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ACTIVATION OF INNATE IMMUNITY BY HUMAN VACCINE ADJUVANTS AT INJECTION SITE

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SUMMARY

Oil in water emulsions are potent human adjuvants used for effective pandemic influenza vaccines, however their mechanism of action is still unknown. By combining microarray and immunofluorescence analysis we monitored the effects of the adjuvants MF59 oil in water emulsion, CpG and alum in the mouse muscle. MF59 induced a time-dependent change in the expression of 891 genes, while CpG and alum regulated 387 and 312 genes respectively. All adjuvants modulated a common set of 168 genes and promoted antigen presenting cell recruitment. MF59 was the stronger inducer of cytokines, cytokine receptors, adhesion molecules involved in leukocyte migration and antigen presentation genes. In addition, MF59 triggered a more rapid influx of CD11b+ blood cells compared to other adjuvants. The early biomarkers selected by microarray, JunB and Ptx3, were used to identify skeletal muscle as a direct target of MF59. We propose that oil in water emulsions are the most efficient human vaccine adjuvants because they induce an early and strong immunocompetent environment at injection site by targeting muscle cells.

INTRODUCTION

The mammalian immune system is comprised of two arms: innate and adaptive immunity. The innate immunity is the first line of defense against pathogens providing non-specific microbial killing mediated by leukocytes and by the complement system. The adaptive immunity is involved in elimination of pathogens in the late phase of infection as well as the generation of immunological memory and is mediated by antigen specific lymphocytes (1). Two types of lymphocytes exist: B lymphocytes originate in the bone marrow and secrete antibodies; T lymphocytes originate in the thymus and can be divided into two subclasses; CD8, are cytotoxic T cells able to kill infected cells, CD4 T cells are required for the activation of specific B lymphocytes (helper function) and can also activate other cells like macrophages (effector function) (**Fig. 1**). There is also another class of lymphocytes called natural killer (NK) cells, these cells do not have specific antigen-receptor and so they belong to innate immune system (2).

Dendritic cells (DCs) are specialized leukocytes, which act at the interface between innate and adaptive immunity. During infection DCs are activated by pathogen derived structures and capture the antigen, migrate into the draining lymph nodes, where they activate specific T lymphocytes. This step is required for both humoral and cellular adaptive immune response and is the target of many compounds used to potentiate the response to vaccine formulations called vaccine adjuvants.

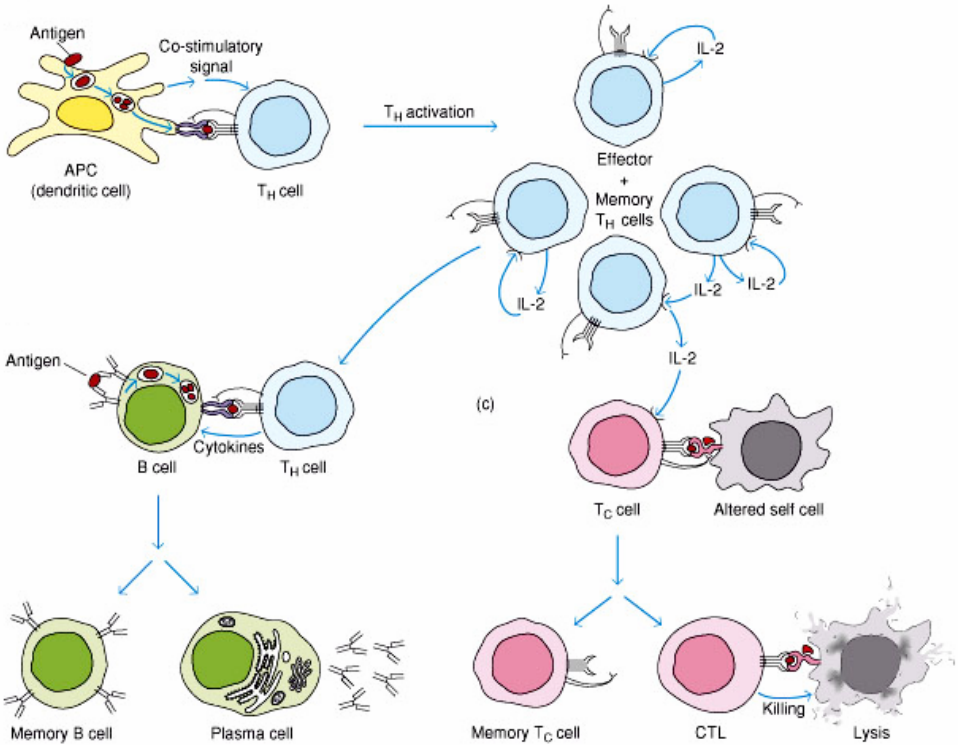

Figure 1 Immune system. Activation of T and B lymphocytes in response to an antigen

1. Activation of innate immune system

1.1 PRRs pathways

The innate immune system can recognize microorganisms directly via pattern-recognition receptors (PRRs) including toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs). PRRs, expressed constitutively in the host, recognize microbial components, known as pathogen associated molecular patterns (PAMPs) that are essential for the survival of the microorganism (3). TLRs sense extracellular signals and are expressed on various immune cells, including macrophages, DCs, B cells, specific type of T cells; even on non-immune cells such as fibroblasts and epithelial cells. TLRs can be divided into other subfamilies: TLR1, TLR2 and TLR6 recognize lipids; TLR3 recognizes double stranded (ds) RNA; TLR4 recognizes lipopolysaccharide (LPS); TLR5 recognizes flagellin; TLR7/8 and TLR9 recognize imidazoquinolines and unmethylated CpG oligonucleotides, respectively. All TLRs subfamilies are characterized by a leucin-rich-repeat (LRR) extracellular or luminal domain and a tail containing toll-interleukin 1 receptor (TIR) domains, that can

interact with downstream signaling molecules (4). The common adaptor protein myeloid differentiation primary-response gene 88 (MyD88) binds the TIR domain of all TLRs except TLR3. MyD88 triggers the activation of transcription nuclear factor-κB (NF- κB), the JNK and p38 kinases inducing the production of pro-inflammatory cytokines. In some cell subsets including DCs, MyD88 signaling also activates interferon regulatory factors inducing the production of type1 interferons α and β. TLR3 binds an alternative adaptor protein called TIR-domain-containing adaptor protein inducing type 1 interferons (TRIF), which triggers both NF- κB and IRF1 signaling pathways leading to production of proinflammatory cytokines and interferon type 1. TLR4 can stimulate both MyD88 and TRIF dependent pathways (1, 4), (**Fig. 2**).

PAMPs are also recognized by NOD-like receptors (NLRs) that sense signals from intracellular bacteria. NLRs have a C-terminal LRR domain, a central nucleotide-binding domain and N-terminal protein-protein interaction CARD (caspase activation and recruitment domain) and a pyrin domain. They include proteins such as NOD (nucleotide-oligomerization domain) 1 that senses *meso*-diaminopimelic acid (*meso*-DAP) in Gram-negative bacteria and NOD2, which detects muramyl dipeptide (MDP) from Gram-negative and Gram-positive bacteria. NALP (NACHT-, LRR- and pyrin domain-containing proteins) 3 is a general sensor for danger signals, molecules like uric acid crystals that alert the innate immune system and trigger defensive immune responses. NALP3 interacts with the CARD-domain-containing adaptor protein ASC and the protease caspase 1 to form a complex called inflammasome that, upon activation induces the release of activated IL1b and IL18. IPAF (ICE-protease activating factor) can also activate the inflammasome and is another protein included in NLRs family (4, 5).

PRR signaling results mainly in the activation of transcription factors such as NF-κB and IRFs (interferon regulatory factors), which provide the inflammatory context for the rapid activation of host defenses (6), (**Fig. 2**). PRR engagement on antigen presenting cells such as DCs plays a fundamental role in the establishment of an adaptive immune response. In fact, PRR like TLRs induces in DCs the up-regulation of co-stimulatory molecules and MHC class II and cell migration to the draining lymph nodes, where naïve T cells are primed.

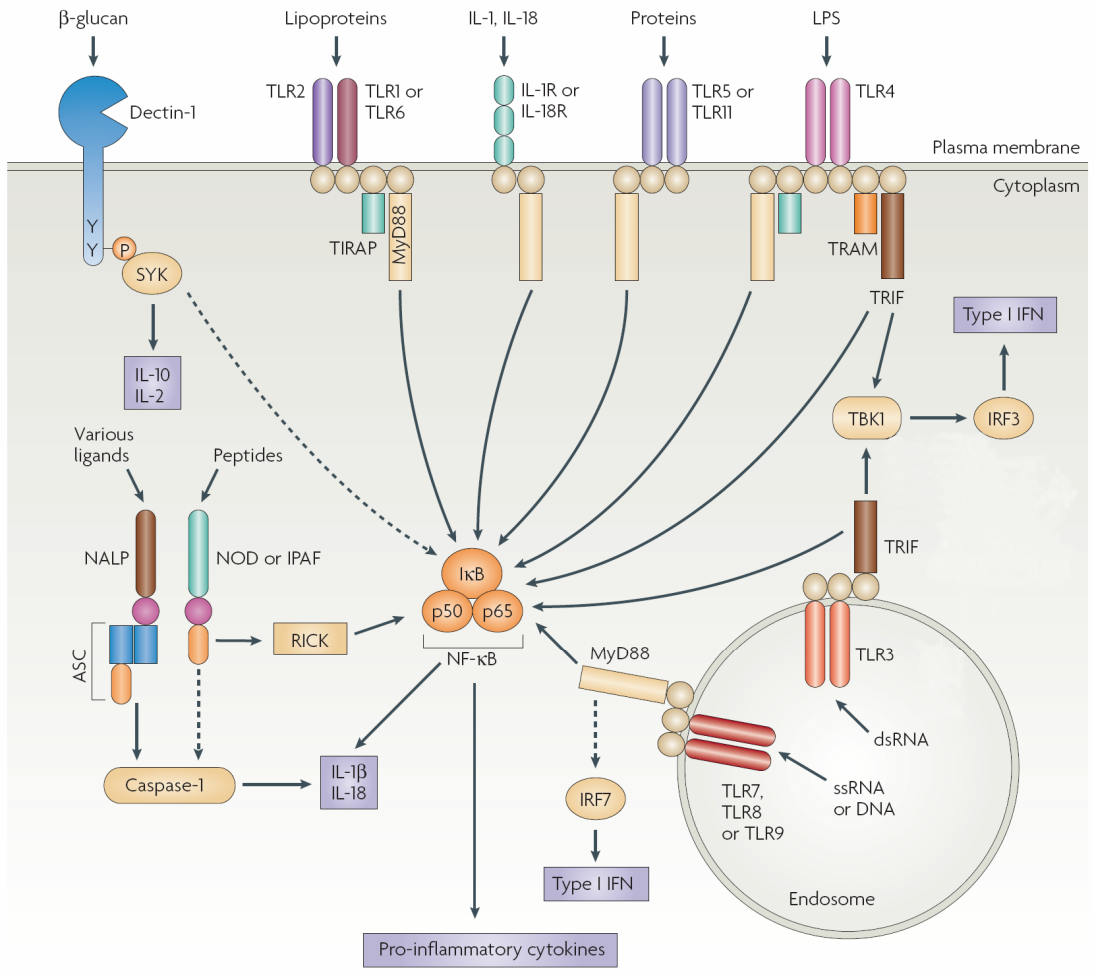

Figure 2 Signaling pathways of the PRRs families. Dectin-1 (a β-glucan receptor) is shown as an example of various cell-surface PRRs (adapted from (7)).

1.2 *Complement system*

Local inflammation and phagocytosis of bacteria can be also made by activation of the complement on the surface of bacterium. The complement system consists of plasmatic proteins that opsonize the pathogens and trigger a cascade of reactions on their surface that enable to be recognize by macrophages. These reactions release peptides that contribute to make an inflammatory environment.

There are three different ways to activate the complement system: *classical pathway* is activated through antibodies bound on the surface of microorganism; *mannan-binding lectin pathway* is activated by a lectin that binds mannose on the surface of the pathogens; the last way is the *alternative pathway* that begins when a complement

component binds the surface of a bacterium. All of these three pathways lead to activation of a protease C3 convertase that cleaves the inactive C3 component to produce the active component C3b; many molecules of C3b bind on the surface of microorganism, opsonize it and allow the binding of receptors of phagocytes. In addition complement can directly kill pathogens through deposition of the membrane attack complex (2).

2. Adaptive immune response

Peripheral lymphoid tissues are specialized not only to trap phagocytic cells that have ingested antigen but also to promote their interaction with lymphocites that are needed to initiate an adaptive immune response. The spleen and lymph nodes in particular are highly organized for the latter function.

Circulating mature T cells, that have not encountered their specific antigens are called naïve T cells; to participate in an adaptive immune response, a naïve T cell must first encounter an antigen presenting cell (APC) that presents to the T cell receptor (TCR) a specific epitope through specialized host-cell glycoproteins encoded in a large cluster of genes called major histocompatibility complex (MHC). After priming by APCs naïve T cells can proliferate and differentiate into effector or memory T cells. T cells fall into two classes, CD4 and CD8 that recognized peptide antigens derived from different types of pathogen. Peptides from intracellular pathogens that multiply in the cytoplasm are carried to the cell surface by MHC class I molecules and presented to CD8 T cells. These differentiate into cytotoxic T cells that kill infected target cells. Peptide antigens from pathogens multiplying in intracellular vesicles, and those derived from ingested extracellular bacteria and toxins, are carried to the cell surface by MHC class II molecules and presented to CD4 T cells. These can differentiate into two types of effector T cell, called T helper 1 (Th1) and T helper 2 (Th2). Pathogens that accumulate in large numbers inside macrophage and DC vesicles tend to stimulate the differentiation of Th1 cells, whereas extracellular antigens tend to stimulate the production of Th2 cells.

Effector T cells have only a limited life-span and, once antigen is removed, most of the antigen specific cells generated by the clonal expansion of small lymphocytes undergo

apoptosis. However, some APCs persist as memory cells, which ensure a more rapid and effective response on a second encounter with a pathogen and thereby provide lasting protective immunity.

Three cell types are able to process and present epitopes to CD4 naïve T cells trough MHC class II molecules. DCs, machrophages and B cells. DCs have an important role in the initiation of adaptive immune responses. Tissue DCs ingest antigen at site of infection and are activated as part of the innate immune response. This induces their migration to local lymphoid tissue and their maturation into cells that are highly effective at presenting antigen to recirculating T cells. These mature DCs are distinguished by surface molecules known as co-stimulatory molecules that synergized with T cell receptor signaling in the activation of naïve T cells. Macrophages mediate the innate immunity; they provide a first line of defense against infection, can also be activated to express co-stimulatory and MHC class II molecules. B cells contribute to adaptive immunity by presenting peptides from antigens they have ingested and by secreting antibody.

Antibodies produced by B cells cause the destruction of extracellular microorganisms and prevent the spread of intracellular infections. Similarly to T cells, B cells that have not encountered the antigen are called naïve B cells. B cells activation requires both binding of the antigen by the B cell surface immunoglobulin, the B cell receptor (BCR) and interaction of B cell with antigen-specific CD4 helper T cells. The BCR delivers the antigen to intracellular sites where it is degraded and returned to the B cell surface as peptides bound to MHC class II molecules. Helper T cells stimulate the B cell, through the binding of CD40L on the T cell to CD40 on the B cell and by the directed release of cytokines. Helper T cells induce a B cell proliferation and direct the differentiation of the clonally expanded progeny of the naïve B cells into either antibody secreting plasma cells or memory B cells. Helper T cells also direct isotype switching of antibodies, leading to the production of antibody of various isotypes that can be distributed to various body compartments. IgM is produced early in the response and has major role in protecting against infection in the bloodstream, whereas more mature isotypes such as IgG diffuse into the tissues. IgG antibodies are usually of higher affinity and are found in blood and in extracellular fluid, where they can neutralize toxins, viruses and bacteria activating complement system. Multimeric IgA is produced in the lamina propria and transported across epithelial surfaces, whereas IgE is made in small amounts and binds to the surface of the mast cells (2).

2.1 Adaptive immune response to infection

The adaptive immune response is required for effective protection of the host against pathogenic microorganisms. The response of the innate immune system to pathogens helps initiate the adaptive immune response, as microbial infection leads to the activation of DCs. which transport the antigen of the pathogens to local lymphoid organs and then present it to the naïve T cells. T cells priming and the differentiation of effector T cells occur here on the surface of antigen loaded DCs, and effector T cells either leave the lymphoid organs to effect cell-mediated immunity in sites of infection in the tissues or remain in the lymphoid organs to participate in humoral immunity by activating B cells. The type of adaptive response to pathogen infection is determined by the differentiation of CD4 T cells into Th1 or Th2 cells. The mechanisms that control this step are influenced by cytokines induced by pathogens that are present during the initial proliferative phase of T cell activation. T cells initially stimulated in the presence of interleukin (IL) 12 and interferon (IFN) γ tend to develop into Th1 cells, in part because IFN-γ inhibits the proliferation of Th2 cells. As IL-12, produced by DCs and macrophages, and IFN-γ, produced by NK cells and CD8 T cells, predominate in the early phase of the response to viruses and some intracellular bacteria, CD4 T cell responses in these infections tend to be dominated by Th1 cells. By contrast, CD4 T cells activated in the presence if IL-4, especially in the presence also of IL-6, tend to differentiate into Th2 cells. This because IL-4 and IL-6 promote the differentiation of Th2 cells, and IL-4 or IL-10, either alone or together, can also inhibit the generation of Th1 cells.

Within the immune system, a series of anatomically distinct compartments can be distinguished, each of which is specially adapted to generate a response to pathogens present in a particular set of body tissues. Besides the lymphoid nodes and spleen where adaptive immunity is activated in response to antigens that have entered the tissues or spread into the blood, there is a second compartment of the adaptive immune system located near the surfaces where most pathogens invade, the mucosal immune system (MALT). The adaptive immune system of the mucosa associated lymphoid tissues differs from that of the rest of the peripheral lymphoid system in several aspects. The types and distribution of T cells differ; the major antibody type secreted across the epithelial cells is secretory polymeric IgA; while Th2 cells promote the production of

IgG1 (in mice) and IgE (in human and in mice) in subsequent humoral immune responses.

Protective immunity against reinfection is one of the most important consequences of adaptive immunity operating through the clonal selection of lymphocytes. Protective immunity depends not only on pre-formed antibody and effector T cells, but most importantly on the establishment of a population of lymphocytes that mediated long lived immunological memory. The capacity of these cells to respond rapidly to restimulation with the same antigen can be transferred to naïve recipients by primed B and T cells (2).

2.2 Adaptive immune response to vaccination

Probably the most important consequence of adaptive immune responses is the establishment of an immunological memory. Immunological memory is the ability of immune system to respond more rapidly and effectively to pathogens that have been encountered previously, and reflects the preexistence of a clonally expanded population of antigen-specific lymphocytes. In the normal course of an infection, a pathogen first proliferates to a level sufficient to elicit an adaptive immune response and then stimulates the production of antibodies and effector T cells that eliminate the pathogen from the body. Most of the effector T cells then die, and antibody levels gradually decline after the pathogen is eliminated, because the antigens that elicited the response are no longer present at the level needed to sustain it. Memory T and B cells remain, however, and maintain a heightened ability to mount a response to a recurrence of infection with the same pathogen (2).

Adaptive immunity to a specific infectious agent can be achieved in several ways. One early strategy was to deliberately cause a mild infection with the unmodified pathogen. Unfortunately, infections were not always mild. One of most successful invention of medicine in the modern immunology against infectious disease has come from vaccination. The general principles for a vaccine are that it must be safe and effective. A vaccine includes the antigen, against which adaptive immune responses are elicited; immunepotentiators because the stimulation of innate immune system has an important role in the evolution of the adaptive immune response, and delivery systems to ensure that the vaccine is delivered to the right place at the right time (6).

The most effective vaccines are based on attenuated live microorganisms, but these carry some risk and are potentially dangerous to immunosuppressed or immunodeficient individuals. The development of vaccines was based on killed organisms and, subsequently, purified components of organisms that would be as effective as live whole organisms. The particular requirements for successful vaccination vary according to the nature of the infecting organism. For extracellular organisms, antibody provides the most important adaptive mechanism of host defense, whereas for control of intracellular organisms, an effective CD8 T lymphocytes response is also essential. The ideal vaccination provides host defense at the point of entry of the infectious agent; stimulation of mucosal immunity is therefore an important goal of vaccination against those many organisms that enter through mucosal surface.

Immune responses to infectious agents usually involve antibodies directed at multiple epitopes and only some of these antibodies confer protection. An effective vaccine must induce an antibodies and T cells production against correct epitopes of the infectious agent. There are many requirements to obtain an effective vaccination. A vaccine must be safe, must be able to produce protective immunity in a very high proportion of the people to whom it is given, it must generate long-lived immunological memory. This means that both B and T cells must be primed by the vaccine. Finally, vaccines must be very cheap if they are administered to large populations.

Most antiviral vaccines currently in use consist of inactivated or lived attenuated viruses. Inactivated or killed viral vaccines consist of viruses treated so that they are unable to replicate. Live attenuated viral vaccines are generally far more potent, perhaps because they elicit a greater number of relevant effector mechanisms, including cytotoxic CD8 T cells: inactivated viruses can not produce proteins in the cytosol, so peptides from the viral antigens can not be presented by MHC class I molecules and thus CD8 T cells are not generated by these vaccines. Similar approaches are being used for bacterial vaccine development (2).

Unlike inactivated or attenuated pathogens, purified antigens are usually not immunogenic on their own and most vaccines require the addition of compounds that can increase and modulate their intrinsic immunogenicity; these substances are called adjuvants.

3. Vaccine adjuvants

Vaccine adjuvants are represented by different classes of compounds that display adjuvant activity in pre-clinical models; among them microbial products, mineral salts, emulsions, microparticles, nucleic acids, small molecules, saponins and liposomes, which exert their function by diverse and often poorly characterized mechanisms of action (4, 6, 8). However, only a few of them have been licensed for human use, while the vast majority failed due to an unacceptable safety profile (9). Based on recent findings a classification in two major functional groups, TLR-dependent and TLR-independent can be made (10, 11). TLR-dependent adjuvants act directly on DCs, inducing the up-regulation of cytokines, MHC class II and co-stimulatory molecules, and promoting DC migration to T cell area of the lymph node (8, 12). One example of TLR-dependent adjuvant is represented by non-methylated CpG oligonucleotide (CpG) used as vaccine adjuvant in both preclinical and clinical studies (13, 14). CpG acts through TLR9, expressed by human plasmacytoid DCs and B cells (3). Monophosphoryl lipid A (MPL), a derivative of the bacterial component LPS, is probably the most characterized human licensed adjuvant. MPL activates TLR4, a receptor expressed on APCs. Engagement of TLR4 promotes cytokine expression, antigen presentation and migration of APCs to T cell area of draining lymph nodes, allowing for an efficient priming of naïve T cells. Other TLR agonists, such as flagellin and poly (I:C) double stranded RNA induce a similar process and are validated vaccine adjuvant in pre-clinical models (9).

Among TLR-independent adjuvants, alum has been widely used in human vaccines for more than 70 years, while the squalene-based oil in water emulsion MF59 has been licensed for human use a decade ago. The molecular mechanism of action and the target cells of alum and MF59 are still unknown. Alum is employed in Diphteria, Tetanus, Pertussis and Hepatitis A and B vaccines; its adjuvanticity is associated with enhanced antibody responses. It has been proposed that alum acts through the formation of a depot because the adsorption to alum increases antigen availability at injection site inducing a gradual release and allowing an efficient uptake by APCs (15). Alum could also increase antigen uptake by DCs *in vitro*, further supporting an antigen delivery function (16). However, several studies suggested that, in addition to antigen delivery, alum might have immunostimulating activities *in vivo*. Alum intramuscular

administration resulted in cell recruitment events at injection site (17, 18). Another suggested mechanism of alum adjuvanticity includes the activation of complement cascade and the generation of a local inflammatory environment at injection site characterized by the recruitment of blood cells. Several *in vitro* studies have tried to identify the primary target cells of alum immunostimulating activity. Unlike TLR agonists, alum does not activate directly DCs *in vitro*, but acts on macrophages inducing their differentiation into DC-like cells with enhanced antigen presenting capacity. Monocytes purified from human peripheral blood mononuclear cells (PBMCs) after treatment with alum produced pro-inflammatory cytokines, up-regulated MHC class II, CD86 and CD83, and down-modulated CD14 expression, thus reflecting the acquisition of mature DC phenotype (19, 20). More recently it has been demonstrated that intraperitoneal injection of alum induced the recruitment of monocytes, which could uptake the vaccine antigen, migrate to the draining lymph nodes and differentiate into fully competent inflammatory DCs (21).

Similarly to alum, MF59 could promote antigen uptake by dendritic cells *in vivo* (22). After 48h of intramuscular injection, MF59 is internalized by cells expressing DEC-205 and MHC class II molecules, markers of activated DCs. Furthermore, at the same time point, MF59 co-administered antigen localized inside the DC vesicular organelles more efficiently than antigen administered alone. These data showed that after intramuscular injection, MF59 is internalized by APCs that migrate to the lymph node (23). Besides promoting antigen delivery, MF59 might also act as a local pro-inflammatory adjuvant. It was observed that MF59 intramuscular injection induces the influx of blood mononuclear cells expressing the surface markers CD11b and F4/80. (23). In addition, 2 days after injection, MF59 was shown to localize in the subcapsular sinus of draining lymph nodes in cells expressing the CD80 and CD86 co-stimulatory molecules, and the I-A^d, CD11c and CD11b markers. Probably, MF59 induces in the muscle an infiltration and activation of mature macrophages, which engulf the antigen and transport it to draining lymph nodes where they differentiate into DCs. Mononuclear cell recruitment was shown to be dependent on chemokine receptor 2 (CCR2) and its ligands, monocyte chemoattractant proteins (CCL2, CCL7, CCL8, CCL12 and CCL13). However, other factors may also be involved, since influx of $CD11b^+$ and $F4/80^+$ cells at injection site was not completely abolished in $CCR2^{-/-}$ mice compared to wild-type animals (23). These data are in agreement with recent *in vitro* results confirming that MF59 stimulates human macrophages, monocytes and granulocytes to release monocyte and granulocyte

attracting chemokines like CCL2, CCL3 and CCL4 and CXCL8 (Seubert et al., 2008). In addition, flow cytometric analysis of human PBMCs, showed that MF59 induces the differentiation of monocytes towards DCs as revealed by the up-regulation of the costimulatory molecule CD86 and the down-regulation of the monocyte marker CD14. MF59 also enhanced CD86 up-regulation and CD14 loss in pure monocyte cultures supplemented with GM-CSF and IL4, factors which induce the differentiation of monocytes into immature DCs.

Several mouse studies reported that MF59 enhances immunogenicity of soluble antigens better than alum and CpG. It has been demonstrated that in Balb/c mice, after intramuscular administration, MF59 induced a greater IgG titer than CpG alone while the combination of both provided significantly greater titers than either CpG or MF59. CpG is a potent adjuvant for the induction of Th1 response as consequence that induces an increase of IgG2a antibody isotypes while MF59 induces an increase of IgG1 antibody isotypes and thus leads to Th2 response (24-27). Furthermore, recent clinical data have demonstrated that MF59 is safe and enhances human humoral and cellular immune responses to various antigens derived by different pathogens such as influenza virus, HSV and HIV. MF59-adjuvanted vaccine showed significantly increased antibody titers and enhanced cross-reactivity compared to non-adjuvanted vaccine formulations. Pandemic flu vaccines formulated with oil in water emulsions induce superior sero-conversion and cross neutralization compared to non-adjuvanted vaccines or to vaccines formulated with alum (28-30). The adjuvanticity of alum and MF59 is modulated by the addition of CpG (24, 26). In particular, the addition of CpG to MF59 or alum induces a dramatic shift from a Th2 to a Th1 response in BALB/c mice (24, 27).

4. Aim of the project

To better understand the molecular mechanism of action of oil in water emulsions and their relative potency when compared to other adjuvants, we performed microarray analysis of the whole muscle injected with MF59, alum, CpG and with a combination of MF59 and CpG. Genes selected by microarray data analysis were used in immunofluorescence experiments to identify MF59 target cells and to monitor cell

recruitment events triggered by vaccine adjuvants at injection site. Finally, the systemic effects of all adjuvants tested were investigated by measuring cytokine concentration in the serum.

RESULTS

1. Differential modulation of gene expression at injection site by human vaccine adjuvants

To analyze the local effects on gene expression induced by MF59, alum, CpG and a combination of MF59 and CpG, mice quadriceps were injected with 50 μl of each adjuvant diluted in PBS and processed at 3, 6, 12, 24, 48 and 96 hours for whole mouse genome microarray analysis as described in materials and methods. The same volume of PBS was used as control. A total number of 1260 genes have been selected with an average log2 ratio $\geq |2|$ compared to untreated quadriceps, and a p-value ≤ 0.05 calculated on the three replicates of at least one time point. Among these genes, 79 were modulated by all adjuvants and by PBS. The injury produced by the needle and by the injection of a relatively large volume of liquid into the muscle might be responsible for regulating this group of transcripts, which included Ccl7, Timp1, Socs3, Mt1 and Mt2. All other genes selected with the threshold criteria described previously were regulated by at least one adjuvant but not by the injection of PBS and therefore were considered adjuvant-responsive genes. MF59 regulated a larger number of genes (891) compared to other adjuvants and among these 489 were MF59-selective. Alum regulated 312 genes and only 24 were alum-selective. CpG modulated 387 genes, of which 85 selective **(Fig. 3A)**.

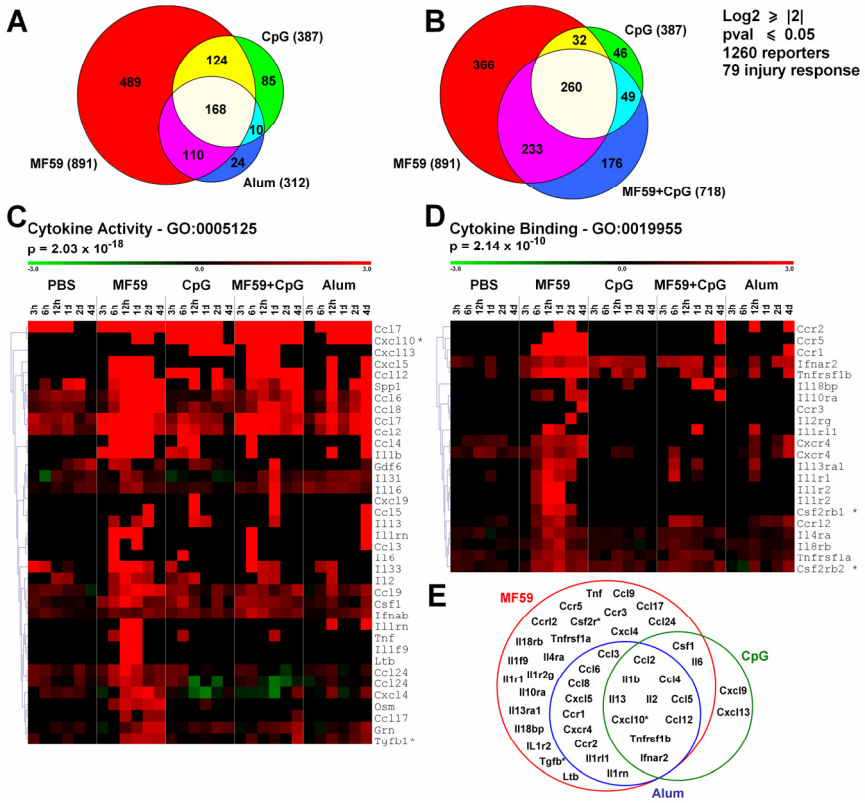

Figure 3 Microarray analysis of transcription profiles induced by vaccine adjuvants in mouse muscle. 1260 genes have been selected with an average log2 ratio ≥ |2| and a p-value ≤ 0.05 in at least one time point. 79 genes were modulated log2 ratio ≥ |2| by PBS and were considered injury response genes as indicated. (A, B) Venn-diagram showing the distribution of genes modulated by MF59, CpG and alum (A) or by MF59, CpG and MF59+CpG co-administered (B). In parenthesis the total number of genes modulated by each treatment is indicated. The area of each sector is proportional to the number of genes. (C, D) Cluster analysis of the expression profiles of genes encoding proteins with Cytokine Activity (C) and Cytokine Binding activity (D) after treatment with PBS, MF59, CpG, MF59+CpG and alum for the indicated times. Asterisks indicate genes not identified by Gene Ontology database and manually added to the cluster. Each column represents one time point. Each row represents the average kinetic of expression of one gene. Some genes, such as Ccl24, appear more than once in the cluster because they are represented by multiple unrelated probes in the Agilent 44k whole mouse genome array. The expression values are shown as log2 ratio. Color scale ranges from -3 (green, down-regulation) to 3 (red, up-regulation). (E) Venn-diagram showing the responsiveness of cytokines and cytokine receptor genes to MF59, CpG and alum.

Interestingly, 168 genes were responsive to all adjuvants, therefore they were defined as "adjuvant core response genes". Functional analysis of this group of genes identified three categories that were significantly enriched: "cytokine-cytokine receptor interaction" (KEGG database; p-value = 0.00127), "host-pathogen interaction" (GO:0030383, p-value= 1.07 x 10^{-18}) and "defense immunity protein activity" (GO: 0003793, p-value=9.58 x 10^{-4}). We also identified 19 genes related to type 1 interferon response **(Table 1)**.

Table 1. Core response gene list.

Gene Symbol						
Cytokines	**Interferon Response**	**Others**				
Ccl12	Mx2	1700019J19Rik	Chit1	H28	Ms4a6d	Rsad2
Ccl2	Psmb8	1700055M20Rik	Chrm1	H28	Ms4a7	Rtp4
Ccl4	Stat2	3110003A17Rik	Coro1a	Herc5	Msn	Saa3
Ccl5	Gvin1	4921505C17Rik	Cst12	Hils1	NAP007796-001	Shc2
Cxcl10	Ifi202b	4930482L21Rik	Ctss	Hp	NAP030542-1	Slc16a11
Il13	Ifi204	4930584F24Rik	Cybb	Hpse	Ncf2	Slfn10
Il1b	Ifi204	9230002F21Rik	D11Lgp2e	Hrbl	Ncf4	Slfn2
Il2	Ifi47	Adam8	D17H6S56E-5	Ighv1-56	Nkg7	Soat1
Immune cell receptors	Ifit2	AI451617	Ddx58	Ighv1-9	Olfr411	Sp110
Csf2rb1	Ifit3	AI607873	Dnahc2	Insl3	Olfr502	Spn
Klra18	Ifit3	Aif1	EG632412	Irg1	OTTMUSG00000000971	Tacr1
Lilrb3	Ifnar2	AK006628	EG665317	Isg15	Parp14	Tgtp
Lilrb3	Igtp	AK037592	Emr1	Itgb2	Pglyrp1	Tmem139
Lilrb4	Iigp1	Asb9	ENSMUST00000042610	Kiss1r	Phf11	Tor3a
Ltb4r1	Irf7	BC013712	ENSMUST00000051565	Lcp1	Plac8	Trim30
Msr1	Mx1	BC057170	ENSMUST00000094405	Lcp1	Plek	Trim30
Tnfrsf1b	Oas1f	Bcl2a1b	Fbxo39	LOC232875	Plek	Trim6
Fcer1g	Oasl1	BG067131	Fbxo8	LOC433772	Plekhq1	Tsga8
Fcer1g	Oasl2	C130026I21Rik	Fpr-rs2	LOC668139	Prm1	Tsp50
Fcgr1	**Lectins**	Cadm3	Gbp1	LOC668139	Ptpn6	Tyrobp
Fcgr4	Clec4a1	Casp1	Gbp3	Lrrc50	Ptprc	Ube1l
Hrh2	Clec4a2	Cd52	Gbp4	Lst1	Reg4	Ucp1
	Clec4a2	Cd53	Gmfg	M60510	Retnlg	Usp18
	Clec4a3	Cfb	Gucy2e	Marcksl1	Rffl	
	Lgals3bp	Chi3l3	Gyk	Ms4a6d	Rnf213	

As expected, the majority (542) of the genes regulated by co-administered MF59 and CpG were also modulated by MF59 or CpG alone. However, some genes (176), including interferon type I Ifnab, Stat6 and Il16, were regulated only when MF59 and CpG were co-administered **(Fig. 3B)**. Other interferon pathway genes responsive to both MF59 and CpG, such as Irf1, Irf7, Irf8, Stat1 and Stat2, were further up-regulated in the combination treatment **(Fig. 4)**. Interferons are natural glycoproteins produced by cells of the immune system in response to foreign agents involved in activation of NK cells and macrophages. They can be divided in two subsets: type 1 interferons (IFN-α and IFN-β) expressed in many cell types and induced by viral infection, inhibit with replication of viral RNA or DNA, promote presentation of viral antigens via MHC class II and promote CD4 T cell responses and antibody production (31). Type 2 interferon (IFN-γ) released by CD4 Th1 cells and CD8 cytotoxic cells down-modulates Th2 response and recruits leukocytes to the site of infection, resulting in increased inflammation (32).

We found that CpG regulated the expression profile of a large number of MF59-responsive genes. Indeed, 366 genes modulated by MF59 were not regulated by the combination treatment **(Fig. 3B)**. In particular, CpG inhibited the activation of many inflammatory genes including Tnf, Il1b, Ltb, Ccr1, Ccr3 and Il1r2 **(Fig. 3C and D)**.

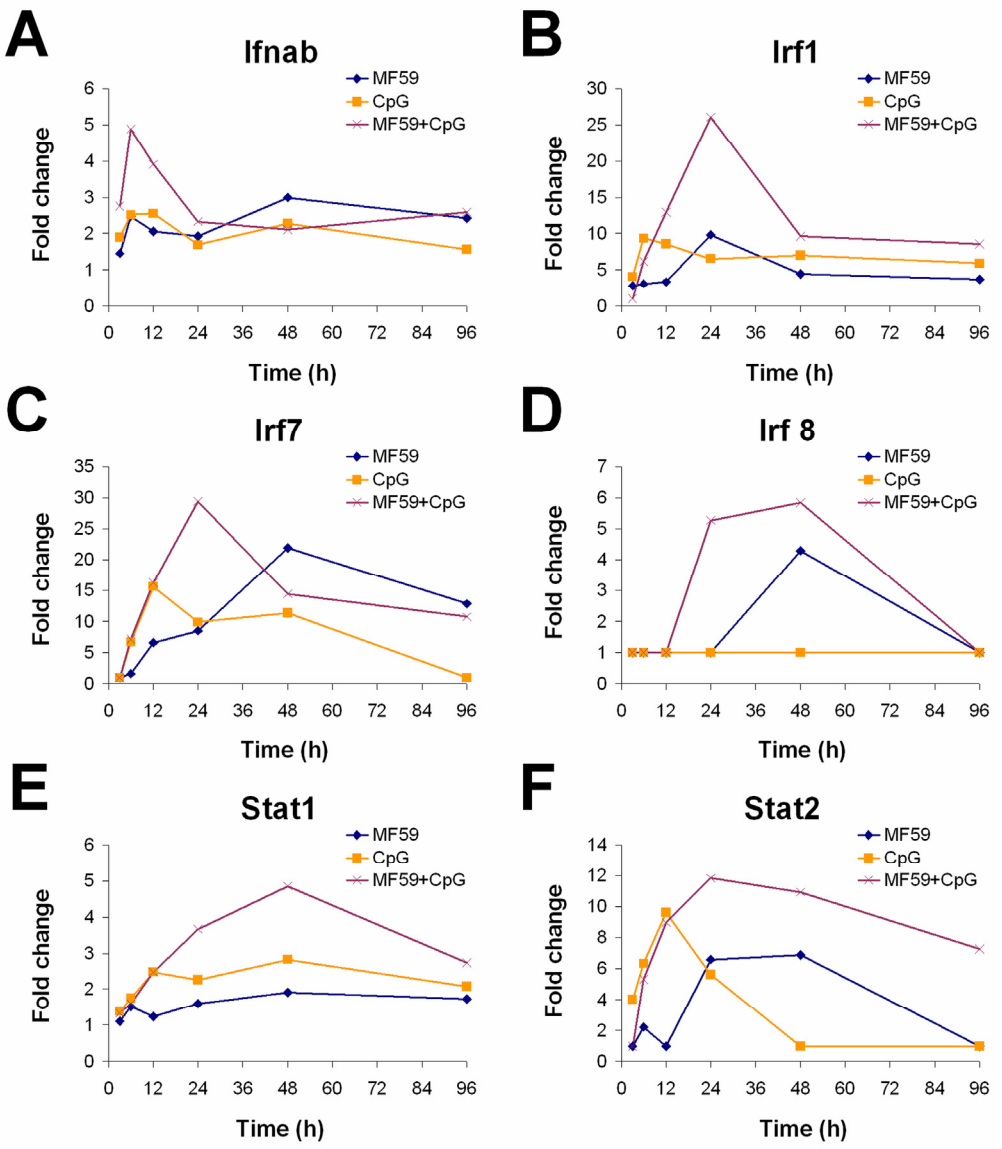

Figure 4 CpG and MF59 synergize in the activation of Interferon pathway genes. Expression profile of Ifnab, Irf1, Irf7, Irf8, Stat1 and Stat2 in response to administration of MF59, CpG and MF59+CpG as measured by microarray analysis.

2. MF59 activates multiple inflammatory and host defense pathways at injection site

All 1260 genes selected by microarray have been subjected to functional analysis using the Gene Ontology, KEGG and GenMapp databases. The genes belonging to the most significantly enriched categories have been clustered based on the expression profile. All adjuvants regulated the local expression of cytokines and cytokine receptors **(Fig. 3C, D and E)**. A group of cytokines (Ccl2, Ccl4, Ccl5, Ccl12, Cxcl10, Il1b and Il2) was up-regulated at early time points by MF59 and CpG, and later also by alum **(Fig. 3C and E)**. Several other cytokines, such as Tnf, Ccl17, Ccl24, Ltb and Tgfb1 were specific for MF59, Cxcl9 and Cxcl13 were specific for CpG, while we failed to detect cytokines specific for alum **(Fig. 3C and E)**. MF59 was a more potent inducer of chemokine receptors compared to CpG and alum, triggering the sequential up-regulation of Ccr1 and Cxcr4 (6 h); Ccr5 (12 h); Ccr2 (1 d) and Ccr3 (4 d) **(Fig. 3D)**. In addition, the receptors for Il1, Il2, Il4 and Il10 were induced selectively by MF59. Several transcription factors known to regulate cytokine expression like Irf1, Irf7, Stat1 and Stat2 were modulated by all adjuvants **(Fig. 5)**.

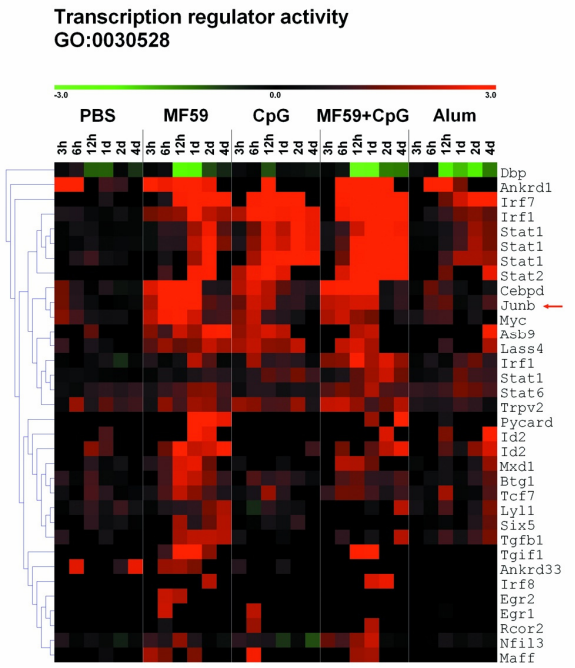

Figure 5 Adjuvant responsive genes with transcription activity. Cluster analysis of the expression profiles of genes with transcription regulator activity. Data are expressed as in Fig. 3. The arrow indicates JunB, selected for the immunofluorescence analysis shown in Fig.11.

By functional analysis we identified other significantly enriched gene clusters linked to inflammation, preferentially activated by MF59. In these clusters we identified genes involved in complement activation, prostaglandin synthesis, Il1 signaling and genes encoding matrix metalloproteinases. This class of proteins is involved in degradation of extracellular matrix promoting cellular extravasation. Matrix metalloproteases, are also known to be involved in the cleavage of cell surface receptors, the release of apoptotic ligands and chemokine activation **(Fig. 6)**.

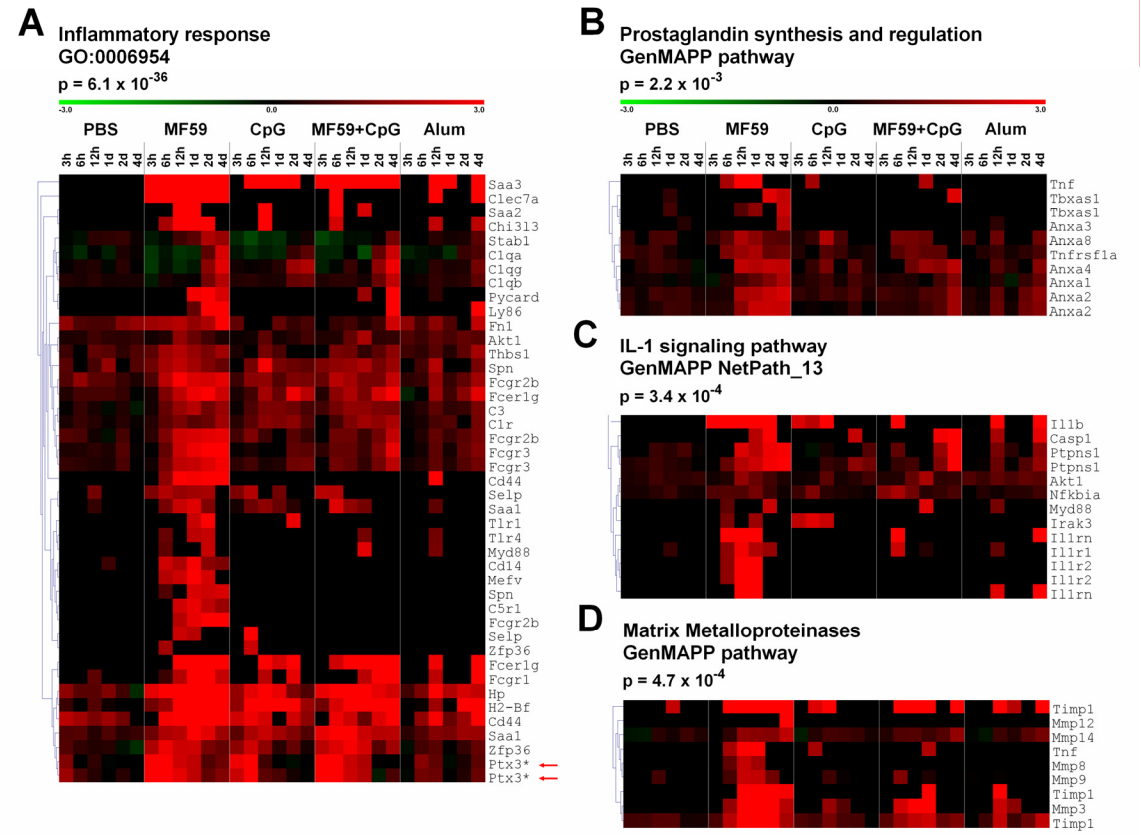

Figure 6 Adjuvant responsive genes involved in innate immune pathways. Cluster analysis of the expression profiles of genes encoding inflammatory response signals **(a),** prostaglandin synthesis and regulation factors **(b)**, IL-1 signaling pathway molecules **(c)** and matrix metalloproteinases genes **(d)**. Data are expressed as in Figure 1. The arrows indicate Ptx3, selected for the immunofluorescence analysis shown in Fig.10.

3. MF59 induces the recruitment of MHC class II+ and CD11b+ cells at injection site

The up-regulation of pro-inflammatory genes at the injection site suggests that vaccine adjuvants could also drive cell recruitment from the bloodstream into the muscle. This hypothesis was further supported by the significant enrichment of genes involved in leukocyte transendothelial migration. This cluster is composed mainly of integrins that are important for the recruitment of phagocytes in the site of infection **(Fig. 7)**. MF59 was the most potent inducer of genes involved in this cluster including Itgam, also know as CD11b, a marker expressed on cell surface of monocytes, macrophages, DCs and granulocytes. CD11b was up-regulated at high levels by MF59 already at 12 h. These results suggested a recruitment of cells at injection site.

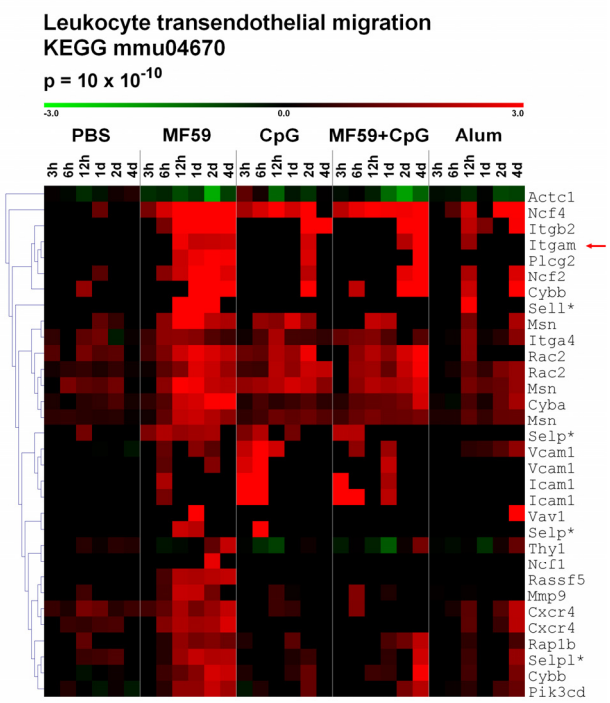

Figure 7 MF59 induces the expression of genes involved in leukocyte migration. Cluster analysis of the expression profiles of genes encoding leukocyte transendothelial migration factors. Data are expressed as in Fig. 3. The arrow indicates the gene selected for the immunofluorescence analysis shown in Fig. 8 (Itgam/CD11b).

To verify this hypotesis, we monitored the recruitment of CD11b+ cells by immunofluorescence analysis of muscle cryosections after i.m. administration of PBS, MF59, CpG and alum using an anti CD11b antibody. The structure of the muscle was visualized using an antibody specific for utrophin, a cytoskeletal protein playing a role in anchoring the cytoskeleton to the plasma membrane and located in the sarcolemma of muscle cells. We found that, at 1 day after injection, only MF59 induced influx of CD11b+ cells into the muscle **(Fig. 8 *Left*)**. This finding is consistent with previous data obtained from muscle single cell suspension, which showed that at 1 day after injection MF59 induced a influx of mononuclear cells (23). All adjuvants induced the recruitment of CD11b+ cells with similar efficiency 4 days after injection **(Fig. 8 *Right*)**.

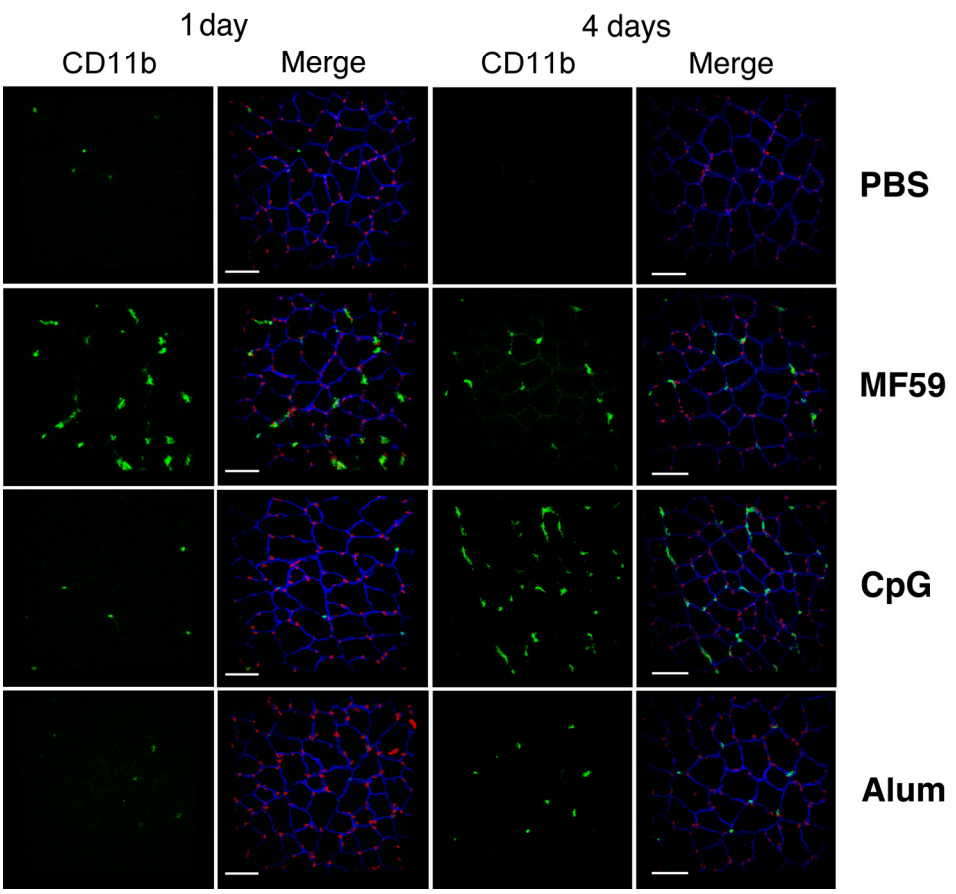

Figure 8 Analysis of CD11b+ cell recruitment at injection site by MF59, CpG and alum. Confocal microscopy analysis of muscles collected 1 or 4 days after treatment with PBS, MF59, CpG or alum and stained with anti-CD11b (green), anti-UTRN (blue) and the nuclear tracker ToPro3 (red). (*Left)*: CD11b+ stain. (*Right)*: merge images. (Scale bar, 60 μm.)

Another group of genes selected by the functional analysis of microarray data is involved in antigen processing and presentation **(Fig. 9A)**. Within this cluster, MHC class I genes (H2-Q, H2-K, H2-T, H2-D) were up-regulated by all adjuvants, even though with different kinetics: MF59 and CpG induced an up-regulation already at 6/12 h after treatment, while alum at 1/2 days. MHC class II genes, including H2-Aa, H2-Ea and H2-Eb1, were up-regulated by all adjuvants at 4 days. MF59 was a more potent inducer of MHC class II transcripts compared to CpG or alum. Interestingly, at earlier time points, CpG down-regulated MHC class II genes both when administered alone and in combination with MF59. Other genes involved in antigen processing and presentation like cathepsins, endosomal-lysosomal proteases that when are released into the cytoplasm start their proteolytic activity and contribute to NLRP3 inflammasome activation a member of NLRs family (33), and B2m, a protein associated with MHC class I heavy chain, were also up-regulated. The up-regulation of MHC class II genes might result either from activation of resident APCs or from APC recruitment driven by the local expression of chemoattractants and adhesion molecules. To monitor the APC recruitment events following adjuvant injection we performed immunofluorescence analysis of muscle cryosections, similar to that performed on CD11b, after i.m. administration of PBS, MF59, CpG and alum using an anti-MHC class II I-A/I-E antibody. However, at 4 days after injection, all vaccine adjuvants increased the local concentration of MHC class II+ cells in muscle tissue compared to the PBS control **(Fig. 9B)**. Interestingly, the kinetic of MHC class II+ cell recruitment was consistent with the MHC class II gene expression data **(Fig. 9A)**.

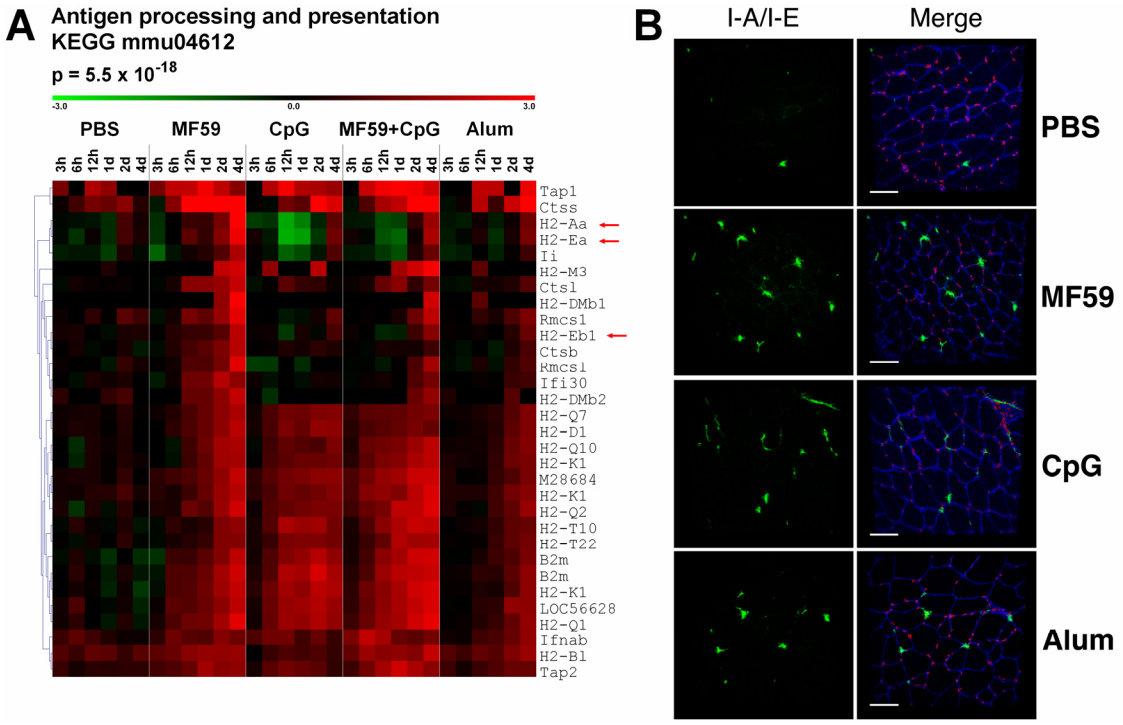

Figure 9 MHC class II+ cell recruitment at injection site by MF59, CpG and alum. (a) Cluster analysis of genes encoding antigen processing and presentation proteins. The arrows indicate the MHC class II genes. **(b)** Confocal analysis of muscles collected 4 days after treatment and stained with anti-MHC class II (anti-IA/IE) (green) and anti-Utrophin (anti-UTRN) (blue) antibodies and with the nuclear tracker propidium iodide (PI) (red). Left panels: IA/IE stain. Right panels: merge images. Scale bar, 60 μm.

4. MF59 activates the expression of the early biomarkers Ptx3 and JunB in muscle fibers

The data described above demonstrate that MF59 acts as a strong immune potentiator at injection site, however the target cell of MF59 immunostimulating activity is not known. In the attempt to identify MF59 target cell, Pentraxin3 (Ptx3) and JunB, induced by MF59 and CpG 3 h after treatment, were selected as biomarkers for immunofluorescence analysis on muscle cryosections.

The long pentraxin 3 (PTX3) is a member of a superfamily of proteins that are highly conserved during evolution. The classical short pentraxins, C-reactive protein (CRP) and serum amyloid P component (SAP), produced in the liver, are structurally related but distinct from long pentraxins. They share the capacity to bind C1q, the recognition subunit of the classical component pathway (34).

The long Pentraxin 3 (Ptx3) is a soluble pattern recognition receptor that recognizes pathogens such as *Aspergillus fumigatus*, *Salmonella typhimurium* and *Pseudomonas aeruginosa* facilitating the interaction with mononuclear phagocytes and DCs. It is rapidly produced and released by several cell types, in particular mononuclear phagocytes, DCs, fibroblasts and endothelial cells, in response to primary inflammatory signals such as IL1β, TNFα, microbial components like LPS and other agonists for different members of the TLR family (35).

Expression profile **(Fig. 10A)** showed that MF59 and CpG but not alum or PBS control induced Ptx3 expression at early time points Consistent with microarray data, immunofluorescence analysis showed an increased expression of PTX3 in muscle fibers at 12 h in both MF59 and CpG-treated mice, while there was no significant difference between alum and control **(Fig. 10B)**.

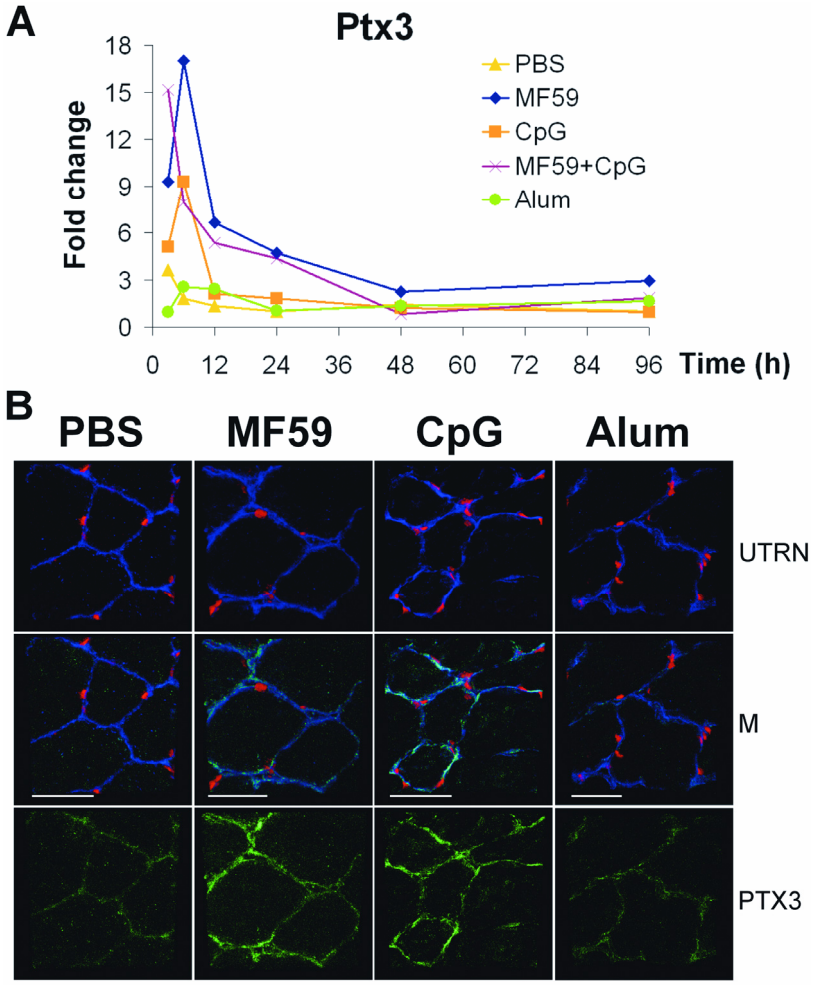

Figure 10 (A, B) MF59 and CpG up-regulate PTX3 in muscle fibers. (A) Microarray analysis of Ptx3 expression profile in MF59, CpG, alum, MF59+CpG or PBS-treated muscles after 3, 6, 12 h, 1, 2 and 4 days. Expression levels are shown in fold change compared to untreated muscles. (B) Confocal microscopy analysis of muscles collected 12 h after treatment with PBS, MF59, CpG or alum and stained with anti-PTX3 (green), anti-UTRN (blue) and PI (red). M: merge. Scale bar, 40 µm.

Similar results were obtained using an antibody against JUNB; expression profile of JunB showed that MF59 induced a higher up-regulation than CpG, while alum or PBS did not induce any change in the expression of the gene **(Fig. 11A)**.The results of microarray reflected the up-regulation of the protein in the nuclei of skeletal muscle in response to MF59 and CpG detected by immunofluorescence analysis **(Fig. 11B)**.

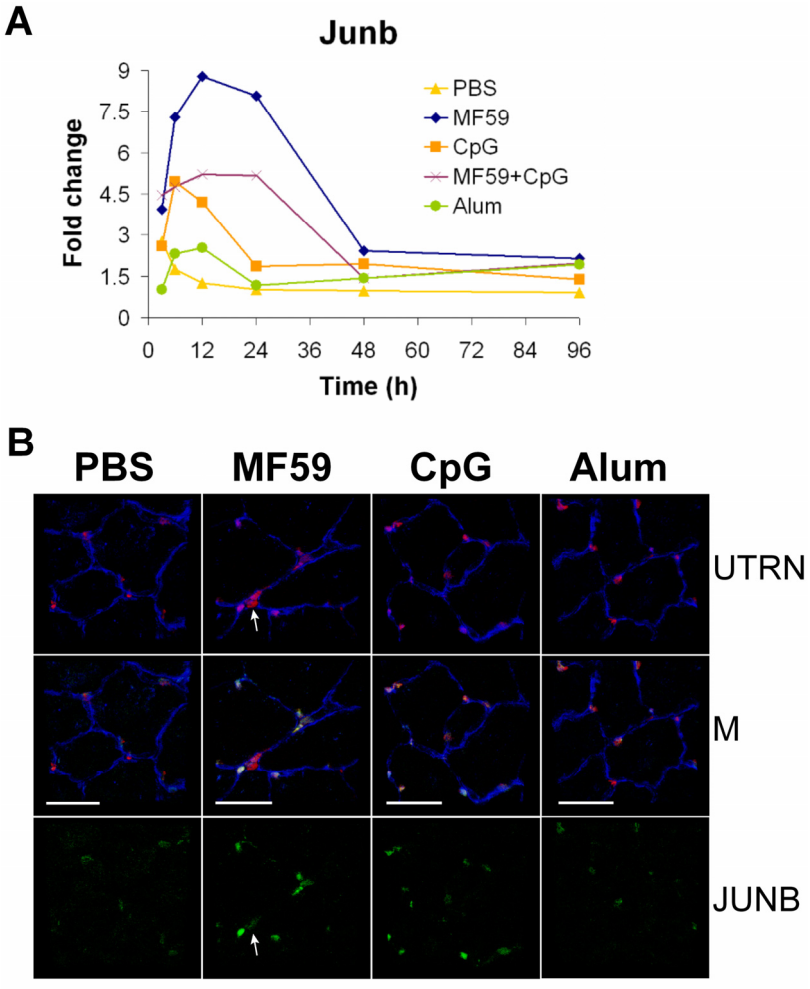

Figure 11 MF59 and CpG increase JUNB expression in muscle fiber nuclei. (A) Microarray analysis of JunB expression profile in MF59, CpG, alum, MF59+CpG or PBS-treated muscles after 3, 6, 12 h, 1, 2 and 4 days. Expression levels are shown in fold change compared to untreated muscles. (B) Confocal

microscopy analysis of muscles collected 12 h after treatment with PBS, MF59, CpG and alum and stained with anti-JUNB (green), anti-UTRN (blue) and PI (red). M: merge. Nuclei of cells external to the fibers are shown by the arrow. Scale bar, 40 µm.. In agreement with mRNA expression, MF59 and CpG induced an up-regulation of JUNB protein in the nuclei, while alum had no significant effect. The up-regulation of JUNB was specific for muscle fibers since no effect was detected on the nuclei of cells external to the muscle (see arrow).

The induction of early response proteins JUNB and PTX3 suggests that MF59 activated directly muscle fibers. To study the interaction of MF59 with muscle cells, we injected a DIO-labeled form of MF59. At 3 h, MF59 localized inside muscle fibers further supporting the hypothesis that MF59 directly targets the muscle **(Fig. 12A and B)**.

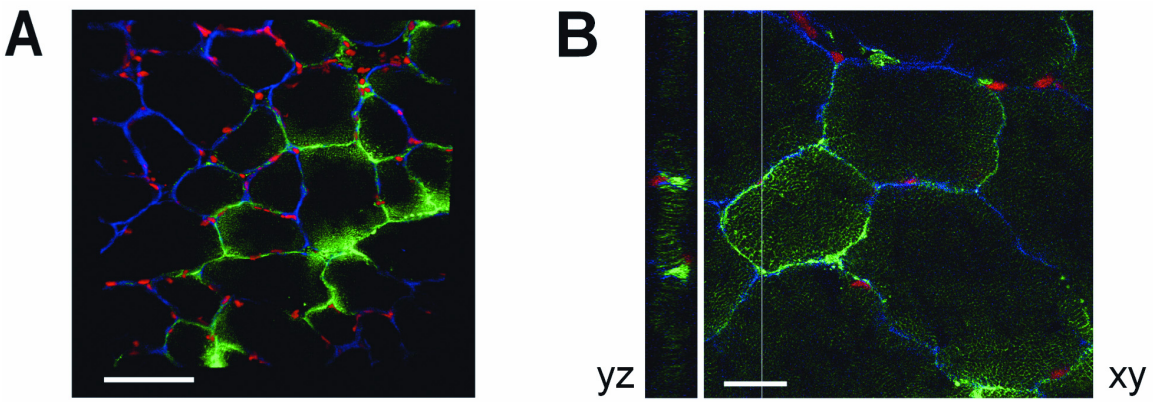

Figure 12 (A, B) MF59 enters muscle fibers at 3 h. Confocal microscopy analysis of muscles collected 3 h after injection of DIO-labeled MF59 (green) and stained with anti-UTRN (blue) and PI (red). (A) 40x magnification and 60 µm scale bar. (B) 100x magnification, 20 µm scale bar.

5. Systemic response to vaccine adjuvants

In order to dissect the local and systemic effects of vaccine adjuvants we collected the sera of the same mice used for microarray analysis and measured cytokines concentration by a multi-cytokine detection system (See Materials and Methods). CpG was the most potent inducer of a large number of cytokines including IL12(p40), CCL5, CCL2, CXCL1, while MF59 up-regulated IL5. Alum did not induce any of the tested cytokines **(Fig. 13)**.

The systemic expression of IL12(p40) and IL5 is in agreement with the Th1 and Th2 immune responses elicited by CpG and MF59 respectively in BALB/c mice (24, 27). In

addition, IL12p40 and IL5 mRNAs were not up-regulated at injection site, suggesting that the increase in cytokine levels in the serum derived from the activation of cells of the draining lymph nodes or from circulating blood cells.

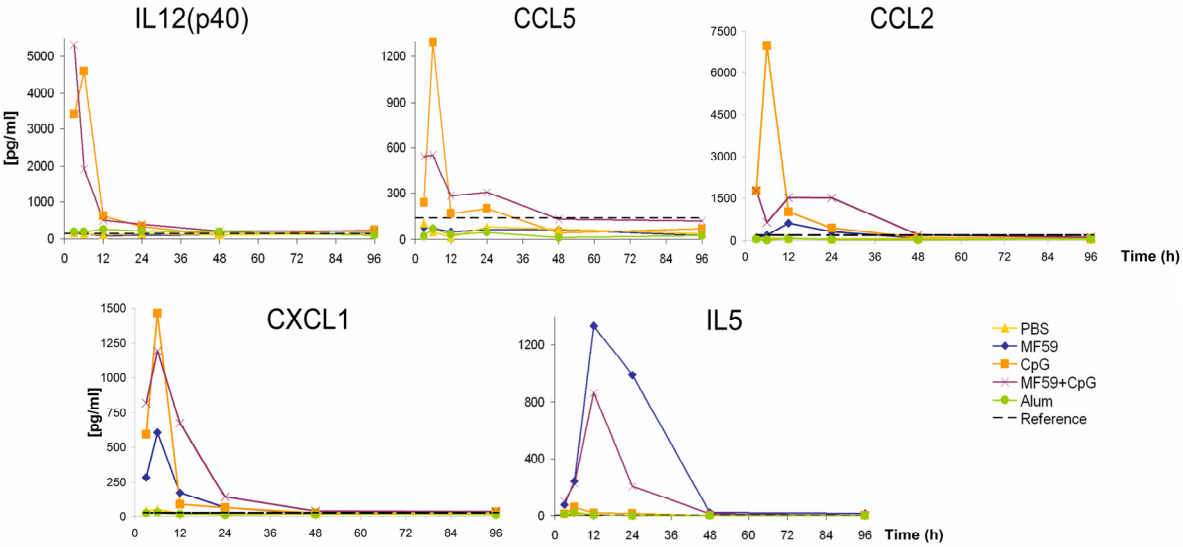

Figure 13 Systemic expression of cytokines after vaccine adjuvant administration. Cytokine expression profiles were measured in the sera of the same mice subjected to microarray analysis. IL12(p40) (A); IL5 (B) and CCL5 (C) protein expression were measured at the indicated times (expressed in h) after adjuvant administration. The dashed lines indicate the cytokine concentration in untreated mice.

DISCUSSION

Although oil in water emulsions are considered the best adjuvants for flu and promising candidates for new human vaccines, their mode of action is still unclear. Here we show that oil in water emulsions, similarly to alum and CpG, activated innate immune reactions at the injection site. The cluster of genes modulated by all adjuvants named "adjuvant core response gene" was characterized by the up-regulation of cytokines, chemokines and adhesion molecules, suggesting that the establishment of a local immunocompetent environment associated to a non-pathogenic inflammatory process is generally associated to vaccine adjuvanticity. Indeed, we could monitor the recruitment in the muscle of CD11b+ and MHC class II+ blood cells 4 days following administration of all adjuvants. These data are in agreement with previous reports showing that the injection of alum results in local inflammation (17, 18) and with more recent data showing that alum induced monocyte recruitment in the peritoneum (21). Furthermore, two of the adjuvant core response genes identified in mouse muscle, CCL2 and IL1b, were also up-regulated in the peritoneum after alum injection (21). MF59 was a more potent activator of immune related genes than alum and CpG and promoted a more rapid recruitment of CD11b+, the marker of mononuclear cells, and MHC class II+ blood cells in the muscle. This finding is consistent with previous data obtained from muscle single cell suspension, which showed that at 1 day after injection MF59 induced an influx of mononuclear cells. It has been shown that monocytes chemoattractant proteins which bind to CCR2 play a significant role in monocyte and DC trafficking and it has been demonstrated that MF59-mediated cell recruitment was partially driven by CCR2 (23). Accordingly, in our microarray analysis, MF59 induced Ccr2 at 1-2 days. Moreover, MF59 up-regulated the Ccr2 ligands Ccl2 and Ccl7 at 3 h

and Ccl8 at 12 h.

It has been previously reported that CpG oligonucleotides can modulate the adaptive immune response elicited by MF59 in mice (24, 27). Here we show that CpG regulated the expression profile of a large number of MF59-responsive genes at the injection site, which may contribute to the modulation of the adaptive response. Moreover, we found that CpG induced stronger systemic responses compared to MF59 and alum, probably reflecting the capability of oligonucleotides to directly activate circulating blood cells such as DCs and B cells.

By using two early biomarkers identified by microarray analysis, JunB and Ptx3, we could identify the skeletal muscle as a target of MF59 immunostimulating activity. Furthermore, we detected labeled MF59 in muscle fibers, supporting a direct activation of the muscle by MF59. Interestingly, also CpG could activate PTX3 and JUNB expression in muscle cells, suggesting that they might respond directly to TLR9 agonists. However, we cannot rule out that early cytokine expression induced by MF59 or CpG in hematopoietic cells contributes to muscle activation. It is well known that skeletal muscle can actively participate in local immune reactions by expressing pro-inflammatory cytokines, chemokines, adhesion molecules and TLRs. In the inflamed muscle, in fact, various immune cell populations express and release different chemokines and muscle fibers seem to be a cellular source of these chemotactic cytokines. Also matrix metalloproteinases appear to be crucial in initiating, perpetuating and resolving the inflammatory response in muscle tissue (36). Our data suggest, for the first time, that the skeletal muscle could play an important role in enhancing the efficacy of intramuscularly administered human vaccines. Unlike MF59 and CpG, alum failed to activate muscle fibers and more work must be performed to identify the target cell responsible for alum-dependent local immunostimulation in the muscle. Recent data suggest two independent mechanisms of action for alum: it can enhance antigen immunogenicity and delivery to APCs, and, like MF59, it can promote, by a TLR-indipendent mechanism, a local pro-inflammatory environment, which results on blood cell recruitment and DC differentiation. Although the molecular target of alum is still unknown, there are some data demonstrating that this target is NOD-like receptor protein 3; a member of NLRs proteins belong to inflammasome system. It has been hypnotized that alum might activate NLRP3 through the induction of necrosis, which causes the release of danger signals such as uric acid and ATP [37].

We hypothesize that MF59 is a very efficient adjuvant because it combines antigen delivery function with strong immune stimulating activity at injection site. We propose that MF59 induces in muscle fibers the production of immune mediators (TNFα, IL1β, CCLs) that establish a local immunocompetent environment and in turn activate tissue resident DCs. MF59 may also promote a sustained antigen presentation process following vaccination by triggering the recruitment of CD11b+ monocytes which might differentiate in functional inflammatory DCs expressing high levels of MHC class II as previously described for alum (21) **(Fig. 14)**.

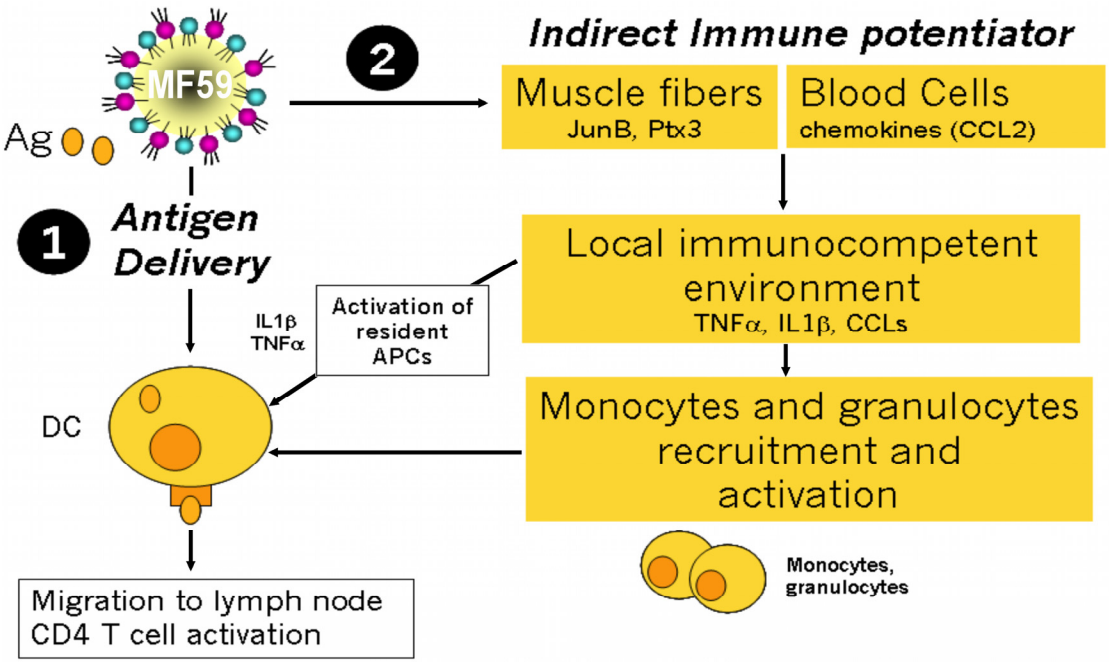

Figure 14 Possible mechanism of action of MF59. We propose two functions for MF59: 1) as antigen delivery that promotes antigen uptake by DCs that upon activation migrate to draining lymph nodes; 2) as indirect immune potentiator which targets muscle fibers and blood cells to induce a local immunocompetent environment that contribute to the recruitment and the activation of monocytes and granulocytes; and also to the activation of resident APCs.

Our findings strongly suggest that the mechanism of action of vaccine adjuvants must be addressed *in vivo* where different cell types cooperate in establishing an integrated immunocompetent environment. The up-regulation of IL1 signaling pathway genes such as IL1β and caspase 1 might suggest that MF59, like other particulate adjuvants, activates the inflammasome through NLRP3 protein which results in the cleavage of

pro-caspase 1 that activates IL1β and IL18. This is an assumption that needs to be verified.

In summary this study has shown that both alum and MF59, although not capable of activating directly DCs *in vitro*, could trigger a local immunostimulatory environment characterized by the expression of several cytokines, which may indirectly activate DCs through a TLR-independent mechanism. These adjuvants and also other particulate adjuvants enhance antigen uptake, activate innate immune pathways and induce a local recruitment of blood cells. More work must be performed to understand the different contribution of each of these mechanisms in the activation of an optimal adaptive immune response.

MATERIALS AND METHODS

1. Mice

Pathogen-free female BALB/c mice 6-8 weeks of age were obtained from Charles Rivers Laboratories. All animals were housed and treated according to internal animal ethical committee and institutional guidelines. Mice were injected i.m. in both quadriceps with 50 μl/quadriceps of PBS alone (control experiment) or supplemented with MF59 (1:1 dilution); 10 μg of CpG; 10 μg CpG and MF59 diluted 1:1 or 100 μg $Al(OH)_3$ (Pierce). We choose the amount of adjuvant that gave optimal adjuvanticity in previous studies conducted with various antigens (24-27). Muscles and sera were taken from three mice/group at 3, 6 and 12 hours, and 1, 2 and 4 days after treatment**.**

2. Adjuvants

MF59 (5% squalene, 0.5% Tween 80, 0.5% Span 85) was prepared in distilled water with a Microfluidizer 110S (MFIC Corporation, Newton, MA) as previously described (37, 38). The CpG oligonucleotide sequence used was 5' -TCC ATG ACG TTC CTG ACG TT- 3' with all phosphorothioate backbones (CpG1826). MF59-DIO was prepared by diluting chloroform-re-suspended DIO (Invitrogen) in MF59, final concentration 0.25 μg/ml.

3. Muscle RNA extraction and purification

Whole muscles were homogenized in TRIzol (Invitrogen) with an Ultra-Turrax T25 (IKA) and the total RNA was extracted from the tissue.

Protocol:

1) Place the muscle in 7.5ml TRIzol and break down the tissue.
2) Incubate the samples for 10 minutes at room temperature.
3) Add 1.5 ml of chloroform.
4) Vortex for 15 seconds and incubate at room temperature for 2 minutes.
5) Centrifuge at 9500rpm for 15 minutes at 4 °C. (The RNA stays in the aqueous phase).
6) Transfer the aqueous phase (on top) into a new 15 ml falcon tube.
7) Add 3.75ml of iso-propanol and incubate for 5 minutes at room temperature.
8) Centrifuge at 9500rpm for 10 minutes at 4 °C.
9) Remove the supernatant by pipetting.
10) Wash the pellet by adding 7.5 ml of 75% ethanol without disturbing the pellet.
11) Centrifuge at 9500rpm for 5 minutes at 4 °C.
12) Remove the supernatant.
13) Dry the RNA pellet for a few minutes.
14) Dissolve the pellet in 103 µl RNase-free water pipetting up and down a few times.
15) Measure the RNA concentration by putting 2 µl of RNA on the top of Nanodrop. A ratio more than 1.8 is good.
16) Prepare 100 µg of RNA into a final volume of 100 µl by adding RNase-free water use.

100 µg RNA from each couple of muscles were purified using the RNeasy RNA purification columns (QIAGEN) following the producer's protocol. RNA quality was assessed using the automated Experion electrophoresis system (Bio-Rad) coupled with the RNA StdSens kit following the producer's protocol.

4. RNA labeling, microarray hybridization and data acquisition

Microarray cDNA probes were prepared from total RNA obtained from treated muscles (test) or from a pool of RNAs extracted from the muscles of 15 naïve mice (reference) using Cy5 and Cy3 dyes respectively. Total RNA was retro-transcribed, degraded and then the labeled cDNA was purified using the QIAquik PCR Purification Kit (Qiagen) following the manufacturer's protocol.

4.1 Synthesis of labelled cDNA using the in-house probe preparation protocol

Pre-annealing of the primer to the RNA:

1) Dilute 25 μg of total RNA in 16 μl RNase free water.
2) Add 5 μl of oligo dT ([0.5 μg/μl], Invitrogen) – (use 1 µg of dT / 10 μg of RNA)
3) Heat at 70 °C for 5min.
4) Cool at room temperature for 5 minutes.
5) Centrifuge briefly.

Prepare the mix for Reverse Transcriptase reaction:

Reagent	Vol/sample (µl)
5x First Strand Buffer (Invitrogen)	8
DTT ([0.1M], Amersham)	4
dNTP mix (2 mM dATP, dGTP, dTTP + 1 mM dCTP) (Amersham)	2
RNAsin ([40 U/µl], Promega)	1

To the eppendorf add:

1) 15 μl of the mix.
2) 2 nmol of Cy3 or Cy5 labelled dCTP (Amersham) and mix gently.
3) 2 μl of SS (Super Script II Reverse Transcriptase, Life technologies., [600U/μl] special order) and mix gently.
4) Incubate at 42 °C for 2h.
5) Centrifuge briefly.

Prepare a mix for RNA degradation:

Reagent	Vol/sample (µl)
RNase One ([9U/µl], Promega)	0.22
RNase H ([2U/µl], Invitrogen)	2
5 X First Strand Buffer	4
RNAse free water	13.89

6) Add 20 µl of the mix/eppendorf.
7) Incubate 30 minutes at 37 °C.

The efficiency of incorporation of the Cy5 or Cy3 dyes was measured by Nanodrop analysis.

4.2 Hybridization onto the Agilent array

Equal amounts of labeled Cy5 and Cy3 cDNAs were hybridized onto the Agilent 44k Whole Mouse Genome Microarray, detecting over 40000 transcripts.

Protocol:

1) Dissolve each sample in 100 µl of RNase-free MilliQ water.
2) Add to each Cy5-labeled experimental sample the same amount of Cy3-labeled reference sample and mix.
3) Heat at 98 °C for 3minutes.
4) Cool down at room temperature for 3 minutes. Spin down briefly.
5) Add 50 µl of 10X control oligonucleotides (Agilent).
6) Add 250 µl of 2X hybridization buffer (Agilent).
7) Mix by flipping the tube and then centrifuge briefly.
8) Pipette 490 µl of the sample onto the slide and put into the oven at 60 °C O.N.
9) Transfer immediately the slide in a jar containing wash buffer 1 (Agilent) and put it on a magnetic stir plate and stir at medium speed for 1 minute at room temperature.
10) Prepare a second jar filled with wash buffer 2 (Agilent) at 37°C. Transfer the slide into the washing buffer 2 and stir at medium speed for 1 minute.
11) Prepare a third jar filled with acetonitrile and transfer the slide into the jar for 1 minute.

12) Transfer the slide into a fourth jar containing the tabilization and drying solution (Agilent). Incubate for 30seconds and then very slowly take the slide out of the solution.

Images were acquired using the ScanArray Express microarray scanner (Perkin Elmer).

5. Microarray data analysis

Microarray images were first analyzed using the GenePix 6.0 software (Molecular Devices), and the data were then transferred to the BASE 1.2 database/analysis software (39). For each spot, local background was subtracted and spot intensities were normalized by the mean fluorescence intensity for each channel. Spots with a signal-to-noise ratio ≤ 3 in both channels or manually flagged for bad quality were filtered. Four additional hybridizations using the same reference RNA labeled with Cy5 and Cy3 were processed in the same way to determine the dye incorporation bias and to correct the baseline of each spot. The average intensity ratio of each spot from experimental replicates was estimated by geometric mean and the accuracy and statistical significance of the observed ratios were determined using the Student's t-test. Spots with less than two values in the same time point were considered "not found" and we assigned a log2 ratio of zero. Only genes having t-test p-values lower than 0.05 and average intensity ratios greater than 4 (log2 ratio $\geq |2|$) in at least one time point were selected. Genes were considered responsive to each stimulus if modulated with a fold change of log2 ratio $\geq |2|$ compared to untreated muscles. Hierarchical clustering was performed with the TMEV 3.1 software (40) on the log2 ratio transformed dataset applying the Euclidean distance matrix and the average linkage clustering method. Some genes appear more than once in clusters because they are represented by multiple unrelated probes in the Agilent 44k whole mouse genome array. Functional analysis of the dataset was performed with GeneSpring GX version 7 software (Agilent Technologies) using Gene Ontology (GO), GenMAPP and Kyoto Encyclopedia of Genes and Genomes (KEGG).

6. Immunofluorescence experiments

14 μm cryostat-cut muscle sections were mounted, fixed in PBS, 3% p-formaldehyde for 10 min and then incubated for another 10 min in blocking and permeabilization solution (PBS, 3% BSA, 1% saponin). The structure of the muscle was visualized using an antibody specific for utrophin, a cytoskeletal protein located in the sarcolemma of muscle cells. Tissue sections were incubated for 1 h with the following primary antibodies: goat anti-human utrophin (Santa Cruz Biotechnology), rabbit anti-mouse PTX3 (Santa Cruz Biotechnology), rabbit anti-mouse JUNB (Santa Cruz Biotechnology), FITC-conjugated rat anti-mouse I-A/I-E (BD Pharmingen,) and rat anti-mouse CD11b (AbD Serotec). After washing, sections were incubated 30 min with the following secondary antibodies: donkey anti-goat IgG-Alexa Fluor 647 (Molecular Probes), donkey anti-rabbit IgG-Alexa Fluor 488 (Molecular Probes), chicken anti-goat IgG-Alexa Fluor 488 (Molecular Probes), goat anti-rat IgG-Alexa Fluor 555 (Molecular Probes). Nuclei were stained with propidium iodide (PI) in all experiments with the exception of CD11b staining in which ToPro3 (Invitrogen) was used. Sections were washed and mounted in Vectashield mounting Medium (Vector Labs) and viewed by confocal microscopy (Bio-Rad).

7. Cytokines concentration in the serum

Cytokine's concentrations in the serum have been determined using the Bio-Plex Cytokine Assay (23-Plex Bio-Rad). Bio-Plex cytokine assays are mulptiplex bead-based assays designed to quantify multiple cytokines in diverse matrices in a single microplate well. Bio-Plex assays contain dyed beads conjugated with monoclonal antibodies specific for a target cytokine. Each of the 100 spectrally addressed bead sets can contain a capture antibody specific for a unique target cytokine. The antibody-conjugated beads are allowed to react with sample and a secondary, or detection, antibody in a microplate well to form a capture sandwich immunoassay. The constituents of each well are drawn up into the flow-based Bio-Plex array reader, which illuminates and reads the sample. When a red diode classification laser (635 nm) in the Bio-Plex array reader illuminates a dyed bead, the bead's fluorescent signature identifies it as a member of one of the 100

possible sets. Bio-Plex Manager software correlates each bead set to the assay reagent that has been coupled to it (for example, an IL-2 capture antibody coupled to bead n° 36). In this way the Bio-Plex system can distinguish between the different assays combined within a single microplate well. A green reporter laser (532 nm) in the array reader simultaneously excites a fluorescent reporter tag (PE) bound to the detection antibody in the assay. The amount of green fluorescence is proportional to the amount of analyte captured in the immunoassay. Extrapolating to a standard curve allows quantification of each analyte in the sample.

We determined cytokines concentration as an average of three replicates each time point following the producer's protocol. Three naïve mice were used to detect background level for each cytokine.

APPENDIX

1. Publications

Molecular and cellular signatures of human vaccine adjuvants.

Mosca F, Tritto E, Muzzi A, Monaci E, Bagnoli F, Iavarone C, O'Hagan D, Rappuoli R, De Gregorio E.

Proc Natl Acad Sci U S A. 2008 Jul 29;105(30):10501-6. Epub 2008 Jul 23.

Mechanism of action of licensed vaccine adjuvants.

Tritto E, Mosca F, De Gregorio E.

Vaccine. 2009 Feb 5. [Epub ahead of print]

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