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Involvement of the CXC Chemokines Mig and IP-10 in Response to *M. bovis* BCG in Mice

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The non-ELR-containing CXC chemokines Mig and IP-10 have been shown to function as chemotactic cytokines for activated T lymphocytes. In this study, we examined the potential involvement of Mig and IP-10 in antimycobacterial response of mice immunized or infected with *M. bovis* BCG. The accumulation of Mig and IP-10 mRNA in resident peritoneal monocytes (RPMΦ) was slightly reduced by stimulation with vBCG, and the degree was greater for 24 hr culture even though IFN-γ was added. Expression of *Mig*, *IP-10*, and *IFN-γ* in 24 hr delayed-type hypersensitivity (DTH) response was stronger in vBCG-immune mice than in the non-immune. The increase of DTH measured by foot-pad thickness appears to be clearly related to the levels of chemokines Mig and IP10 messages and those of IFN-γ and IL-12. Stimulation with vBCG for 2 days decreased or completely dropped the levels of Mig message in non-immune or immune splenocytes, respectively, whereas IP-10 message was slightly decreased in 2 days culture. Moreover, messages for IL-12 (p40) showed similar kinetics for Mig. The levels of Mig and IP-10 mRNA during the course of infection with BCG were not readily changed in lungs, livers, and spleens from BCG-infected mice. Although there was no obvious changes of Mig and IP-10 messages in the target organs during infection process, we found that the infection progressed over the first 3 wk before being contained by the emerging immune response suggested from detectable amount of IFN-γ mRNA around this time. In view of selectivity of chemokines Mig and IP-10 for activated T cells, these data suggest that chemokine Mig and IP-10, especially in collaboration with IL-12 and IFN-γ, may play a role as T cell recruiters in immune response against mycobacterial infection.

Key Words: non-ELR CXC chemokines, Mig, IP-10, Delayed-type hypersensitivity (DTH), *Mycobacterium bovis* BCG

INTRODUCTION

The increasing prevalence of tuberculosis (TB) in many areas of the world, coupled a rise in drug-resistant *Mycobacterium tuberculosis* (MTB) strains, presents a major threat to global health (28). While host defense against mycobacterial infection involves natural resistance, the acquired immune response conferred by Ag-specific T cells and macrophages are required for successful control of MTB infection. Cell-mediated immunity to MTB infection is characterized by the sensitization of T cells that subsequently release cytokines which activate parasitized macrophages and recruit monocytes

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from the blood to the site of infection (27). Although it is generally accepted that gamma interferon (IFN-γ), secreted by $CD4^+$ T cells, is one of the most critical cytokines involved in the acquired response to mycobacterial infection (7) and that chemokines Mig and IP10 are inducible in macrophages by IFN-γ (9-11, 24, 34) and attract activated T cells (5, 22, 23, 33), little is known about how these two chemokines are regulated not only during the course of mycobacterial infection but also in delayed-type hypersensitivity response against mycobacterial antigen.

Mig and IP-10 belong to a family of small, inducible, secreted factors whose best described activities are as chemotactic factors and that have been termed chemokines (25). Chemokines exert their biological activities through G protein-coupled receptors on the surface of target cells (25, 26). The activities of chemokines are not limited to selective and specific chemotaxis and leukocyte trafficking and include effects on T cell activation (2), angiogenesis (1, 4, 16, 31), and HIV replication (4). Thus, the chemokines may play an important role in host defense against various infections, in the pathogenesis of chronic inflammatory disorders, and in wound healing.

Chemokines are divided into four groups, CXC, CC, C, and CX3C, based on the number and arrangement of conserved cystein motifs (25). The CXC chemokines are primarily active on neutrophils. The CXC subfamily is subdivided into ELR and non-ELR CXC chemokines based on the presence or absence of this Glu-Leu-Arg tripeptide sequence adjacent to the CXC motif. The non-ELR CXC chemokines include Mig, IP-10, I-TAC, and SDF-1. Among the members of these non-ELR CXC chemokines, all of Mig (22, 23, 33), IP-10 (22, 23, 33), and I-TAC (5) have a unique selectivity for T cells that have been activated by IL-2, whereas SDF-1 has a broader range of activities on resting and activated memory T cells, monocytes, and granulocytes (3). It has been shown that IP-10 (17) and Mig (30) are expressed in inflammatory responses in which IFN-γ involves with high local concentration.

Thus, it is likely that chemokines Mig and IP-10 would be expressed following exposure of the host to infection with *M. bovis* BCG (BCG) and that these molecules are regulated in delayed-type hypersensitivity response to mycobacterial antigen. In this study, we have demonstrated that *Mig* and *IP-10* genes were expressed with patterns of regulation by BCG in macrophage cultures and splenocyte cultures, and in the lungs, livers, and spleens following intravenous infection of mice, that the induction of *Mig* and *IP-10* genes were regulated in the delayed-type hypersensitivity response to BCG, and that the message level of Mig and IP-10 was related to the strength of the DTH. We suggest that infected mice mobilize chemokines Mig and IP-10 which may contribute to host defense by recruiting T cells in cell-mediated immunity to mycobacterial infection.

MATERIALS AND METHODS

Mice. Specific pathogen-free BALB/C mice purchased from Daehan Laboratory Animal Research Center (Chungbuk) and maintained in conventional state were used at ages of 5 to 6 wk.

Bacteria. *M. bovis* BCG (French strain) maintained in our laboratory was grown, prepared, and counted as described previously (21). *M. bovis* BCG (French strain) was grown in Dubos broth (Difco Laboratories, Detroit, MI) supplemented with Bacto Dubos Medium Albumin (Difco) and 5.0% glycerol for 3 wk. Bacteria were collected by centrifugation at 7000 x *g* for 20 min, washed three times, resuspended in phosphate-buffered saline (PBS), and stored in ampules frozen at -70℃ until use. The number of bacteria was determined by plating the bacterial suspension on Middlebrook 7H11 agar

(Difco) plates and counting the colonies after 4 wk. Killed bacteria (kBCG) was prepared by heat treatment at 60℃ for 1 hr.

Cell culture. Resident peritoneal monocytes (RPMΦ) and splenocytes were harvested from adult BALB/c mice in RPMI 1640 containing 10 U/ml heparin. Adherent peritoneal cells (95% macrophages) and splenocyes were cultured in 6-well cluster plates in RPMI 1640 supplemented with 20 mM HEPES buffer (GIBCO), 0.2% sodium bicarbonate, 2 mM glutamine, 50 μg/ml gentamicin and containing 10% lipopolysaccharide (LPS)-free heat-inactivated fetal bovine serum (FBS, Sigma Chemical Company. St. Louis, MO). RAW 264.7 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in the same medium as above. Cells were suspended at approximately $5x10^5$/ml, and stimulated with viable BCG (vBCG, $2.5x10^8$ CFU/ml) and other reagents for the indicated times at 37℃ and 5% CO_2. IFN-γ was murine recombinant protein purchased from Sigma (specific activity of $>1x10^7$ U/mg) and was added 4 hr before cell harvest.

Experimental infections. Mice were infected iv with vBCG ($5x10^5$ CFU) via lateral tail vein and the course of infection was monitored against time by harvesting target organs and enumerating viable bacilli. Briefly, serial dilutions of pooled organ homogenates were plated in triplicate on nutrient 7H11 agar, and bacterial colony formation was counted 3 to 4 wk later after incubation at 37℃. The data were expressed as the $\log_{10}$ value of the mean number of bacteria recovered from the triplicate of pooled organ homogenates.

Generation of DTH against BCG. Mice were immunized by sc injection of $5x10^4$ vBCG cells in PBS at dorsal neck on day 0 and day 70, respectively. One wk after the last injection, Mice were challenged by sc injection of $5x10^6$ kBCG in a volume of 0.05 ml into right hind footpad. Footpad swellings were measured using micrometer just before (T_0) challenge and at 24 (T_{24}) or 48 hr (T_{48}) after challenge. Percent change in footpad thickness was calculated using the following formula. Percent change = (T_{24} or T_{48} - T_0) x $100/T_0$. Just after measuring footpad thickness, skins of the kBCG-challenged footpads were excised and pooled for the preparation of total RNA.

Preparation of cDNA. Total cellular and tissue RNA was extracted using Trizol reagent (Bethesda Research Laboratories, Gaithersburg, MD) according to the vendor's protocol. Total RNA was collected and reverse transcribed into cDNA by priming 1 μg of total RNA at 42℃ in a final volume of 20 μl using 0.5 μg of an oligo dT primer using kit and protocols from Bethesda Research Laboratories.

PCR amplification of cDNA. Aliquot (1 μl) of cDNA was used per 20 μl reaction for amplification by PCR using a thermocycler running a program of 37 cycles for chemokines and cytokines or 30 cycles for β-actin, each cycle consisting of 1 min of denaturation at 94℃, annealing at 57℃ (64℃ for IFN-γ, 58℃ for murine β-actin), and extension at 72℃. The PCR product was subjected to a final extension period of 5 min at 72℃. The sequences of oligonucleotide primers specific for murine chemokines Mig and IP-10 (23), and for murine β-actin (24) have been previously published. Semiquantitative RT-PCR for IFN-γ and IL-12 was also included. Primers for murine IFN-γ were GGGGAAGAGATTGTCCCAAT and CGAGTTATTTGTCATTCGGG, for murine IL-12 (p35) were ATCGATGAGCTGATGCAGTC and GATGGGAGAACAGATTCTG, for murine IL-12 (p40) were GAAGGTCACACTGGACCAAA and AATAGCGATCCTGAGCTTGC, and for murine β-actin were GTTGGATACAGGCCAGACTTTGTTG and GATTCAACTTGCGCTCATCTTAGGC. All primers were used at a final concentration of 1 μM. PCR product was subjected to electrophoresis on 1.5% agarose gels and visualized by staining with ethidium bromide.

RESULTS

Induction of chemokine Mig and IP-10 mRNA from macrophages is reduced by BCG stimulation. It is known that both Mig and IP-10 are induced from macrophages by IFN-γ stimulation and that mycobacteria multiply within macrophages. To investigate whether vBCG induce Mig and IP-10 mRNA form macrophages, RPMΦ and RAW 264.7 cells were treated with vBCG for 4 or 24 hr. Chemokine mRNA production was evaluated by visual comparison of the intensity of the band representing PCR product from bacteria-stimulated cells with that from cells cultured in media alone or with that form cells cultured in the presence of IFN-γ (100 U/ml) for the final 4 hr of culture. For RPMΦ, basal level of expression of chemokines Mig and IP-10 was identified with different intensities (Fig. 1A). For RAW 264.7 cells, IP-10 mRNA only was detectable without stimulation (Fig. 1B). IFN-γ-stimulated RPMΦ and RAW 264.7 cells strongly expressed Mig and IP-10 mRNA. The induction pattern of IL-12 was differ depending on cell types. The message level for IL-12 (p40) was detectable when RAW 264.7 cells was stimulated with vBCG for 24 hr in the presence of IFN-γ for final 4 hr. The accumulation of Mig and IP-10 mRNA in RPMΦ was slightly decreased by stimulation with vBCG, and the degree was greater for 24 hr culture even though IFN-γ was added (Fig. 1A), whereas Mig and IP-10 mRNA levels of RAW 264.7 cells were maintained at around controls (Fig. 1B).

Chemokines Mig and IP-10 are induced with different patterns of expression in DTH against BCG. It has been reported that there is a direct correlation between DTH and resistance to bacterial infection (18). Thus, we have elicited footpad swelling reaction to BCG and detected expression of Mig and IP-10 in the

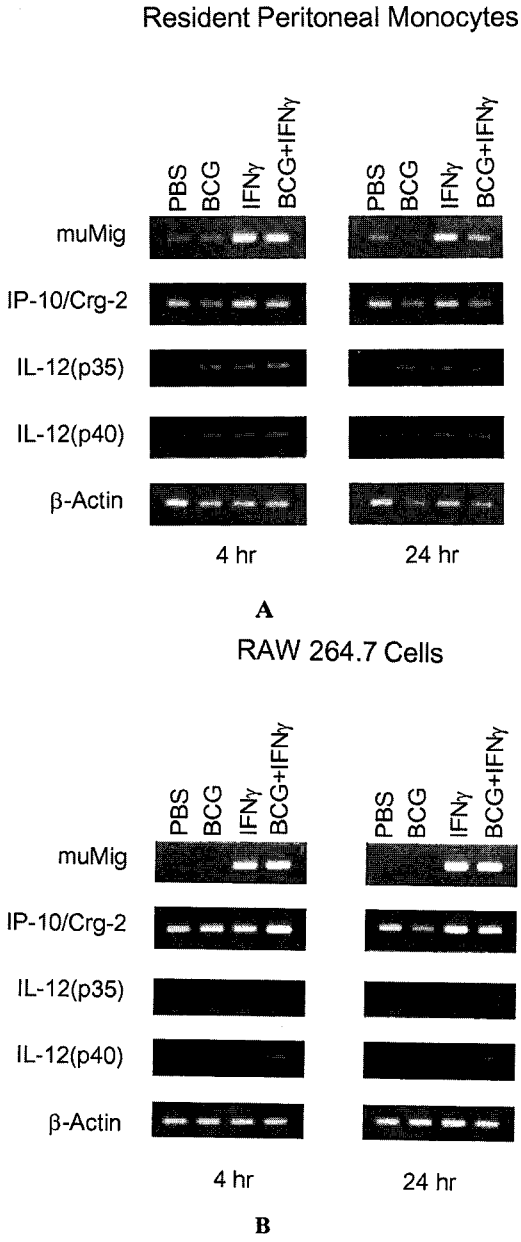

Figure 1. Chemokine and cytokine mRNA in macrophages shown by semiquantitative RT-PCR analysis. **A)** Resident peritoneal monocytes (RPMΦ) or **B)** RAW 264.7 cells ($5x10^6$ cells/ml) was cultured in the presence of viable BCG (vBCG, $2.5x10^8$ CFU/ml) for the indicated times, and total cellular RNA was subjected to RT-PCR analysis. IFN-γ (100 U/ml) was added into the culture medium at 4 hr before cell harvest.

response sites. Mice were immunized sc with $5x10^4$ vBCG for two times (on days 0 and 70)

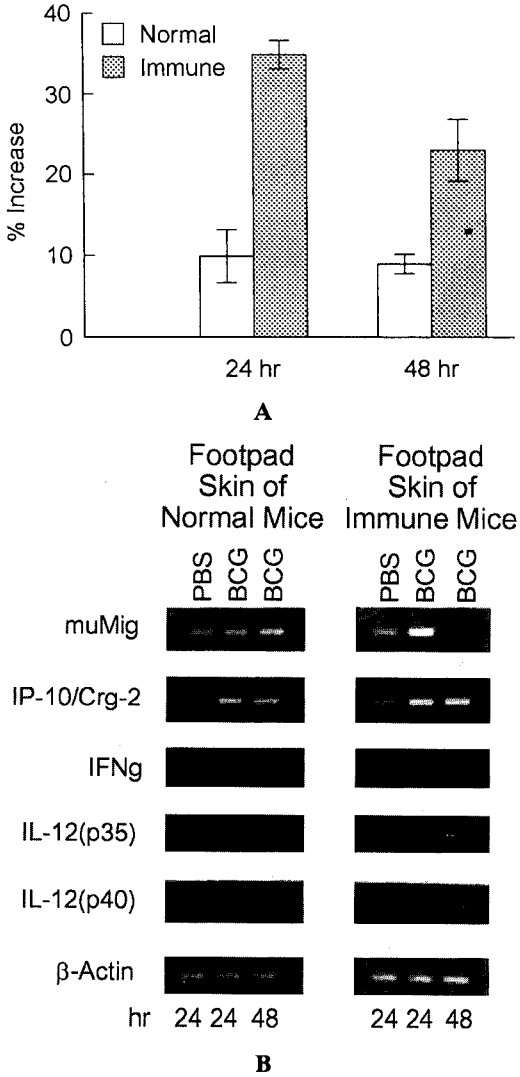

Figure 2. A) Footpad swelling reaction in vBCG-immune Balb/c mice. Mice were immunized by sc injections of vBCG ($5x10^4$ CFU) at dorsal neck on day 0 and day 70. Footpad swelling reactions were elicited by sc injection of $5x10^6$ killed BCG (kBCG, 0.05 ml) into right hind footpad at 7 days after the second immunization. Footpad swellings were measured using micrometer just before (T_0) challenge and at 24 hr (T_{24}) or 48 hr (T_{48}) after challenge. % change in footpad thickness was calculated using the following formula. % change = (T_{24} or T_{48} - T_0) x 100/ T_0. Each point represents mean + SE from 3 (normal) or 4 (immune) mice. The footpad skins were used for the following RT-PCR analysis. **B)** Chemokine and cytokine mRNA in footpad skins from normal or vBCG-immune BALB/c mice shown by semiquantitative RT-PCR analysis. Footpad skins excised at the indicated times were pooled, and total cellular RNA was subjected to RT-PCR analysis.

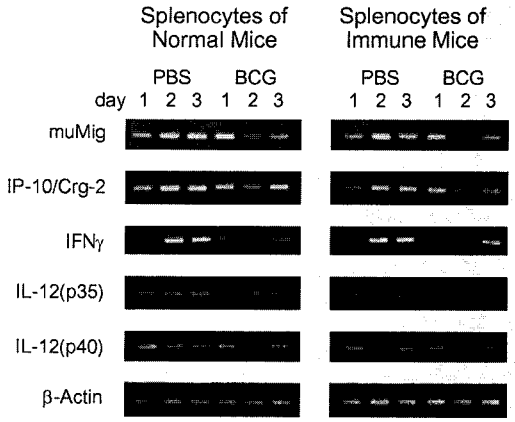

Figure 3. Chemokine and cytokine mRNA in splenocytes from normal or vBCG-immune BALB/c mice shown by semiquantitative RT-PCR analysis. Mice were immunized by sc injections of vBCG ($5x10^4$ CFU) at dorsal neck on day 0 and day 70. Splenocytes ($5x10^6$ cells/ml), prepared 8 days after the second immunization, was stimulated with vBCG ($2.5x10^8$ CFU/ml) for the indicated times, and total cellular RNA was subjected to RT-PCR analysis.

and DTH was measured by eliciting the response by injection of $5x10^6$ kBCG 1 wk after the last immunization. As shown in Fig. 2A, both of the 24 and 48 hr DTH responses in vBCG-immune mice were significantly higher than those in the control groups. The responses were higher in 24 hr DTH compared with the 48 hr. Thus, total RNA obtained from the footpad skins was subjected to semiquantitative RT-PCR analysis. Expression of *Mig*, *IP-10*, and *IFN-γ* in 24 hr DTH was stronger in vBCG-immune mice than in the non-immune, whereas Mig message in 48 hr DTH was barely detectable in vBCG-immune mice (Fig. 2B). The increase of DTH measured by foot-pad thickness appears clearly related to levels of the chemokines Mig and IP-10 messages and those of IFN-γ and IL-12. Simultaneous amplification of cDNA for β-actin confirmed that total cellular mRNA and efficiency of reverse transcription were comparable for footpads stimulated with PBS alone or kBCG.

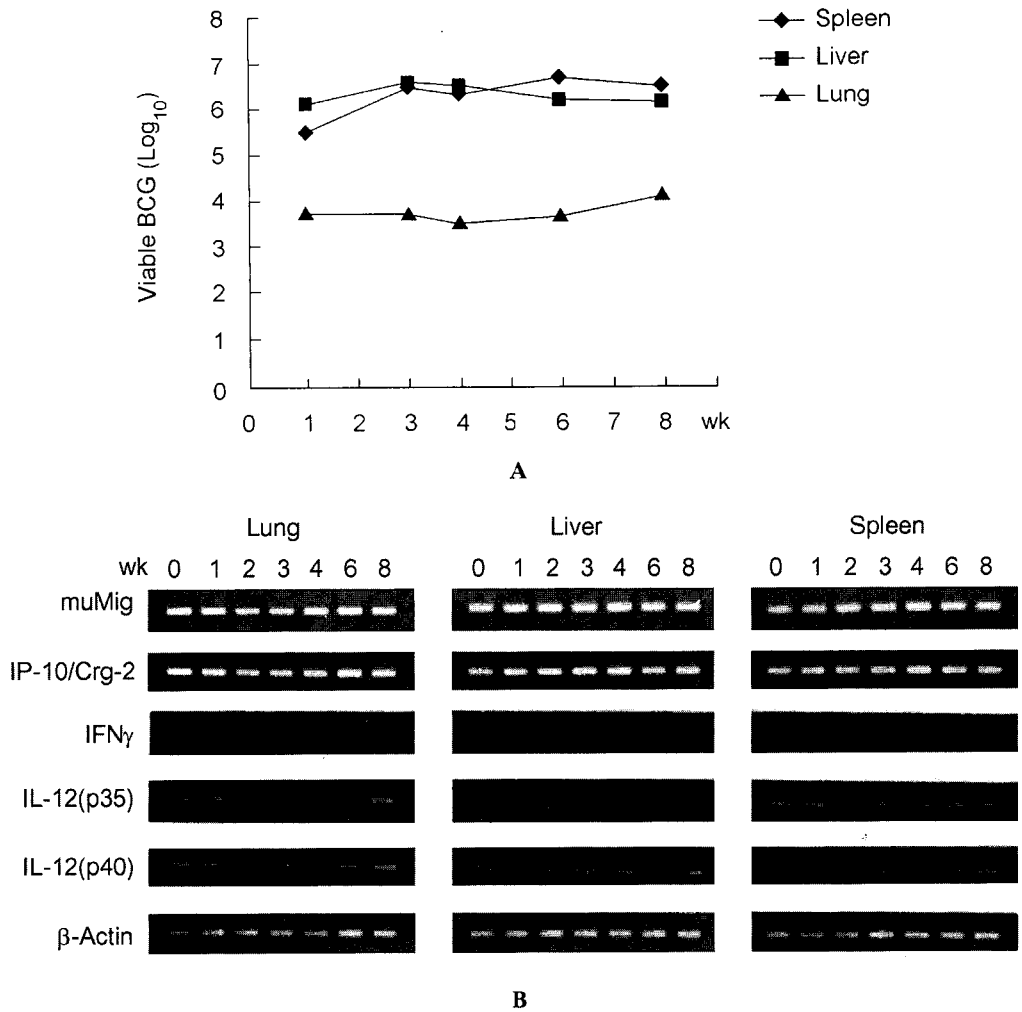

Figure 4. A) Growth of BCG in lungs, livers, and spleens of BALB/c mice at different intervals following iv infection with $5x10^5$ vBCG bacilli. Target organs were pooled and homogenated for plate counting. Each point represents mean of triplicate. **B)** Chemokine and cytokine mRNA in lungs, livers, and spleens of BALB/c mice shown by semiquantitative RT-PCR analysis. Mice were iv infected with $5x10^5$ vBCG bacilli. Target organs were isolated and pooled from 5 mice at different intervals. Total cellular RNA was subjected to RT-PCR analysis.

Induction of chemokines Mig and IP-10 mRNA by stimulation with vBCG in splenocytes from vBCG-immune and non-immune mice. To compare *Mig* and *IP-10* expression of splenocytes originated from immune and non-immune mice, splenocytes were stimulated with vBCG for varying durations. Chemokine and cytokine gene expression, except for IFN-γ of 1 day culture, was detected without vBCG stimulation. Interestingly, stimulation with vBCG for 2 days decreased or completely dropped levels of Mig message in non-immune or immune splenocytes, respectively, whereas IP-10 message was slightly decreased in 2 days culture (Fig. 3). Moreover, messages for IL-12 (p40) showed similar kinetics for Mig. It appears, however, that there was no difference in message levels of chemokines and cytokines between immune splenocytes and the non-immune.

Bacterial isolation and expression of *Mig* and *IP-10* in BCG-infected mice. Having demonstrated the expression of *Mig* and *IP-10* genes in DTH, mice were i.v. infected with vBCG in order to determine growth of BCG and expression of chemokines and cytokines in lungs, spleens, and livers. As shown in Fig. 4A, BCG grew quickly in the livers and spleens over the first 3 wk, after which the bacterial load maintained. In contrast, the bacterial load in the lungs increased with the infection course. The pathologies of each organ with the infection course of vBCG-infected mice (data not shown) were similar to other report (29). When the expression of chemokines and cytokines was measured by semiquantitative RT-PCR for the organs, there was constitutive expression with the exception of IFN-γ, for which levels of IFN-γ message was detectable at 2 wk and peaked at 3 wk postinfection with decreasing tendency of message level thereafter (Fig. 4B). Although there was no obvious changes of Mig and IP-10 messages in organs during infection process, we found that the infection progressed over the first 3 wk before being contained by the emerging immune response suggested from detectable amount of IFN-γ mRNA around this time.

DISCUSSION

The results of this study show that both Mig and IP-10, which are inducible by IFN-γ in human and mouse cells (9, 11) but also can act in vitro as chemotactic factors for activated T cells (5, 22, 23, 33), are involved and regulated in DTH against BCG of vBCG-immune mice, and BCG may evade host responses by modulating the chemokines response during infection. In DTH measured by foot-pad thickness, the increase of DTH appears clearly related to the levels of both Mig and IP-10 messages of vBCG-immune mice, and chemokine Mig and IP-10 genes show differences in their patterns of expression. We also found regulated pattern of Mig and IP-10 expression in splenocytes stimulated with vBCG, especially in immune-splenocyes. The level of Mig and IP-10 messages was decreased in RPMΦ stimulated with vBCG, while IP-10 message only was decreased in RAW 264.7 cells stimulated with vBCG. Although the levels of Mig and IP-10 mRNA in the organs from BCG-infected mice was not changed clearly during the course of infection, the expression of IFN-γ coincided with slowing of bacterial growth rate in livers, spleens, and lungs at around 3 wk after infection. Of note, variable levels of expression of *Mig* as well as *IP-10* are detected in RPMΦ, footpad skins, splenocytes, lungs, livers, and spleens of normal mice.

We have shown that accumulation of Mig and IP-10 mRNA in RPMΦ was slightly decreased by stimulation with vBCG, and the degree was greater for 24 hr culture even though IFN-γ was added (Fig. 1A). In other studies, however, *M. tuberculosis* stimulated IL-8 (13), MCP-1 (14), and both Mig and IP-10 production (21) in monocytic cell lines. Chemokine levels were elevated in bronchoalveolar lavage from persons with tuberculosis (19). The infection of murine macrophages with various strains of *M. tuberculosis* found to induce chemokine gene expression in vitro (30) suggests that mycobacteria can induce chemokine expression. Although the precise mechanism of mycobacterial involvement in decreased induction of these chemokine genes is unknown, we speculate that BCG may somehow modulate chemokine response of macrophages for its intracellular survival.

The finding that the increased expression of *Mig* and *IP-10* in DTH responses against in vBCG-immune mice (Fig. 2B) is both interesting and surprising in view of the nature of the T-cell-monocyte granulomatous response to mycobacterial infection and chemotactic activity of Mig and IP-10 for activated T cells. Since

cooperation between macrophages and T cells is required for protective immunity against mycobacterial infection in vivo, these cells must be recruited to infection foci from blood. It has been reported that IP-10 (17) and Mig (30) are expressed in inflammatory responses in which IFN-γ involves with high local concentration. The findings that Mig and IP-10 share a receptor expressed on the surface of activated T cells (5, 22, 23) and antigen-specific T cells are required for protective immunity against mycobacterial infection (27) suggest that Mig and IP-10 may be recruiters of T cells to the site of infection and in their activation which may further amplify protective immunity through recruitment of Ag-specific T cells to the inflammatory foci and then further activation of macrophages of the site by cytokines including IFN-γ and IL-2 produced by T cells.

In non-immune or immune splenocytes stimulated with vBCG for 2 days, Mig message was decreased or completely dropped, respectively, whereas IP-10 message was slightly decreased. It appears, however, that there was no difference in message levels of chemokines and cytokines between immune splenocytes and the non-immune (Fig. 3). Although both Mig and IP-10 levels were decreased, similar kinetics between Mig and IL-12 (p40) messages observed in this experiment. This has led to speculation that a certain kind of network may exist between Mig and IP-10 chemokines and IL-12 in immune reaction against mycobacteria. It is interesting to note that IL-12 has been produced by macrophages in response to mycobacterial infection (12, 15) and detected in tuberculous pleuritis (35), and that antitumor effect of IL-12 is dependent on Mig and IP-10 chemokines (16, 32).

While there were the regulated patterns of *Mig* and *IP-10* expression in DTH response to kBCG and in macrophages and splenocytes stimulated with vBCG, the levels of Mig and IP-10 mRNA during the course of infection with BCG were not readily changed in lungs, livers, and spleens from BCG-infected mice (Fig. 4B). It is of interest that constitutive expression of *Mig* and *IP-10* with variable levels was seen in all of target organs of normal mice, suggesting nonspecific activation of monocytes by any general stimulation of the mononuclear phagocyte system. Of note, in this experiment, the growth pattern of BCG in mice was somewhat similar to the report by Collins and Montalbine (6). We found that containment of progression of infection occurred around the period of *IFN-γ* expression (Fig. 4), suggesting that immune response against BCG infection emerges around this period. In the context of chemotactic activity of Mig and IP-10 on activated T cells (5, 22, 23, 33) and their inducibility from macrophages by IFN-γ (9, 11), it is likely that local production of Mig and IP-10 may contribute to this containment of progression of infection.

This study provides indirect clues to the involvement of chemokines Mig and IP-10 in immune response against mycobacterial infection. The selective accumulation of memory T cells inside inflammatory foci of tuberculosis may be dependent on the in situ production of Mig and IP-10 chemokines or/and on that of I-TAC, which has also been shown recently to share CXCR3 and to attract activated T cells selectively (5), acting in concert with additional chemokines including RANTES (35). Further studies of the local immune response in mycobacterial infections is necessary to determine the relative contribution of each of non-ELR CXC chemokines (Mig, IP-10, and I-TAC) to protective immunity against mycobacteria and will enhance our understanding of the complex host-pathogen interaction and facilitate development of immunomodulatory therapy to reduce morbidity and mortality from mycobacterial infection.

Acknowledgements

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