-1beta are thought to contribute to an anti-tuberculosis response by sequentially activating inflammatory cells and following cytokine secretion [44, 45]. The two cytokines are seem to act at the upstream of the cytokine network, because their functional loss lead to not only the loss of inflammatory cell attraction but also the compromised secretion level of other cytokines, such as IFN-gamma and IL-12, and chemokines [46-52].

The functions of such TNF-alpha- or IL-1beta-regulated cytokines, including IFN-gamma and IL-6, in TB lesions are well characterized. IFN-gamma is a Th1 cytokine that is secreted by Th1 cells, cytotoxic-T cells and NK cells, and activates the cellular immunity. The role of the cytokines in granulomatous lesions is also demonstrated by an IFN-gamma-knockout mouse model [53-55]. In genetically IFN-gamma-disrupted mice, MTB infection causes almost normal granuloma but poor ability to eliminate the bacteria [53]. This suggests the role of IFN-gamma for the macrophage-activation, not for the granuloma-induction. IL-6 is a multifunctional cytokine and secreted mainly from macrophages and lymphocytes. The role of IL-6 in TB lesions is less clear than that of cytokines above because the results of previous studies using IL-6-deficient mice or mice treated with IL-6-neutralizing antibodies are

still conflicting [56-58]. For example, a report suggested that IL-6 depletion caused granulomas within a shorter period [56], but another reported no remarkable difference in histopathology between IL-6-depleted mice and control mice [57]. Therefore, it is likely that IL-6 is essential to induce proper immune response against MTB and is required for granuloma maintenance.

Not only such pro-inflammatory cytokines, anti-inflammatory cytokines also play important role in inflammatory process. The histopathological lesions reflect the balance of such factors with the opposite functions. Anti-inflammatory cytokines include IL-10 [59], TGF-beta and IL-27. The role of IL-10 in TB lesions is comparably well characterized among them. IL-10-knockout mice show early and enhanced cytokine production and macrophage infiltration in MTB infection, which results in an increased resistance against MTB [60-62]. Such reports suggest that IL-10 has the function limiting the anti-MTB immunity. IL-27 was identified as an IL-12-related cytokine in 2002 [63] and subsequent studies have revealed an immunosuppressive function of IL-27 [63]. Because this cytokine was identified recently, the relationship between IL-27 and TB lesion is scarcely unlabeled [64-66]. Transforming growth factor (TGF)-beta is thought to function as a down-regulator of Th1 response, an inducer of fibrosis and a suppressor of excessive inflammation [67]. TGF-beta is therefore considered to be an important inflammation regressive factor while its fibrosis-inducing activitiy may contribute to the long-term persistence of the granulomatous lesions. In fact, suppression of TGF-beta function by using a TGF-beta antagonist resulted in an enhanced Th1 activity and decreased fibrosis in a murine tuberculosis model [68].

The expression profile of chemokines is also important to understand the course of granulomatous lesions, because the trafficking and migration of leukocytes are

controlled by chemokines. At present, over 50 kinds of chemokines and about 20 chemokine receptors have been identified. Multiple chemokines bind to one receptor and a single chemokine can bind multiple receptors. Because each inflammatory cell-type has a specific pattern of chemokine receptors, the chemokine profile of the inflammatory lesion determines the infiltrating cell types. For example, macrophage inflammatory protein (MIP)-1α, also known as CCL3, is secreted from macrophages and attracts CCR-1-, 4- or 5-expressing polymorphonuclear leukocytes (PMNs) [69]. In tuberculosis, infiltrating macrophages rapidly produce multiple chemokines soon after the contact with MTB. And then recruited leukocytes produce other cytokines. As a result, granulomatous lesions are organized [42, 70].

In this thesis, therefore, I tried to clarify the kinetics of cytokines and chemokines in the TDM-induced granulomatous lesion and their relation with the histopathologic changes, and after that, tried to unravel the mechanism that alters such cytokine and chemokine profile.

Chapter I

Histopathological features and expression profiles of cytokines, chemokines and negative regulators of cytokine signaling in trehalose 6, 6' - dimycolate induced granulomatous lesions.

Sumary

To investigate the immunological response in the granulomatous lesion, I injected granulomagenic mycobacterial cell wall lipid, TDM into mice. First, I examined the cytokine and chemokine expression levels comparing with histopathological course of the lesion. The size of the lesions was increased toward 7 dpi and after that decreased. The infiltrating cell population in the granulomatous lesions was changed from PMN-dominant until 7 dpi to lymphocyte-dominant after 14 dpi. The number of TUNEL-positive apoptotic cells reached at the peak at 7dpi and decreased thereafter. The expression levels of pro-inflammatory cytokines such as TNF-alpha, IL-1beta and IL-6 showed the similar kinetics to that of the size of the lesion. This may show the importance of suppression of pro-inflammatory cytokines for the reduction of lesion sizes. On the other hand, anti-inflammatory cytokines except for TGF-beta were down-regulated at all time points. The result indicates the IL-10 and IL-27 have few roles in the TDM-induced granulomatous lesion. The chemokine profiles changed from PMN-attracting chemokine-predominant to lymphocyte-attracting chemokine-predominant. The change of chemokine profiles may explain the change of infiltrating cell population. I also examined the negative regulators of cytokine signaling to know how such down-regulation of pro-inflammatory cytokines occurrs. As a result, SOCS-3, A20 and ABIN-3 were highly expressed at 7 to 14 dpi. Therefore, I concluded that the factors play some roles on the regression of TDM-induced granulomatous lesions.

Introduction

As written in the General Introduction, the roles of various cyokines and chemokines in TB lesions have been already described in previous reports [41-58, 60-62, 64-66, 68] Though, the kinetics of the cytokines from the development to regression of the lesions is scarcely studied. It also seems to be important to grasp the kinetics of cytokines in correlation with histopathology of the lesions. The histopathological and clinical profiles of the lesions are not caused by the role of a single cytokine but resulted from the network of various cytokines in the lesions. It is important to know the expression profile of cytokines in the progressing and regressing lesions. Therefore, in this chapter, I tried to clarify the cytokine profile in TDM-induced granuloma. I also investigated the expression kinetics of IL-17 and IL-27, which have been discovered recently and the role in the TB lesion is still unknown.

In addition to cytokines and chemokines, I also analyzed negative regulators of the cytokine signal transduction. These include suppressors of cytokine signaling (SOCS) proteins, A20 and A20-binding inhibitors of NF-kappa B (ABIN) proteins.

Among eight SOCS family proteins (SOCS1-7 and CIS), the role of SOCS-1-3 and CIS are comparably well investigated [71]. Therefore, I investigated the timing of their beginning of expression and contribution to granuloma regression. A20 and ABIN form a complex and mediate the proteasomal degradation by editing the ubiquitination status of the target proteins, most of which are NF-kappaB signaling proteins [72]. Three members of ABIN family proteins have been identified. However, their importances in *in vivo* inflammatory diseases are still not unveiled. Thus, I aimed to confirm the relationship between the granulomatous lesions and ABIN family proteins.

This chapter includes investigations first, to clarify the TDM-induced lesions histopathologically; second, to identify the cell population in the lesions at ; third, to

analyze the expression profiles of cytokines, chemokines, SOCS family proteins, A20 and ABIN family proteins; and finally to determine which mediator most contributes to the development and regression of TDM-induced granulomatous lesions.

Materials and Methods

Animals

Six-week-old female BALB/c mice were purchased from SLC (Shizuoka, Japan). The animals were housed in isolator cages under a specific pathogen-free condition. The experimental procedures in the present study were approved by the Experimental Animal Committee of the University of Tokyo.

Preparation of water/oil/water (w/o/w) emulsion

W/o/w emulsion containing TDM was prepared as described in a previous report [30]. Briefly, 100 μg of TDM (Nakalai Tesque, Tokyo, Japan) was dissolved in 3.2 μℓ of Freund's incomplete adjuvant (Chemicon, Temecula, CA), and then, 3.2 μℓ of 0.1M PBS were added. The mixture was then homogenized with a glass homogenizer. Finally, 93.6 μℓ of saline containing 0.2% Tween 20 were added to the homogenized w/o emulsion.

Induction of granuloma

The mice were twice injected i.p with a 100 μℓ of w/o/w emulsion at a 1 week-interval, before being sacrificed at days 0, 3, 7, 14, or 21 after the last injection. Three mice were used per group. At 0 day post injection (dpi), mice were sacrificed just after the last injection.

Histopathological examination and immunohistochemistry

Formalin-fixed paraffin sections and acetone-fixed frozen sections were obtained from granulomatous lesions in the peritoneum. Paraffin sections were stained with hematoxylin and eosin (HE). To measure the area of the lesion, an image analysis

software, Image J was used. The number of PMN in 10 areas in a section was counted in each mouse.

Some sections were subjected to immunohistochemical staining using the LSAB method. In this procedure, autoclave-pretreatment in citrate buffer (pH6.0) was performed for antigen retrieval. Rabbit antibody against CD3 (A0452 ; Dako, Carpenteria, CA) as a T-cell marker, goat antibody against mouse IgG (KPL, Aurora, OH) as a plasma-cell marker, and rabbit antibody against Granzyme B (Springbioscience, Fremont, CA) as a cytotoxic T-cell marker were applied to the paraffin sections, and rat antibody against CD68 (FA-11; Abcam, Cambridge, UK) as a macrophage marker to the cryosections as primary antibodies. Positive signals were visualized by a peroxidase-diaminobenzidine reaction. The number of positive cells in 10 areas was counted in each mouse.

TUNEL stain

DNA fragmentation caused by apoptosis was detected on a paraffin section by using a commercial apoptosis detection kit (ApopTag® Peroxidase *In situ* Apoptosis Detection Kit; Chemicon, CA) according to the manufacturer's instruction. In brief, multiple fragmented DNA 3'-OH ends on a deparrafinized section were labeled with digoxigenin-dUTP in the presence of terminal deoxynucleotidyl transferase (TdT) enzyme. Peroxidase-conjugated anti-digoxigenin antibody was then reacted with the section. Apoptic nuclei were visualized by the diaminobenzidine (DAB) reaction. The number of positive cells in 10 areas was counted in each mouse.

RNA extraction, RT-PCR, and real time PCR

Tissues were homogenated in ISOGEN fluid (Nippongene, Tokyo, Japan) using

Tissue-ruptor (Qiagen, Valencia, CA). RNA extraction was performed through the treatment of sample tissues with chloroform, precipitation in isopropanol, and washing with 75 % ethanol. The resulting total RNA was eluted in Rnase-free water and stored at -80 ℃ until use.

Reverse transcription (RT) was performed using the PrimeScript RT reagent kit (Takara Biotechnology, Dalian, China), according to the manufacturer's instructions. Briefly, 400 n g of total RNA were mixed with PrimeScript buffer, Oligo dT primer, dNTP mix and PrimeScript RT Enzyme, and RNase-free water was added to make a total volume of 20 μ ℓ. The mixture was kept at 42 ℃ for 50 min, and the reaction stopped at 70 ℃. The primers used for RNA amplication are shown in Table 1. Either RT-PCR or real time PCR was performed for the detection of specific mRNA sequences.

For RT-PCR, I used the ExTaq Enzyme Hot Start PCR kit (Takara Biotechnology), according to the manufacturer's instructions. After PCR, agarose gel electrophoresis was performed and bands of PCR products were stained with ethidium bromide. The ratio of the intensity of a specific band to that of GAPDH was calculated.

For real time PCR, cDNA was mixed with the SYBR Green Realtime PCR Master Mix (TOYOBO, Osaka, Japan). cDNAs were preheated at 95 ℃ for 3 min and then subjected to 40 cycles of amplication. The reaction was monitored and analyzed with the ABIprism 7700 sequence detection system (Perkin Elmer, Foster city, CA). After normalization to GAPDH mRNA levels, relative mRNA levels compared with that at 0 dpi were calculated. The results are shown as the mean ± standard deviation (SD).

Results

Histology

The granulomatous lesions grew larger until 7 dpi and became smaller thereafter with time (Figs. 1 and 2). The lesions consisted of CD68-positive macrophages and PMN at 3 dpi (Figs. 2a, 4a). At 7 dpi, the major constituents of the lesions were still CD68-positive macrophages and PMNs, but the proliferation of fibroblasts was also found (Figs. 2b and 4a). At 14 dpi, the number of CD3-positive T-cells began to increase, while that of PMNs had decreased by about 50% of 3 dpi (Figs. 2c and 4a). At 21 dpi, the number of PMNs had decreased to less than 10% of all cells (Fig. 4a). At that time point, the major constituents were CD3-positive T-cells and CD68-positive macrophages (Figs. 2d and 4a). In some parts of the lesions, only lymphocytes and fibrous tissue were found. The numbers of Granzyme B-positive cells and IgG-positive cells were very low at all time points (Fig. 4a). Apoptosis might contribute to the reduction of lesion size, because the frequency of TUNEL positive apoptotic cells became high at 7 and 14 dpi (Fig. 3).

Expression profiles of cytokines

The mRNA expression of two major pro-inflammatory cytokines, TNF-α and IL-1β, increased after the injection of TDM, peaked 7 dpi, and then decreased toward 21 dpi (Fig. 5a). The expression intensities paralleled to the sizes of the lesions. Thus, 14 dpi was thought to be the time point when the resolution process overcame the pro-inflmmatory reaction. Among the three anti-inflammatory cytokines investigated (IL-10, IL-27 and TGF-β), only the expression of TGF-β was upregulated (Figs. 5a and 5b). The expression of the cytokine peaked at 7 dpi as TNF-α and IL-1β did. At 14 dpi, notably, the expression level of TGF-β was still high (Fig. 5a). The expression of IFN-γ, a representative Th1 cytokine with a role in macrophage activation, was

upregulated at all time points examined, while the expression levels of a Th2 cytokine, IL-4, and a Th17 cytokine, IL-17, were downregulated (Fig. 5c). IL-6, a Th2 cytokine, was upregulated at all time points. Different from IL-4, IL-6 is secreted from not only Th2 cells but also activated macrophages. Therefore, I considered that not only Th lymphocytes but also macrophages may play the effector function in TDM-induced glaucomatous response.

Expression of chemokines

The expression of RANTES peaked at 3 dpi and those of MIP-1α and MIP-2α peaked at 7 dpi (Fig. 6a). At 3 and 7 dpi, PMNs were most frequently observed. The expression levels of IP-10 and MCP-1 were highest at 14 dpi (Fig. 6b), at which time point the number of lymphocytes in the lesions was significantly high.

SOCS family proteins

I also investigated the mRNA expression of four members of SOCS family proteins. The expression of SOCS-3 was upregulated at all time points, and the expression levels of the others, except for SOCS-1 at 21 dpi, were downregulated (Fig. 7). Therefore, among the SOCS family proteins, only SOCS-3 is suggested to plays an important role for the regulation of TDM-induced granulomatous lesions.

A20 and ABINs

In addition to SOCS-family proteins, I investigated mRNA levels of A20 and ABIN-family proteins, which are other negative feedback regulators of cytokine signaling. The expression levels of A20 quantified by the Real-time PCR at 3, 7, 14 and 21 dpi were 5 times higher than that at 0 dpi (Fig. 8). The expressions of ABIN-1 and ABIN-3 were also up-regulated after granuloma induction while that of ABIN-2 was down-regulated at all time points. The up-regulation of ABIN-3 at 7 and 14 dpi were 30 times higher than that at 0 dpi while the fold change of ABIN-1 was

about 3.7 times.

Discussion

In this chapter, I revealed histopathological features and expression profiles of cytokines, chemokines, SOCS family proteins, A20 and ABIN family proteins in TDM-induced granuloma for 3 weeks after the induction. The sizes of the granulomatous lesions increased up to 7 dpi and then became smaller. The expression of three pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) rose up to 7 dpi and decreased thereafter as well. These results indicate that TDM-induced granulomatous inflammation is promoted until 7 dpi and goes into the regression phase thereafter. As described in General Introduction, TNF-α, IL-1β, IFN-γ and IL-6 are thought to be crucial molecules for anti-mycobacterial immunity during granuloma formation in MTB-injected mice [41-58, 60-62, 64-66, 68]. In this chapter, the reduction of granuloma size coincided with decreases in TNF-α or IL-1β expression. IFN-γ maintained a high expression level throughout the course. IL-6 also showed high expression levels at 3 and 7 dpi. Therefore, I could affirm the importance of TNF-α, IL-1β, IFN-γ and IL-6 in the promotion of granulomatous inflammation.

On the other hand, the results of anti-inflammatory cytokines did not exhibit a clear correlation with the granuloma regression. As the expression levels of IL-10 and IL-27 were downregulated at almost all time points, I discarded the idea that these cytokines promote the regression of TDM-induced granuloma. The expression profile of TGF-β, another anti-inflammatory cytokine, showed a similar pattern to that of TNF-α and IL-1β. However, the expression of TGF-β was more than 15 times higher at 14 dpi, when the lesions had already regressed, than that at of 0 dpi. TGF-β is known to promote the proliferation of fibroblasts and to inhibit inflammation [67, 68]. Moreover, it was reported that TGF-β suppressed cytokine production and

cellular infiltration in a guinea pig model of TB [73]. Also in this study, TGF-β may have contributed to the proliferation of fibroblasts and the regression of the lesions because the expression of the cytokine was high not only at 7 dpi but also 14 dpi, at which time points fibroblast proliferation and regression of the lesions were observed.

I also investigated the expression profiles of SOCS family proteins. Among the four SOCS family proteins, the expression of SOCS-3 was upregulated at all time points. This protein is known as a negative regulator for IL-1, IL-6, IL-10, IL-12 , TGF-β, and IFN-γ, and is produced in mice injected with MTB [71,74-75]. SOCS-3 is suggested to regulate the regression of TDM-induced granuloma by a negative-feedback mechanism, according to the present results in which its targets, IL-1β, IL-6, IFN-γ, and TGF-β were upregulated at 3 to 7 dpi. SOCS-1 is also thought to be involved in the formation of TB lesions, according to a previous study, which described the inflammatory process during several days after the injection of BCG [76]. In the present study, however, the expression of SOCS-1 was downregulated at all time points. These incongruous results may come from the difference of the time course of the two experiments. SOCS-1 may play a role in the early phase before 3 dpi, not in the chronic phase. Previous reports have outlined the immunosuppressive roles of SOCS-2 and CIS during inflammation [77, 78]. However, the present results showed these two proteins have little importance in the formation and regression of TDM-induced granuloma, because their expression levels were downregulated at all time points. Therefore, it is concluded that TGF-β and SOCS-3 are the possible candidates that contribute to granuloma.

In this chapter, I also investigated the expression of IL-17 in TDM-induced granuloma. Its roles in the lesions of various diseases were recently unveiled [79], but the roles in TB or TDM-induced granuloma are poorly understood. In the present

study, the expression of the cytokine was downregulated at all time points. This suggests that the cytokine is unrelated to the regression of TDM -induced granuloma. The function of IL-17 in TB granuloma is thought to be the activation of memory T-cells and PMN attraction [80], which occur in the early phase of the disease.

At 3 and 7 dpi, PMNs were the most predominant component in the TDM-induced lesions, and the expressions of PMN-attracting chemokines such as RANTES, MIP-1α, and MIP-2α was higher at these time points. Thereafter, the expression levels of the mononuclear cell-attracting chemokines, IP-10 and MCP-1, increased. Such chemokine expression profiles can explain the changes in the inflammatory cell population in the granulomatous lesions. However, the factors that induce changes in the chemokine expression profiles observed were still unknown. The expression of chemokine is known to be promoted by TNF-α, IL-1β and IFN-γ [81-83], but this mechanism cannot explain the changes observed in this study. Therefore, it is reasonable to consider other regulatory factors that may be responsible for the change in chemokine expression profiles. One possible candidate is TGF-β, because this cytokine matintained a high expression level at 14 dpi, and there are reports regarding the induction of MCP-1 [84] and IP-10 [85] by the expression of TGF-β. In addition, knocking out of SMAD3, a transducer of the TGF-β signaling, resulted in higher expression of MIP-1α suggesting thet TGFβ suppresses the expression of MIP-1α [86].

I next examined the expression levels of A20 and ABIN family proteins, because they are also known as negative feedback factors for cytokine signalings. Among them, mRNA levels of A20 and ABIN-3 showed marked elevations at 7 and 14 dpi. In addition between 7 and 14 dpi, at which time points the expression of A20 and ABIN-3 levels were reached at peak, the size of the lesions were markedly reduced.

These results implied that the increased expression of A20 and ABIN-3 affected the reduction of the granuloma size.

The result of TUNEL stain chapter suggested apoptosis of leukocytes contributed to the granuloma regression. TUNEL-positive cells were increased toward 14 dpi, in which time point the size of the lesions markedly decreased. Leukocytes undergo apoptosis through almost the same way as other cell types do, extrinsic or intrinsic pathway [87-89]. Whichever pathways induce apoptosis, the cell fates are determined by the balance of survival and apoptotic signals. In general, pro-inflammatory cytokines act as survival signals for leukocytes by activating the NF-kappa B pathway. However, TNF-alpha has both pro-apoptotic and anti-apoptotic effects. In recent studies, ubiquitination status of a receptor-interacting protein serine-threonin kinase (RIP)-1 is suggested to determine the destination of TNFR activation [90]. RIP-1 promotes caspase-8 activation by recruiting Fas-associated death domain (FADD) to TNFR-associated death domain (TRADD). On the other hand, Lys-63 ubiquitinated RIP-1 cannot promote this reaction but activate the NF-kappaB signaling [90]. A20 is demonstrated to negatively regulate Lys-63 ubiquitination and to promotes ubiquitination of other locus of RIP-1 resulting proteasomal degradation [91]. Therefore, down-regulation of pro-inflammatory cytokines, such as IL-1β and IL-6, and up-regulation of A20 may contribute to induce apoptosis to infiltrating cells.

The regression of TDM-induced granuloma began between 7 and 14 dpi. The increased expression of SOCS-3, A20, ABIN-3 and TGF-β combined with the down-regulation of TNF-α and IL-1β may correlate with granuloma regression and apoptosis in the lesion. TGF-β may also contribute to the changes in infiltrating leukocyte profiles by modulating chemokine expression. As mentioned above, the

present study revealed the expression levels of cytokines and chemokines in the development and regression process of the granulomatous lesion combined with histopathological process. I convince the importance of the present demonstration of the possible relation of cytokine kinetics to histopathological findings of granuloma regression.

Table 1 Primers for PCR

gene		primer sequence	gene		primer sequence
TNF-α	sense	TCTCATCAGTTCTATGGCCC	MIP-1α	sense	GCCCTTGCTGTTCTTCTCTGT
	antisense	GGGAGTAGACAAGGTACAAC		antisense	GGCAATCAGTTCCAGGTCAGT
IFN-γ	sense	GCTCTGAGACAATGAACGCT	MIP-2α	sense	GAACAAAGGCAAGGCTAACTGA
	antisense	AAAGAGATAATCTGGCTCTGC		antisense	AACATAACAACATCTGGGCAAT
TGF-β	sense	ACCGCAACAACGCCATCTAT	IP-10	sense	ACCATGAACCCAAGTGCTGCCGTC
	antisense	GTAACGCCAGGAATTGTTGC		antisense	GCTTCACTCCAGTTAAGGAGCCCT
IL-1β	sense	TTGACGGACCCCAAAAGATG	SOCS-1	sense	ACCTTCTTGGTGCGCGAC
	antisense	AGAAGGTGCTCATGTCCTCA		antisense	AAGCCATCTTCACGCTGAGC
IL-4	sense	TCGGCATTTTGAACGAGGTC	SOCS-2	sense	GGTTGCCGGAGGAACAGTC
	antisense	GAAAAGCCCGAAAGAGTCTC		antisense	GAGCCTCTTTTAATTTCTCTTTGGC
IL-6	sense	GTTCTCTGGGAAATCGTGGA	SOCS-3	sense	GCGAGAAGATTCCGCTGGTA
	antisense	TGTACTCCAGGTAGCTATGG		antisense	CGTTGACAGTCTTCCGACAAAG
IL-10	sense	ATGCAGGACTTTAAGGGTTACTTG	CIS	sense	CCAGCCATGCAGCCCTTA
	antisense	TAGACACCTTGGTCTTGGAGCTTA		antisense	CGTCTTGGCTATGCACAGCA
IL-17a	sense	CTCCAGAAGGCCCTCAGACTAC	A20	sense	CATCCACAAAGCACTTATTGACA
	antisense	GGGTCTTCATTGCGGTGG		antisense	GAGTGTCGTAGCAAAGTCCTGTT
IL-27	sense	TGTTCAAAGGAGGAGGAGGAC	ABIN-1	sense	GCATGGAGCTGCTGGAA
	antisense	GGATGACACCTGATTGGGG		antisense	CATGGACCGGAAATGCT
RANTES	sense	CATATGGCTCGGACACCACT	ABIN-2	sense	AACAGAAGGTGACTCATGTAGAAG
	antisense	ACACACTTGGCGGTTCCTTC		antisense	TCCCTACTTGCATCATAACG
MCP-1	sense	ATGCAGGTCCCTGTCATG	ABIN-3	sense	GGACTTGACAAATTCTCTTGAACGA
	antisense	GCTTGAGGTGGTTGTGGA		antisense	CGGAATTGCTGGTCCCATTG

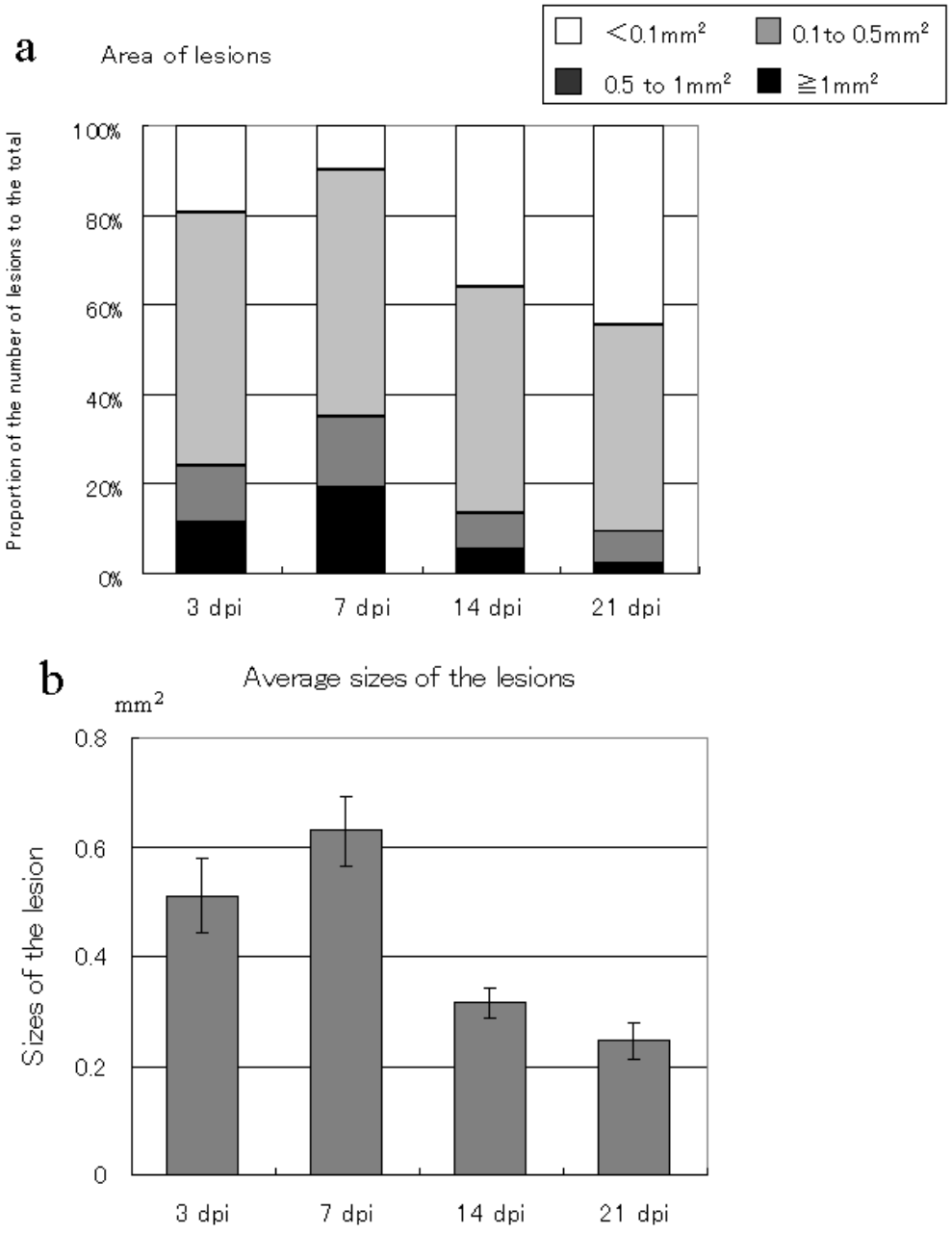


Figure 1. Size of the granulomatous lesions.

a. The size of each lesion was graded into 4 groups. The largest was ＞1 mm^2, followed by that between 1 m ㎡ and 0.5 m ㎡ and that between 0.5 and 0.1 mm^2, and the smallest was ＜ 0.1 mm^2. The graph shows the proportion of the number of lesions to the total.

b. Average size of the lesions at each time points. Mean area ± SD. The differences between groups were confirmed as significant (P<0.05) except for 3 dpi vs. 7 dpi and 14 dpi vs. 21 dpi by by ANOVA and Tukey-Kramer test as post-hoc test .

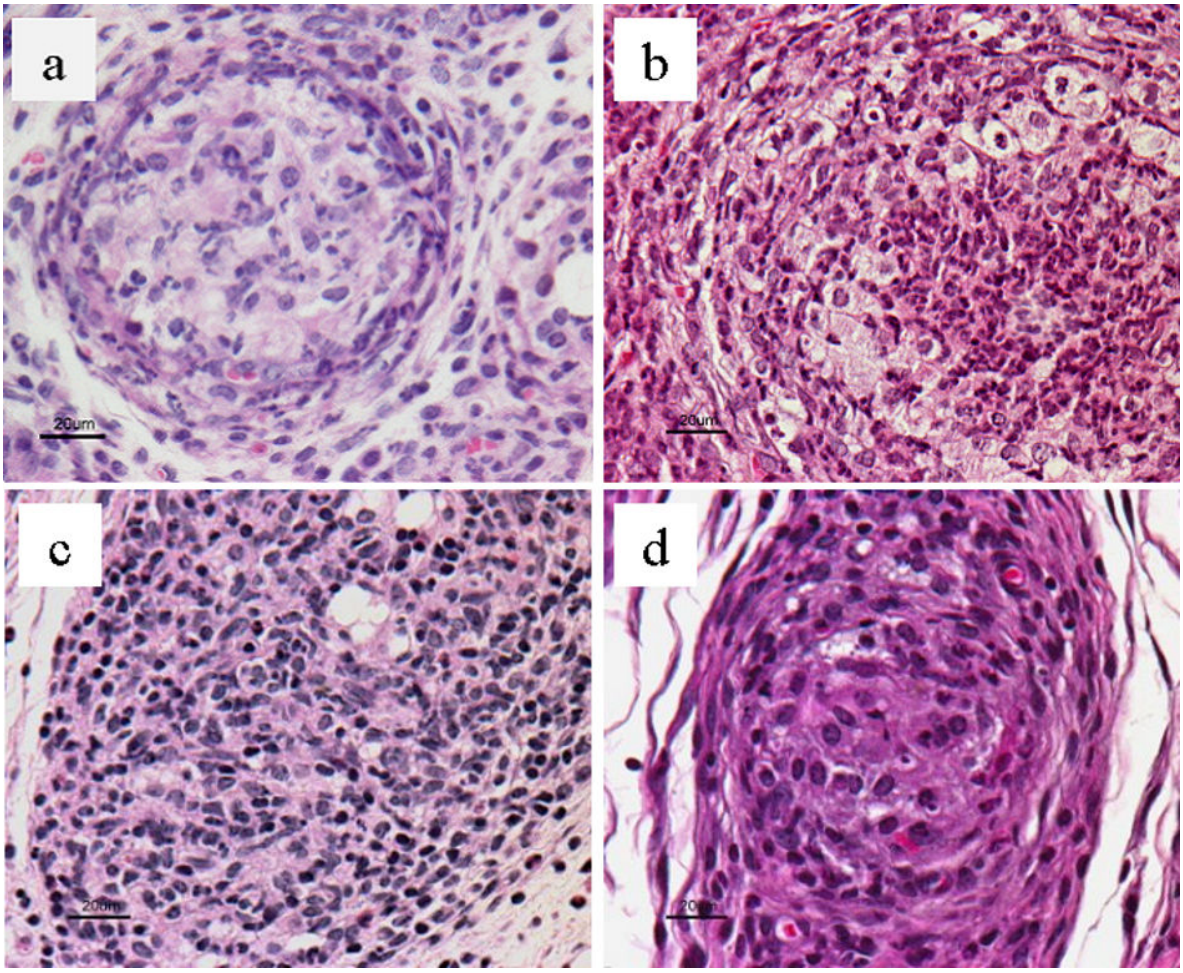


Figure 2. Histopathology of granuloma.

A granuloma consisting mainly of PMNs and macrophages. 3 dpi (a). Fibroblast's proliferation is seen in addition to PMNs and macrophages. 7 dpi (b). The major consistuents are macrophages and lymphocytes rather than PMNs. 14 dpi (c). A granuloma has been reduced in size, and only mononuclear cells are seen. 21 dpi (d). HE stain. Scale bar = 20 μ m.

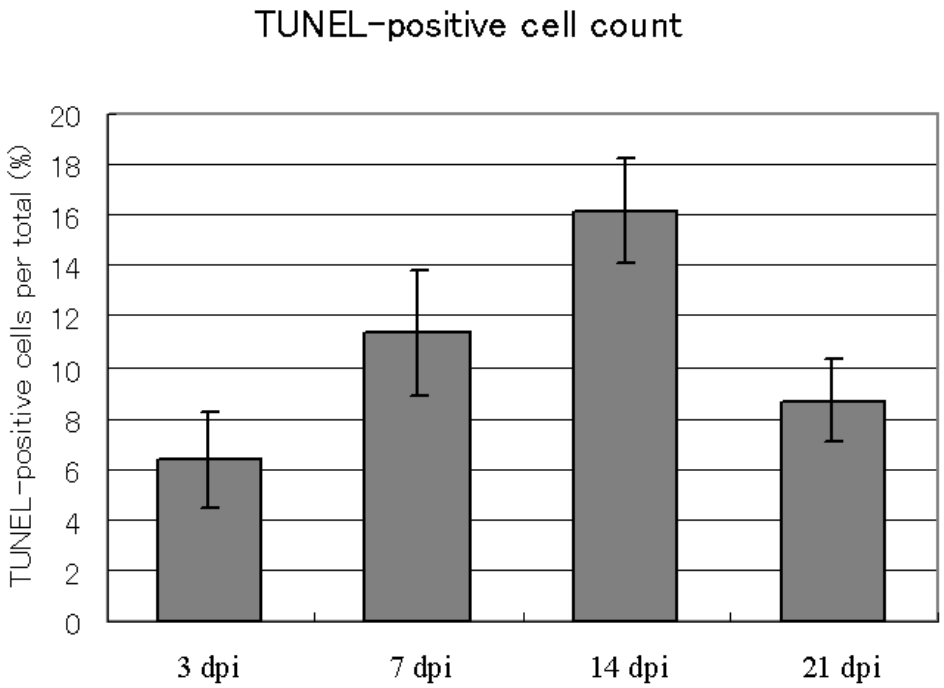


Figure 3. TUNEL-positive cells.TUNEL staining was performed for paraffin sections. The number of TUNEL-positive cells was counted, and percent to the total cell number was calculated. Mean ±SD. 3 mice were used per time point and ten lesions were counted per mouse.

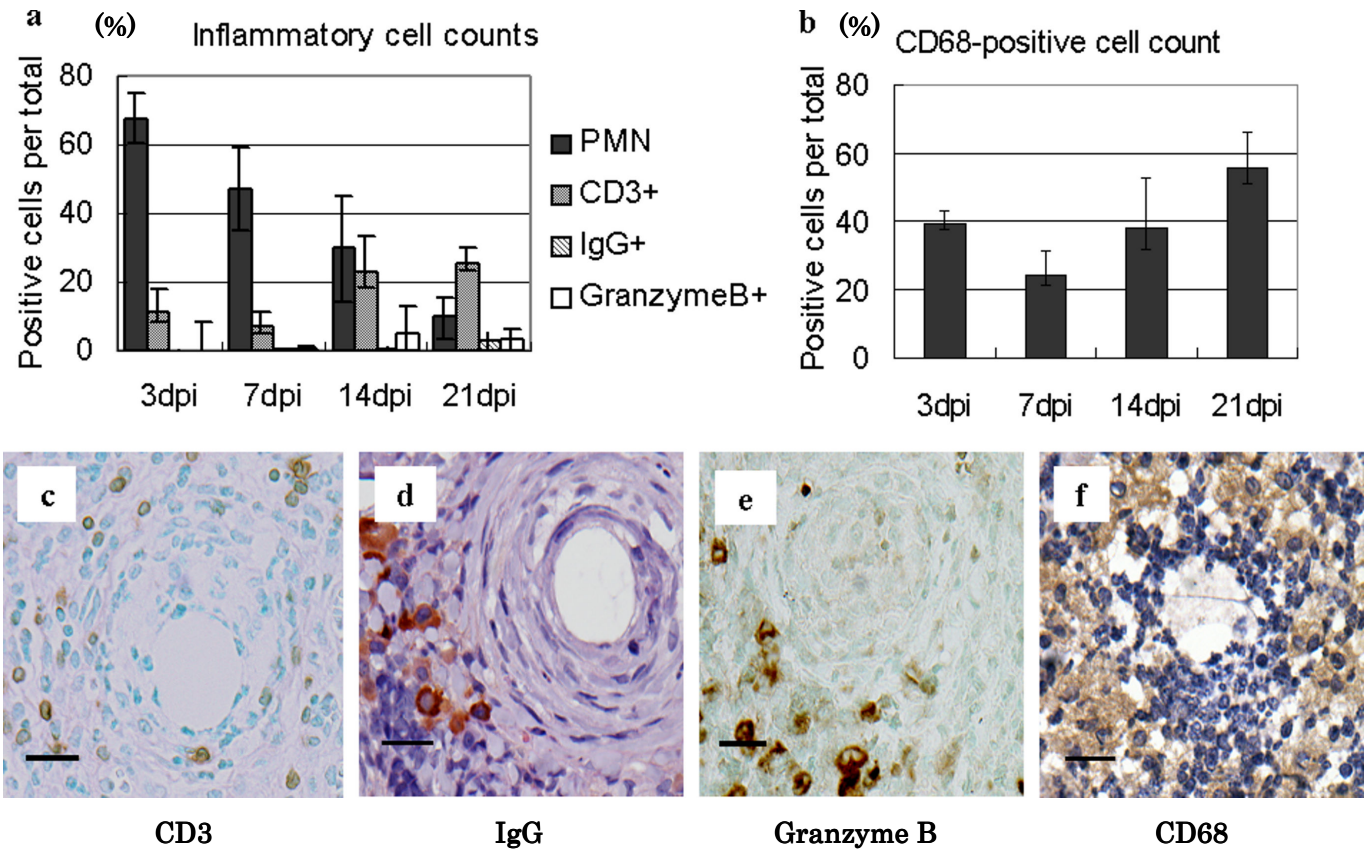


Figure 4. Results of immunohistochemical staining and cell counts. Immunohistochemical staining was performed on paraffin sections (a). The number of inflammatory cells were counted, and the proportion relative to the total cell number was calculated. Mean value ±SD are shown. Ten lesions were counted per mouse, and 3 mice were used per time point. Immunohistochemical staining was performed for cryosections (b). The numbers of CD68-positive macrophages were counted, and the proportion relative to the total cell number was calculated. Mean value±SE are shown. Ten lesions were counted per mouse, and 3 mice were used per time point. Immunohistochemical staining of CD3 (c), IgG (d), Granzyme B (e), and CD68 (f). Scale bar = 20 μ m.

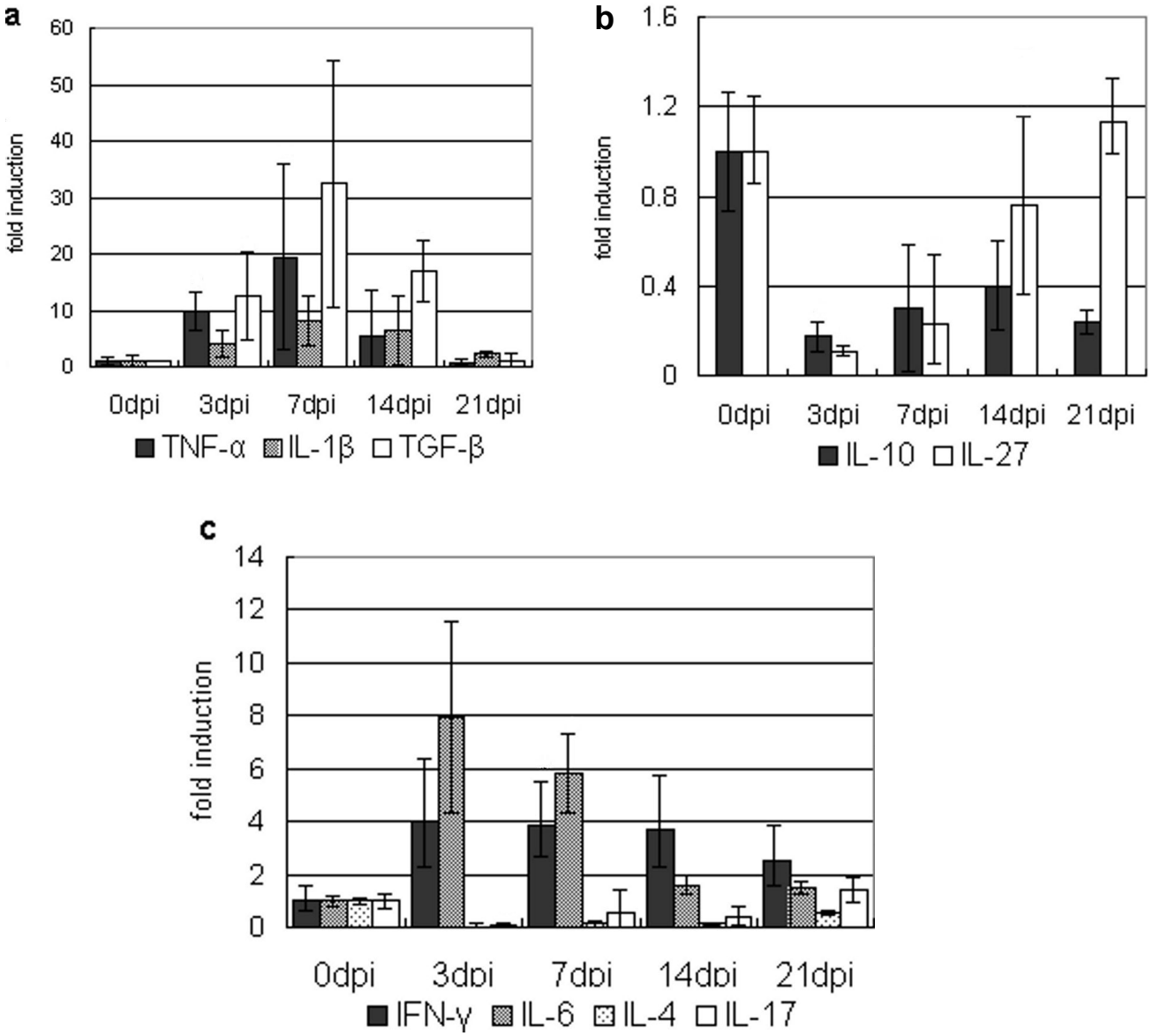


Figure 5. Expression of cytokines

mRNA Expression levels were measured by real time PCR . Relative values against GAPDH were calculated, and fold changes relative to 0 dpi are represented. Mean ±SD (n = 3 per group per time point). Differences among groups were examined by one-factor ANOVA followed by Tukey- -Kramer multiple comparison test. $P < 0.05$ was considered statistically significant.0 dpi vs. 7dpi and 7 dpi vs. 14 dpi for TNFα, 0 dpi vs. 7dpi and 7dpi vs. 21dpi for TGFβ, 0dpi vs. 3dpi, 0dpi vs. 7dpi, 0dpi vs. 14dpi and 0dpi vs. 21dpi for IL10, 0 dpi vs. 3 dpi, 0 dpi vs. 7 dpi and 0 dpi vs. 14 dpi for IL-4, 0 dpi vs 3 dpi, 3 dpi vs. 21 dpi, 7 dpi vs. 21 dpi, and 14 dpi vs. 21 dpi for IL-17 were statistically significant. For IL-1β, IL-27, IFNγ and IL-6, statistically no significant result was obtained.

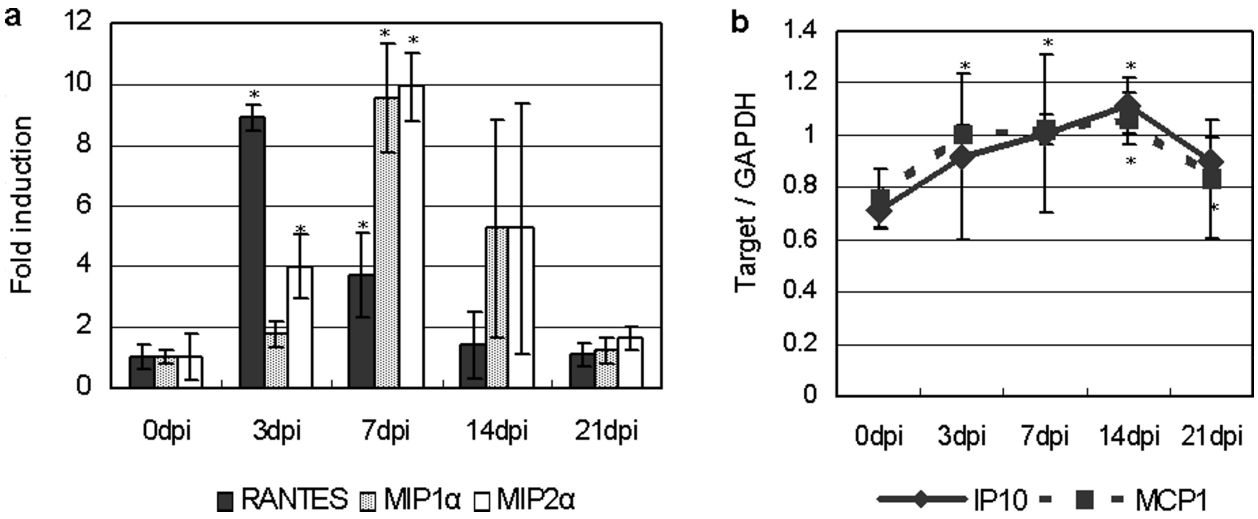


Figure 6. Expression of chemokines

(a) Real time PCR for RANTES, MIP-1α, and MIP-2α. Relative values against GAPDH were calaulated, and fold changes relative to 0 dpi are represented. Mean ± SD (n = 3 per time point). Significant differences among groups were examined by one-factor ANOVA followed by Tukey -Kramer multiple comparison test. $P < 0.05$ was considered statistically significant. 0 dpi vs. 3 dpi, 0 dpi vs. 7 dpi, 3 dpi vs. 7 dpi, 3 dpi vs. 14 dpi, 3 dpi vs. 21 dpi and 7 dpi vs. 14 dpi for RANTES, and 0 dpi vs. 7 dpi, 3 dpi vs. 7 dpi and 7 dpi vs. 21 dpi for MIP1α, 0 dpi vs. 7 dpi, 3 dpi vs. 7 dpi, 7 dpi vs. 14 dpi and 7 dpi vs. 21 dpi for MIP2α were statistically significant. (b) Semi – quantitive RT-PCR for IP-10 and MCP-1. Electrophoresis of PCR products was performed, and band intensity was measured. Relative values against GAPDH are represented. Mean ± SD (n = 3 per time point). Significant differences among groups were examined by one-factor ANOVA followed by Tukey -Kramer multiple comparison test. $P < 0.05$ was considered statistically significant. For MCP-1 and IP-10, no statistically significant result was obtained.

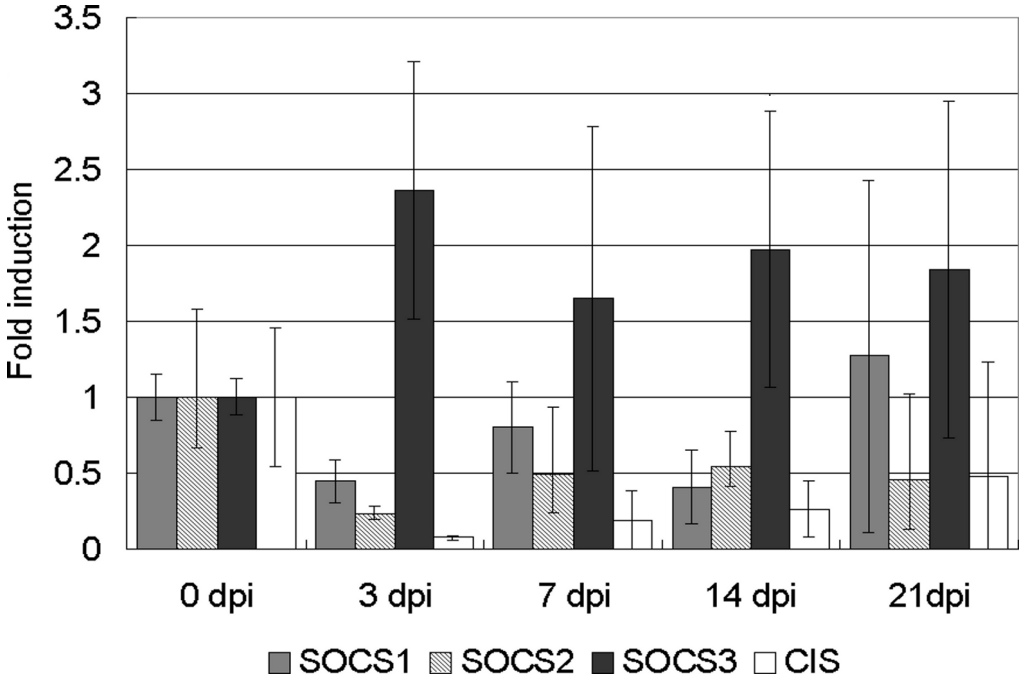


Figure 7. Expression of SOCS family proteins.

The levels of mRNA expression were measured by real time PCR. Relative values against GAPDH were calculated, and fold changes relative to 0 dpi are represented. Mean ± SD (n = 3 per time point). Significant differences among groups were examined by one-factor ANOVA followed by Tukey--Kramer multiple comparison test. $P < 0.05$ was considered statistically significant. 3 dpi vs. 21 dpi for SOCS1, 0 dpi vs. 3 dpi for SOCS2, 0 dpi vs. 3 dpi for SOCS3 and 0 dpi vs. all other time points for CIS were statistically significant.

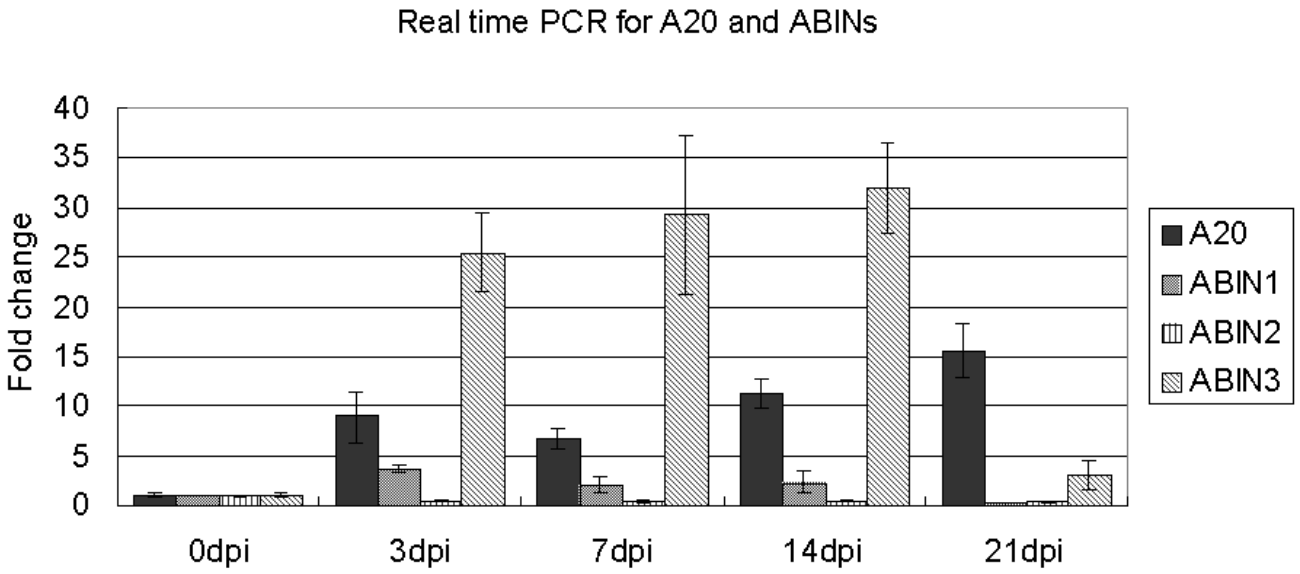


Figure 8. Real-time PCR for A20 and ABINs

mRNA Expression levels were measured by real time PCR . Relative values against GAPDH were calculated, and fold changes relative to 0 dpi are represented. Mean ±SD (n = 3 per group per time point). The 95 % level significance of differences were analyzed using ANOVA and Tukey-Kramer test as post-hoc test. For A20, the differences between groups were confirmed as significant except for 3 dpi vs. 7 dpi, 3dpi vs. 14dpi and 14 dpi vs. 21 dpi. For ABIN-1, 0 dpi vs. 3 dpi, 0 dpi vs. 14dpi, 3 dpi vs. 21 dpi and 14 dpi vs. 21dpi were confirmed as significant. For ABIN-2, 0 dpi vs. all the other time points were confirmed as significant. For ABIN-3, the significance of differences between groups were confirmed as significant except for 3 dpi vs. 7dpi and 14 dpi vs. 21 dpi.

Chapter Ⅱ

A20 and ABIN-3 negatively regulate trehalose 6'-6-dimycolate (TDM)-induced granuloma by interacting with an NF-kappa B signaling protein, TAK-1.

Summary

Purpose of this chapter is to confirm the correlation of NF-kappa B and A20-ABIN-3 in the formation and regression of the TDM-induced granulomatous lesion. I first examined the activation of NF-kappa B by immunohistochemical and Western blotting methods. The results demonstrated the increase of NF-kappa B toward 7 dpi and the decrease after that. In addition, the level of NF-kappa B activation correlated with the size of the lesion. Next, I quantified protein levels of A20 and ABIN-3 in the lesion. The similar kinetics of the proteins to that of their mRNA levels demonstrated in Chapter 1 was observed in a milder manner, i.e., high expression levels of A20 and ABIN-3 at 7 to 14 dpi. Finally, I tried to identify factors that interact with ABIN-3 by co-immunoprecipitation method. As a result, TAK-1 and A20 were immunoprecipitated with ABIN-3, demonstrating that TAK-1 and A20 interact with ABIN-3. Therefore, it can be concluded that the ABIN-3 and A20-mediated suppression of TAK-1 induces the suppression of NF-kappa B, and this may lead to the regression of the granulomatous lesion.

Introduction

As demonstrated in Chapter 1, A20 and ABIN-3 were markedly up-regulated in the TDM-induced granulomatous lesion. ABIN-3 was originally identified as a factor upregulated in monocytes infected by *Listeria monocytogenes* [92]. It is demonstrated that the stimulation of Toll-like receptor 4 (TLR4) by lipopolysaccharide (LPS) induces the expression of ABIN-3 in human and murine macrophages and also more weekly in other cell lines such as HepG2 and Hela [93-95]. The transcription of ABIN-3 is thought to depend on NF-kappa B. Therefore, ABIN-3 is considered as a negative feedback regulator of NF-kappa B signaling [95]. However, the regulation of ABIN-3 is not well characterized under conditions other than *Listeria* infection or LPS stimulation. In addition, there have been few *in vivo* experiments regarding the regulation of ABIN-3.

The function of ABIN3 is now gradually being unlabeled. ABIN-3 suppresses the NF-kappa-B signaling pathway, which finally activates inflammatory leukocytes, by forming a complex with an ubiquitin editing protein, A20 [93, 96]. A20 also functions as a negative regulator of the NF-kappa-B signaling by making a complex with ABIN-3 homologous proteins, ABIN-1 and ABIN2. Each ABIN is considered to interact with different counterparts. For example, ABIN-1 interacts with NEMO/IKK-gamma [93, 97]. However, the counterpart of ABIN-3 is not well clarified.

Thus, in this chapter, I examined the protein levels of A20 and ABIN-3 and molecules in the NF-kappa B signaling.

Materials and Methods

Animals

Six weeks old female BALB/c mice were purchased from SLC (Shizuoka, Japan). The animals were housed in isolator cages under a specific pathogen-free condition. The experimental procedures in the present study were approved by the Experimental Animal Committee of the University of Tokyo.

Preparation of water/oil/water (w/o/w) emulsion

W/o/w emulsion containing TDM was prepared as described in previous reports [9]. Briefly, 100 μg of TDM (Nakalai Tesque, Tokyo, Japan) was dissolved in 3.2 μℓ of Freund's incomplete adjuvant (Chemicon, Temecula, CA), and then, 3.2 μℓ of 0.1M PBS were added. The mixture was then homogenized with a glass homogenizer. Finally, 93.6 μℓ of saline containing 0.2% Tween 20 were added to the homogenized w/o emulsion.

Induction of granuloma

The mice were twice injected intraperitoneally(i.p) with a 100 μℓ of w/o/w emulsion at a 1 week-interval, before being sacrificed at 3, 7, 14, or 21 dpi. Three mice were used per group. At 0 dpi, mice were sacrificed just after the last injection.

Histopathological examination and immunohistochemistry

Formalin-fixed and paraffin-embedded sections were obtained from granulomatous lesions in the peritoneum. Some sections were subjected to immunohistochemical staining using the LSAB method. In this procedure, autoclave-pretreatment in citrate buffer (pH6.0) was performed for antigen retrieval. Antibody used in this study were rabbit antibody against phospho-NF-kappa-B (Serine 536) (93H1, 1:50, Cell Signaling Technology, Danvers, MA,) and Rabbit antibody against phospho-IKK alpha / beta (Serine 176 / 180) (16A6, 1:50, Cell Signaling Technology).

Western blotting

Granulomatous lesions were collected and homogenized in 10mM Tris-HCl buffer (pH 7.8) containing 150mM NaCl, 1mM EDTA, 2mM Na3VO4, 10mM NaF, 1% NP-40 and Proteinase Inhibitor Cocktail (Roche Applied Science, Penzberg, Germany). The homogenate was centrifuged at 12,000 g, at 4℃ for 20 min to exclude debris. The supernatant was mixed with laemmli sample buffer (Bio-Rad, Hercules, CA) and loaded onto a SDS-PAGE gel, and electrophoresed. Proteins on the gel were transferred to a PVDF membrane. The blotted membrane was probed with antibodies to phospho-NF-kappa-B (Serine 536) (93H1, Cell Signaling Technology), A20 (ab74037 ; Abcam, Cambridge, UK) , ABIN-3 (SAB3500087, Sigma-Aldrich, Saint Louis, MO), RIP (Cell Signaling Technology), phospho-IKK alpha / beta (Serine 176 / 180) (16A6, Cell Signaling Technology), TAK-1 (4505 ; Cell Signaling Technology), IKK-gamma (2685 ; Cell Signaling Technology) or beta-actin (4967, Cell Signaling Technology). After incubation with the appropriate secondary antibodies, positive immunoreaction was visualized with the ECL Plus Western Blotting Reagent (Amersham, Buckinghamshire, UK), and with the ChemDoc XRS-J (Bio-Rad).

For quantification of the Western blot analyses, immunoreactive bands were quantified by densitometry. The intensity of each band was normalized by the level of beta-actin. The fold changes relative to the value in the 0 dpi group are shown as the mean ± SD.

Co-immunoprecipitation

Protein-A Sepharose beads were pre-incubated at 4℃ for 3 hours with Rabbit antibody against ABIN-3 antibody (SAB3500087, Sigma-Aldrich) or Rabbit isotype control IgG at the dilution of 1:100 with 0.1% BSA in TBST. 250 μℓ of protein homogenates were incubated at 4℃ overnight with 50 μℓ of the protein-A sepharose

beads. Then the mixtures were centrifuged and the supernatants were collected. The supernatants were incubated with anti-ABIN-3 antibody-coated beads or isotype control IgG-coated beads at 4℃ for 3 hours. Thereafter, the beads were picked up, and proteins were resolved in laemli sample buffer. The extracts were used for Western blotting to detect the protein that interacts with ABIN-3.

For detection of ABIN-3 through Western blotting, conformational specific anti-rabbit IgG (Cell Signaling Technology) was used to avoid the cross reaction of a secondary antibody with rabbit IgG light chain.

Results

Activation of NF-kappa B signaling

The expression of NF-kappa B signaling proteins were quantified by immunohistochemical staining and Western blotting using anti- phospho NFκB and anti- phospho IKK α / β antibodies. Both phospho NF-kappa B- and phospho IKK α / β -positive cells in the lesions increased toward 7 dpi and gradually decreased after that (Fig. 1). The results of Western blotting demonstrated that the amount of phospho NF-kappa B reached the peak at 3 dpi (Fig. 2). Therefore, we concluded that the sizes of the lesions might relate with the activation status of NF-kappa B signal proteins.

Protein levels of A20 and ABINs

I performed Western blotting to examine whether the mRNA up-regulation observed in Chapter 1 leads to the elevation in the protein level. The results confirmed the up-regulation of A20 and ABIN-3 in the protein level, although the fold changes were smaller than those of mRNA levels(Fig. 3).

Co-immunoprecipitation with ABIN-3 antibody

To confirm the formation of an A20 and ABIN-3 complex in the granulomatous lesions, co-immunoprecipitation using anti-ABIN-3 antibody was performed. A20 protein was co-immunoprecipitated with anti-ABIN-3 (Fig. 4), indicating the formation of the complex.

Next, we tried to identify the proteins involved in the NF-kappaB pathway and interacting with ABIN-3. Because RIP and IKK- γ were identified as interacting partners of an ABIN-3 homologous protein, ABIN-1 [93, 97], we first performed Western blotting of immunoprecipitated samples using anti-RIP and IKK- γ antibodies. However, no positive signals were gained (Fig. 4). RIP and IKK- γ were not considered to interact with ABIN-3. TAK-1 mediates signals from IL-1R / TLR [98,

99], TNF-α [98] and TGF-β [100] and ,as a result, activates NF-kappa B. This protein acts at the upstream of IKK complex and downstream of TRAF-6 [101-103]. Therefore, TAK-1 is considered to be one of candidates for ABIN-3 interacting protein. As a result, TAK-1 positive band was observed and, therefore, TAK-1 were considered to be an ABIN-3 interacting protein (Fig 4).

Discussion

In this chapter, we first examined NF-kappa B activation because A20 and ABIN-3 target the NF-kappa B signaling pathway. The NF-kappa B activation was first indicated by the increased number of the phospho-NF-kappa B- and phospho-IKK alpha / beta-positive cells up to 7 dpi, when the size of the lesion reached at peak, and it was then reduced toward 21 dpi. The result of Western blotting showed the similar kinetics with that of positive cell counts in a milder manner. Therefore, we assumed the involvement of NF-kappa B signaling in the development and regression of the granulomatous lesions.

Previous studies well characterized the role of NF-kappa B in inflammation. NF-kappa B acts as a transcriptional factor and promotes the expressions of various cytokines, chemokines, cyclooxygenase 2 (COX-2) and leukocyte's adhesion molecules in various cell types [104, 105] including macrophages [106]. As NF-kappa B has such pro -inflammatory functions, an aberrant expression of NF-kappaB molecules leads to the immunodeficiency and increased susceptibility to pathogens [107]. The involvement of NF-kappa B in the lesions of tuberculosis or Th1-mediated delayed type hypersensitivity is also well characterized. In tuberculosis mice, NF-kappa B is activated through TLR signaling or pro-inflammatory cytokine signaling [108], and leads to the secretion of a key cytokine for Th-1 response such as IFN-gamma [109]. In addition, TDM is known to stimulate TLR-2 [110]. Therefore NF-kappa B is thought to play important role in TDM-stimulated mice as well as in tuberculosis mice. Therefore, it is concluded that the development and regression of granulomatous lesions and NF-kappa B activation are strongly corelated.

We next examined the protein levels of A20 and ABIN family proteins, because their mRNA levels showed marked elevation in Chapter 1. Protein levels of A20 and

ABIN-3 were elevated at 7 and 14 dpi, mimicking the mRNA kinetics of in a milder manner. On the contrary, NF-kappa B activation was remarkably decreased at that time point. These results indicated that the NF-kappa B activation was affected by A20 and ABIN-3 in the granuloma lesions.

There are several proteins that suppress NF-kappaB function by post-transcriptional modification [111]. A20 is one of such negative feedback regulators. A20 ubiquitinates or de-ubiquitinates proteins involved in NF-kappa B signaling pathway and, as a consequience, promotes proteasomal degradation of target proteins [91]. Previous studies suggested a crucial role of A20 in the pathogenesis of some diseases in which NF-kappa is deeply involved. For instance, A20-mediated NF-kappa B suppression is involved in the suppression of tumor [112] and cardiac fibrosis [113]. A mutation of A20 causes various autoimmune diseases and chronic inflammatory diseases [114]. The present study demonstrated the relevance of NF-kappa B in TDM-induced granulomatous lesion, therefore A20 likely to promote the granuloma regression.

ABIN family proteins have been identified as A20 binding proteins [93, 115, 116] and are thought to function as adaptors between A20 enzyme and target proteins. Also in this study, A20 protein was co-immunoprecipitated with ABIN-3, suggesting that it makes a complex with ABIN-3. The functions of ABIN-3 have recently been reported in several articles [93-96]. ABIN-3 suppresses NF-kappa B signaling by linking A20 and an unknown counterpart [93]. Previous studies observed ABIN-3 induction by LPS stimulation only in cell lines of macrophage-origin [93] or also in some non-macrophage cell lines weakly [95]. Therefore, the prominent elevation of ABIN-3 in the TDM-induced granuloma may be due to the accumulation of macrophages that highly expresse ABIN-3.

Murine ABIN-3 molecule lacks the ABIN homology domain (AHD)-2, which is considered to be necessary for a NF-kappa B suppressing role of ABIN-family proteins [93]. Some reports, therefore, concluded that murine ABIN-3, not like human ABIN-3, has no effects on NF-kappa B pathway [93, 117]. The discussion about the role of ABIN-3 in human tuberculosis and its murine model is still controversial.

Finally, we tried to find proteins that interact with ABIN-3 by co-immunoprecipitation. The results of Western blotting demonstrated that A20 was immunoprecipitated with ABIN-3. Thus, we confirmed the interaction of ABIN-3 with A20.

TAK-1 is a protein that originally identified as a mediator of the TGF-beta signaling [100], and then, the role of this protein has been unlabelled in other signaling pathway such as IL-1R / TLR and TNF-alpha pathways [98, 99]. Cytokines like IL-1beta and TNF-alpha plays an important role in granulomatous diseases. For example, IL-1 receptor or TNF-alpha knock-out mice fail to form granulomatous lesions [44, 46-52]. Therefore, TAK-1 is assumed to act as an important mediator for pro-inflammatory signalings also in the TDM-induced granuloma model. Mediating these signalings consequently activates NF-kappa B. Thus, suppression of TAK-1 function by interacting with ABIN-3 possibly suppresses NF-kappa B activation as consequence.

The present study confirmed the importance of the NF-kappa B signaling in TDM-mediated granulomatous inflammation, including the induction of ABIN-3 by TDM stimulation. Although, the efficacy of ABIN-3-dependent suppression of NF-kappaB is disputable in the mice model, ABIN-3 is assumed to be induced in human tuberculosis and to suppress the NF-kappa B signaling. Additionally, we identified TAK-1 as an ABIN-3 interacting protein. As the AHD-2 domain is not necessarily

required for the protein-protein interaction of ABIN-3, TAK-1 can be a murine candidate for ABIN-3 interacting protein also in human.

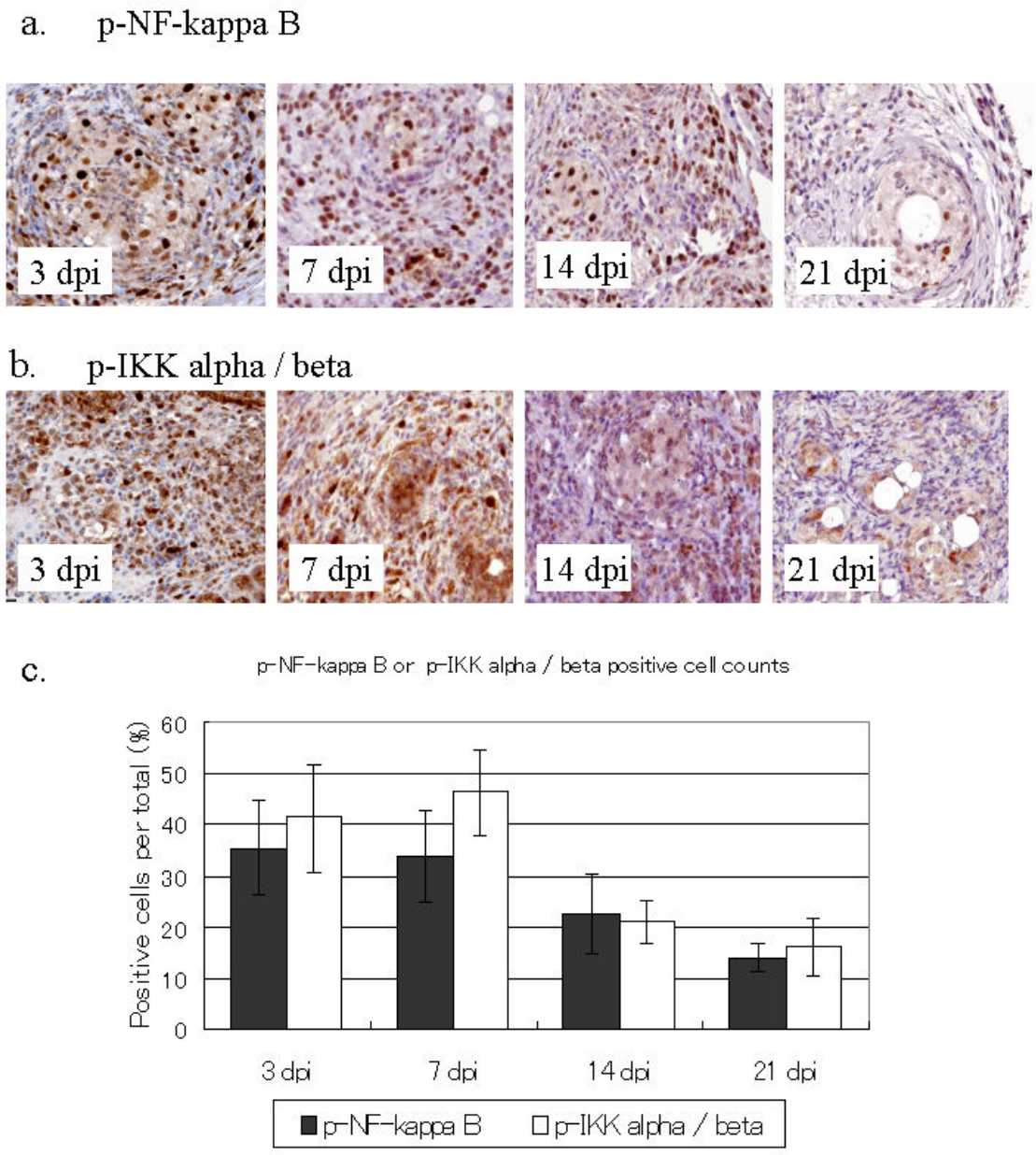


Figure 1. Immunohistochemistry of p-NF-kappa B and p-IKK alpha / beta

Results of immunohistochemistry of p-NF-kappa B(a) and p-IKK alpha / beta (b). Representative pictures of each time points are shown. Positive cells decrease from 3 dpi toward 21 dpi. (c) The numbers of p-NF-kappa B- or p-IKK alpha / beta-positive cells were counted, and the proportions relative to total cells were calculated. Mean ±SD. Ten lesions were counted per mouse, and 3 mice was used per time point. The significance of differences was analyzed using ANOVA and Tukey-Kramer test as post-hoc test. The significance of differences between groups were confirmed as significant ($P<0.05$) except for 3 dpi vs. 7 dpi and 14 dpi vs. 21 dpi in both p-NFκB and p-IKKα/β.

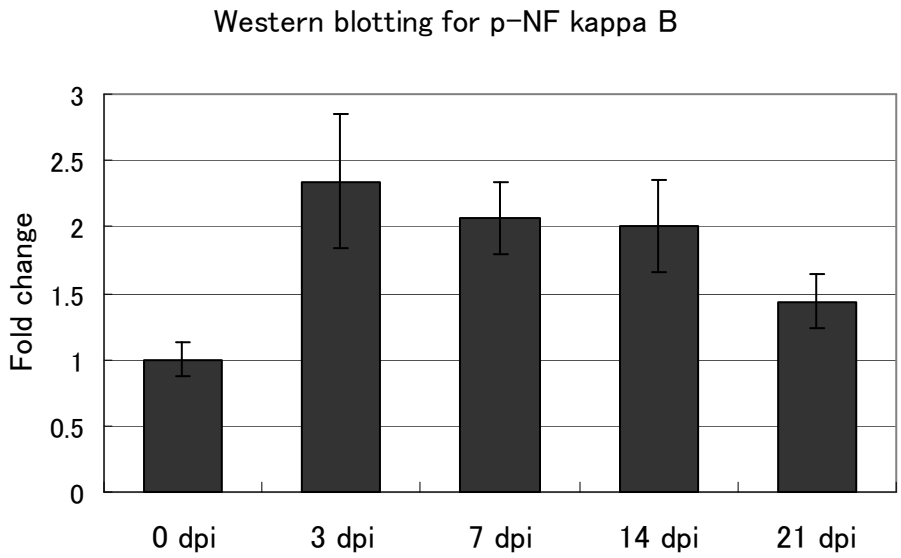


Figure 2. Western blotting for p-NF-kappa B.

Relative values against beta-actin were calaulated, and fold changes relative to 0 dpi are represented. Mean value ± SD. The significance of differences was analyzed using ANOVA and Tukey-Kramer test as post-hoc test. The differences of 0 dpi vs. all the other time points and 3dpi vs. 21dpi were confirmed as significant ($P<0.05$).

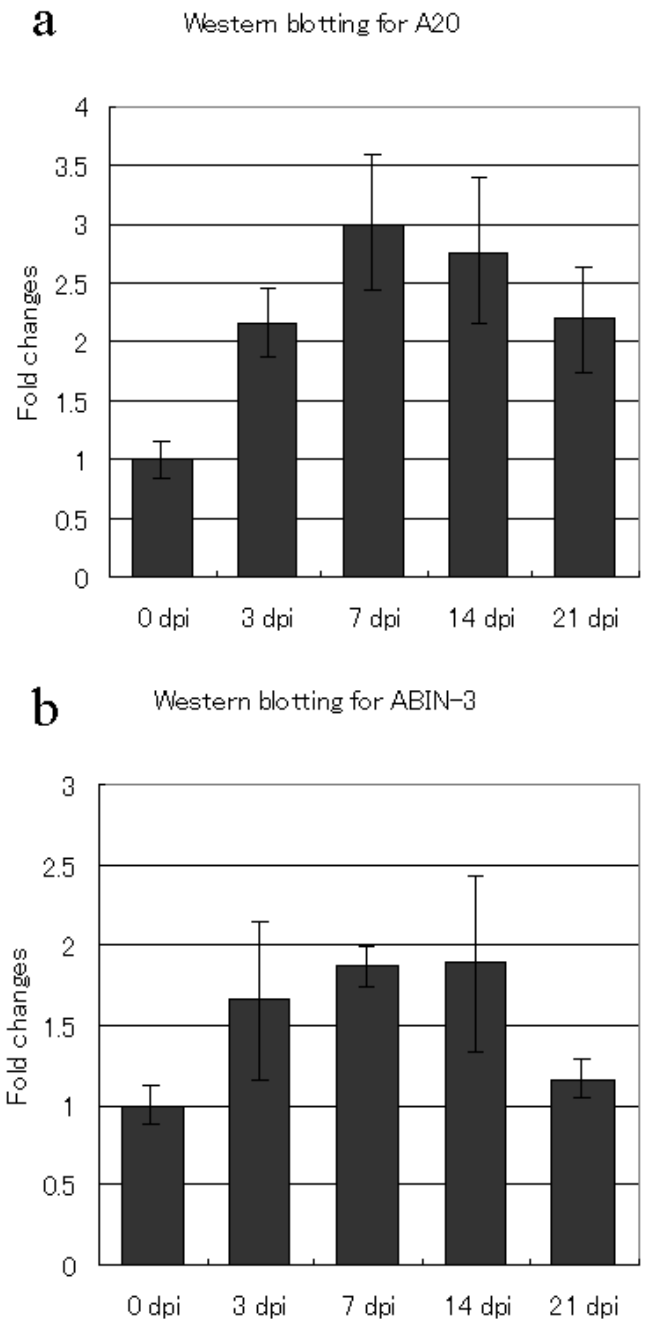


Figure 3. Protein levels of A20 and ABIN-3. Western blotting for A20 and ABIN-3. Relative values against β-actin were calculated, and fold changes relative to 0 dpi are represented. Mean value ± SD. The significance of differences was analyzed using ANOVA and Tukey-Kramer test as post-hoc test. **a.** For A20, 0 dpi vs. 7dpi and 0 dpi vs. 14 dpi were confirmed as significant ($P < 0.05$). **b.** For ABIN-3, 0 dpi vs. 7dpi was confirmed as significant ($P < 0.05$).

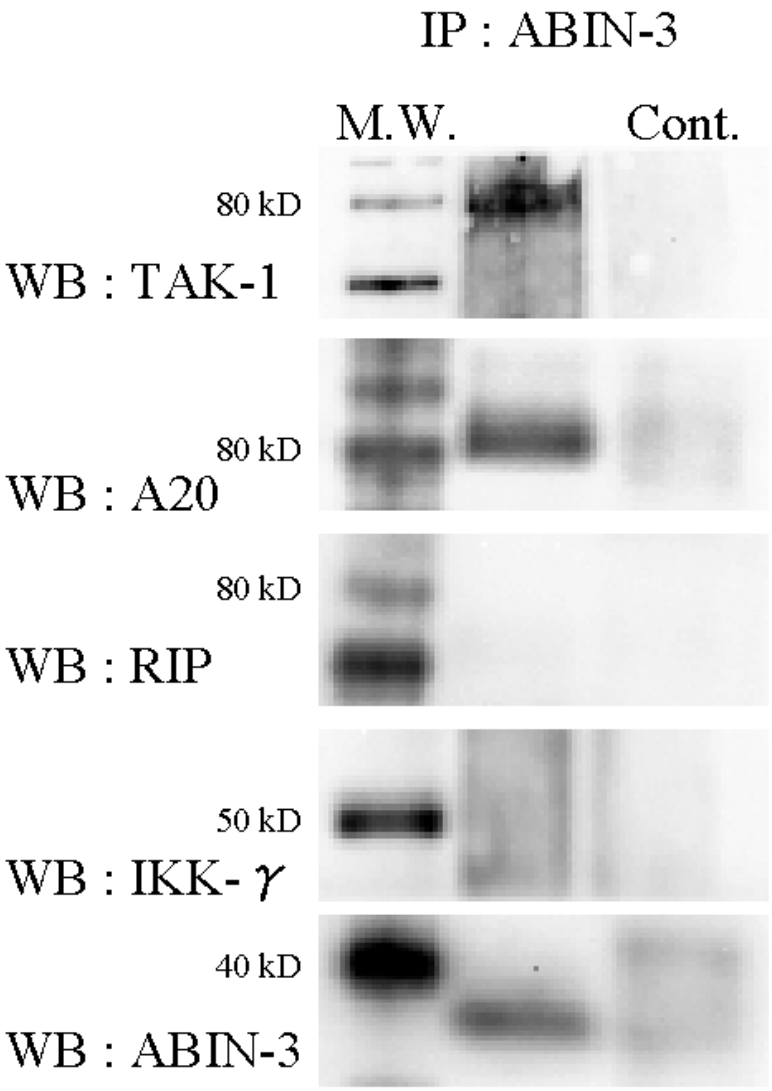


Figure 4. Interaction of ABIN-3 with A20 and TAK-1 The protein extracts from the lesions at 7 dpi were immunoprecipitated with anti-ABIN-3 antibody. The figure shows the results of Western blotting of immunoprecipitated samples using anti- TAK-1, A20, RIP, IKK-gamma or ABIN-3 antibodies. The antibodies used for Western blotting are indicated at the left of each picture. The left lane : Molecular markers. The middle lane : Samples immunoprecipitated with anti-ABIN-3 antibody. The right lane : Samples immunoprecipitated with Rabbit isotype-control antibody.

Conclusions

In this thesis, I observed the expression profiles of cytokines, chemokines and negative regulators of cytokine signalings in the TDM-induced granulomatous lesion comparing with the histopathological course of the lesion. The size of the lesion was reached peak at 7 dpi and decreased thereafter. The expression of the pro-inflammatory cytokines such as TNF-alpha, IL-1beta and IL-6 showed the similar kinetics, i.e., increasing toward 7 dpi and decreasing thereafter, suggesting the correlation between the expression of pro-inflammatory cytokines and the size of the lesion. On the other hand, the expressions of anti-inflammatory cytokines such as IL-10 and IL-27, except for TGF-beta, were suppressed at all time points. It suggest that the decrease of pro-inflammatory cytokines is more important than the expression status of anti-inflammatory cytokines for the regression of the TDM-induced granulomatous lesion. This leaded me to an assumption that regulatory-T-cells play few roles in the formation and regression of granulomatous lesion, because IL-10 and IL-27 are mainly produced by regulatory-T-cells. While TGF-beta, one of an anti-inflammatory cytokines is likely to play some roles in the granulomatous lesions because this cytokine is kept at high level at 7 dpi and 14 dpi. Until 7 dpi, the expression levels of PMN-attarcting chemokines such as RANTES, MIP-1alpha and MIP-1beta exceeded those of lymphocyte-attracting chemokines including IP-10 and MCP-1. After 14 dpi, their balance became inverted. Such change reflected histopathological changes of inflammatory cell population, i.e., PMN-dominant granuloma to lymphocyte-dominant granuloma

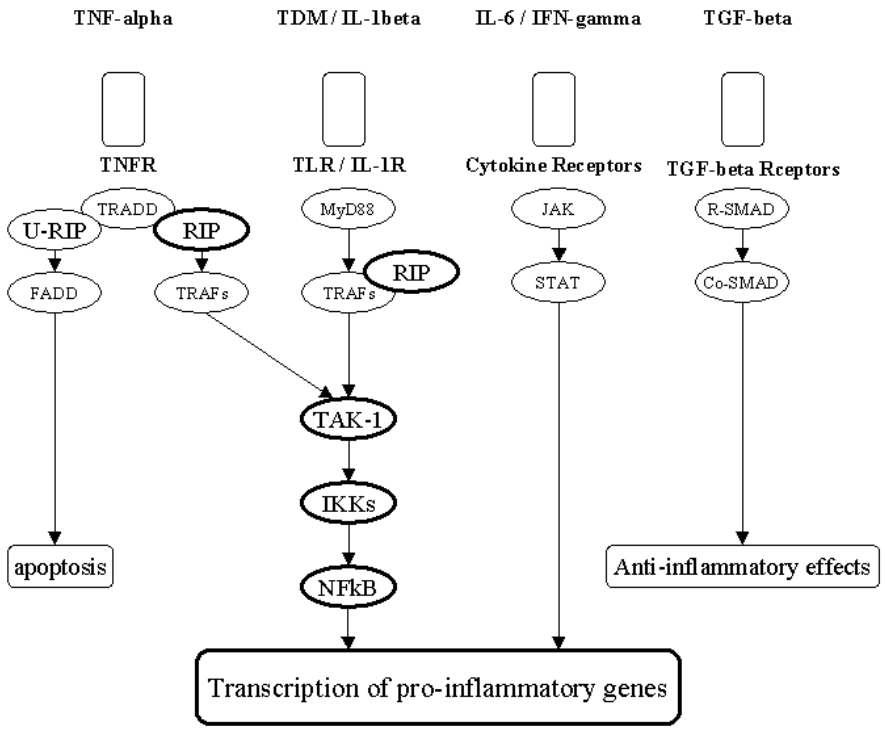
To investigate why such time-dependent down-regulation of pro-inflammatory cytokines occurs, I analyzed some negative feedback regulators for cytokine signaling. Among them, the expression levels of SOCS-3, A20 and ABIN-3 were markedly elevated at 7 and 14 dpi. The elevations of these factors may suppress the

leukocyte-activating signaling and further pro-inflammatory cytokine secretion. Such suppression of pro-inflammatory signal then reduced the TDM-induced granulomatous lesions at the time point.

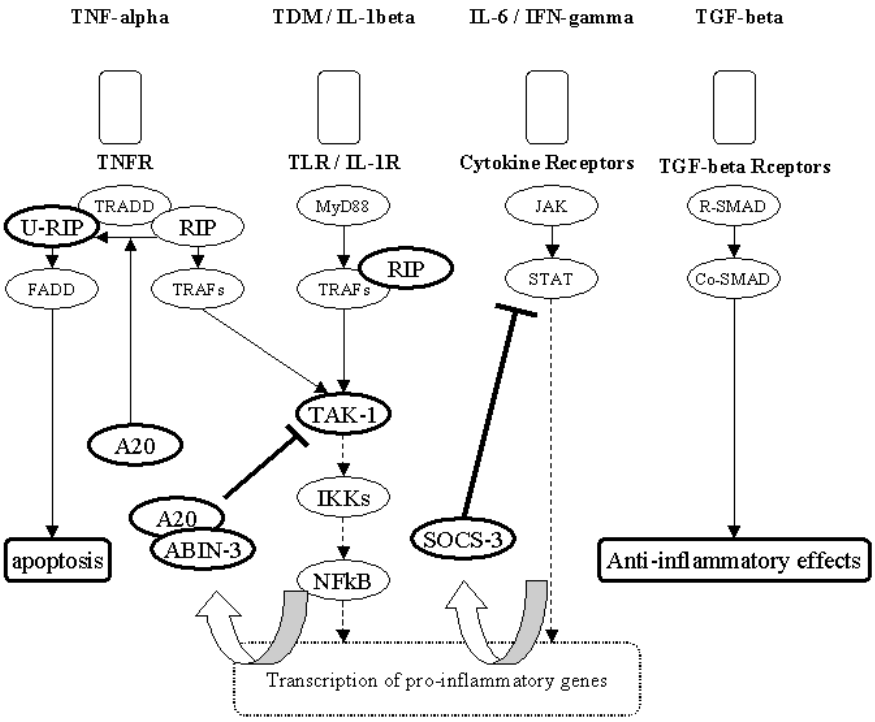
I further investigated details of the role of A20 and ABIN-3, because the function of ABIN-3 is sill scarcely known. The activation of the NF-kappa B signaling, which is a target of A20 and ABIN-3 and is involved in the regulation of inflammatory cytokines, was decreased from 7 dpi. On the contrary, the amounts of A20 and ABIN-3 were high at 7 and 14 dpi. Thus, it may show the involvement of the A20 and ABIN-3 in the suppression of NF-kappa B signaling. The increased levels of A20 and ABIN-3 may also induce apoptosis. Because NF-kappa B is known to promote the pro-inflammatory cytokines secretion inhibit the apoptosis of leukocytes.

Through co-immunoprecipitation, I identified TAK-1 as a protein that interacts with ABIN-3. This protein functions as one of signal transducers from cytokine receptors to NF-kappa B. The interaction of TAK-1 and ABIN-3 may inhibit the NF-kappa B signaling. Therefore, the termination of inflammation may be regulated by such negative feedback factors.

This is the first demonstration of the induction of ABIN-3 in the TDM-induced granulomatous lesion. The ABIN-3-dependent self-limiting regulation of inflammation may not be a TDM-specific but universal mechanism in stimulated macrophages. I hope this the present study contribute the future researches on ABIN-3 as an important anti-inflammatory factor in macrophages.



Granuloma developing phase



Granuloma regression phase

Fig 1. Kinetics of pro-inflammatory and anti-inflammatory factors in the development and regression of TDM-induced granuloma.

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