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Mycobacterial Lipomannan Induces Granuloma Macrophage Fusion via a TLR2-Dependent, ADAM9- and $\beta_1$ Integrin-Mediated Pathway$^1$

Marie-Pierre Puissegur,$^{2,*}$ Guillaume Lay,$^{2,*}$ Martine Gilleron,$^\dagger$ Laure Botella,$^*$ Jérôme Nigou,$^\dagger$ Hedia Marrakchi,$^*$ Bernard Mari,$^{3,8}$ Jean-Luc Duteyrat,$^\$ Yann Guerardel,$^1$ Laurent Kremer,$^8$ Pascal Barbry,$^{3,8}$ Germain Puzo,$^\dagger$ and Frédéric Altare$^{3,*}$

Tuberculous granulomas are the sites of interaction between the host response and the tubercle bacilli within infected individuals. They mainly consist of organized aggregations of lymphocytes and macrophages (Mf). A predominant role of mycobacterial envelope glycolipids in granulomas formation has been recently emphasized, yet the signaling events interfering with granuloma cell differentiation remain elusive. To decipher this molecular machinery, we have recently developed an in vitro human model of mycobacterial granulomas. In this study, we provide evidence that the mycobacterial proinflammatory phosphatidyl-1-myoinositol mannosides and lipomannans (LM), as well as the anti-inflammatory lipoarabinomannan induce granuloma formation, yet only the proinflammatory glycolipids induce the fusion of granuloma Mf into multinucleated giant cells (MGC). We also demonstrate that LM induces large MGC resembling those found in vivo within the granulomas of tuberculosis patients, and that this process is mediated by TLR2 and is dependent on the $\beta_1$ integrin/ADAM9 cell fusion machinery. Our results demonstrate for the first time that the Mf differentiation stage specifically occurring within granulomatous structures (i.e., MGC formation) is triggered by mycobacterial envelope glycolipids, which are capable of inducing the cell fusion machinery. This provides the first characterization of the ontogeny of human granuloma MGC, thus resulting in a direct modulation by a particular mycobacterial envelope glycolipid of the differentiation process of granuloma Mf. The Journal of Immunology, 2007, 178: 3161–3169.

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4 Abbreviations used in this paper: TB, tuberculosis; Mf, macrophage; Ly, lymphocyte; GGC, multinucleated giant cell; TDM, trehalose-6,6'-dimycolate; PIM, phosphatidyl-1-myoinositol mannoside; LM, lipomannan; LAM, lipoarabinomannan; infection, the inhaled bacilli arrive to lung alveoli where they are thought to enter and replicate in alveolar macrophages (Mf). The infected Mf trigger an inflammatory response leading to the recruitment of blood and tissue Mf as well as lymphocytes (Ly) at the infectious site. The resulting cellular accumulations are called tuberculous granulomas. These granulomas mainly contain Mf, epithelioid cells (differentiated Mf), and multinucleated giant cells (MGC), also called giant Langhans cells (fused Mf), which are all surrounded by a rim of T Ly (2, 3). Their major function is now generally considered to be the containment of the infection to a localized area, thus avoiding bacterial spread to surrounding healthy tissues and to other organs, and to concentrate the immune response to a limited infectious area. TB disease outbreak appears upon bacilli growth and granuloma disruption, leading to lung cavitations (4, 5).

Studies dedicated to identify the mycobacterial compounds enabling granulomatous structures formation and modulating granuloma cells physiology, would certainly lead to a better understanding of persistent bacilli. Important insights into the mechanism of granuloma formation have been recently reported using the Mycobacterium marinum/zebrafish embryo model, which enables a real-time study of the formation of fish granulomatous structures that strongly resemble the human ones (for review, see Ref. 6). Yet, the precise molecular events that are triggered by the mycobacteria and give rise to granulomatous structures still remain to be clearly defined.

ManLAM, mannosae-capped LAM; PI, phosphatidyl inositol; ITG8β1, $\beta_1$ integrin; MGG, May-Grünnwald-Giemsa; s.e.m., scanning electron microscopy; aRNA, antisense RNA; n.s., nonstimulated.

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Mycobacterial envelope lipids have long been suspected to be implicated in the interplay between the bacilli and host cells. One of the first reasons for this interest is the extraordinary high lipid content of the mycobacterial envelope, with ~60% of the bacilli dry weight as compared with 20% for the lipid-rich envelope of Gram-negative organisms (7). In addition, it is known that the capacity of Mycobacterium tuberculosis to induce granuloma formation is notably attenuated by delipidation of the mycobacteria (8). Hence, this delipidation significantly hampers M. tuberculosis survival within mouse Mf. Thus, M. tuberculosis lipids were largely explored during the past decades for a role in the modulation of immune responses (9–11). Yet, only a few lipids were studied for their modulation of the granulomatous response.

Among cell wall glycolipids, trehalose-6,6′dimycolate (TDM) as well as phosphatidyl-myo-inositol mannosides (PIMs) have been reported to induce granuloma formation in mice (12–17). PIMs and their multiglycosylated counterparts, lipomannan (LM) and lipoarabinomannan (LAM), are major lipoglycans interspersed within the mycobacterial envelope and are noncovalently attached to the plasma membrane by their PIM anchor. Although PIMs and LM are direct biosynthetic precursors of LAM, they all trigger important immunomodulatory functions (18). LM and PIMs have been reported to induce a strong proinflammatory response by inducing TNF-α, IL-8, and IL-12 secretion (19, 20). Mannose-capped LM (ManLM) found in M. tuberculosis down-regulates LPS-induced IL-12 production (21) and inhibits the phagosome/lysosome fusion (18, 22).

Because PIMs appear as potent granuloma inducers, and because they are direct precursors of LM and LAM, two lipoglycans presenting biological antagonistic properties, we have investigated the ability of PIM, LM, and ManLM to 1) trigger human granuloma formation, and 2) modulate the physiology and maturation of granuloma cells. For this purpose, we have used our previously reported model, which enables the formation of granulomatous-like cellular aggregation around mycobacterial compound-coated artificial beads in vitro (23). Although this model does not enable the study of very late and highly differentiated necrotic granulomatous structures such as those found in vivo, it represents a powerful tool to analyze the very early steps of the granulomatous response. These steps include the recruitment of Ly and Mf around mycobacterial Ag-coated artificial beads as well as the cellular differentiation (epithelioid cell and MGC formation) within the structures. Using this model, we have herein investigated the molecular mechanisms involved in granuloma cell differentiation induced by the major M. tuberculosis glycolipids.

Materials and Methods

Lipoglycans and other activators

PIM1, PIM2, PIM3, and ManLAM were extracted from M. tuberculosis H37Rv, LM from Mycobacterium chelonae, Mycobacterium smegmatis, and Mycobacterium kansasii PHRI 901, and AraLAM from M. chelonae, as described previously (15, 24). TDM from M. smegmatis is a gift from M. Daffe (Toulouse, France). Phosphatidylinositol (PI) (P5766) was obtained from Sigma-Aldrich. The TLR2 activator (25) Mycobacterium leprae 19-kDa hexameric peptide was home-synthesized. The repurified LPS of Escherichia coli K12 was obtained from Invitrogen Life Technologies.

Blood samples

Human blood samples, purchased from the Etablissement Français du Sang of Toulouse (Toulouse, France), were collected from non-tuberculous control donors. This study was conducted according to the principles expressed in the Helsinki Declaration, with informed consent obtained from each donor.

Isolation of PBMC

PBMC were purified from heparinized blood of donors by density gradient using Ficoll-Hypaque (Amersham Pharmacia). PBMCs were diluted to a concentration of 10⁶ cells/ml in RPMI 1640 plus Glutamax (Invitrogen Life Technologies) supplemented with 10% human AB serum (Sigma-Aldrich). This medium will be referenced as culture medium.

Isolation and activation of blood monocytes

A total of 2 × 10⁶ isolated PBMCs were incubated in RPMI 1640 for 1 h in 24-well culture plates containing glass coverslips. The adherent monocytes were washed and stimulated with LM or ManLM (10 μg/ml) for 5 days, with or without previous incubation for 1 h using 10 μg/ml of the following mAbs: anti-TLR2 (TL2.1, HBT), anti-ADAM9 (MDC9, HBT), anti-β2-integrin (ITGb1 (PSD2, R&D Systems), or a IgG1 isotype control Ab (DakoCytomation). The formation of cellular aggregates was followed every day under light microscopy.

Coating of polystyrene beads

A total of 4 × 10⁴ polystyrene beads (90-μm diameter) (SD91; Sigma-Aldrich) was washed two times with 10 ml of 20% NaOH (w/v), rinsed with a water:ethanol mixture (7:3, v/v) to neutrality, removed using a water:ethanol mixture (99:1, v/v), and collected by centrifugation at 500 × g. Beads were then washed, and 500 μg of lipoglycans was added in 0.5 ml of a water:ethanol mixture (2:1, v/v). The beads were weighted and strongly agitated in 10 ml of PBS, then sonicated for 2 min. The lipoglycan-coated beads were then stored at 4°C.

In vitro granuloma formation

In vitro granulomas were obtained as described previously (23). Briefly, 10⁶ freshly isolated cells were incubated in 24-well culture plates with 200 mycobacterial lipoglycan-coated beads for 21 days at 37°C in a 5% CO₂ atmosphere, in RPMI 1640 + Glutamax supplemented with 10% human AB serum.

Cell counting

One hundred granulomatous structures were collected for each condition, and the cells were recovered and counted under an inverted light microscope using a Mallassez cell. For cell-type quantification, cells were recovered from 50 granulomas and stained with May-Grünewald-Giemsa (MGG), and the proportions of multinucleated cells. MGC, MF, and Ly were manually scored under a light microscope.

Scanning electron microscopy (s.e.m.)

For s.e.m. analysis, the granulomas were rapidly collected under light microscopy at different time points and prepared for s.e.m. by fixing in 2% glutaraldehyde in 0.1% phosphate buffer for 4 h. After two washes in the same buffer, the granulomas were removed, dehydrated in a graded ethanol series, dried by critical point drying with ESMCPE CPD 750, coated with gold-palladium for 3 min at 100 A min⁻¹, and observed with a S4500 scanning electron microscope (Hitachi) at an accelerating voltage of 15 kV.

Immunohistochemistry

Granuloma cells were collected and plated onto glass slides with a cytopsin (Cytobuckets; Jouan) and submitted to MGG and/or immunohistochemical staining as follows. After a 10-min incubation in cold acetone, the slides were stained by MGG reagents (Sigma-Aldrich) according to the manufacturer’s instructions. For alkaline phosphatase staining, LSAB-2 (label-ε streptavidin biotin reagents; DakoCytomation) was used. Rapidly, specimens were fixed for 10 min in cold acetone and incubated with the corresponding primary mouse mAb (CD163 (clone Ber-Mac3); DakoCytomation) for 15 min, followed by three washes in 0.05 M Tris-HCl (pH 7.5). The slides were then incubated with rabbit anti-mouse Ig antiserum (DakoCytomation) for 15 min, washed in Tris-HCl, and incubated with streptavidin–alkaline phosphatase complex for 15 min. The Fuchs chromogen was added for 15 min, and the slides were then counterstained with hematoxylain (DakoCytomation) and 1% NH₄OH (Sigma-Aldrich) and mounted. Stained slides were observed by inverted microscopy (Nikon TE 300).

Visualization of Mf fusion

PBMC-isolated monocytes (2 × 10⁵ cells/cover slip) were incubated for 5 days with 10 μg/ml LM or ManLM, the slides were then washed twice with PBS, fixed with acetone, and permeabilized with 0.1% Triton X-100 in 0.1% BSA in PBS. The slides were then incubated with different markers: nuclei were stained with TOPRO-3 (Molecular Probes) and F-actin.
with rhodamin-phalloidin (Sigma-Aldrich). After the staining step, the slides were washed twice with PBS, prepared with fluorescent mounting medium (DakoCytomation), and analyzed with a Leica scanning confocal microscope equipped with an argon-krypton laser.

**Target preparation and hybridization**

**RNA extraction**

Total RNA was isolated from LM- and ManLAM-induced granulomas after 10 days of culture using a filter-based RNA isolation system (Ambion). Quality of total RNA was assessed by the 28S:18S RNA ratios, checked on an Agilent 2100 Bioanalyser (Agilent Technologies).

Cy3- and Cy5-labeled antisense RNA (aRNA)

RNAs were labeled using an amplification protocol, following the manufacturer’s recommended protocol (MessageAmp II aRNA Amplification kit; Ambion). Cy3- and Cy5-labeled aRNA were fragmented with the Ambion aRNA fragmentation reagents, purified, and hybridized in Agilent hybridization buffer in an oven at 62°C for 16 h. Microarrays were washed and scanned with a Genepix scanner (Axon Instruments, Molecular Devices).

**Probe design and microarray printing**

Experiments were performed using a home-made microarray consisting of 1,696 distinct oligonucleotide probes. Genes were selected based on their relevance in inflammation, apoptosis healing, cell cycle and differentiation, and cytoskeleton, according to bioinformatics criteria explained by Le Brigand et al. (46). The final list of the 1,696 probes spotted on the microarray is available on http://www.microarray.fr (follow the link to “local purpose,” all probes (ranging from 50 to 52 bases) were diluted to a final concentration of 35–50 μM in 35% DMSO, 100 mM potassium phosphate (pH 8.0). Microarrays were printed with a ChipWriterProarrayer (Bio-Rad) on commercial HydroGel slides (Schott) and processed according to the manufacturer’s instructions. Each oligonucleotide was spotted four times on each slide (two distinct pairs of spots), to reduce positional bias of the fluorescence readout.

**Microarray analysis**

TIF images containing the data from each fluorescence channel were quantified with the Genepix Pro 6.0 program (Axon Instruments). At least two independent experiments were performed. Normalizations were performed using the limma package available from Bioconductor (http://www.bioconductor.org) (26). Background was evaluated according to Kooperberg et al. (27). Interslide normalization was performed using the scale method. Differentially expressed mRNAs were identified either by the limma package from Bioconductor (28), or by ANOVA using the GeneANOVA software (29).

**Results**

**Analysis of the cellular aggregation around lipoglycan-coated beads**

To assess the capacity of the various *M. tuberculosis* glycolipids (PI, LM, and ManLAM) to induce cellular aggregation, lipids were first coated to polystyrene beads. The respective binding properties of these lipids was then checked and compared. For this purpose, *M. tuberculosis* was grown in the presence of 14C-acetate. After purification, radiolabeled PIMs, LM, and ManLAM were quantified and coated to polystyrene beads. Analysis of the amount of radioactivity remaining in the binding solution after the reaction in comparison to the one present for the binding reaction, confirmed that comparable amounts of each glycolipid was bound onto the beads and could thus be compared for their intrinsic activity (data not shown). A dose response was done to determine whether there could be a dose effect. This did not point out any dose effect for any of the compounds under study, and we thus decided to use a single dose for each glycolipid. Polystyrene beads coated with PIMs, LM, or ManLAM extracted from *M. tuberculosis* were incubated for 21 days with human PBMC from a healthy donor, and the cellular aggregation formed around the beads were analyzed by s.e.m. (Fig. 1). In this experiment, beads coated with PI were used as a negative control, whereas TDM was used as a positive control because this latter has been shown to induce any cellular recruitment. Thus, different envelope glycolipids appear to share an equivalent ability to induce cellular recruitment in vitro, independently of their structure, and more interestingly independently despite being either pro- (PIM, LM, TDM) or...
anti-inflammatory (ManLAM) molecules. These results prompted us to assess whether these molecules could also comparatively modulate granuloma cells functions.

**Modulation of granuloma cell maturation by M. tuberculosis glycolipids**

To investigate whether these glycolipids share a common ability to modulate the cellular differentiation and activity within the granulomatous structures, cells from cellular aggregates were collected at day 21 of the reaction and the different cell populations were analyzed. As shown in Fig. 2, MGC were found around PIM2-, at day 21 of the reaction and the different cell populations were ulomatous structures, cells from cellular aggregates were collected modulate the cellular differentiation and activity within the gran.

To investigate whether these glycolipids share a common ability to induce MGC formation to the same extent as PIMs in this in vitro experiment, isolated Mf were used instead of a granulomatous structure to shorten the time of reaction, thus avoiding eventual Ab processing by the cells, which would have impaired their blocking effect.

A representative picture showing the number of Mf aggregates for each stimulation condition is presented in Fig. 4A. Many cell aggregates were formed with the LM- or PIM2-stimulated Mf, whereas the 19-kDa hexameric peptide only poorly induced the formation of cell aggregates. No cellular aggregates were observed when Mf were incubated with either ManLAM or LPS, or in the control unstimulated (n.s.) cells. As shown in the lower panel, the stimulation of isolated Mf with LM or PIM in the presence of a blocking anti-TLR2 mAb completely abolished the formation of

**Table I. Cell proportions within glycolipids-induced granulomas**

<table>
<thead>
<tr>
<th></th>
<th>PIM2</th>
<th>PIM6</th>
<th>LM</th>
<th>ManLAM</th>
<th>AraLAM</th>
<th>TDM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>5.10^4 (±100)</td>
<td>2.5.10^4 (±100)</td>
<td>2.5.10^4 (±100)</td>
<td>3.10^4 (±200)</td>
<td>2.5.10^4 (±100)</td>
<td>3.10^4 (±200)</td>
</tr>
<tr>
<td>Phagocytes</td>
<td>5.10^4 (±100)</td>
<td>1.10^4 (±200)</td>
<td>1.10^4 (±200)</td>
<td>1.10^4 (±300)</td>
<td>1.10^4 (±200)</td>
<td>1.10^4 (±300)</td>
</tr>
<tr>
<td>Small giant cells (5&gt; nuclei&gt;7)</td>
<td>15 (±5)</td>
<td>15 (±5)</td>
<td>3 (±3)</td>
<td>0</td>
<td>0</td>
<td>13 (±5)</td>
</tr>
<tr>
<td>Large giant cells (~15 nuclei)</td>
<td>0</td>
<td>0</td>
<td>55 (±15)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Cells were collected from 50 granulomas for each condition and stained with May-Grünwald Giemsa, and cell proportions were estimated under a light microscope. The mean cell number per 100 granulomas corresponding to three parallel experiments is shown for each condition (±SD). The results are representative of five independent experiments performed on samples from five unrelated control individuals.

**LM-induced MGC formation is TLR2-dependent**

Because LM and PIMs have been shown to mediate cellular activation via the TLR2 receptor (20, 32, 33), we analyzed whether the MGC formation induced by LM and PIMs was dependent on TLR2. For this purpose, Mf were either kept nonstimulated (n.s.), or were incubated for 5 days with the various glycolipids (ManLAM, LM, PIM2), the 19-kDa hexameric peptide, or LPS. These compounds were used either alone, in the presence of anti-TLR2 blocking mAbs, or with the corresponding isotype control Ab. In this particular experiment, isolated Mf were used instead of a granulomatous structure to shorten the time of reaction, thus avoiding eventual Ab processing by the cells, which would have impaired their blocking effect.

**FIGURE 3.** Giant cell formation by LM is not species specific. A. A total of 10⁶ PBMCs were incubated for 21 days with polystyrene beads coated with LM from either *M. kansasii*, *M. chelonae*, or *M. smegmatis*. A. The cellular aggregates were then collected under a light microscope, prepared for s.e.m., and observed using a scanning electron microscope (S450 Hitachi). Scale, 50 μm. B. The aggregated cells were separated from the beads, plated, and stained with May-Grünwald Giemsa stain. A picture of a representative multinucleated cell is shown for every condition. Scale, 10 μm.
any cellular aggregates, thus supporting the essential role of TLR2 in mediating Mf aggregation.

A quantitative analysis of the different cell aggregates is presented in Table II. Approximately 200 Mf aggregates per well were found when Mf were stimulated with LM or PIM2, whereas only 8–10 aggregates were induced per well in the presence of the 19-kDa hexapeptide. No cellular aggregates could be found when Mf were incubated with either ManLAM or LPS, or remained unstimulated (n.s.).

Interestingly, LPS did not induce Mf aggregation, thus excluding a parallel mechanism mediated by TLR4. More surprisingly, the N-terminal hexameric peptide from the 19-kDa Ag of *M. lepraee* mediated cell activation via TLR2 (25), induced 20 times less aggregates, which were also usually smaller, than those induced by PIM2 or LM (Table II). Moreover, the discrepancy between the capacity of PIM, LM, and ManLAM to induce comparable granuloma formation, when incubated with Mf plus Ly (Fig. 1), and the different ability of these molecules to induce Mf aggregation in the absence of Ly (Fig. 4A), was an unexpected finding. It emphasizes the critical role of Ly in glycolipid-induced granuloma formation, and highlights the T cell-independent ability of LM and PIM to induce the first stages of granuloma formation (Mf aggregation and fusion).

Confocal microscopy analysis of a LM-induced Mf aggregate compared with an equivalent region from LM-activated Mf in the presence of anti-TLR2 mAbs (second image). Scale, 25 μm. The third image shows LM induced Mf in the presence of an isotype Ab. For confocal microscopy, Mf were stained with rhodamin-phalloidin (red, F-actin) and Topro 3 (blue, nuclei). Pictures are merged Z-series images.

Table II. Size and number of Mf aggregates

<table>
<thead>
<tr>
<th></th>
<th>LM</th>
<th>PIM2</th>
<th>ManLAM</th>
<th>19 kDa</th>
<th>LPS</th>
<th>LM + Anti-TLR2</th>
<th>PIM2 + Anti-TLR2</th>
<th>LM + Isotype Control</th>
<th>PIM2 + Isotype Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggregates/well</td>
<td>200 (±20)</td>
<td>170 (±30)</td>
<td>0</td>
<td>8 (±5)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>190 (±20)</td>
<td>165 (±30)</td>
</tr>
<tr>
<td>Small aggregates (50–200 cells)</td>
<td>20% (±2)</td>
<td>30% (±4)</td>
<td>100% (±2)</td>
<td>0%</td>
<td>80% (±2)</td>
<td>70% (±2)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mf obtained from 2 × 10⁶ PBMCs were activated with the corresponding molecules, either alone or in the presence of the corresponding Ab. Mf aggregate numbers and sizes were estimated under a light microscope. The mean aggregate number and aggregate size per well estimated in three parallel wells are shown for each condition (±SD). The results are representative of three independent experiments performed on samples from unrelated individuals.
Molecular mechanism of LM-induced Mf aggregation and fusion

PIMs, LM, and, to a lesser extent, ManLAM trigger cellular recruitment within granulomatous structures, yet only PIMs and LM, but not ManLAM, are able to induce the Mf fusion leading to the formation of MGC. We anticipated that a comparison of gene expression profiles between cells stimulated with either LM or ManLAM, at the precise time point when Mf start fusing to form MGCs, would help identifying, among all the genes differentially regulated by LM, the ones specifically involved in the Mf fusion process. Therefore, PBMCs were activated with either LM- or ManLAM-coated beads, and cells were recovered from the corresponding granulomas at day 10 of the reaction, which corresponds to the onset of the Mf fusion time point. Total RNA were extracted and comparatively analyzed on a dedicated glass slide DNA microarray containing 1,696 oligonucleotides. These probes were selected against human transcripts encoding proteins involved in Mf and Ly activation pathways. Fig. 5 summarizes the results from two independent experiments performed with PBMCs from two

FIGURE 5. Differential gene expression of LM-induced granuloma cells. Twenty-three genes from the 1,696 probes were found to be differentially up-regulated by LM as compared with ManLAM at day 10 of granuloma formation. The 23 genes are grouped by functional families, with a special emphasize on orange lanes for known LM-induced proinflammatory molecules, blue for the two genes belonging to the cell-fusion machinery, and yellow for genes involved in cell adhesion. A column corresponds to the logarithm (base 2) of the average fluorescence intensity for each probe. M column corresponds to the logarithm (base 2) of the LM:ManLAM ratio.

FIGURE 6. LM-induced Mf fusion is mediated by ADAM9 and ITGβ1. A total of 2 × 10^5 cells were either not stimulated (NS) or stimulated for 5 days with 10 μg/ml LM, without (LM) or with blocking Abs to either ITGβ1 or ADAM9 as indicated. A, Light microscopy global view of the corresponding wells. Scale, 200 μm. B, Confocal microscopy analysis of a Mf aggregate from the different conditions. Scale, 10 μm. For confocal microscopy, Mf were stained with rhodamin-phalloidin (red, F-actin) and Topro 3 (blue, nuclei). Pictures are merged Z-series images.
unrelated healthy donors, which were found to be similar for a third unrelated donor subsequently analyzed. Because the expression profile was established at the onset of Mf fusion, a large percentage of the cells were not yet involved in the fusion process. Due to that point, ratios between cells stimulated with LM vs cells stimulated with ManLAM are usually small. However, it was possible to establish a list of differentially expressed genes, which were selected due to their highly significant p value in the comparison between LM and ManLAM (Fig. 5). The list of genes differentially expressed in response to LM is organized by functional groups. The first group includes the IL-8 and IL-12-inducing factor genes (orange box), which is consistent with previous work demonstrating that LM are potent IL-8 and IL-12-inducing factors (19, 20). Genes from the second group (blue box) appear to be involved in cell fusion processes. It is noteworthy that the two corresponding molecules (ADAM9 and ITGβ1), which are up-regulated by LM have been previously described through the use of blocking mAbs, to be strictly necessary for homotypic cell fusion mediated by the fusion-regulatory-protein-1 (FRP-1/CD98) (34–36). ADAM9 has been directly reported to be necessary for the induction of Mf fusion in vitro (37). The ITGβ1 has also been reported to be strongly expressed by fusing Mf, but only weakly during Mf maturation, thus confirming its predominant role for the Mf fusion process (38). Interestingly, the third group contains genes known to be involved in cellular adhesion/aggregation processes (RAP1A/B, CD36, STAB1, ITGA4; yellow box), and are thus very likely involved in a preliminary stage to MGC formation. In the last group, genes are known to participate in general cell activation processes and/or inflammation, which are also differentially up-regulated in response to LM and not to ManLAM. Yet, to our knowledge, their eventual participation in the cell fusion process has not been established in any model.

**Table III. Effect of anti-ITGβ1 and anti-ADAM9 on LM-induced Mf aggregation**

<table>
<thead>
<tr>
<th>Aggregates/well</th>
<th>LM</th>
<th>LM + Anti-ITGβ1</th>
<th>LM + Anti-ADAM9</th>
<th>LM + Isotype Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small aggregates (50–200 cells)</td>
<td>200 (±20)</td>
<td>3 (±1)</td>
<td>40 (±1)</td>
<td>200 (±20)</td>
</tr>
<tr>
<td>Large aggregates (&gt;200 cells)</td>
<td>80% (±2)</td>
<td>5% (±5)</td>
<td>25% (±3)</td>
<td>80% (±2)</td>
</tr>
</tbody>
</table>

* Mf obtained from 2 × 10^6 PBMCs were incubated with LM, either alone or with the corresponding Ab. On day 5, the aggregate number and the respective proportions of small and large aggregates were estimated in three different wells for each condition. The mean values are presented (±SD).

**Discussion**

Since their first description in the lung by Ghon in the beginning of the last century, granulomatous structures are classically considered by pathologists as morphological correlates of pulmonary TB (4). Yet, despite a good knowledge of the structural organization of these multicellular structures (39), very little is known about the molecular and cellular machinery leading to mycobacterial containment. Granulomatous structures contain specific cell types, such as epithelioid Mf and MGC, which presumably play an important role in controlling infection. However, those particular cell populations still remain poorly characterized. One major reason for the lack of information concerning the function of these cells is due to the difficulty in isolating these cells from in vivo granulomas present in animals. In addition, human biopsy samples only consist of dead material, thus precluding any subsequent functional analysis.

Easier access to such cellular populations can be achieved by using in vitro models that enable the formation of inflammatory structures that mimic natural granulomas. Toward this end, we have recently developed an in vitro model that enables the analysis of the very early steps of granuloma formation, i.e., the Mf and Ly aggregation and the Mf differentiation into epithelioid cells or MGCs (23). Using this model, we herein describe that some of the major lipoglycans from *M. tuberculosis* envelope are responsible for the aggregation and fusion of granulomas Mf, and that this induction is mediated by the TLR2 and involves several partners of the regular cell fusion machinery.

The ability of mycobacterial lipids to induce granuloma formation was already shown several years ago in mice models. The TDM is known since the early 1970s to be able to trigger a transient granulomatous response in mice after i.v. injection in an oil emulsion (12). More recently, it was reported that this granulomatogenic activity was caused in mice by the secretion of various chemokines including IL-1, MCP-1, MIP-1α, and TNF-α, all known as potent granuloma-inducing factors (30). Finally, it was recently shown that trehalose-containing mycolates were the most bioactive peripheral cell wall lipids, which mediate an intense proinflammatory cytokine production and granuloma formation, in a TLR2- and TLR4-independent, but MyD88-dependent, manner, suggesting the possible participation of an IL-1R or other TLR-mediated pathways for TDM activation (13). Interestingly, in this study, Geisel et al. (13) also observed MGC in the periphery of PIM- and TDM-induced granulomatosus structures, indicating that cellular differentiation can also be triggered by isolated lipids. The ability to induce granulomatous responses is not limited to TDM, because PIMs injected into mice were also shown to trigger in vivo granuloma formation and to recruit NKT cells in the lesions (17). Interestingly, PIMs have also recently been shown to induce intense TNF-α secretion by primary Mf in a TLR2- and MyD88-dependent mechanism (33, 40).
In our human in vitro model, TDM also stimulated MGC formation within cellular aggregates. This granuloma induction activity of TDM was previously described as a TLR2- and TLR4-independent mechanism, yet the TLR/IL-1R-associated intracellular cofactors such as MyD88 were necessary for this activity, thus still pleading for a TLR/IL-1R-mediated phenomenon (13). The important role of TLR in immunity to mycobacteria has been extensively investigated with knockout mice. TLR2 and to a lesser extent TLR4 appear to be the major mediators of M. tuberculosis-mediated cellular activation. Even if both receptors do not appear to be necessary for resistance to mycobacterial infection (knockout mice being as susceptible as wild-type mice), their role in the recognition of mycobacterial Ags and the development of the immune response to mycobacteria is now demonstrated (for review, see Ref. 41).

In the experiments described in this study, it appears that the major M. tuberculosis cell-wall extractable lipopolysaccharides induce comparable levels of cellular aggregation and that TLR2-mediated MGC formation correlates with the extent of glycosylation of the lipids. Hence, this cellular aggregation process can presumably be abolished by the arabinan core contained in ManLAM and AraLAM, arabinan being proposed to mask the proinflammatory activity of LAM (18). It was recently proposed that binding of ManLAM to C-type lectin receptors, such as dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin, was mediated by direct contact with the mannose cap decorating the arabinan domain, which is presented at the periphery of ManLAM clusters (42). It is therefore tempting to speculate that the arabinan domain masks the capacity of ManLAM to bind to TLR2, presumably through steric hindrance, at the same time that it favors its binding to C-lectin type receptors.

In addition, LM from the different mycobacterial species (M. kansasii, M. chelonae, and M. smegmatis) share comparable MGC induction activities. Although they possess an overall similar structure characterized by a linear α1,6-linked Manp backbone, they present subtle structural differences, notably the presence of α1,2-linked Manp side chains in M. kansasii and M. smegmatis, and an α1,3-linked Manp unit in M. chelonae (24). Moreover, characterization of the mannan core of LM from M. kansasii also revealed the presence of 4-linked 5-deoxy-5-methylthio-xylofuranose residues that were described to substitute the arabinan moiety from M. tuberculosis LAM (43–45). Together, these results suggest that the structural differences between these LM molecules do not affect their ability to induce MGC formation and are pleading for the implication of the α1,6 mannosyl backbone.

This study shows for the first time that the induction of granuloma MGC by envelope lipoglycans is mediated by TLR2, although the cell machinery involved in the TLR2 signaling and leading to cell fusion remained to be identified. Microarray analysis of the genes differentially activated by LM, as compared with LAM, at the very starting point of MGC formation period within granulomas, has enabled the identification of 23 genes potentially involved in the fusion process. Among these genes, two of them (IL-8 and IL-12p35 transcription activator) have previously been proposed as potent LM- but not LAM-induced proinflammatory molecules thereby validating the microarray analysis. More interestingly, the analysis pointed out two other genes that were previously shown to be involved in homotypic cell fusion. The gene products, namely ADAM9 and ITG81, have been shown to be required for FRP-1/CD98-induced cell fusion, during the fusion of muscle cells into muscle fibers, or during virus-induced cell syncytia formation (36, 37). Altogether, our study proposes a novel link between the TLR2-dependent mycobacterial recognition pathway and an ubiquitous cell machinery dedicated to the cell fusion process.

In conclusion, we have identified the molecular machinery triggered by TLR2-inducing mycobacterial lipids, which mediates granuloma Mf fusion. Our results also suggest the existence of a common cell activation pathway possibly mediated by MyD88, which leads to MGC formation in response to either TDM or LM; although the definitive implication of MyD88 in a TDM-induced FRP-1/CD98-mediated cell fusion, which could definitely confirm the central role of MyD88 in mycobacterial glycolipid-induced MGC formation, remains to be established. A novel step toward the characterization of the host/mycobacteria interplay within granulomatous structures has been made. This will fully justify the extensive analysis of the cellular functions of MGC, to better determine their role and which advantage there is for the mycobacteria, or for the host, to promote their formation within granulomatous structures, is now justified. Future studies are needed to examine the respective functions of MGC with low number of nuclei as compared with MGC with large nuclei numbers, such as those found within human tuberculous granulomas, and their influence with regard to the global activity of granulomas for the control of mycobacterial infection.

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Disclosures

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