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Th1-skewed tissue responses to a mycolyl glycolipid in mycobacteria-infected rhesus macaques

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ABSTRACT

Trehalose 6,6′-dimycolate (TDM) is a major glycolipid of the cell wall of mycobacteria with remarkable adjuvant functions. To avoid detection by the host innate immune system, invading mycobacteria down-regulate the expression of TDM by utilizing host-derived glucose as a competitive substrate for their mycolyltransferases; however, this enzymatic reaction results in the concomitant biosynthesis of glucose monomycolate (GMM) which is recognized by the acquired immune system. GMM-specific, CD1-restricted T cell responses have been detected in the peripheral blood of infected human subjects and monkeys as well as in secondary lymphoid organs of small animals, such as guinea pigs and human CD1-transgenic mice. Nevertheless, it remains to be determined how tissues respond at the site where GMM is produced. Here we found that rhesus macaques vaccinated with *Mycobacterium bovis* bacillus Calmette–Guerin mounted a chemokine response in GMM-challenged skin that was favorable for recruiting T helper (Th)1 T cells. Indeed, the expression of interferon-γ, but not Th2 or Th17 cytokines, was prominent in the GMM-injected tissue. The GMM-elicited tissue response was also associated with the expression of monocyte/macrophage-attracting CC chemokines, such as CCL2, CCL4 and CCL8. Furthermore, the skin response to GMM involved the up-regulated expression of granulysin and perforin. Given that GMM is produced primarily by pathogenic mycobacteria proliferating within the host, the Th1-skewed tissue response to GMM may function efficiently at the site of infection.

1. Introduction

Mycobacteria possess highly lipid-rich cell walls that are critical not simply for their acid-fast properties but also for their pathogenesis. Outside the peptidoglycan structure, mycobacteria-specific, long-chain fatty acids, referred to as mycolic acids, are aligned densely in covalent association with the 6-position of arabinose termini of the underlying arabinogalactan sugar layer, thereby forming the rigid skeleton of the cell wall [1]. Mycolic acids also exist at the surface of the cell wall as free molecules complexed to sugars. The extremely hydrophobic cell wall architecture constructed by interactions between the arabinogalactan-linked mycolic acids and carbon chains of the surface-exposed glycolipids is essential for protection from a variety of chemical agents, such as reactive oxygen intermediates and hydrolytic enzymes, derived from the host cells [2].

Among mycolyl glycolipids, trehalose 6,6′-dimycolate (TDM) has been studied extensively because of its abundance in the cell wall of mycobacteria and its potent adjuvancy [3]. TDM is recognized by host innate immune receptors, including the macrophage-inducible C-type lectin (Mincle), and strongly activates macrophages to secrete proinflammatory cytokines [4]. However, pathogenic mycobacteria appear to have evolved an evasive maneuver to down-regulate TDM expression to avoid unnecessary stimulation of the host innate immunity. Mycobacteria-derived mycolyltransferases catalyze the final step of TDM biosynthesis, using trehalose 6-monomycolate as a substrate, but upon entry into the host, mycobacteria utilize host-derived glucose as a competitive substrate for the enzymes, resulting in down-regulation of TDM expression and up-regulated production of glucose monomycolate (GMM) [5]. As glucose is present at high concentrations in the host and scarce in external environments, only mycobacteria

Abbreviations: Ag, antigen; BCG, *Mycobacterium bovis* bacillus Calmette–Guerin; GMM, glucose monomycolate; TDM, trehalose 6,6′-dimycolate.

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that sustain reasonable metabolism within the host, not those that are killed by the host or viable only in environments, can produce GMM. Thus, GMM is regarded as a marker glycolipid for pathogenic mycobacteria that actively proliferate within the host.

Notably, the acquired immune system is equipped with T cells that react to GMM specifically. A GMM-specific, CD1b-restricted T cell line was established from a patient with leprosy [6], and GMM-specific, CD1c-restricted T cell lines were obtained from *Mycobacterium bovis* bacillus Calmette–Guerin (BCG)-vaccinated rhesus macaques [7]. In addition, T cell responses to GMM in the peripheral blood, lymph nodes, and spleen have been detected in humans [8], rhesus macaques [7], cattle [9], guinea pigs [10], and human CD1-transgenic mice [11]. However, these studies, including ours [7,10], did not address directly how tissues responded at the site where GMM production occurred. Therefore, the present study was designed to determine the quality of the tissue response elicited at the site challenged with GMM, using BCG-vaccinated rhesus macaques. We found that GMM provoked highly Th1-skewed local responses.

2. Materials and methods

2.1. Animals and vaccination

The rhesus macaques (*Macaca mulatta*) used in this study were treated humanely in accordance with institutional regulations, and experimental protocols were approved by the Committee for Experimental Use of Non-human Primates at the Institute for Virus Research, Kyoto University. Vaccination with the Tokyo 172 strain of BCG (Japan BCG Laboratory, Tokyo, Japan) was performed as described previously [7].

2.2. Purification of GMM and preparation of liposomes

GMM was purified from cultured mycobacteria and integrated into stearylated octaarginine-containing liposomes as described previously [10]. The purity of the GMM sample was confirmed by TLC, using two different solvent systems, and its molecular identify was confirmed by mass spectrometry. Protein contamination was not detected by silver staining of SDS–PAGE gels or by the Bradford assay.

2.3. DNA microarray

Peripheral blood mononuclear cells (PBMCs) were obtained from BCG-vaccinated monkeys (MM553 and MM556) and placed in wells of 24-well tissue culture plates (6×10^6/well). The cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT) in the presence of either GMM in liposomes (1 μg/ml) or empty liposomes. After 24 h at 37 °C, total RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany) and sent to HaploPharma, Inc. (Okinawa, Japan), where a DNA microarray analysis was performed as instructed by the Expression Analysis Technical Manual, 2005 (Affymetrix, Santa Clara, CA). Briefly, biotinylated complementary RNAs (cRNAs) were prepared from 250 ng of total RNA, and the cRNA fragments were hybridized for 16 h at 45 °C on the GeneChip rhesus macaque genome array (Affymetrix). The GeneChips were subsequently washed, stained with the Affymetrix Fluidics Station 450, and scanned using the Gene Chip Scanner 3000 7G. The data were analyzed with Microarray Suite version 5.0 using Affymetrix default analysis settings. The obtained microarray data were deposited in the GEO database (accession number GSE44963).

Table 1
Primers used for RT-PCR.

Targets	Primers
CCL1	5′-CAA GAC GTG GAC AGC AAG AG-3′(sense) 5′-CAT CTA GCC TGG TTC AAG GC-3′ (anti-sense)
CCL2	5′-TCT GTG CCT GCT GCT CAT AG-3′ (sense) 5′-CGG AGT TTG GAT TTG CTT GT-3′ (anti-sense)
CCL3	5′-TTG CTG TCC TCC TCT GCA C-3′ (sense) 5′-CGT ATT TCT GGA CCC ACT CC-3′ (anti-sense)
CCL4	5′-GTT CTG TAG CCT CAC CTC TG-3′ (sense) 5′-GAC TTG CTT GCC TCT TTT GG-3′ (anti-sense)
CCL7	5′-CAC CTC TGT GTC TGC TGC TC-3′ (sense) 5′-CAT GGC TTG GTT TCA GTT CA-3′ (anti-sense)
CCL8	5′-CTT CTG TGC CTG CTG CTC AT-3′ (sense) 5′-ATC CCT GAC CCA TCT CTC CT-3′ (anti-sense)
CCL11	5′-ACC TGC TGC TTT ACC CTG AC-3′ (sense) 5′-AGT TGG AGA TTT TCG GTC CA-3′ (anti-sense)
CCL17	5′-CTT CTC TGC AGC ACA TCC AT-3′ (sense) 5′-AAC AGA TGG CCT TGT TCT GG-3′ (anti-sense)
CCL24	5′-AAC CAG CCT TCT GTT CCT TG-3′ (sense) 5′-GGC ATC CAG GTT CTT CAT GT-3′ (anti-sense)
CXCL9	5′-TTT TCC TCT TGG GCA TCA TC-3′ (sense) 5′-TTT GGC TGA CCT GTT TTT CC-3′ (anti-sense)
CXCL10	5′-CAT TCT GAT TTG CTG CCT TG-3′ (sense) 5′-TTG ATG GCC TTA GAT TCT GGA-3′ (anti-sense)
CXCL11	5′-GAG TGT GAA GGG CAT GGC TA-3′ (sense) 5′-TGG GAT TTA GGC ATC GTT GT-3′ (anti-sense)
Granulysin	5′-ACC CAG AGA AGC ATT TCC AA-3′ (sense) 5′-GGG AAG GGA GAC TGG AGA GT-3′ (anti-sense)
Perforin	5′-CCC TCT GTG AAA ATG CCC TA-3′ (sense) 5′-GAT GAA GTG GGT GCC GTA GT-3′ (anti-sense)
GAPDH	5′-ACC ACA GTC CAT GCC ATC AC-3′ (sense) 5′-TCC ACC ACC CTG TTG CTG TA-3′ (anti-sense)

2.4. RT-PCR

To assess tissue responses to GMM, liposome containing 5 μg of GMM as well as an equivalent amount of empty liposome was dissolved in 100 μL of phosphate-buffered saline and injected into the skin of BCG-vaccinated monkeys. After 2 days, the monkeys were sacrificed and the excised skin was deep-frozen in liquid nitrogen. Each skin sample (approximately 100 mg) was disrupted by operating the Tomy MS-100R beads cell disruptor (Tomy Seiko Co., Tokyo, Japan) with 5 mm beads, and total RNA was extracted using the RNeasy fibrous tissue midi kit (Qiagen). The first-strand cDNA

Table 2
Primers used in this study for real time PCR.

Targets	Primers
IFNG	5′-GAC ATC TTG AGG AAT TGG AAA G-3′ (sense) 5′-TTT GGA TCC TCT GGT CAT CTT-3′ (anti-sense)
T-bet	5′-CAC CTG TTG TGG TCC AAG TTT-3′ (sense) 5′-TGA CAG GAA TGG GAA CAT CC-3′ (anti-sense)
IL10	5′-TGC CTT CAG CAG AGT GAA GA-3′ (sense) 5′-GCA ACC CAG GTA ACC CTT AAA-3′ (anti-sense)
GATA3	5′-ACT ACG GAA ACT CGG TCA GG-3′ (sense) 5′-GGC AGG GAT CCA TGA AGC AG-3′ (anti-sense)
IL17F	5′-TGG GAA GAC CTC ATT GGT GC-3′ (sense) 5′-GGA TTT CGT GGG ATT GCT AT-3′ (anti-sense)
RORγt	5′-CAG CGC TCC AAC ATC TTC T-3′ (sense) 5′-CAC AGC GTT CCC ACA TCT C-3′ (anti-sense)
CD3E	5′-AGA TGC AGT CGG GCA CTC-3′ (sense) 5′-TAC CAT CTT GCC CCC AAA C-3′ (anti-sense)

Table 3
Chemokines up-regulated in GMM-stimulated PBMCs.[a]

Genes	Probe set ID	MM553	MM556
Cytokines			
IFNG	MmuSTS.173.1.S1_at	4.9	7.8
IFNG	MmugDNA.41414.1.S1_at	6.6	5.4
IL26	MmuSTS.4352.1.S1_at	2.4	4.9
IL6	Mmu.12240.1.S1_at	3.8	4.2
IL6	MmuSTS.4354.1.S1_at	4.3	3.3
LTA	MmuSTS.1446.1.S1_at	2.2	3.9
C chemokines			
None			
CC chemokines			
CCL2 (MCP-1)	Mmu.11912.1.S1_at	18.4	4.8
	MmuSTS.3317.1.S1_at	2.1	14.6
CCL8 (MCP-2)	MmugDNA.3158.1.S1_at	15.5	3.6
CCL7 (MCP-3)	MmuSTS.3575.1.S1_at	3.5	11.0
CCL3 (MIP-1α)	Mmu.6471.1.S1_at	3.8	2.7
	Mmu.6471.1.S1_x_at	2.5	3.7
CXC chemokines			
CXCL9	Mmu.11358.1.S1_at	93.1	8.4
	MmuSTS.4003.1.S1_at	88.5	3.3
CXCL10	Mmu.11363.1.S1_at	17.8	2.1
CXCL11	Mmu.11366.1.S1_at	57.0	3.6
	MmugDNA.15618.1.S1_s_at	44.2	2.8
	MmugDNA.19126.1.S1_at	37.9	2.2
CX3C chemokines			
None			

[a] Values indicate the fold increase in transcription levels after GMM stimulation.

was synthesized from 1 μg of RNA using oligo(dT) and the PrimeScript reverse transcriptase (Takara Bio, Inc., Otsu, Japan). The samples were then subjected to PCR for 25 s at 94 °C, 30 s at 58 °C, and 45 s at 72 °C using Taq DNA polymerase. The primers used are listed in Table 1.

2.5. Real-time PCR

PCRs were performed using the THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Briefly, a reaction mixture (20 μL) containing 0.4 μL of each cDNA sample, 10 μL of the THUNDERBIRD SYBR qPCR Mix, 0.4 μL of the ROX reference dye, and 0.6 μm of each primers was subjected to PCR using the Applied Biosystems 7500 real-time PCR system. The reactions were run in triplicate samples, and the absolute mean values for each gene were normalized to that of the CD3ε gene. The primers used are listed in Table 2.

2.6. Histochemistry

The skin samples were fixed for 1 day with 4% paraformaldehyde, dehydrated, and deep-frozen in OCT compound. The cryosections were stained with hematoxylin and eosin and observed under a microscope. Some cryosections were processed for immunohistochemistry as described previously [12], and labeled with an anti-rhesus macaque CD14 monoclonal antibody (MF396) generated in our laboratory, or a negative control antibody (RPC5.4) [13]. Signals were visualized with the standard protocol using a Vector Elite ABC kit.

2.7. Statistics

Statistical analysis was performed, using the one-way Analysis of Variance (ANOVA).

3. Results and discussion

3.1. Peripheral blood cytokine and chemokine responses to GMM

Cytokines and chemokines play a pivotal role in determining the quality of immune responses in tissues. We assumed that cytokine/chemokine profiles detected for GMM-stimulated PBMCs might provide a clue to tissue responses to GMM. Therefore, to grasp an overview of these responses, we performed a DNA microarray analysis, using RNA derived from GMM-stimulated PBMCs. PBMCs were isolated from 2 BCG-vaccinated monkeys (MM553 and MM556) that contained a significant pool of GMM-specific circulating T cells and stimulated in vitro with either the GMM liposome or empty liposome. After 24 h, total RNA was extracted from the cells and subjected to the GeneChip analysis. On the basis of the microarray data (GEO database accession number, GSE44963), transcriptional levels for each of the known 59 cytokines and 40 chemokines were analyzed, and those with more than a 2-fold increase in both monkeys in response to GMM were listed. As shown in Table 3, the listed cytokines included Th1 cytokines (IFNG and LTA) and a proinflammatory cytokine (IL6), but the up-regulated expression of representative Th2 cytokines, such as IL4 and IL10, was not observed. In addition, the up-regulation of Th17-related cytokines, such as IL17A, IL17F, IL21, and IL22, was

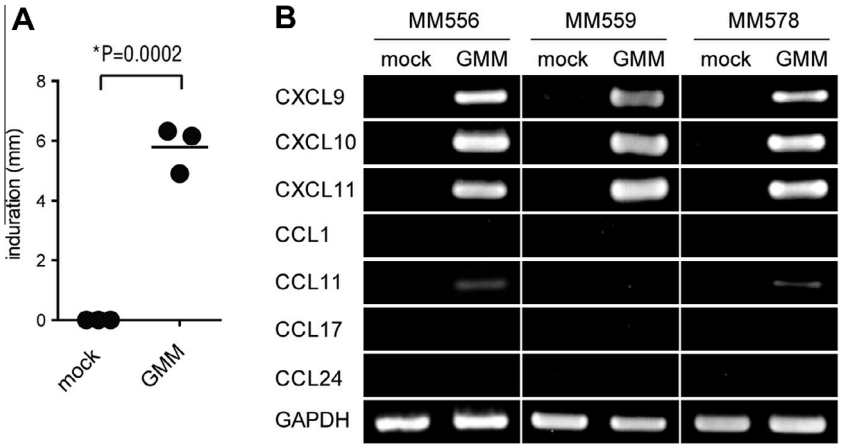

Fig. 1. Up-regulated expression of Th1-attracting chemokines in GMM-challenged skin. (A) Three BCG-vaccinated rhesus macaques (MM556, MM559, and MM578) received an intradermal injection of either the GMM liposome (GMM) or empty liposome (mock). After 2 days, the skin induration at the site of injection was measured. (B) RNA was extracted from the skin, and RT-PCR was performed for detection of chemokine expression.

not apparent except for IL26, a potential Th17 cell-associated cytokine. In parallel with these cytokine responses, skewed chemokine responses were also indicated. Besides monocyte chemotactic proteins (MCPs), such as CCL2, CCL7, CCL8, and macrophage inflammatory protein (MIP)-1α (CCL3), the expression of CXCL9, CXCL10, and CXCL11, a typical set of CXC chemokines with the potential to attract Th1-type T cells, was prominently up-regulated by stimulation with GMM. On the basis of these microarray data, we predicted that the tissue response to GMM may involve the expression of Th1 cytokines and chemokines. To address this directly, tissue responses to GMM were evoked in the skin of BCG-vaccinated rhesus macaques and analyzed for their cytokine/chemokine expression profiles.

3.2. GMM-induced tissue chemokine responses

Our previous study [7] indicated that GMM-specific T cell responses could be detected in BCG-vaccinated, but not unvaccinated rhesus macaques. Therefore, liposome containing 5 μg of GMM as well as an equivalent amount of empty liposome was injected separately into the skin of 3 BCG-vaccinated rhesus macaques (MM556, MM559, and MM578). After 2 days, significant skin induration was induced at the site of GMM challenge in all 3 animals whereas no apparent response was elicited toward empty liposome (Fig. 1A). The skin was excised and total RNA was extracted, followed by RT-PCR for expression of representative T cell-attracting chemokines. As shown in Fig. 1B, CXCL9, CXCL10, and CXCL11, a group of chemokines known to preferentially attract Th1 cells [14], were highly transcribed in the GMM-injected tissue. In sharp contrast, transcription of Th2-attracting chemokines [15], such as CCL1, CCL11, CCL17, and CCL24, was either marginal (for CCL11) or barely detectable (for CCL1, CCL17, CCL24) (Fig. 1B). Therefore, the predominant expression of the Th1-attracting chemokines over the Th2-attracting chemokines predicts a Th1-skewed local response in the GMM-injected skin.

3.3. IFN-γ dominant tissue responses to GMM

Hematoxylin and eosin staining of the GMM-challenged skin of the MM556 rhesus macaque revealed prominent infiltration by mononuclear cells (Fig. 2A, right), which was not observed in the mock-challenged skin (left). In order to assess the net cytokine response at the site of the antigen (Ag) challenge, total RNA was extracted from the GMM-challenged and mock-challenged skin of the 3 rhesus macaques, and transcriptional levels for hallmark cytokines, such as IFNG (for Th1), IL10 (for Th2), and IL17 (for Th17), were determined by real-time PCR. As shown in Fig. 2B, IFNG expression was significantly up-regulated in response to GMM ($P = 0.0002$) whereas no up-regulation was observed for IL10 and IL17. In parallel with the skewed cytokine response, the expression of T-bet, a Th1 cell-specific transcription factor that controls IFNG expression, appeared to be up-regulated in the GMM-challenged skin although the increase was not statistically significant ($P = 0.066$). In sharp contrast, the expression of GATA-3, a Th2-specific transcription factor, and RORγt, a transcription factor promoting T cell differentiation into Th17 cells [16], was decreased significantly ($P = 0.021$ and $P = 0.046$, respectively) (Fig. 2B). Taken together, these results pointed to the IFN-γ dominant response in the GMM-challenged skin.

Our previous study indicated that the GMM-specific T cell population developed in BCG-vaccinated rhesus macaques contained $CD8^+$ cytotoxic T cells [7], and therefore, we predicted that the expression of cytotoxic granule proteins, such as granulysin and perforin, might be up-regulated in the GMM-challenged skin. Indeed, we found that the transcription of the granulysin and perforin genes was readily detectable by RT-PCR in the GMM-challenged, but not mock-challenged skin of the 3 rhesus macaques analyzed (Fig. 2C).

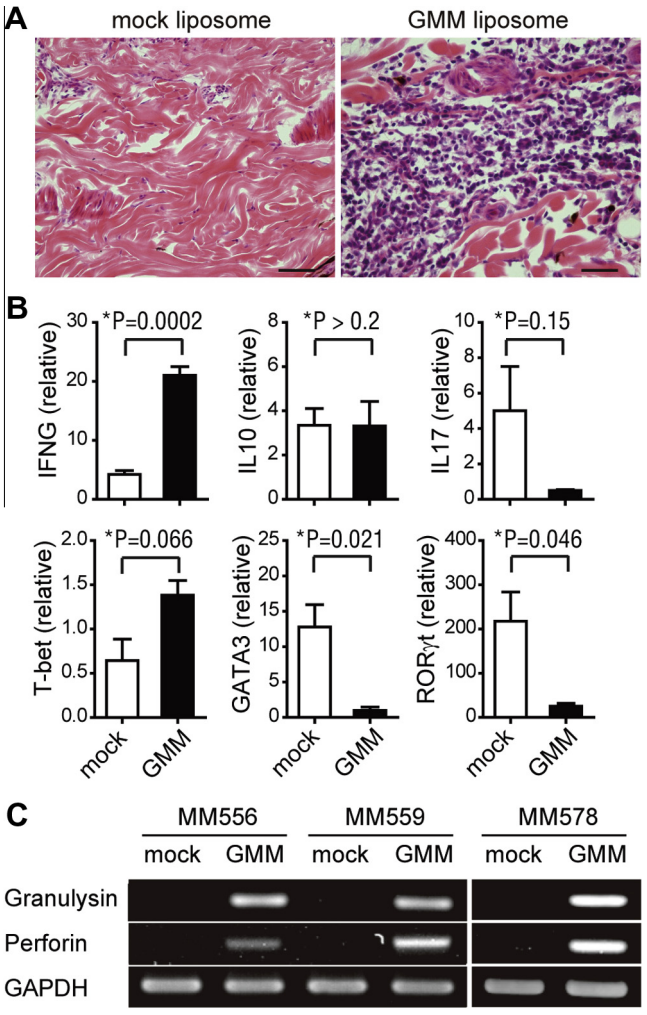

Fig. 2. Th1-skewed responses in the GMM-challenged skin. (A) The skin sections derived from MM556 were stained with hematoxylin and eosin, and viewed under a microscope. Scale bars, 50 μm. (B) Gene expression of cytokines and related transcription factors was analyzed by quantitative real-time PCR, using RNA extracted from the GMM-challenged and mock-challenged skin. The absolute values for each gene were normalized to that of the *CD3ε* gene. Statistical assessment of the real time data obtained form the 3 animals was performed. (C) RNA was extracted from the GMM liposome-challenged or mock-challenged skin as in Fig. 1, and RT-PCR was performed for transcription of the granulysin and perforin genes.

3.4. Macrophage recruitment

IFN-γ orchestrates the trafficking of specific immune cells, including monocytes and macrophages, to sites of inflammation through up-regulating expression of a group of chemokines. Indeed, an immunohistochemical analysis of the GMM-challenged skin with an anti-rhesus macaque CD14 monoclonal antibody detected a local accumulation of $CD14^+$ mononuclear cells that represented macrophages (Fig. 3A). Besides the Th1-attracting CXC chemokines described above, the expression of a fraction of CC chemokines capable of attracting monocytes and macrophages is known to be up-regulated by IFN-γ [17]. Therefore, we examined if the expression of such chemokines might be up-regulated in the GMM-challenges skin. As shown in Fig. 3B, the transcription of CCL2 (MCP-1), CCL4 (MIP-1β), and CCL8 (MCP-2) was induced in the skin challenged with the GMM liposome, but not with empty liposome, in all 3 monkeys analyzed.

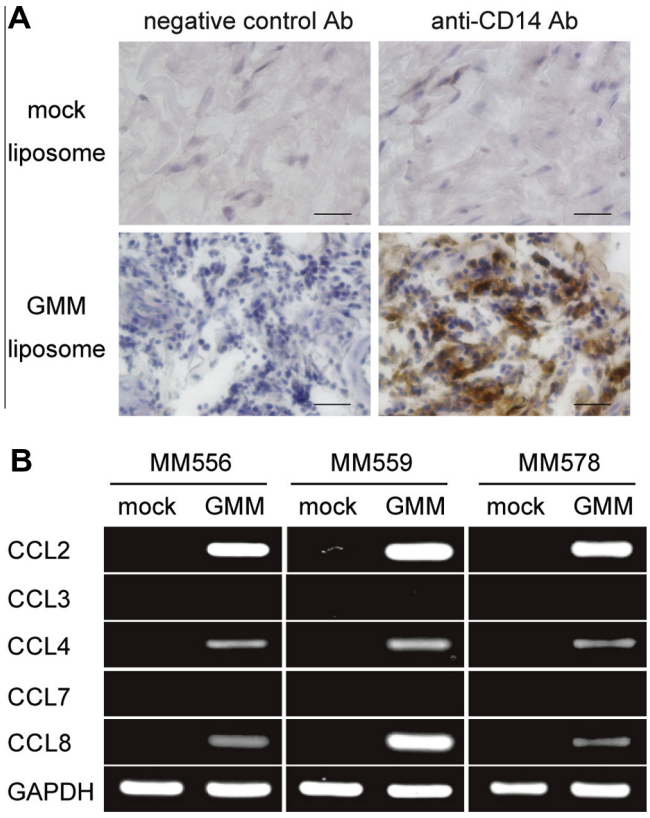

Fig. 3. Up-regulated expression of macrophage-attracting chemokines in the GMM-challenged skin. (A) The GMM-challenged and mock-challenged skin sections derived from MM556 were immunolabeled with either anti-rhesus macaque CD14 antibody or negative control antibody, and viewed under a microscope. Scale bars, 50 μm. (B) Gene expression of indicated CC chemokines was analyzed by RT-PCR, as in Fig. 1.

3.5. An integrated model for the GMM-induced tissue response

As illustrated in Fig. 4, the tissue response to GMM involved up-regulated expression of a set of Th1-attracting chemokines (Fig. 1B) and local accumulation of Th1 T cells that produced IFN-γ (Fig. 2B). This was also associated with up-regulated expression of cytotoxic granule proteins (Fig. 2C) and macrophage-attracting chemokines (Fig. 3B). However, the initial event that triggers these tissue reactions remains to be determined. Although TDM exhibits outstanding adjuvant functions, such activities have not been noted for GMM [5], and indeed, no tissue reactions manifest at the site of GMM injection in unsensitized animals [7], making it unlikely that GMM activates tissue-resident innate immune cells directly. Alternatively, we propose that tissue dendritic cells take up GMM and stimulate GMM-specific T cells localized in their vicinity to release IFN-γ (Fig. 4). Subsequently, tissue-resident macrophages respond to IFN-γ and produce a set of chemokines that are up-regulated by IFN-γ, including those attracting Th1 T cells (CXCL9, CXCL10, and CXCL11) and those attracting macrophages (CCL2, CCL4, and CCL8). The transcriptional expression of CXCR3, the major receptor for CXCL9, CXCL10, and CXCL11 [14], was readily detected in rhesus macaque IFN-γ-producing, GMM-specific T cell lines (D. Morita and M. Sugita, unpublished data), and indeed, circulating GMM-specific T cells were able to extravasate and approach the site of mycobacterial infection where GMM was produced [7]. Therefore, GMM-specific T cells could contribute to the establishment and maintenance of the IFN-γ dominant local milieu, favoring the host to control mycobacterial infections. Similar patterns of chemokine responses are induced in lungs infected

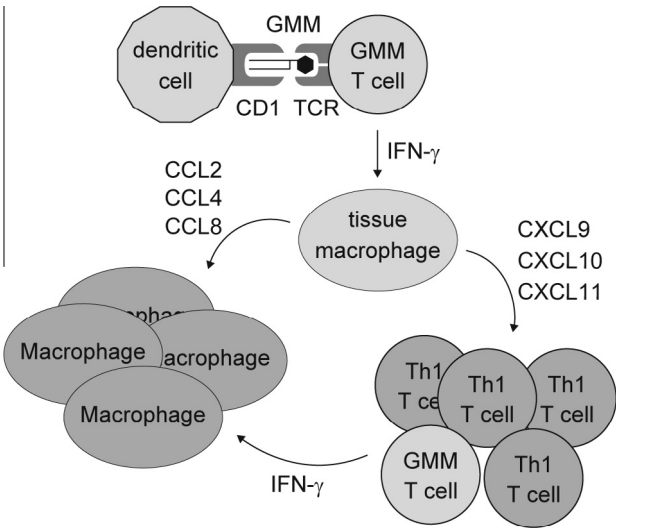

Fig. 4. A proposed model for tissue responses to GMM. IFN-γ produced by activated GMM-specific T cells triggers the up-regulated expression of Th1-attracting chemokines (CXCL9, CXCL10, and CXCL11) and those attracting macrophages (CCL2, CCL4, and CCL8) by tissue-resident macrophages. The local accumulation of IFN-γ-producing T cells, including GMM-specific T cells, further contributes to the establishment of an IFN-γ-dominant niche.

with pathogenic mycobacteria [18], and GMM-specific T cells, including those expressing the high affinity germline-encoded, mycolyl lipid-reactive (GEM) T cell receptor [19], expand prominently in patients with tuberculosis [8]. Taken together, these results suggest that the GMM-elicited tissue response demonstrated in the present study could potentially occur at the site of infection with pathogenic mycobacteria.

The highly potent Th1-inducing peptide, designated Peptide-25, derived from Ag85B has been discovered and studied extensively [20]. The present study indicates that GMM may function as a CD1-presented lipid version of Th1-inducing Ag. Interestingly, the production of GMM depends on mycolyltransferase activities of the Ag85 proteins [5]. Thus, up-regulated expression of Ag85 by proliferating mycobacteria is associated with the creation of potent Th1-inducing peptides (Peptide-25) and lipids (GMM). Given that Ag85 is critical for survival and virulence of mycobacteria, we predict that tissue responses to such Th1-inducing peptides and lipids could function efficiently at the site of infection with pathogenic mycobacteria.

Acknowledgments

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