The Adjuvants Aluminum Hydroxide and MF59 Induce Monocyte and Granulocyte Chemoattractants and Enhance Monocyte Differentiation toward Dendritic Cells

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Aluminum hydroxide (alum) and the oil-in-water emulsion MF59 are widely used, safe and effective adjuvants, yet their mechanism of action is poorly understood. We assessed the effects of alum and MF59 on human immune cells and found that both induce secretion of chemokines, such as CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), and CXCL8 (IL-8), all involved in cell recruitment from blood into peripheral tissue. Alum appears to act mainly on macrophages and monocytes, whereas MF59 additionally targets granulocytes. Accordingly, monocytes and granulocytes migrate toward MF59-conditioned culture supernatants. In monocytes, both adjuvants lead to increased endocytosis, enhanced surface expression of MHC class II and CD86, and down-regulation of the monocyte marker CD14, which are all phenotypic changes consistent with a differentiation toward dendritic cells (DCs). When monocyte differentiation into DCs is induced by addition of cytokines, these adjuvants enhanced the acquisition of a mature DC phenotype and lead to an earlier and higher expression of MHC class II and CD86. In addition, MF59 induces further up-regulation of the maturation marker CD83 and the lymph node-homing receptor CCR7 on differentiating monocytes. Alum induces a similar but not identical pattern that clearly differs from the response to LPS. This model suggests a common adjuvant mechanism that is distinct from that mediated by danger signals. We conclude that during vaccination, adjuvants such as MF59 may increase recruitment of immune cells into the injection site, accelerate and enhance monocyte differentiation into DCs, augment Ag uptake, and facilitate migration of DCs into tissue-draining lymph nodes to prime adaptive immune responses. The Journal of Immunology, 2008, 180: 5402–5412.

The use of vaccines is one of the most cost-effective public health measures, providing a huge impact on lowering disease burden and increasing life expectancy (1). The basic principle of most vaccines is to elicit an immune response that largely resembles that induced by real infections while avoiding unwanted side effects of infectious diseases. The desired result of vaccination or infection is immunity, that is, the induction of memory in the adaptive, Ag-specific arm of the immune response. Immunity is characterized by increased titers of specific Abs and memory in the adaptive, Ag-specific arm of the immune response.

It has been clear for a long time that both the magnitude and the quality of the adaptive immune response largely depend on the efficient induction of a more ancient system of immune surveillance, the innate arm of the immune system (2, 3). One of the central interfaces between the innate and the adaptive immune system is dendritic cells (DCs), which are considered sentinels for pathogen- or danger-related signals. DCs are activated by such signals and at the same time are the only cells fully equipped to prime naive T cells. Many of the conventional vaccines in use today are made of attenuated or killed pathogens, thus containing naturally a number of signals able to activate the innate immune response. However, a growing number of more recent vaccines is made of recombinant proteins devoid of such signals and therefore poorly immunogenic. To overcome this inadequacy, adjuvants are added to vaccine formulations whose role is to increase the immunogenicity of the Ag. Whereas a vast number of substances, including many TLR agonists, are under investigation for their effectiveness as adjuvants, the range of currently approved adjuvants comprised for a long time only two compounds, namely aluminum hydroxide (alum) or aluminum phosphate and the oil-in-water emulsion MF59 (4–7), with the recent addition of a detoxified form of LPS, the monophosphoryl lipid MPL (8). Preclinical and clinical testing and the use in millions of individuals in approved vaccines show that alum and MF59 are safe and well-tolerated adjuvants with the ability to enhance immune responses to a wide range of Ags (9, 10), but their mechanism of action is largely unknown. As for alum, numerous factors have been brought forward to explain its adjuvant effects, among which the depot effect (6), a local persistence of Ag at high concentrations instead of systemic dissipation, the stabilization of Ag by adsorption to alum, the increased uptake of alum-adsorbed Ag due its more particulate nature (11), and the activation of macrophages and other immune cells (12, 13). More recently, the Ab-inducing activity of alum was shown to be mediated by cells expressing Gr-1, which may be granulocytes or a monocyte subset (14). A corresponding cell type in humans may be CD14high CD16+ “inflammatory” monocytes (15).

Water-in-oil emulsions have been used in animals as Freund’s adjuvant for decades. MF59 is an oil-in-water emulsion and an...
approved adjuvant used so far on over 20 million individuals (9). Similar oil-in-water emulsions are candidate adjuvants currently tested in clinical trials. The mechanism of action of this adjuvant is poorly understood. In contrast to alum, the clearance kinetics of MF59 and Ag are very different, arguing against a major contribution of the depot effect to MF59 adjuvanticity (16). In mice, it has been shown that MF59 induces macrophage recruitment into the injection site, which is partially CCR2-dependent (17). Both macrophages and DCs loaded with MF59 are subsequently found in draining lymph nodes between 3 and 48 h after injection, and uptake of Ag together with MF59 was observed (18). Thus, it is clear that MF59 has an impact on the migration of macrophages and DCs in vivo, but the direct, primary effect and the target cell populations of MF59 are not clear. In particular, no studies on the effects on human immune cells exist.

In this study, we analyze the effects of MF59 on a number of human cell types and compare the effect to that of alum, another widely used adjuvant. We show that MF59 induces pure macrophages, monocytes, and granulocytes to secrete chemokines associated with cell recruitment but not proinflammatory cytokines. Alum induces a similar range of cytokines and appears to act mainly on macrophages and monocytes but not granulocytes. Cells that are unresponsive to MF59 include T, B, and NK cells, sorted myeloid DCs, monocyte-derived DCs, monocyte-depleted PBMCs, and epithelial or endothelial cell lines. In addition, monocytes are induced both by MF59 and alum to increase endocytosis, to up-regulate MHC class II and CD86, and to lose expression of the monocyte marker CD14, which are changes resembling the differentiation of monocytes into DCs. Thus, we identify macrophages, monocytes, and granulocytes as three target cell populations of MF59, and we show that alum and MF59 induce a similar but not identical pattern of phenotypical and chemokine responses in monocytes.

### Materials and Methods

#### Reagents

Human recombinant GM-CSF and IL-4 were purchased from GENTAUt and IL-1β, TNF-α, and IL-6 were from R&D Systems. PGE2 was purchased from Sigma-Aldrich. Ficoll-Paque Plus was purchased from GE Healthcare. Cell culture material is from Invitrogen Life Technologies and LPS (serotype R515) from Alexis Biochemicals.

**MF59 preparation**

MF59, an emulsion consisting of 5% squalene, 0.5% Tween 80, and 0.5% Span 85 (Sigma-Aldrich) in water, was prepared by homogenization at 12,000 psi with a Microfluidizer (model 110Y; Microfluidics). The emulsion was sterilized by passage through a polysulfone filter (0.22-μm pore size; Gelman Sciences) and stored at 4°C. The mean particle size of the emulsion droplets was determined with a Mastersizer X (Malvern Instruments) at 194 ± 76 nm.

#### Cell isolation and culture

Buffy coats were obtained from healthy blood donors upon informed consent and fractionated by Ficoll density centrifugation. The PBMC layer was recovered, washed three times with RPMI 1640 medium, and resuspended in complete medium (RPMI 1640 supplemented with 10% FCS [HyClone Laboratories] and 1% penicillin/streptomycin/glutamine). PBMCs were cultured in 96-well flat-bottom plates with DC medium (RPMI 1640 supplemented with 10% FCS [HyClone Laboratories], 1% penicillin/streptomycin/glutamine, and 100 ng/ml M-CSF) in 96-well plates at a density of 5 × 10^5 cells/ml. After 1 wk, cells were washed, placed in complete medium, and stimulated with MF59, alum, or LPS at varying doses and time spans.

Monocyte-derived DCs were obtained by culturing monocytes in DC medium (RPMI 1640 supplemented with 10% FCS [HyClone Laboratories], 1% nonessential amino acid supplement, 1% pyruvate, 1% Glutamax, 1000 U/ml IL-4, and 500 U/ml GM-CSF) in 75-cm² cell-culture dishes at a density of 5 × 10^5 cells/ml. At the end of the incubation period, cells were detached by gentle pipetting and cultured in complete medium in 96-well plates. For time course experiments of DC differentiation, the monocytes were directly cultured in 96-well flat-bottom plates with DC medium in the presence of MF59 (1/3, v/v), alum (15 μg/ml), or LPS (100 pg/ml). Where indicated, fluorescent latex beads (1 μm, Fluoresbrite YG Microspheres; Polysciences) were added to monocytes at a concentration of 5 beads/cell, and bead uptake by the cells was visualized by FACS. Cells were analyzed without further maturation as immature DCs (iDCs) or were matured by addition of an inflammatory mix (10 ng/ml IL-1β, IL-6, and TNF-α) and 1 μM PGE2 during the last 24 h of incubation.

Myeloid DCs were isolated from PBMCs by FACS sorting for lineage negative (CD3−, CD14−, CD19−, CD56+) CD11cpositive cells. Sorted cells were cultured in complete medium at a density of 1 × 10^5 cells/well.

To analyze cells from fresh total blood, venous blood was collected from healthy volunteers upon informed consent. RBCs were lysed with ACK lysis buffer (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA (pH 7.3)) for 5–10 min, and the remaining cells were washed and cultured in complete medium in 15-ml Falcon tubes.

Granulocytes were isolated from buffy coats by Ficoll centrifugation upon which the granulocyte fraction and erythrocytes separate in the bottom pellet. The cells were washed and resuspended with PBS and diluted with an equal volume of dextran solution (TS50; Dextrane). The solution was left for sedimentation at room temperature for 30 min, and supernatant was taken and centrifuged. Remaining RBCs in the cell pellet were lysed, and granulocytes were washed with PBS suspended in HBSS.

T cells for MLR were isolated from PBMCs by magnetic cell separation using the MACS Naive CD4+ T Cell Isolation kit (Miltenyi Biotec).

**MLR analysis**

APCs were placed in serial dilutions (1/3) into 96-well flat-bottom plates at a starting cell density of 4 × 10^5 cells/well. A total of 2 × 10^5 naïve CD4+ T cells were added per well, and cells were cocultured for 5 days. At 24 h before the end of the incubation period, 50 μl of culture supernatant was drawn for cytokine analysis, and the cell cultures were pulsed with 0.5 μCi of [3H]thymidine (Amersham Biosciences). The incorporation of [3H]thymidine in proliferating cells was measured by a liquid scintillation counter (Packard).

**Endocytosis assays**

PBMCs were cultured with MF59, alum, or LPS for 24 h. Phagocytosis of medium latex beads (1 μm, Fluoresbrite YG Microspheres; Polysciences) was analyzed after cocultivation with 10 beads/cell throughout the total cultivation period. Cells were stained for CD14 with anti-CD14-allophycocyanin, and the mean fluorescence intensity of CD14+ monocytes in the FITC channel (FL-1) was analyzed.

Endocytosis was assessed likewise by analysis of CD14+ monocytes in PBMCs cocultured with MF59, alum, or LPS together with the fluid phase dye Lucifer Yellow CH (Invitrogen Life Technologies) or Alexa Fluor 488-conjugated dextran for 24 h.

**Analysis of dead or apoptotic cells**

To identify dead or apoptotic cells at the end of the culture period, cells were stained with Annexin V-PE Apoptosis Detection kit I (BD Pharmingen), according to the manufacturer’s instructions. Apoptotic cells stain positive for Annexin V, whereas dead cells are Annexin V/7-aminoacti-

**Cytokine secretion**

Cell-free culture supernatants were collected and stored at −20°C until analysis. Cytokine secretion was measured by Bio-Plex analysis (Bio-Rad), according to manufacturer’s instructions using the human 27-plex panel. The following soluble proteins are assayed: IL-1β, IL-1ra, IL-2, IL-4, IL-10, IL-12p70, IL-13, IL-15, IL-17, eotaxin, basic fibroblast growth factor, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), platelet-derived growth factor-BB, RANTES (CCL5), TNF-α, and vascular endothelial growth factor.

**Flow cytometry**

Expression of surface markers was assessed using CellQuest analysis software on a FACSCalibur (BD Biosciences). For staining, cells were transferred into 96-well round-bottom plates and preincubated for 5 min with
PBS containing 20% rabbit serum to prevent nonspecific binding. Staining was performed for 30 min at 4°C using the following Abs: CD14-FITC, CD14-allophycocyanin, CD80-PE, CD86-allophycocyanin, HLA-DR-PerCP (all from BD Biosciences), CD71-FITC, CD1a-FITC, CD1a-PE, CD80-FTTC, CD83-PE (all from BD Pharmingen), receptors CCR2-PE (clone FAB 151P) and CCR7-PE (R&D Systems).

Cell culture supernatants or chemokine containing medium was placed in the lower chamber of a 96-well Transwell plate (pore size 5 μm; Millipore MultiScreen). Cells were added to the upper chamber at a density of 2 × 10^4 cells/well and allowed to transmigrate for 3 h. The number of transmigrated cells was assessed by FACS.

Results

Induction of cytokine and chemokine release by MF59 in macrophages, monocytes, and granulocytes

No direct effect of MF59 on human or mouse cells has been described so far. Therefore, we aimed to assess the potential activation of a variety of human cell types that reflect putative MF59 target cells. In the vaccination site, these target cell candidates include tissue-resident macrophages and DCs, endothelial and epithelial cells. As activation of immune cells is usually accompanied by the release of cytokines, we assessed a broad panel of cytokine tested in response to MF59. As for monocytes, granulocytes and macrophages, monocytes, and granulocytes do not produce any other cytokines, we assessed a broad panel of cytokines, chemokines, and growth factors in culture supernatants of putative target cells incubated for varying time periods with a range of concentrations of MF59. Effects were compared with alum, LPS, and medium alone. Monocyte-derived macrophages released a variety of different cytokines and chemokines upon stimulation with MF59. Fig. 1A shows that this response is both dose- and time-dependent. In contrast, MF59 did not induce cytokine release in endothelial and epithelial cells lines or in monocyte-derived DCs, which are models for tissue-resident DCs (data not shown and Fig. 1C). We conclude that macrophages are a major target of MF59 in the vaccination site. A similar set of cytokines was induced by alum (data not shown).

Surprisingly, the majority of the factors released by macrophages are chemotactants for granulocytes such as CXCL8 (IL-8) or for monocytes, iDCs, NK cells, and activated T cells like CCL2 (MCP-1), CCL3 (MIP-1α), or CCL4 (MIP-1β).

We hypothesized that these cells may be recruited to the vaccination site from the bloodstream and represent additional target cells to be activated by MF59. To test this hypothesis, we analyzed also the cytokine secretion of PBMCs, purified monocytes, or monocyte-depleted PBMCs in response to the different stimuli. Supernatants from MF59-treated PBMCs contain increased concentrations of the same chemotactants as those found for macrophages (Fig. 1B). Pure monocytes, but not monocyte-depleted PBMC cultures, are induced to produce these chemokines, indicating that the main if not only target cell populations of MF59 present within PBMCs are in fact monocytes. The induction of CCL2 was reconfirmed in expression profiling experiments in which the messenger for this cytokine was consistently found up-regulated (data not shown). Alum additionally induces CCL5 (RANTES), and more pronounced CCL3 (MIP-1α) and IL-1ra (Fig. 1B). In contrast, LPS induces, among others, a number of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α in endothelial and epithelial cells, macrophages, monocyte-derived DCs, and PBMCs. However, these were never found after incubation with MF59 (data not shown). To extend our observations to other cell types, we also assessed whole blood, granulocytes, and myeloid DCs sorted from PBMCs. CCL2 production was induced in cultures of whole blood and pure granulocytes, but not in myeloid DCs (Fig. 1C). Myeloid DCs did not produce any other cytokine tested in response to MF59. As for monocytes, granulocytes also appear more responsive to MF59 than alum regarding CCL2 secretion. RANTES is also induced by MF59 in whole blood culture (data not shown). Taken together, our data indicate that MF59 induces a range of chemotacticants but not classical proinflammatory cytokines and that macrophages, monocytes, and granulocytes represent their three principal target populations. Alum, in contrast, induces a wider range of chemokines, including proinflammatory cytokines like IL-1β (data not shown), and appears to act mainly on monocytes and macrophages and not on granulocytes.

MF59 induces phenotypic changes and increases endocytotic activity in monocytes

As already outlined, most of the cytokines induced by MF59 have been described as potent chemoattractants. Therefore, we assessed whether MF59-conditioned culture supernatants are in fact able to induce directed migration of monocytes or granulocytes. Fig. 2A shows that both cell types are attracted toward the chemokines present in cell culture supernatants of MF59-stimulated PBMCs. To analyze whether contact with MF59 would then induce additional changes in the recruited cells, total human PBMCs or purified monocytes were incubated with MF59 or control stimuli, and changes in surface marker expression were assessed. Fig. 2B shows that after incubation of PBMCs with the indicated agents, the granularity of cells within a monocyte gate (based on scatter parameters, cells painted in red) is greatly increased both by MF59 and by alum (Fig. 2B, top). By light microscopy, large granules were observed within alum and MF59-treated monocytes (data not shown). Gated cells up-regulate the costimulatory molecule CD86 (B7-2) and down-regulate the monocyte lineage marker CD14 (Fig. 2B, bottom). None of these changes were induced by LPS. Similar effects were found on pure monocytes (data not shown).

No other cell type present in PBMCs showed any phenotypic change (data not shown). In Fig. 2D, the dose-dependent changes of a wider panel of surface markers in response to MF59, alum, and LPS are shown. Besides the change in CD14 and CD86 expression, both MF59 and alum slightly increased expression levels of MHC class II (as revealed by an Ab specific to HLA-DR) on monocytes gated as described. These changes can already be seen at 24 h (data not shown) and are more pronounced at 48 h of incubation. LPS leads to a different profile of surface marker expression characterized by up-regulation of CD14, CD40, and CD80 and the down-regulation of MHC class II, which are effects that are even more pronounced at higher concentrations (data not shown). Effects that are common to all three compounds are the up-regulation of CD54 and CD71. Interestingly, all changes induced by MF59 and alum are consistent with changes described for monocyte differentiation into iDCs. To confirm that neither MF59 nor alum is toxic to cells in the concentrations tested, we assessed apoptosis and cell death at the end of a 48-h incubation period. No detrimental effects were observed, and instead, MF59 and LPS lead to an overall better survival of stimulated cells (data not shown).

We also observed that MF59 enhances all forms of endocytosis, namely pinocytosis, mannose receptor-mediated uptake, and phagocytic uptake, as measured by the percentage of fluorescent cells after endocytosis of fluid phase dye Lucifer Yellow, dextran-conjugated Alexa Fluor 488, and FITC latex beads, respectively (Fig. 2C). Due to the high fluorescence of FITC beads, uptake of a single bead is sufficient to render a cell positive. Therefore, mean fluorescence intensity was chosen as the adequate readout to quantify phagocytosis.

In conclusion, MF59 induces chemoattractants able to recruit monocytes and granulocytes and induces phenotypic changes in monocytes consistent with their differentiation toward DCs.
MF59 accelerates acquisition of a mature DC phenotype by monocytes

As described, MF59 induces phenotypic changes in monocytes that are compatible with differentiation toward iDCs. However, full maturation as measured, e.g., by CD83, is not achieved by MF59 alone within 2 days, which is the period that is permissive for monocyte cultivation without growth factors added. To assess the effect of MF59 on differentiation in a more extended culture period, we used a number of well-established systems of monocyte differentiation. First, we assessed the effect of MF59 on monocyte differentiation toward macrophages (in the presence of GM-CSF) or toward iDCs (by the combination of GM-CSF and IL-4). Although monocytes in GM-CSF alone do not up-regulate CD86 and retain a certain level of CD14 expression, addition of MF59 enhances loss of CD14 and CD86 up-regulation (Fig. 3A). At later time points of culture in GM-CSF alone, CD14 expression is regained, a hallmark of macrophage differentiation, whereas the presence of MF59 keeps CD14 levels low at all time points. Thus,
The phenotype of these cells more resembles that of iDCs generated in the presence of GM-CSF and IL-4, suggesting that MF59 alone can counteract macrophage differentiation. Compared to monocytes, iDCs are characterized by loss of CD14, gain of CD1a, and slightly elevated levels of MHC class II, CD86, and CD80. Addition of MF59 or alum during GM-CSF/IL-4 culture leads to earlier loss of CD14 and to earlier and stronger up-regulation of MHC class II and CD86 (Fig. 3, A and B, left panels). However, these cells differ in some aspects from canonical iDCs, as they acquire CD1a expression to a much lesser extent. In addition, alum induces CD71 expression already on iDCs.

As previously noted, even at early time points during the differentiation of monocytes into immature DCs, the addition of an inflammatory cytokine mix (TNF-α, IL-6, IL-1β, PGE₂) drives differentiation toward mature DCs (19). In our experiments, this mix was added during the last 24 h of culture for each time point. However, the total culture time is identical with that of the respective iDCs (Fig. 3B). Comparison between immature and mature canonical DCs (Fig. 3B, open symbols, left vs right panels) shows that maturation entails a further up-regulation of MHC class II, CD86, and CD80 and the induction of CD71 and CD83, the hallmark of mature DCs. It becomes clear that some of the changes induced by MF59 and alum on iDCs, such as high MHC class II and CD86 expression, contribute to a phenotype closer to that of mature DCs.

When we compare the phenotype of the resulting mature DCs, we observe considerable differences depending on the adjuvant added: mature DCs generated in the presence of either adjuvant express less CD1a and more CD71 and CD86 than canonical DCs, and in addition, alum strongly inhibits CD80 up-regulation. MF59-treated mature DCs, however, show normal levels of CD80 and increased expression of CD83, the marker of mature DCs. Strikingly, only MF59 is able to induce high levels of CCR7 specifically on mature DCs. CCR7 has been implicated in chemotactic migration of DCs from peripheral tissue to draining lymph nodes. Taken together, MF59 and alum accelerate the differentiation toward mature DCs, yet they induce cells with a phenotype that differs in some aspects from that of conventional mature DCs.

Chemokine production by DCs differentiated in the presence of adjuvants

We were interested to see whether the altered phenotype of DCs described is also reflected in a changed pattern of chemokines secreted by these cells. Monocytes were differentiated for different time periods with IL-4 and GM-CSF as described either in the absence or presence of MF59, alum, LPS, or beads (Fig. 4A, immature, left halves of panels). The matured DCs (Fig. 4A, right halves of panels) were generated by addition of the proinflammatory cytokine mix for the last 24 h of culture as described before. Whereas classical iDCs do not secrete CCL2 at any time point,
those differentiating in the presence of MF59 and alum produce high amounts of CCL2 from day 4 onward. A similar effect is seen for CCL4, but not for CCL3 or CCL5 (Fig. 4A). Maturation of MF59- and alum-treated iDCs suppresses CCL2 secretion, whereas CCL2 secretion in response to LPS is not suppressed by maturation. In contrast, CCL3, CCL4, and CCL5 production...
increases in mature DCs compared with iDCs. This effect is both accelerated and enhanced when cells are cultured in MF59 or alum. Importantly, the presence of latex beads during culture does not change the cytokine pattern observed, indicating that particle uptake alone is not a sufficient stimulus. The effects of LPS in this culture system appear to be in contrast to effects of MF59 and alum: LPS addition leads to an immediate production of CCL2 that is not suppressed by maturation, whereas production of the other chemokines by iDCs is induced very early but subsequently lost during culture. This inhibition of cytokine production at late time points cannot be overcome by maturation.

**FIGURE 4.** DCs differentiated in the presence of MF59, alum, or LPS secrete chemokines acting on monocytes, macrophages, or NK cells. Cells were incubated in medium containing GM-CSF and IL-4 to induce the differentiation toward DCs (G4). As described in Fig. 3, this differentiation was performed in the presence of MF59 (1/300, v/v) (G4-M), alum (13 μg/ml) (G4-A), or LPS (100 pg/ml) (G4-L) or differentiated in the presence of FITC latex beads (G4-B). Culture supernatants were taken on each day (from 2 d, open bars, extreme left to 7 d, black bars, extreme right of each group), and cytokine content was analyzed by multiplex analysis (27-plex). A, Comparison between immature and matured DCs cultured for the same total time period, but the inflammatory cytokine mix was added for the last 24 h of each time point of incubation. B, Additional panel of cytokines produced by immature DCs. Data shown are from one experiment representative for a total of two performed.

As DCs differentiated in the presence of MF59 show a clearly altered profile in surface marker expression and cytokine secretion, we asked whether this alteration is also reflected in functional assays. The observed changes could render these DCs more prone to prime naive T cells in draining lymph nodes. Because we observed MF59-dependent up-regulation of the lymph node homing receptor CCR7 on mature DCs, we tested whether these cells have an increased ability to migrate toward its ligands CCL19 and CCL21. Fig. 5A shows that only matured DCs respond to these ligands with chemotaxis and that this response is not significantly different in cells differentiated in the presence of MF59. We also found that the presence of MF59 cannot substitute for components of the inflammatory mix leading to mature migratory DCs (Fig. 5B). In summary, in the assays used in this experiment, we did not detect changes in the migratory ability of DCs as expected by the observed up-regulation of CCR7.

We also tested the ability of DCs to stimulate proliferation and skew cytokine production in naive T cells in a MLR. Fig. 5C shows that both iDCs and, more potently, mature DCs induce proliferation of naive CD4⁺ T cells. When comparing mature DCs differentiated in the presence of different stimuli, we found that they stimulated T cells with similar efficiency but with small differences found throughout all experiments: T cell proliferation induced by DCs generated in the presence of MF59 is consistently but not significantly higher than that induced by the other DC groups. Similarly, for all donors tested, the cytokine profile induced in naive CD4⁺ T cells is slightly but consistently skewed when activated by DCs generated in the presence or absence of MF59. Fig. 5C shows IFN-γ and IL-5 production, and comparable results were found for the other Th2 cytokines IL-4 and IL-13 (data not shown).
In conclusion, our data show that macrophages, monocytes, and granulocytes are target cells of MF59 and that CCL2 and other chemoattractants are induced by MF59 and alum, which is in line with our observation that immune cells like monocytes and granulocytes are efficiently attracted by MF59-conditioned cell supernatants. These adjuvants also enhance and accelerate differentiation of monocytes into DCs, key players during the onset of adaptive immunity. Although DCs generated in the presence of adjuvants show phenotypic differences from canonical DCs that should render them more prone to migrate to draining lymph nodes and prime naive T cells, we were not able to detect significant differences in our in vitro assays. The observed adjuvant effect of substances like MF59 and alum may therefore assist in inducing chemokine secretion in the vaccination site that in turn leads to a potent recruitment of additional immune cells to amplify the immune response to the vaccine Ag.

Discussion
To identify efficient adjuvants and to rationally design new and better ones, it is indispensable to know their target cells and the effects the adjuvants exert on them. Surprisingly little is known about the mechanism of action of the adjuvants currently in use in huge numbers of individuals. In this study, we identify macrophages, monocytes, and granulocytes as target cells of the widely used adjuvant MF59, whereas alum appears to act mainly on macrophages and monocytes but not on granulocytes. The principal effects of MF59 and alum on these cells are induction to secrete chemokines associated with cell recruitment, the increase of endocytosis in monocytes, and the acceleration and enhancement of monocyte differentiation toward DCs with their unique potential to prime naive T cells. The response pattern induced by MF59 and alum differs greatly from the proinflammatory response induced by most TLR agonists including LPS used in this study, suggesting a TLR-independent mechanism. Accordingly, a recent publication has shown in TLR-signaling-deficient mice that a range of classical adjuvants like alum appears to act independently of any of these signals (20).

Although it is conventionally assumed that adjuvants act on DCs, e.g., by providing danger signals, in this study we show that neither fresh myeloid DCs nor monocyte-derived DCs are very receptive to MF59 or alum. In contrast, these two adjuvants appear to intervene further upstream in the immune response, with an impact mainly on macrophages, monocytes, and granulocytes, on recruitment and on differentiation of precursors into DCs. Macrophages have been found in muscle tissue (21, 22) and likely represent one or the only target cells present at the injection site. All chemokines that we find induced by MF59 and alum are known to mediate the recruitment of immune cells from the blood into peripheral tissue. In particular, CCL2 is considered one of the key chemoattractants for monocytes into sites of inflammation (23). Previous findings in mice closely match our results as the MF59-induced macrophage recruitment into muscle is largely reduced in mice deficient for CCR2, the main receptor for CCL2 (17). In line with these results, we were able to show that MF59-conditioned culture supernatants potently induce chemotaxis of monocytes and granulocytes. As these cell types, in turn, can be activated by MF59 to produce the same set of chemoattractants, we suggest a chemotaxis-driven immune amplification loop leading to a much greater number of immune cells with a high phagocytic potential at the vaccination site, which will increase Ag uptake.
its transport to draining lymph nodes, and eventually the onset of adaptive immune responses. For the first time, we identify granulocytes as target cells of adjuvants, and it will be interesting to assess the contribution of this cell type to adjuvanticity in vivo.

Monocyte recruitment into peripheral tissue is followed by their rapid differentiation toward macrophages, important players in the innate response to fight invading pathogens, or toward DCs, which are crucial for mounting an efficient adaptive immune response. Differentiation of human monocytes into DCs was shown in vitro in a model of transendothelial trafficking in which monocytes give rise to fully blown DCs without addition of external factors within 1–2 days (24), which is a time frame similar to that found when GM-CSF and IL-4 are used for differentiation (19). The factors that are decisive in the differentiation of monocytes into macrophages or DCs are under intense investigation. Proinflammatory cytokines like IL-6 or danger signals like LPS or other TLR agonists were shown to drive monocyte-differentiation toward macrophages and block DC development (25–28). The macrophages generated in such conditions respond to strong inflammatory stimuli and amplify such signals by releasing cytokines like IL-1, IL-6, TNF-α, and IL-12 (2, 3, 29). Accordingly, a number of publications have shown that strong proinflammatory signals such as excessive doses of LPS or bacterial danger signals can be detrimental for the immune response, as DC development is impaired. Such LPS-exposed monocytes do not migrate to draining lymph nodes anymore, but stay in the inflamed site (30). The effects of MF59 and alum on monocyte differentiation were observed in four different experimental settings: First, in the absence of other stimuli, MF59 and alum induce phenotypic changes, that suggest that a differentiation program toward immature DCs is initiated. Second, during monocyte culture in GM-CSF, the presence of MF59 appears to block differentiation into macrophages, inducing instead a phenotype closer to immature DCs. Third, when monocyte differentiation into iDCs is induced by GM-CSF and IL-4, the presence of MF59 and alum accelerates and enhances this process. Fourth, as a consequence, when a cytokine mixture is used to trigger maturation of these iDCs, cells cultured in MF59 or alum show an earlier and overall higher production of chemokines such as CCL3, CCL4, and CCL5, and higher expression of surface markers such as CD83 and CCR7. In conclusion, the changes induced by MF59 and alum share common features, differ from those changes mediated by LPS, and tend in all culture systems to skew monocyte differentiation away from macrophages and more toward DCs. Accordingly, alum was described to induce already differentiated macrophages to acquire a mature DC-phenotype indicated by CD83 expression and loss of CD14 (13), which is in line with recent findings that also macrophages can differentiate into DCs (31, 32).

During differentiation, both the integration of different stimuli and the strength and duration of particular signals influence the phenotype of developing DCs (33), leading to either migratory or proinflammatory type DCs (34). Migratory DCs express a range of chemokine receptors including CCR7, migrate toward CCR7 ligands, and are more efficient in transporting Ag to lymph nodes for efficient presentation to T cells. In contrast, proinflammatory-like DCs secrete cytokines such as IL-10, IL-12, and others. With respect to these DC subsets, we find mixed phenotypes when DCs are matured in the presence of LPS or MF59: LPS-conditioned DCs do not produce IL-10, strongly up-regulate CCR7 but do not migrate more efficiently toward soluble CCR7 ligands. The latter findings are consistent with results showing that the expression level of CCR7 does not necessarily correlate with migratory ability in vitro (34).

However, apart from its role in DC migration, CCR7 has recently been shown to modulate a range of DC functions (reviewed in Ref. 35). One such function is the induction of dendritic protrusions with the function to capture T cells in the lymph node, thus contributing to their enhanced ability to prime T cells. We also observed that DCs generated in the presence of adjuvants but not of LPS secrete elevated amounts of T cell chemoattractants such as CCL3 and CCL4, potentially leading to an increased frequency of T cell–DC interaction (36).

We also tried to determine whether DCs generated in the presence of adjuvants are more efficient at stimulating T cells,
and whether activated T cells are biased toward Th1 or Th2 responses. For this purpose, we performed MLR with naive allogeneic CD4 T cells. We found that DCs generated in any of the conditions were similarly potent at inducing proliferation and cytokine secretion of naive allogeneic T cells, but small differences were found: in each of the four experiments performed, exposure to MF59 consistently led to DCs that stimulate a slightly higher T cell proliferation and secretion of slightly more IFN-γ and slightly less IL-5. This finding is in line with several reports showing that MF59 enhances the immune response without biasing toward Th1 or Th2 (37). The subtle differences we found are in line with the rather complex findings stated in the literature: while alum does enhance recall responses, it does not increase allogeneic T cell stimulation (13). Furthermore, emulsion adjuvants do enhance T cell stimulatory activity in some systems but not in others (38), and in none of these studies, Th1 or Th2 bias was assessed.

It has to be tested in vivo whether the single differences observed in adjuvant-exposed DCs act synergistically to generate more potent APCs in the more complex and competitive situation of an immune response. It will be interesting to see whether Ag-bearing immune cells arrive more efficiently in the draining lymph nodes when using adjuvants or to study whether kinetics of an APC–T cell interaction are altered as suggested by changes in CCR7 expression and CCL3/4 secretion.

In conclusion, we obtained the most solid in vitro results when looking at the induction of chemoattractants and the ability of immune cells to migrate toward these MF59-conditioned supernatants. Therefore, we conclude that during vaccination, adjuvants like MF59 or alum potentially augment the immune response through the mechanism illustrated (Fig. 6). Namely, tissue-resident macrophages are induced to secrete a mixture of chemokines. The chemoattractants produced act on monocytes and granulocytes, which were shown to migrate toward MF59-conditioned supernatants. These recruited target cells also produce the same panel of chemokines like MF59 or alum potentially augment the immune response through the mechanism illustrated (Fig. 6). Namely, tissue-resident macrophages are induced to secrete a mixture of chemokines. The chemoattractants produced act on monocytes and granulocytes, which were shown to migrate toward MF59-conditioned supernatants. These recruited target cells also produce the same panel of chemokines.

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Disclosures
All authors are employees of Novartis Vaccines. MF59 and alum are components of commercial Novartis vaccines.

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