



Local early response to an MVA-mediated vaccination: effects of an MVA infection on skin fibroblasts and macrophages

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Local early response to an MVA-mediated vaccination: effects of an MVA infection on skin fibroblasts and macrophages

Réponse locale précoce à une vaccination par MVA: effets d'une infection par MVA sur des fibroblastes et macrophages de la peau

Par : Julie JOUFFREY

Soutenu à Rennes le 15/06/2015

Devant le jury composé de :

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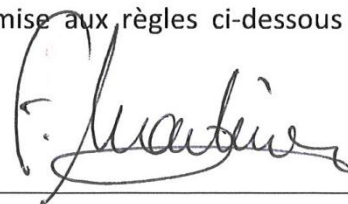
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Local early response to an MVA-mediated vaccination: effects of an MVA infection on skin fibroblasts and macrophages

Commissariat à l'Energie Atomique et aux Energies Alternatives

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Detailed summary

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List of abbreviations and commercial names

BluVid – LIVE/DEAD fixable blue dead, Life Technologies™

CC Chemokines – Chemokines with two adjacent cysteines (C) near their amino terminus.

CPT - BD Vacutainer® CPT™ Cell preparation tube with Sodium Heparin

CD – Cluster of differentiation

DC – Dendritic cells

DMEM – Dulbecco's modified Eagle medium (Gibco®)

DPBS – Dulbecco's phosphate-buffered saline

EDTA – Ethylenediaminetetraacetic acid

eGFP – Enhanced green fluorescent protein

FBS – Fetal bovine serum

Fungizone® - Amphotericin B, antifungal drug

G-CSF – Granulocyte colony-stimulating factor

GM-CSF – Granulocyte macrophage colony-stimulating factor

HeLa – Cervical cancer-derived cell line taken from Henrietta Lacks

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HLA-DR – MHC class II cell surface receptor, $\alpha\beta$ heterodimer

HIV – Human Immunodeficiency Virus

IFN- γ , IFN- α/β – Interferon (IFN) gamma (γ), alpha (α) and beta (β)

IL – Interleukin

IP – Interferon γ -inducible protein

MCP – Monocyte chemoattractant Protein

MDA5 – Melanoma differentiation-associated protein 5

MDDC – Monocyte-derived dendritic cell

MHC – Major histocompatibility complex

MOI – Multiplicity of infection

MyD88 – Myeloid differentiation primary response gene 88

MVA – Modified Vaccinia virus Ankara

NALP3 – NACHT, LRR and PYD domains-containing protein 3 or cryopyrin

NHP – Non-human primate

NK – Natural killer

Pfu – Plaque-forming unit

PSN – Penicillin, Streptomycin, Neomycin

RIG-I – Retinoic acid-inducible gene 1

TLR – Toll-like receptor

TNF – Tumor necrosis factor

VEGF – Vascular endothelial growth factor

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Introduction

Principles of vaccination

Being the interface with the external environment and protecting the body from dehydration and infections, along with a wide variety of immune cells, skin is an important tissue for vaccine inoculation (Frenck Jr. et al., 2011; Fuchs and Raghavan, 2002; Haniffa et al., 2009; Stoitzner et al., 2008; Vogt et al., 2008). Skin is constituted of differently shaped layers. Epidermis is mainly made of keratinocytes and melanocytes, non-immune cells, and some immune cell types as Langerhans cells and lymphocytes. Underneath the epidermis is the dermis, a vascularized conjunctive tissue consisting in numerous and various cell populations including fibroblasts (non-immune cells) in great numbers but also immune cells as T, B and NK lymphocytes, macrophages, interstitial and plasmacytoid dendritic cells, neutrophils and mastocytes. The deeper layer is the hypodermis, mainly constituted of adipocytes (Zaba et al., 2008). Unlike the epidermis and more than the hypodermis, the dermis is rich in blood vessels and nerve endings. This team's recent works show a high diversity of innate immune cells in the skin and their role in inflammatory responses to intradermal vaccination (Epaulard et al., 2014; Romain et al., 2012).

The immune system is able to keep track of its responses in the immunological memory. The aim of vaccination is to use this memory to simulate a first encounter with an antigen (from the target pathogen) and induce an immune response, stored in the immunological memory in prevention of a future encounter with the whole virulent pathogen. There are three kinds of vaccines. The first category is live attenuated vaccines, altered by passages in cells with genetic modifications; the second is inactivated vaccines, altered by heat or chemicals; the third category is subunits purified from pathogens or recombinant vectors. The term vaccination comes from the Latin for "cow" (*vacca*), as the first vaccine was made by Edward Jenner in 1796 from cowpox sore matter inoculated into human patients to prevent the smallpox illness (Downie, 1951). Although the major route of administration of a vaccine is intramuscular, recent methods and devices allow the development of skin routes' administration. The skin inoculation of a vaccine can take several forms: scarification or puncture (e.g. the history of smallpox vaccine (*variola*)), subcutaneously or intradermally (e.g. Bacille de Calmette-Guérin used as vaccine against tuberculosis).

MVA and vaccination

Smallpox (*variola virus*) was eradicated using vaccinostyles and bifurcated needles, showing that the skin route of administration is effective (Liu et al., 2010). Vaccinia virus, another virus from the poxvirus family, is the active constituent of the vaccine used to eradicate smallpox in the late 20th century. If the original host of Vaccinia virus is unknown, the *Choriollantois Vaccine Ankara* strain was studied by Anton Mayr. After 516 passages of this virus in chicken embryo fibroblasts, an attenuated vaccinia virus was obtained, named MVA (Modified Vaccinia virus Ankara) (Verheust et al., 2012). Those passages damaged the MVA genome: 6 large genomic deletions totaling 31kb appear when compared with the parental virus genome (Blanchard et al., 1998). Amongst the damages, MVA mostly lost its replication capacity in mammalian cells and only has a limited cell-to-cell spread (Blanchard et al., 1998; Stittelaar et al., 2001). MVA genome, 178 kb long and fully sequenced, lacks the sequences for some of the known poxviral immune evasion and virulence factors (Verheust et al., 2012). MVA-based recombinant viruses (rMVA) show promises as vaccine candidates due to their capacity to integrate the sequences of the genes of interest into their genome. The good immunogenicity of MVA resides in its loss of virulence factors (Stittelaar et al., 2001). Safety data on the use of recombinant MVA were published concerning the frequent use of MVA as a viral vector basis for new vaccines in the domain of infectious diseases in humans, such as Malaria (Moorthy et al., 2003), AIDS (Howles et al., 2010), Tuberculosis (Brookes et al., 2008), Human Papilloma Virus associated cancer, Melanoma, and other cancers (Verheust et al., 2012).

Human immune responses to MVA

MVA is able to engage both innate and adaptive immunity (Burgers et al., 2008). Engaging a good adaptive immune response (i.e. specific to the pathogen, high affinity and long term memory) is the key that makes MVA such a good vaccine candidate.

As a vaccine candidate, MVA is able to induce the production of memory immune cells and specific antibodies so strongly that stabilized levels of those cells can be detected in humans up to 50 years after vaccination. Human vaccinia specific CD8⁺ T cells and CD4⁺ T cells responses are strong after a smallpox vaccination (Amanna et al., 2006). In Non-human primates (NHP), it was shown that MVA is effective against monkeypox challenges after vaccination (Artenstein, 2008). rMVA-HIV (i.e. rMVA expressing selected genes from HIV) successfully infected human primary cells *in vitro* such as macrophages, T cells, NK cells, monocytes and dendritic cells, but more interestingly, DCs expressing HIV antigens after rMVA infection could activate HIV-specific cytotoxic T lymphocytes and induced a

production of HIV-specific CD4⁺ cells. These results were confirmed in NHP and an MVA-SIV vaccination engages strong SIV-specific T cell response (Brandler et al., 2010). A strong and durable immunological memory is an advantage for a good vaccine candidate such as MVA.

The characteristics of adaptive immunity are determined by innate immunity through recruited cells, their activation states and secreted molecules since the beginning of the inflammatory response. DCs have a primordial role in the junction between innate and adaptive immunity, as T lymphocytes have to be activated by a DC to be able to then activate a B lymphocyte by direct contact. The selection of subpopulations of DCs targeting specific memory cells, the adaptive immunity can be set up by the innate immunity (Cohn and Delamarre, 2014; Coquerelle and Moser, 2010; Weaver et al., 2006).

An *in vivo* MVA infection triggers the rapid immigration of monocytes, neutrophils and CD4⁺ lymphocytes to the injection site (Altenburg et al., 2014). It has been shown that MVA activates two innate immunity pathways in the macrophage upon cell infection: one Toll-like Receptor (TLR)-dependent and the other TLR-independent. The first includes an activation of the TLR2-TLR6 heterodimer receptor, inducing an immune response including Th1 (T helper 1 cells) and Natural Killer (NK) cells through MyD88 interaction and inflammatory cytokines release (Delaloye et al., 2009; Reed et al., 2009; Zhu et al., 2007). The TLR-independent pathways involve the NALP3 inflammasome activation down to IL-1 β production on the one hand and MDA-5, a RIG-I-like receptor activating a cellular feedback using type I IFN (Waibler et al., 2007). Both TLR-dependant and TLR-independent pathways are required to activate innate and adaptive immunity to Vaccinia Virus *in vivo* (Zhu et al., 2007). An MVA infection of whole human blood cells results in an abundant production of chemokines such as IL-8 or IP-10, but a less abundant production of pro-inflammatory cytokines (TNF, IL-1 β and IL-6), which is believed to be due to the attenuation of MVA (Delaloye et al., 2009). More interestingly, RNA breakdown was demonstrated in Monocyte-Derived Dendritic Cells (MDDC) infected with MVA or NYVAC (another attenuated Vaccinia virus strain), showing an induced apoptosis *in vitro* (Guerra et al., 2007). Dendritic cells are known target cells of an MVA infection, as they were shown to be infected by MVA both *in vivo* and *in vitro* in mice (Liu, 2010). MDDC morphology, gene expression profile and maturation state are modified after an MVA infection, and their possible entrance in apoptosis demonstrates the high immunogenicity of MVA-vectored vaccines (Verheust et al., 2012). MVA also affects the migratory capacity of both immature and mature dendritic cells through viral binding proteins modulating the chemokine system of DC migration (Alcami, 2007).

Study Models

Cynomolgus macaque

Cynomolgus macaque (*Macaca fascicularis*) is the major Nonhuman Primate (NHP) used in biomedical research. The macaque's immune system is very close to the human immune system, making it susceptible to human infectious diseases and pathologies. It is a uniquely valuable resource to study human infectious diseases in vaccinology, including influenza virus, flavivirus, arenaviruses, hepatitis E virus, papillomavirus, smallpox virus and *Bacillus anthracis* (Gardner and Luciw, 2008). The similarity of the two immune systems enables researches to explore the experimental conditions of a vaccine's efficiency. Several studies (Barratt-Boyes et al., 2006; Kato et al., 2004; Lu et al., 2003; Querec, 2006; Teleshova et al., 2004; Wonderlich et al., 2011), including some from this team (Epaulard et al., 2014; Malleret et al., 2008a, 2008b; Romain et al., 2012), showed that some of the cynomolgus macaque's cell populations involved in capturing and preparing antigens are similar to those in the human immune system and can be characterized in many ways. Finally, using NHP as animal models is well adapted to assess a vaccine's feasibility and efficiency along with its safety. Those are essential preclinical trials before clinical tests on human patients.

Fibroblasts and macrophages

In vivo imaging of the injection site of an MVA-eGFP (recombinant MVA expressing a fluorescent protein) intradermal injection indicates an expression of GFP in cells whose morphology is compatible with that of macrophages and/or fibroblasts. The GFP expression appears during between approximately 24 and 72 hours following the injection and the local inflammation is characterized by the recruitment of macrophages and neutrophils. In this context, this study aims to identify the immune role of fibroblasts and macrophages in response to MVA and characterize the effects of a possible infection of those cells by MVA on their physiology. To do so, fibroblasts and monocyte-derived macrophages are obtained and cultured from NHP skin biopsies and blood samples, then infected with injection of MVA in the culture medium. Physiological modifications induced by MVA are observed through flow cytometry staining, multiplex analysis of supernatant and culture wells pictures.

Materials and Methods

Animals, skin biopsies and blood withdrawals

Adult cynomolgus macaques (*Macaca fascicularis*) (and weighing 4-8 kg) were housed in Commissariat à l'Energie Atomique facilities. Animals were sedated with ketamine

chlorhydrate during handling. Three punch biopsies per animal (8 mm diameter) were performed on anesthetized animals. Biopsies were then cleaned removing fatty tissues, epidermis and dermis were separated using a dispase solution, and then the dermis of all three biopsies was mixed with the gentleMACS™ Dissociator technology (Miltenyi Biotec) to prepare a cell solution. Blood was drawn in CPT (Cell Preparation Tubes) (8 mL), up to 3 or 4 CPTs per animal in one time.

Fibroblasts cell culture from frozen cells or skin biopsies

Cell culture

Fibroblasts were obtained from skin biopsies prepared as described above and then cultured in 4 mL DMEM solution consisting of DMEM GlutaMAX (Gibco®) with 10% FBS (Eurobio), 1% PSN (Gibco®), 1% HEPES (Gibco®) and 1% Fungizone®(Gibco®). Splitting fibroblasts was performed when cells were approximately 80% confluent, and often overestimating the surface needed (**Figure 1**). Flasks used were 25, 75 and 175 cm². Splitting protocol implied use of 0,25% Trypsin-EDTA (Gibco®) (2 mL for 25cm² flasks, 5 mL for 75cm², 5 mL for 175cm²) during 10 min at 37°C, once the previous culture medium was emptied and the flasks were washed with DPBS (Gibco®) at 37°C. The action of Trypsin-EDTA was stopped by addition of DMEM solution (or DPBS 10% FBS) in excess.

Change of medium was performed every other day for fibroblasts. In case of holiday, more medium was added during the last medium change.

Cell flasks and plates were kept in an incubator set on 37°C, 7% CO₂.

Passages

All splittings were recorded, to avoid reaching the maximum number of passages tolerated by fibroblasts, set around 25 by Hayflick in 1961 (Hayflick and Moorhead, 1961). In case of cells from different passage steps assembled in a new flask, the higher number of passage is recorded.

Frozen cells

Fibroblasts were frozen in Fetal Bovine Serum + 10% Dimethyl Sulfoxide (DMSO) solution in Nunc® cryogenic storage tubes and kept at -135°C. Tubes' content was thawed gently and added to culture medium to prepare a cell solution.

Monocyte-derived macrophages cell culture from whole blood

Monocytes were isolated from whole blood drawn from macaques. Extracted Peripheral Blood Mononuclear Cells (PBMC) were washed and treated with red blood cell lysis solution if needed. PBMC were then set to differentiate following an adhesion protocol in DMEM solution containing 10% FBS 1% PSN and 1% HEPES, 10ng/mL M-CSF and 2ng/mL GM-CSF for six days and then moved to adequate wells for the experiment and changed to a DMEM 10% FBS 1% PSN 1% HEPES medium. On Day 8, well differentiated cells were harvested with DPBS 10mM EDTA (**Figure 2**). It appeared that lymphocytes did not survive a week in monoculture.

Change of medium was limited for macrophages, as differentiated macrophages are easily detached.

MVA-eGFP virus and buffer

MVA-eGFP (recombinant MVA expressing eGFP under the control of p11K7.5 promoter) was provided by Transgene (Illkirch-Graffenstaden, France) with L1 biosafety level. The clone is referred as MVATGN33.1. The viral was shipped in cryotubes of 1 mL of 5×10^8 pfu/mL frozen in dry ice, and virus was kept at -80°C until use.

MVA Buffer is prepared following the virus manufacturer's instruction. It contains 10mM of trizma hydrochloure, 5% sucrose, 10 mM of L-glumatic and monosodium salt monohydrate and 50mM of sodium chloride at pH 8.

***In vitro* MVA Infection of fibroblasts and macrophages**

Fibroblasts previously harvested from culture flasks and counted were stimulated in 6-well culture plates, one well per condition containing 400 000 cells in 4 mL of culture medium. Macrophages were stimulated in 24 or 48-well culture plates depending on their number when harvested and counted as follows: 220 000 cells per well in 48-well culture plates in 150 μL of culture medium or 400 000 cells per well in 24-well culture plates in 400 μL of culture medium. Chosen MOI were set as 0.01, 0.1 and 1 for the different experiments. An aliquot of 100 μL MVA-eGFP at 5.10^8 PFU/mL kept at -135°C was thawed and diluted with MVA Buffer to obtain the selected MOI depending on the cell concentrations in wells. The virus particles were then injected in the culture medium of cells to be infected, according to MOI conditions ("Buffer only" wells received MVA Buffer in the same quantity and "Unstimulated" conditions received DPBS). Two hours after infection, the culture medium was changed to stop the viral infection.

Flow cytometry marking

On due time points (H6, H24 or H48), cells were harvested from the infection wells with Trypsin-EDTA 0.25% (if fibroblasts) or DPBS 10 mM EDTA (if macrophages). Cell mortality was assessed with a blue fluorescent reactive dye from the LIVE/DEAD Fixable Dead Cell Stain Kit “BluVid” (Life TechnologiesTM). Cells were put 10 min at 70°C to induce cell death in BluVid and Annexin V (to assess cell apoptosis) control conditions only. Then all harvested cells were left 15 min to incubate with a BluVid solution (1 mL DPBS 1% BluVid) at 4°C in the dark. After washing with centrifugation (1800 rpm 4°C 5 min), nonspecific cell receptors were saturated with a Macaque Serum solution (50 µL DPBS 5% Macaque Serum) and incubated 20min at 4°C in the dark. An “Unstained” condition was also prepared with cells receiving only DPBS instead of BluVid, Macaque Serum and markers. The detailed antibodies’ list is presented in **Table 1** and **Table 2**. Panels were prepared minimizing the overlapping of fluorochromes. Fluorochrome-free antibodies (“Purified”) were coupled with a secondary antibody coupled to an Alexa Fluor fluorochrome with the Zenon Kit (Life TechnologiesTM). Without washing, cells then received 90 µL of the panel mix or 90 µL of DPBS if unstained, as presented in **Tables 3** and **4**. In case of coupled markers with a Labeled Zenon (e.g. CMH I Purified + IgG2a Zenon PE Texas Red + Component B), cells received 80µL of the mix panel and 10µL of each labeled marker. Cells were incubated 20 min at 4°C in the dark with markers, then centrifugated (1800 rpm 4°C 4 min). Cells were finally fixed with 150 µL BD CellFIXTM solution (BD Biosciences) and analyzed with Flow Cytometry within hours (cells are kept at 4°C in the dark to preserve fluorochromes).

Flow Cytometry analysis

Flow cytometry acquisition was performed on an LSRFortessa cytometer (BD Biosciences, Le Pont de Claix, France) with five lasers (355 nm, 405 nm, 488 nm, 560 nm and 640 nm). Obtained data were then analyzed with FlowJo Single Cell Analysis software (Tree Star, Ashland, OR).

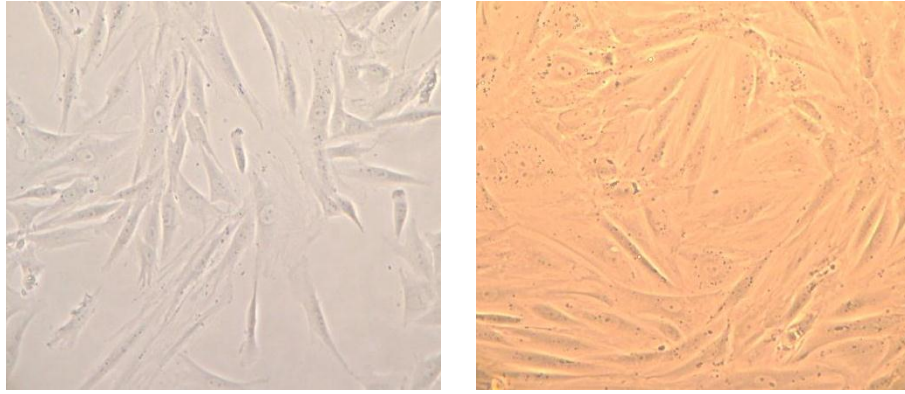


Figure 1 - Fibroblasts (left) and confluent fibroblasts (right). Photographs of dermis fibroblasts taken from usual *in vitro* culture conditions. Fibroblasts were isolated from dermis of skin biopsies. x 100

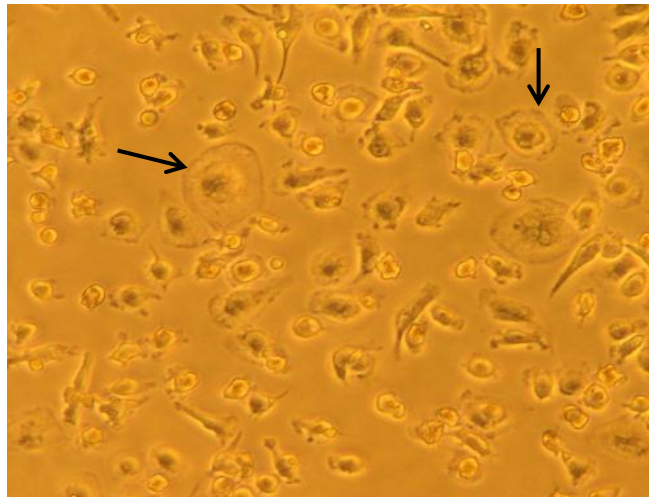


Figure 2 – Monocyte-derived macrophages. Pictures were taken in culture wells at H48 after infection. Differentiated monocytes with macrophages phenotype can be seen (black arrows). x 100

Multiplex immunoassay analysis

Supernatant samples (200 μ L) taken from culture wells at interest time points were analyzed (25 μ L per condition) using Milliplex® MAP Non-Human Primate Cytokine Panel coupled with Luminex xMAP® platform from EMD Millipore Corp., MA. 23 cytokines were tested: G-CSF, GM-CSF, IFN γ , IL-1 β , IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12/23(p40), IL-13, IL-15, IL-17A, MCP-1, MIP-1 α , MIP-1 β , sCD40L, TGF α , TNF α , VEGF and IL-18.

During the fibroblast experiment, the medium volume (4 mL) was sufficient to allow sampling at every time point; on the contrary, macrophages were cultured in small amount of medium (150 or 400 μ L depending on the number of cells per well), limiting the sampling at harvesting only. Supernatant was sampled from fibroblasts cultures at H0, H6, H24 and H48. Fibroblasts were counted and distributed in the experiment wells 24 hours before H0 (infection), therefore the “H0 pool” sample (sampled from a pool of supernatant from every wells belonging to the same individual) was really a H24 sample in normal culture condition.

Multiplex immunoassay data was analyzed with GraphPad Prism Software.

Table 1 - Flow cytometry, Fibroblast Panel

Antibody/Marker	Clone	Host	Isotype	Fluorochrome	Source
CD10	HI10a	Mouse	IgG1, κ	APC	BD Pharmingen™
CD44	IM7	Rat	IgG&b, κ	Brilliant Violet 421™	BD Pharmingen™
CD90	5E10	Mouse	IgG1	BV510	BD Pharmingen™
CMH I	W6/32	Mouse	IgG2a, κ	Purified + Zenon AF594	BioLegend®
Annexin V				PerCP-Cy5.5	BD Pharmingen™
BluVid (LIVE/DEAD Fixable Dead Cell Stain Kit)				Indo-1 hi	Life Technologies™
MVA-eGFP				FITC	

Table 2 - Flow cytometry, Macrophage Panel

Antibody/Marker	Clone	Host	Isotype	Fluorochrome	Source
CD14	M5E2	Mouse	IgG2a, κ	Pacific Blue	BD Pharmingen™
CD80	L307	Mouse	IgG1, κ	Purified	BD Pharmingen™
Zenon® Alexa Fluor 700		Mouse	IgG1		Life Technologies™
CD86	2331	Mouse	IgG1, κ	APC	BD Pharmingen™
CD163	GHI/61	Mouse	IgG1, κ	PE	BD Pharmingen™
CD206	19.2	Mouse	IgG1, κ	Purified + Zenon AF594	BD Pharmingen™
Zenon® Alexa Fluor 594		Mouse	IgG1		Life Technologies™
HLA-DR	G46-6	Mouse	IgG2a, κ	V500	BD Pharmingen™
CCR7	150503	Mouse	IgG2a	PerCP	R&D Systems
BluVid (LIVE/DEAD Fixable Dead Cell Stain Kit)				Indo-1 hi	Life Technologies™
MVA-eGFP				FITC	

Table 3 - Preparation of marking mix for fibroblasts

Marker or Buffer	Quantity for 1 tube (=1 condition)
CD10	5 μ L
CD44	5 μ L
CD90	5 μ L
Annexin V stain	5 μ L
MHCI (coupled with Zenon)	2 μ L of purified MHCI + 5 μ L Zenon IgG2a AF594 + 5 μ L of Component B, 10 μ L in the mix
Brilliant Stain Buffer	100 μ L (total) – 25 μ L = 75 μ L
Total	100 μL

Table 4 - Preparation of marking mix for macrophages

Marker or Buffer	Quantity for 1 tube (=1 condition)
CD14	5 μ L
CD86	5 μ L
CD163	5 μ L
HLA-DR	5 μ L
CCR7	5 μ L
CD80 (coupled with Zenon)	2 μ L of purified MHCI + 5 μ L Zenon IgG1 AF700+ 5 μ L of Component B, 10 μ L in the mix
CD206 (coupled with Zenon)	2 μ L of purified MHCI + 5 μ L Zenon IgG1 AF594 + 5 μ L of Component B, 10 μ L in the mix
Brilliant Stain Buffer	100 μ L (total) – 45 μ L = 55 μ L
Total	100 μL

Results

Fibroblasts – MVA infection, cell activation and induced cell death

Cytometry analysis

Harvested and stained cells were analyzed with flow cytometry. A gating strategy protocol was set up to define the recorded events as cells, based on the morphology of the events and selecting only singlet events (**Figure 3A**).

MVA-induced cell death

BluVid stains both living and dead cells by reacting with free amines, with a 50-fold more intense fluorescence for dead cells. Annexin V is a protein binding phospholipid phosphatidylserine exposed during cell apoptosis. Annexin V marking is used besides BluVid to confirm dead cells as double positive cells. The two populations observed in the “Living cells” dot plot (**Figure 3A**) were previously defined as Annexin V negative cells thanks to a control, and the upper population was verified as mostly cell debris. **Figure 3B** presents a living cell counts expressed in percentage of total cells according to the gating strategy. With an increasing MOI from 0.01 to 1, cell count seems to drop from 90% to about 60%. Without significance, living cells also appear to be fewer with a later time of harvest after the two-hour long infection.

Fibroblasts are infected by MVA

Although fibroblasts naturally show autofluorescence in the FITC channel (same flow cytometry channel as GFP), infected cells express GFP with significantly higher levels (**Figure**

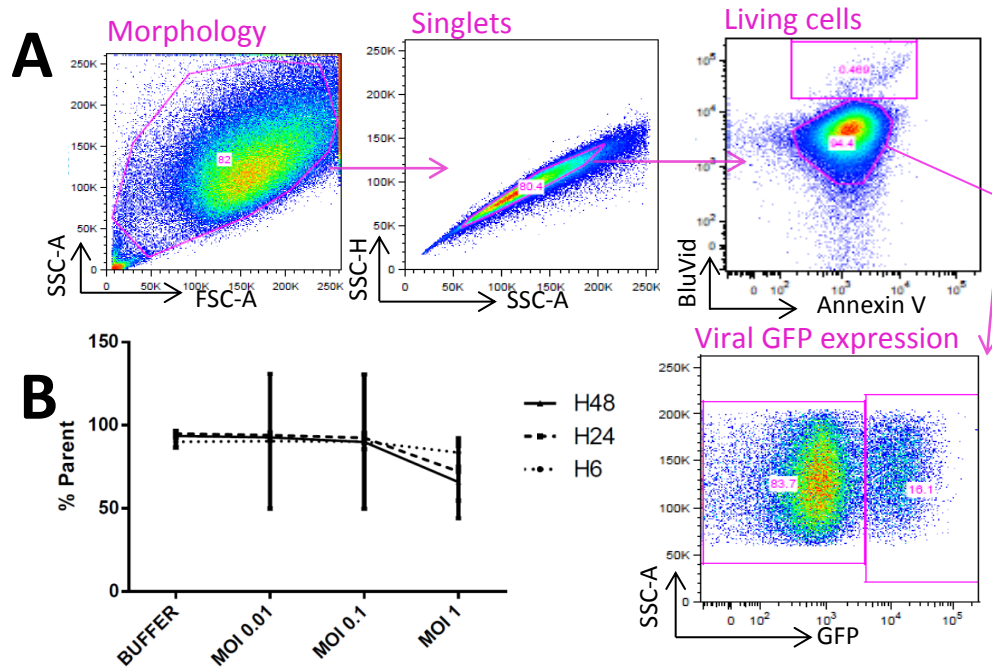


Figure 3 - Fibroblasts gating strategy and living cell count after injection. **A.** Cytometry data were analyzed through the following gating strategy: adequate morphology and singlets were selected based upon flow cytometry parameters, then living cells were defined by being the cell population with the lowest BluVid staining fluorescence and Annexin V negative. The upper population was identified through backgating as not being cells. **B.** Living cells mean count. Negative BluVid-stained cells were expressed in percentage of all cells (singlet signal and with adequate morphology in flow cytometry). Data was obtained from four individuals' fibroblasts

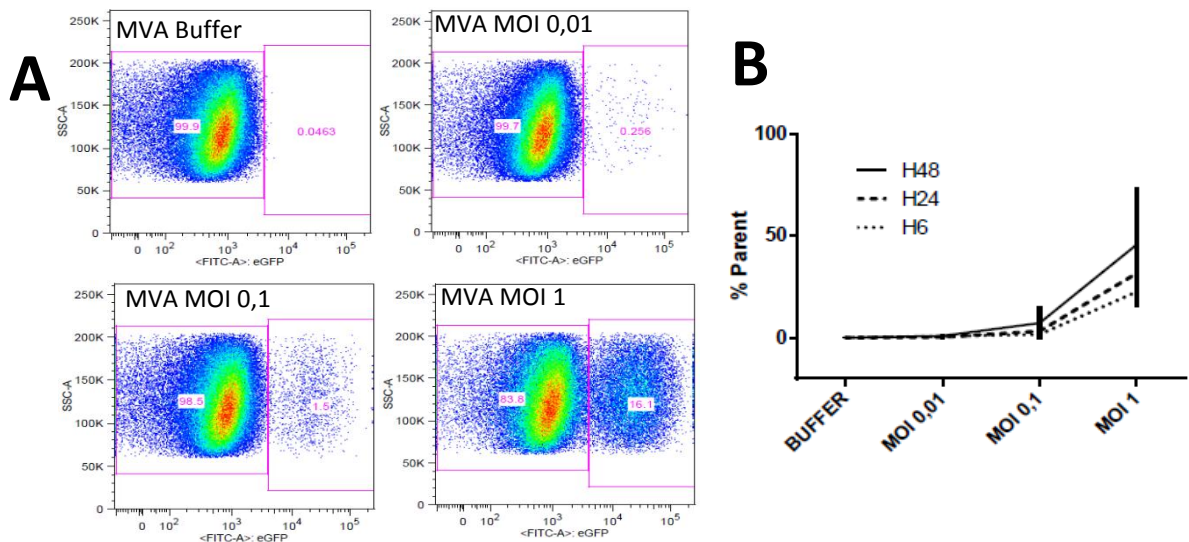


Figure 4 - Fibroblasts are infected by MVA-eGFP. **A.** GFP expression increased with viral load. Cells were separated into naturally GFP-expressing cells (left) and infected and expressing viral GFP cells (right). **B.** Fibroblasts express viral GFP. Viral GFP expression was determined after consideration of the natural fluorescence of fibroblasts using the "Buffer" condition results. GFP positive cell counts were obtained from the flow cytometry data and expressed in percentage of all cells (singlet signal and with adequate morphology). Data here was the mean from four individuals' fibroblasts signal.

4A). The thus defined GFP⁺ population corresponds to infected cells expressing viral GFP. The number of cells in this population increases with the MOI from 0.01 to 1. **Figure 4B** also shows that the longer cells are cultured after infection, the more viral GFP they express.

Fibroblasts markers

Although markers used are not exclusively expressed by fibroblasts, their association in dermis cell suspension allows to mainly characterize this cell population. Markers' expression by long term *in vitro* culture fibroblasts is presented in **Figure 5**, depending on time points and MVA MOI if the cells were in presence of MVA-eGFP. Fibroblasts have been shown to be highly CD10 positive, also called gp100, a type II transmembrane glycoprotein (Kundrotas, 2012). CD10 expression seems to decrease lightly in MVA infected cells 6 hours after infection; but the expression is comparable with uninfected cells at 24 hours after infection. 48 hours after infection, infected cells' expression of CD10 seems slightly higher than that of uninfected cells, especially at an MOI of 1. There appears to have a global decrease in CD10 expression when the MOI gets higher, from 0.01 to 1.

As matrix synthesizer cells, fibroblasts express proteins involved in matrix-cell adhesion, such as CD44, cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration (Kundrotas, 2012) which ligand is hyaluronic acid, or CD90, or Thy-1 for Thymocyte antigen 1, a glycoprotein expressed on many cell types, including T cells, thymocytes, neurons, endothelial cells, and fibroblasts (Rege and Hagood, 2006). Thy-1 has widespread non immunologic functions, such as apoptotic signaling, cell adhesion and migration, and more interestingly fibroblast proliferation and migration. Thy-1 also modulates the fibroblast phenotype involved in wound healing and fibrosis. CD44 and CD90 expressions are similar when comparing uninfected and infected cells: infected cells seem to have a higher expression than uninfected cells at every time points. At 6 hours after infection, the higher the MOI, the higher the CD44 expression, both for infected and uninfected cells. This evolution is similar at 24 and 48 hours after infection but with lower MFI. MFI in buffer condition is also decreasing with time, which shows that this is more due to cell death therefore to a lower mean of fluorescence intensity. An opposite evolution can be observed at 48 hours after infection for CD90 expression: the buffer condition MFI is higher than that of H6 and H24 buffer conditions, but the pairs have decreasing expression with higher MOI. Time seems to increase CD90 expression by uninfected fibroblasts, but if infected by MVA, fibroblasts diminish their CD90 expression.

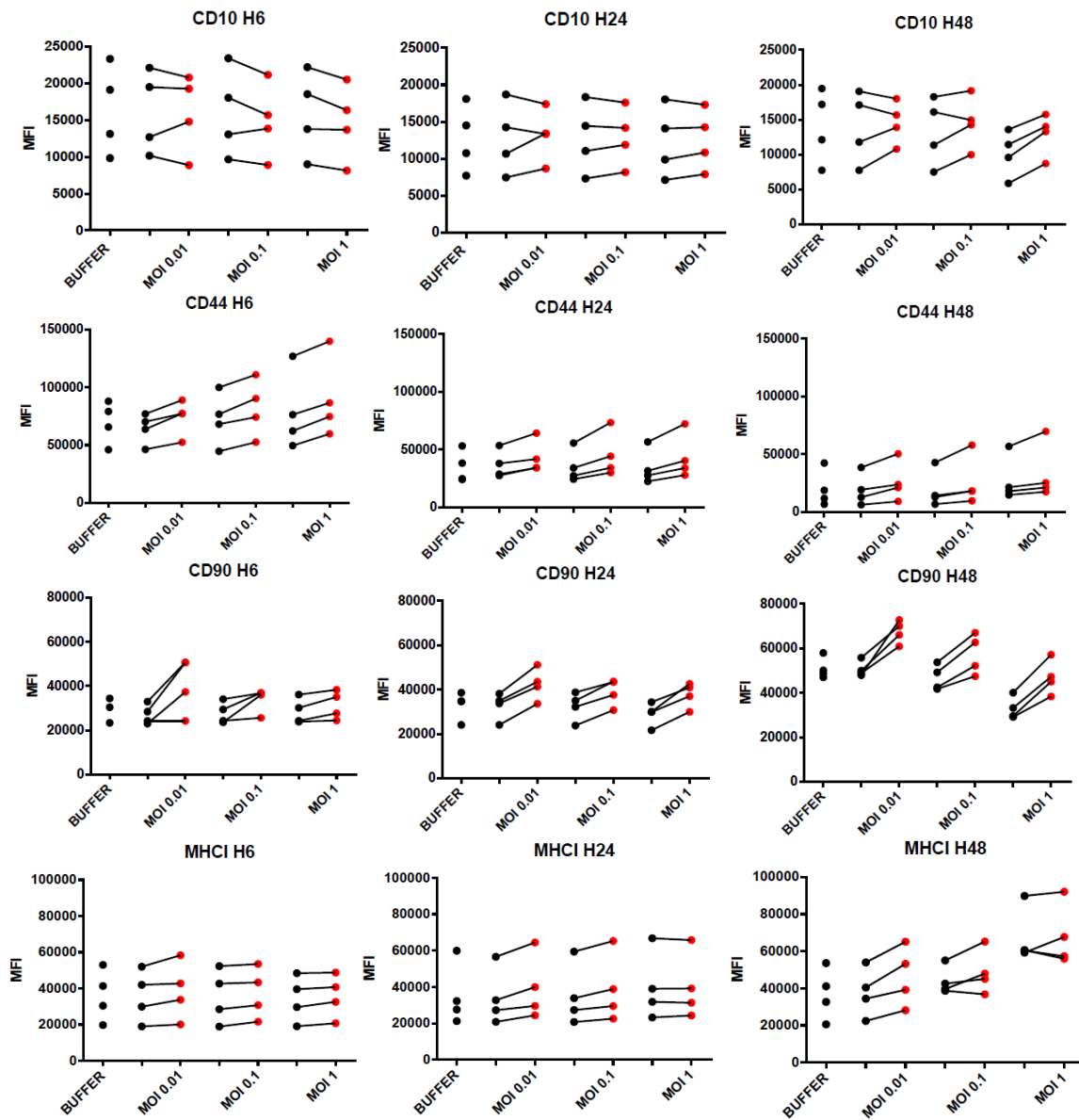


Figure 5 - Fibroblast markers expression. Markers' Mean Fluorescence Intensity (MFI) was recorded from cytometry data. On every graph, black dots represent MFI of cells gated as unexpressing viral GFP or from the buffer condition. Red dots represent cells expressing viral GFP. A couple of black and red dots linked represent one paired cell populations obtained with one condition.

MHC 1 (symbol HLA), or Major Histocompatibility Complex class I, is expressed on nearly every nucleated cell, including fibroblasts. Its functions include intracellular proteins presentation to CTLs, cross-presentation, and inhibitory ligand for NK cells. MHC I expression doesn't seem affected by MVA infection at 6 and 24 hours after infection, but at 48 hours after infection, infected cells tend to express more this marker and the global expression is increased with a higher MOI of MVA.

Macrophages - MVA infection and cell activation

Markers used on macrophages have two objectives: defining the cells as macrophages through a selection of phenotype markers and monitoring their activity via activation markers. Chosen phenotype markers are CD14, CD163 and CD206, macrophages being CD14+ CD163+ CD206+. Although few cells were harvested and analyzed, the majority of living cells are CD206+ HLA-DR+ CD14+ CD163+, confirming a macrophage phenotype as illustrated in **Figure 6**.

Differentiated monocytes are easily detached from the bottom of culture wells, and as presented in **Figure 7**, no cells expressing viral GFP was harvested 48 hours after infection. As for fibroblasts, a buffer condition helped to define the population in the left gate as expressing macrophages' natural fluorescence in the FITC channel instead of viral GFP. The expected cell population expressing viral GFP should be in the right gate.

Chosen activity markers are CD80, CD86, CCR7 and HLA-DR. CD80 and CD86, or B7-1 and B7-2, are two molecules working in tandem to prime T cells by binding T cell CD28 and CTLA-4. CCR7 is a protein of the G protein-coupled receptor family involved in activation and chemotactism. HLA-DR is a Major Histocompatibility Complex class II molecule, working mainly as a peptide antigens presentation receptor and thus is expressed on antigen presenting cells such as macrophages, dendritic cells and B-cells. The expression of the activation markers is compared between the buffer condition and the infected condition. There is a strong variation of results between the four individuals that provided the PBMC (**Figure 8**). The lack of clear trend in the expression of activation markers (**Figure 8**) could be related to the absence of infection of these cells by the MVA-GFP (**Figure 7**).

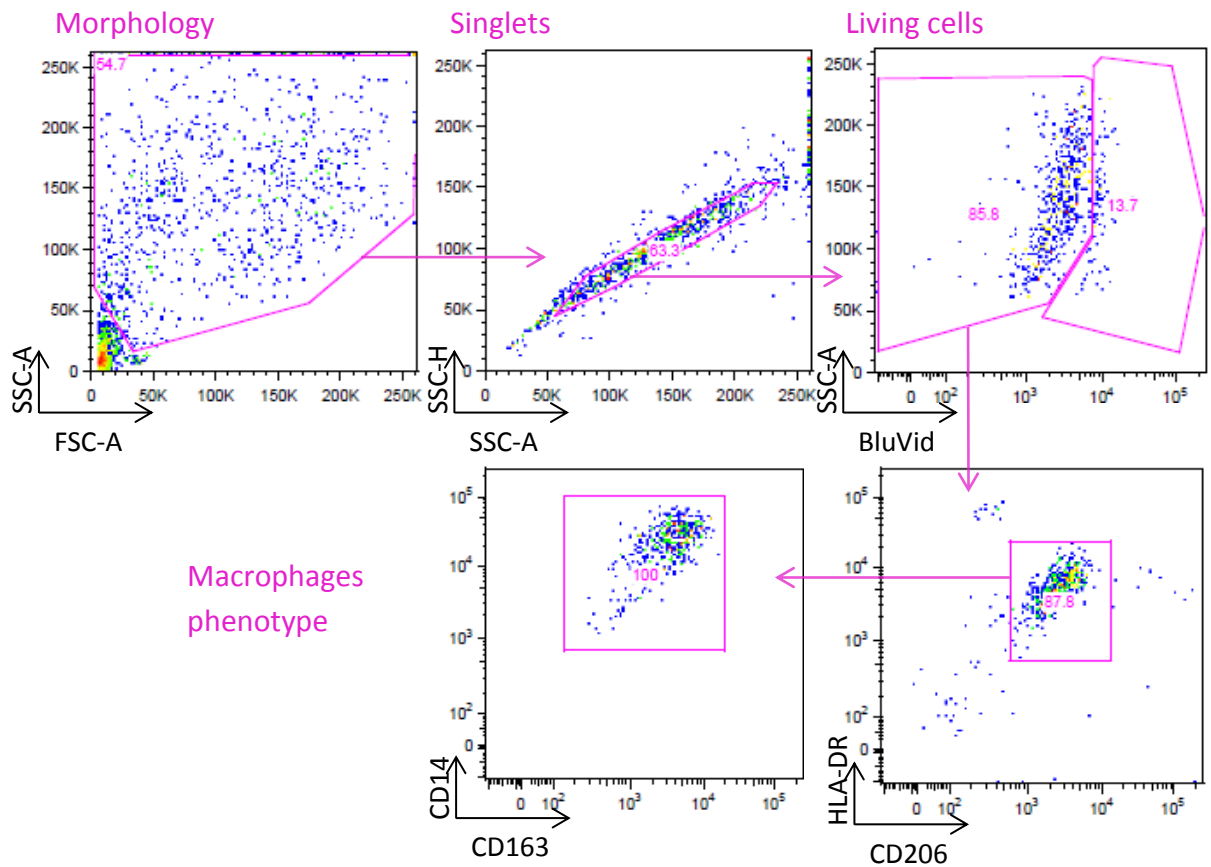


Figure 6 - Gating strategy for macrophages. Macrophages were selecting following a gating strategy based on phenotype markers for macrophages. Adequate morphology and singlets were selected based upon flow cytometry parameters, then living cells were defined by being the cell population with the lowest BluVid staining fluorescence. Among living cells, differentiated monocytes were selected as HLA-DR⁺/CD206⁺/CD14⁺/CD163⁺ cells.

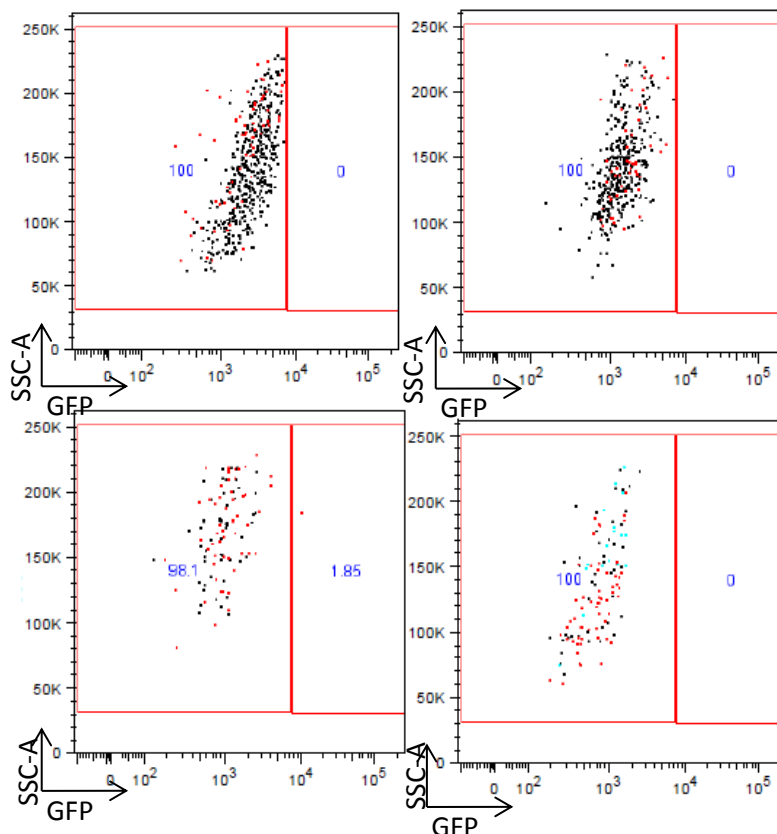


Figure 7 – Expression of GFP in macrophage-like cells. The GFP gate separating non infected cells (left) from MVA-eGFP infected cells (right) was drawn in order not to have any “infected” cells in the buffer condition. Each graph stands for an individual. The colored dots represent the conditions of cells harvested at H48 after infection: buffer condition (black), MOI 0.01 (orange), MOI 0.1 (light blue) and MOI 1 (red).

Supernatant multiplex analysis

Fibroblasts

Out of the 23 cytokines assayed, the cytokines with the higher concentrations or more significant evolutions are presented in **Figure 9**. Exclusions were mostly due to out of range data. Even if fibroblasts are not primarily immune cells, they are able to secrete cytokines *in vitro*; indeed, after changing the medium at H2, the concentration of some cytokines drops near zero and then reach higher levels at H24 and H48 (e.g. IL-6, VEGF and IL-10). The concentration measured is an accumulation of the cytokine secreted by the cells. An increase or decrease of concentration between two conditions can be perceived as a faster or slower production of said cytokine. Concentration variation depending on time can be observed with the fibroblast supernatant analysis. IL-18, IL-10 and IL-6 have higher concentrations (ignoring conditions) at 24 hours after infection than at 6 hours after infection. IL-6 reaches high levels around 1500-2000 pg/mL at H24 and around 2500 pg/mL at H48; there seems to be an MVA-induced decrease of IL-6 secretion, as the concentration is slightly lower when the MOI rises. A difference between H24 and H48 seems to appear without significance between H24 and H48. There appear to be a very limited effect of MVA infection on cytokines' secretion. As an example, MCP-1 concentration seems to be higher with MVA MOI at H48 compared to the MVA Buffer condition. On the contrary, the concentration of TNF α drops from 5-6 pg/mL at MOIs of 0.01 or 0.1 to about 2 pg/mL at an MOI of 1, without significance. The rate of TNF α at H48 MOI 1 is similar to that of H24 Unstimulated, which could imply that an MVA infection at MOI 1 slows the production of TNF α by fibroblasts.

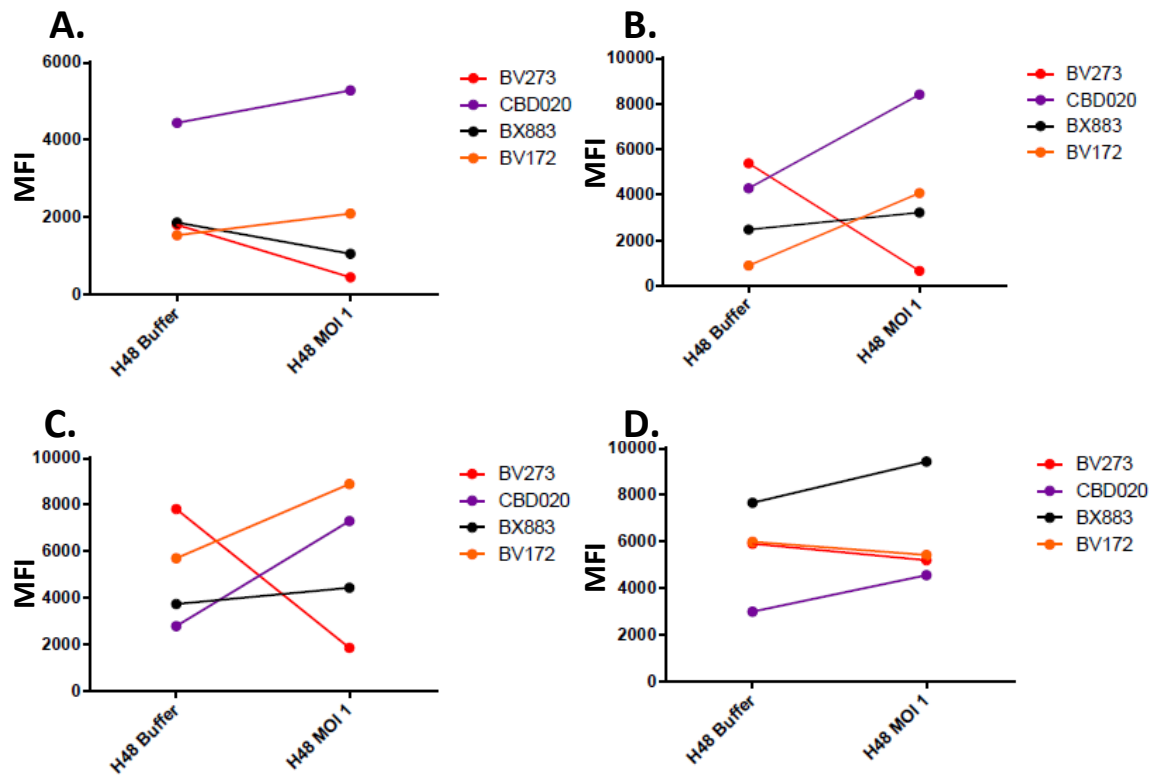


Figure 8 – Expression of activation markers in macrophages. A. CD80 expression. B. CD86 expression. C. CCR7 expression. D. HLA-DR expression. Markers' expression was measured by flow cytometry. The flow cytometry panel was set in order to avoid fluorochrome superposition in their absorption and expression channels.

Macrophages

As for fibroblasts, because of out of range data, only 9 cytokine concentration graphs are presented in **Figure 10**. As macrophages were cultures in low amount of medium, supernatant could only be sampled when cells were harvested. Except for IL-8, MVA seems to induce a cytokine productions in macrophages

IL-8 is secreted by macrophages *in vivo* and is responsible for the recruitment of neutrophils and other granulocytes to the infection site (Harada et al., 1994). MVA seems to decrease this secretion by macrophages *in vitro*, as the concentration drops from about 10000 pg/mL in an unstimulated condition to about 7000 pg/mL in presence of an MOI 1 of MVA.

IL-13 is also a anti-inflammatory cytokine, and its secretion by macrophages *in vitro* appears to remain stable around 10-15 pg/mL when MVA is added to the culture medium.

The secretion of IL-4 appears higher when macrophages were in presence of MVA Buffer or MVA virus for two hours than when left unstimulated.

The concentration of IL-10 is mainly stable, around 140 pg/mL, unaffected by MVA or MVA Buffer.

IL-18, IL-1ra and IL-15 may increase in presence of an MOI 1 of MVA compared to an unstimulated condition or with MVA Buffer.

Finally, the MCP-1 concentration appears stable, slightly decreasing from 2950 to 2900 pg/mL from the unstimulated condition to the MVA MOI 1 condition.

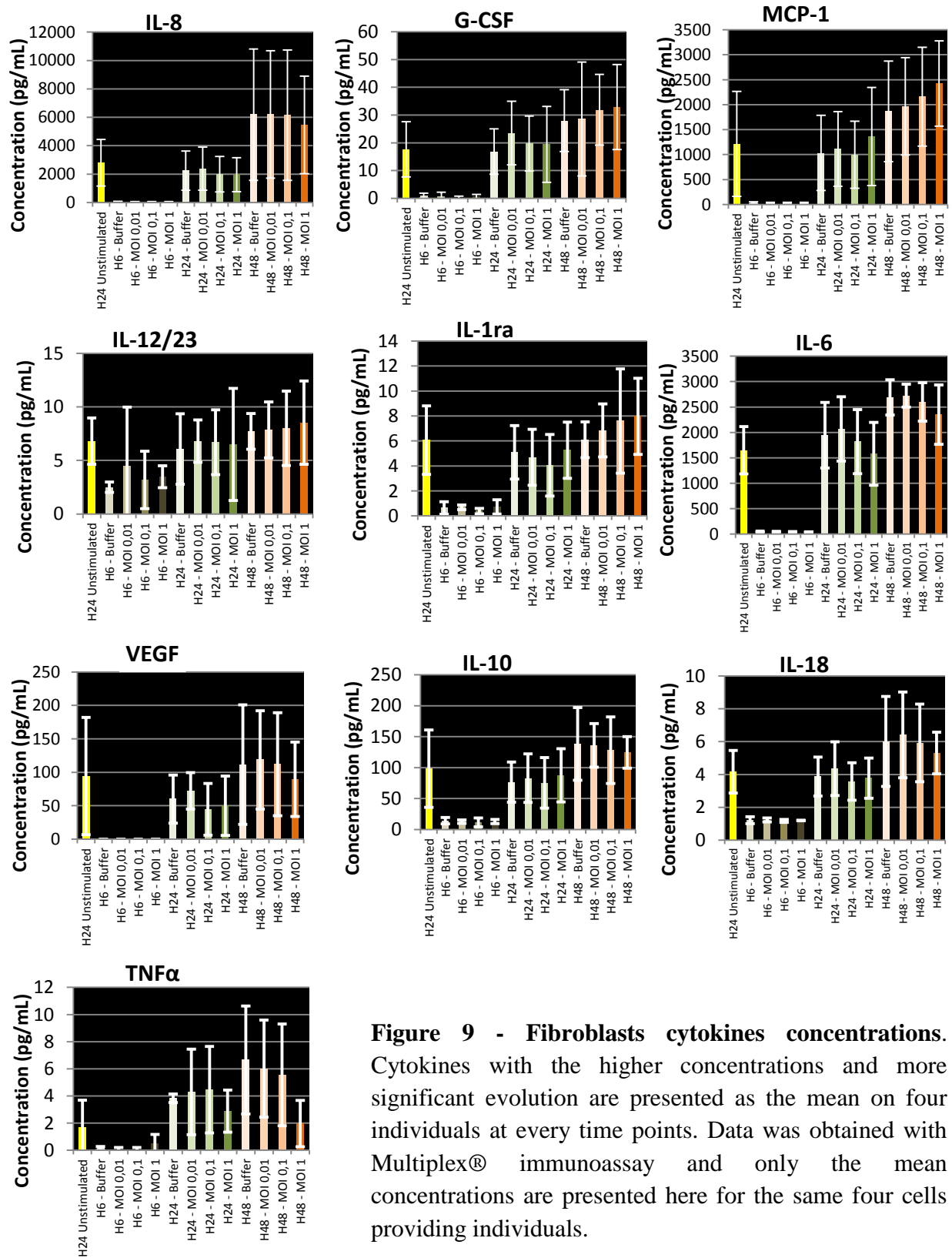


Figure 9 - Fibroblasts cytokines concentrations. Cytokines with the higher concentrations and more significant evolution are presented as the mean on four individuals at every time points. Data was obtained with Multiplex® immunoassay and only the mean concentrations are presented here for the same four cells providing individuals.

Discussion

It was shown here that macrophages and fibroblasts had different behavior to an MVA *in vitro* injection. Fibroblasts emerge as *in vitro* targets of MVA, as they express viral GFP after injection of MVA-eGFP in the culture medium. The infection is enhanced by time and viral load. The later cells are harvested after injection of virus and the higher MOI the well received, the more infected fibroblasts are counted. There seemed to have a dose-dependent effect of MVA on fibroblasts infection. *In vivo*, the encounter of MVA with fibroblasts may be limited by other cell types in dermis, whereas *in vitro*, the fibroblasts form an adherent layer of cells at the bottom of culture wells, improving the access of MVA to the membrane of fibroblasts. Markers used in flow cytometry to monitor the fibroblasts' cell activation (CD10, CD44, CD90 and MHC I) underwent slight change in their expression depending on infection conditions. CD44 is ligand to the hyaluronic acid, component of the extracellular matrix: a higher expression of CD44 by fibroblasts increases the adhesion of those cells to the matrix. CD90 is involved in fibroblasts' migration and proliferation, but its role in this field is vague. Following the idea that the increase observed in CD44 expression means an improved adhesion of fibroblasts to the local matrix, it would be logical to consider CD90 as a reducer of fibroblast migration. CD90 also follows a slight rise in expression by fibroblasts. Moreover, in other cell lines than fibroblasts, CD90 was also shown to be a marker of cell apoptosis, a fact coherent with the result of decreasing living cell numbers when infected (Rege and Hagood, 2006). Dermis fibroblasts infected by MVA would remain at their initial infection site and undergo MVA-induced cell-death; in the meantime their role as direct targets of MVA also improve their expression of viral antigens, triggering the immune response to MVA.

In vitro differentiated macrophages (monocyte-derived macrophages) do not possess the exact phenotype of macrophages differentiated *in vivo*. Moreover, monocyte-derived macrophages are already activated cells by definition, which questions the possibility to observe a real difference in "activation" markers' expression induced by MVA. In spite of their low numbers, the harvested cells were defined as monocyte-derived macrophages through flow cytometry data analysis and thanks to the markers used, such as CD206 (a mannose receptor of the pattern recognition receptors' family), CD14 (Pattern Recognition Receptor) and CD163 (a scavenger receptor cysteine-rich protein). Along with CD14 and

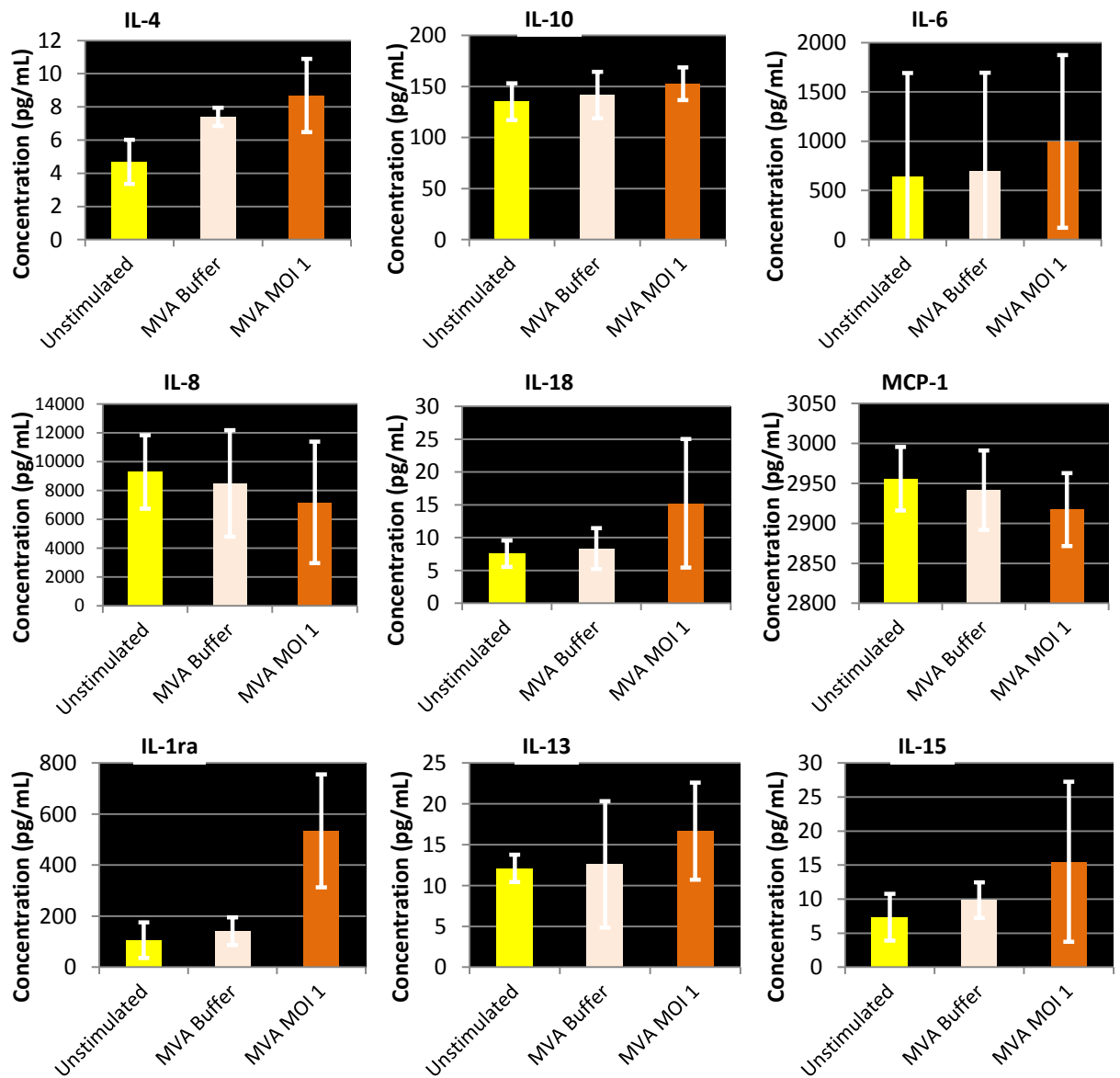


Figure 10 - Macrophages cytokines mean concentrations. Cytokines were assayed from culture medium samples. Cytokines with the higher concentrations and more significant evolution are presented as the mean on four individuals at 48 hours after infection. Data was obtained with Multiplex® immunoassay.

CD206, CD163 is used here as a positive phenotypic marker for monocytes and macrophage-like cells (Vogel et al., 2014). Among harvested cells, none expressed viral GFP, which suggests that either macrophages are not direct targets of MVA infect, or that infected macrophages were lost during the protocol. Indeed, numerous macrophages seemed to have been lost during well washings and medium changes, as differentiated monocytes lose their adhesion capacity to the bottom of the culture well. It is possible that the simple stress of injecting the virus in the culture medium induced a detachment of the macrophages during the first two hours of stimulation, thus leading to a massive loss of cells when washing. Those detached cells may or may not have been infected during those two hours by MVA. The remaining cells harvested at H48 were not infected but showed some slight changes in their activation markers and cytokine secretion. Either signals from infected and detached macrophages or detection of the MVA virus in the culture medium might generate a stress in uninfected macrophages. Previous results of our team showed GFP expression by cells with a phenotype close to that of macrophages. If macrophages are not infected directly by MVA, it is however still possible that the cells identified as expressing GFP be macrophages after their phagocytosis of other infected cells or remains of infected and dead cells.

In vivo, the global effect of several cytokines depends on the balance between the concentrations of antagonist cytokines. The results from the fibroblast supernatants show probable time-dependent evolution of some cytokines, such as IL-10 and IL-6, anti-inflammatory cytokines (Conti et al., 2003) or IL-18, rather a pro-inflammatory cytokine (Kanai et al., 2001). IL-6 however also seems to be affected by the MVA infection, as the concentration is slightly lower when the MOI rises, but overall there appears to be a very limited effect of MVA infection on cytokines' secretion by fibroblasts. Fibroblasts' secretion of cytokines went on during the experiment up to 48 hours after infection; the cytokines secreted by fibroblasts could have a consequence on the immune response, as fibroblasts are present in high numbers in the dermis.

However, the absence of antigen expression in macrophages may be due to the fast degradation of the antigen by those professional antigen presenting cells. On the other hand, macrophages had a higher cytokine secretion than fibroblasts. Unlike fibroblasts, macrophages are immune cells and as such, much more inclined to secrete cytokines. IL-8 secretion for example is responsible for recruiting T cells and neutrophils to the inflammation site, making IL-8 a pro-inflammatory cytokine. IL-8 appears reduced when MVA is added to the culture medium. It is difficult to conclude *in vitro* on isolated cytokines; moreover, there may be more influent and interesting cytokines in an MVA infection than the 23 cytokines

assayed in the Multiplex®. Furthermore, a slight difference in concentration may have important repercussions on the organism through complex domino effects, as explained earlier for fibroblasts.

In a recent study (Kurashima et al., 2014), skin fibroblasts appear to induce dermatitis by upregulating an ATP cellular receptor highly expressed by mast cells. This result shows the importance of the structural cells such as fibroblasts in their regulation of the homeostasis of the skin and the immune consequences of the disruption of this regulation. Targeting structural cells such as fibroblasts in a vaccine strategy may be a lead to explore if the results prove to be a long term adaptive response.

In vivo injections of MVA-eGFP have already been experienced by this team. Now that fibroblasts were identified as *in vitro* targets of MVA, verifying this fact *in vivo* demands a precise and exclusive marking strategy that has not yet been developed. *In vivo* analyzing of an MVA injection and infection includes a lot more information than an *in vitro* injection analysis. First, *in vivo* involves multiple cell types and a multiple-way cell communication between the local cells, the cells arriving on site through blood and those leaving through the lymph canals for example. An intradermal or subcutaneous injection, whatever the formula injected, causes an inflammation by itself, recruiting cells to the inflammation site, including macrophages.

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Annexe I - Résumé

La vaccination est un procédé basé sur le principe de la mémoire immunitaire. En simulant une première rencontre avec un antigène étranger, alors non pathogène, la réponse immunitaire à cet antigène est créée et gardée en mémoire pour constituer la réponse adaptative à cet antigène. Il existe trois catégories de vaccins : les vaccins vivants atténués, qui ont subi des modifications génétiques à la suite de passages successifs dans des cellules, les vaccins inactivés, altérés par la chaleur ou des substances chimiques, et les vaccins composés de sous-unités purifiées de pathogènes ou de vecteurs recombinants. Le vaccin contre le virus de la variole (*smallpox*) est le premier vaccin mis au point à la fin du XVIII^e siècle, en inoculant à des patients sains du pus provenant de lésions de vaches atteintes par la forme bovine du virus (*cowpox*). Le virus de la vaccine, qui tire son nom de son origine bovine, a servi de vaccin lors des campagnes d'éradication de la variole au XX^e siècle. Le MVA (*Modified Vaccinia virus Ankara*) est un virus atténué, obtenu après plus de 500 passages d'un poxvirus dans des fibroblastes de poulet.

La voie d'injection majeure des vaccins est aujourd'hui la voie intramusculaire, mais de nombreux procédés passent par la peau, comme la scarification, l'injection intradermique ou sous-cutanée. La variole fut éradiquée grâce à des vaccinostyles et des aiguilles bifurquées, montrant l'efficacité d'administrer un vaccin par la peau. En effet, la peau comprend une grande variété de cellules immunitaires. Par exemple, le derme est un tissu richement vascularisé constitué de cellules non immunitaires comme les fibroblastes et de cellules immunitaires comme les lymphocytes et les macrophages. De récents résultats du laboratoire ont montré la grande diversité des cellules immunitaires dans la peau et leur rôle dans les réponses inflammatoires à une vaccination intradermique. Le MVA est capable de déclencher les immunités innée et adaptative. Cette dernière reste détectable chez l'homme jusqu'à 50 ans après la vaccination, notamment sous la forme de cellules T CD8⁺ et T CD4⁺ spécifiques au MVA. Le MVA est également efficace chez les primates non humains (NHP) en vaccination contre la variole du singe (*monkeypox*). Afin d'obtenir une réponse adaptative efficace et durable il est possible d'agir sur la réponse innée. Une infection *in vivo* de MVA provoque un recrutement de monocytes, neutrophiles et lymphocytes T CD4⁺ au site d'injection. Il a été montré chez les souris que les cellules dendritiques sont infectées par le MVA, et que leur profil d'expression, leur morphologie et leur stade de maturation en sont affectés. Des résultats préliminaires de l'équipe semblent montrer des cellules infectées par le MVA en injection intradermique ; la morphologie de ces cellules correspond à celle de

fibroblastes et/ou des macrophages. Afin de vérifier l'infection de ces types cellulaires, des fibroblastes et des macrophages sont respectivement mis en culture à partir de derme de NHP et différenciés à partir de monocytes de sang périphérique de NHP. L'utilisation d'un MVA recombinant codant la GFP (MVA-eGFP) permet d'identifier, par cytométrie en flux, les cellules infectées par ce vecteur.

Il est montré dans ce rapport que les fibroblastes sont infectés par le MVA. Le taux d'expression de la GFP virale, c'est-à-dire le taux d'infection, augmente avec la charge virale injectée et avec le temps de culture après les deux heures de contact avec le virus. Le MVA semble également provoquer la mort des cellules en parallèle de l'infection. Les macrophages ne semblent pas être infectés directement, mais la présence de MVA dans le milieu semble modifier les niveaux d'expression de certains marqueurs d'activation de ces cellules. Des échantillons de milieu de culture ont été prélevés afin de mesurer les concentrations de 23 cytokines par technologie Multiplex®. Les résultats semblent montrer des modifications de concentration, donc de sécrétion de plusieurs cytokines par les fibroblastes et les macrophages. Ces changements de sécrétion dépendent du temps après infection et/ou de la charge virale.

Si aucun macrophage n'a été identifié comme exprimant la GFP virale, il est possible que les macrophages se soient décollés de la surface de culture suite au stress induit par l'injection de MVA dans le milieu. En effet les monocytes, une fois différenciés, sont très facilement détachables. Les macrophages décollés entre l'incubation avec le virus et le lavage du milieu deux heures plus tard n'ont pas pu être récupérés séparément du virus et ont été perdus. Cependant, deux hypothèses se présentent : soit les macrophages infectés ont été perdus au lavage du virus soit aucun macrophage n'a été infecté, et le décollage des cellules est dû au lavage en lui-même. Les observations concernant l'expression modifiée de certains marqueurs d'activation et la sécrétion de cytokines par les macrophages s'expliqueraient par une réponse à la présence du MVA dans le milieu.


Les résultats préliminaires de l'équipe montrant des cellules positives pour la GFP virale en cas d'injection intradermique pourraient être interprétés comme l'observation de fibroblastes du derme infectés. Par ailleurs, les macrophages du derme peuvent exprimer directement la GFP après infection ou indirectement après avoir phagocyté une cellule infectée.

Une vérification *in vitro* de ces résultats nécessiterait un autre protocole d'infection par le MVA, qui permettrait de séparer le virus des cellules du milieu, y compris les cellules mortes potentielles. Afin de vérifier ces mêmes résultats *in vivo* sur notre modèle NHP, il faudrait

injecter le virus par voie intradermique, ce qui est déjà réalisé par le laboratoire, puis isoler le derme du site d'injection. La cytométrie en flux peut permettre d'identifier des macrophages infectés, par une stratégie de marquage déjà définie. Cependant, à cause de la nature indifférenciée des fibroblastes, le manque de marqueurs spécifiques uniquement des fibroblastes signifie qu'il faut d'abord mettre en place une stratégie de marquage plus complexe pour identifier les fibroblastes parmi les cellules du derme.

Annexe II - Abstract

MVA is an attenuated poxvirus frequently used in vaccine strategies. Previous results from the lab show unknown dermis cells expressing viral proteins. Those unidentified dermis cells have a morphology alike fibroblasts and/or macrophages. As MVA is routinely used as a recombinant vector in Non-human primates, this model is used here to identify dermis fibroblasts and monocyte-derived macrophages as infection targets of MVA, their cellular response to MVA and their immune role in the response to an MVA infection. Fibroblasts are cultured from skin biopsies and macrophages are derived from peripheral blood monocytes, then cell cultures are incubated with MVA-eGFP. Cells' activity changes are monitored by flow cytometry and Multiplex® immunoassay on medium samples. The results seem to indicate an effective infection of fibroblasts, higher with culture time after infection, along with MVA-induced cell death. Macrophages do not appear infected but their activations markers undergo some changes in their expression. Both cell types, either infected or not, appear to show different cytokine secretions in presence of MVA.

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<p>Résumé (1600 caractères maximum) :</p> <p>Le MVA est un virus atténué de la famille des poxvirus fréquemment utilisé dans des stratégies de vaccination. Des résultats préliminaires de l'équipe montrent des cellules non identifiées du derme exprimant des protéines virales. Ces cellules non identifiées possèdent une morphologie proche de celle des fibroblastes et/ou macrophages. Le MVA étant utilisé en routine comme un vecteur recombinant chez les primates non-humains, ce modèle est utilisé ici pour étudier les fibroblastes du derme et les monocytes différenciés en macrophages comme cibles potentielles du MVA, leur réponse cellulaire au MVA et leur rôle immunitaire dans l'infection au MVA. Les fibroblastes sont mis en culture à partir de biopsies de peau et les macrophages sont obtenus par différenciation de monocytes du sang périphérique. Les cellules sont ensuite incubées en présence de MVA-eGFP. L'activité des cellules est mesurée par cytométrie en flux et immunodosage en technologie Multiplex® sur des échantillons de surnageant de culture. Les résultats semblent montrer une infection des fibroblastes ainsi qu'une mort cellulaire induite par le virus. Les macrophages n'apparaissent pas infectés mais leurs marqueurs d'activation subissent des changements d'expression/ Les deux types cellulaires, infecté ou non, montrent des différences de sécrétion de cytokines en présence de MVA.</p>		
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Mots-clés : MVA-eGFP, fibroblastes, derme, macrophages, PNH, cytométrie, infection Key Words: MVA-eGFP, fibroblasts, dermis, macrophages, NHP, cytometry, infection		