Adjuvance of Influenza virosomes in CTL induction in vitro

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SUMMARY

The induction of cytotoxic T lymphocyte (CTL) responses is of high relevance in immunological defense against intracellular pathogens and tumor cells. While humoral immune responses are successfully induced by a number of vaccines, the activation of cellular immune responses has only been addressed more recently.
The development of novel immunogens from live attenuated vaccines to subunit vaccines demands efficient and safe adjuvants to improve their immunogenicity. Importantly, there are only three adjuvants licensed for human use: aluminium salts, MF59 (microfluidized detergent stabilized oil in water emulsion) and IRIV (immunopotentiating reconstituted influenza virosomes). Aluminium salts are the most widely used adjuvants and their efficacy in enhancement of humoral responses is well documented. They are ineffective in the induction of cellular responses, whereas IRIV and MF59 might be effective, in addition to humoral responses, also in the induction of cellular responses.
The aim of our group, working in the field of cancer immunotherapy, is induction of CTL specific to melanoma associated antigens. The monitoring of a clinical phase I/II trial has demonstrated increased frequencies of specific CTL in peripheral blood upon administration of antigenic epitopes encoded as minigenes with costimulatory molecules in a recombinant vaccinia virus. In the heterologous vaccination protocol adopted, however, high CTL frequencies were not sustained upon administration of the same epitopes as synthetic peptides. This pattern prompted the search for appropriate adjuvants enhancing peptide induced CTL responses.
In this thesis work we focused on the in vitro characterization of immune responses elicited by influenza virosomes and on the in vitro evaluation of influenza virosome adjuvance in HLA class I restricted peptide induced CTL responses. We tested empty IRIV admixed with peptides and influenza virosomes encapsulating peptides, both produced by Pevion Biotech Ltd. Due to the low encapsulation efficiency of IRIV per se, the production of the second formulation required encapsulation of peptides into liposomes and subsequent fusion with chimeric IRIV. Thus, we characterised immune responses elicited by empty IRIV and empty chimeric IRIV fused with empty liposomes (FCIRIV). Then, we evaluated their adjuvant capacity by testing CTL induction in the presence of IRIV admixed with peptides and by peptides encapsulated in FCIRIV as compared to CTL induction by peptides in absence of influenza virosomes.
For IRIV admixed with peptides we addressed induction of CTL specific for the highly immunogenic Influenza matrix $_{58\text{-}66}$ ($IM_{58\text{-}66}$) and to the immunodominant melanoma

associated Melan-A/ Mart-1_{27-35} HLA-A201 restricted epitopes. For peptides encapsulated in FCIRIV we addressed induction of CTL specific for the L_{27}Melan-A/Mart-1_{26-35} HLA-A0201 restricted epitope.

Our results demonstrate that all influenza virosome formulations under investigation induce antigen triggered CD4+ T cell proliferation characterized by a T helper 1 cytokine profile. Further dissection of CD4+ T cells identified CD4+CD45RO+ cells as proliferative responders to IRIV stimulation and no major cell proliferation could be induced in cord blood mononuclear cell cultures. These findings indicate that the majority of CD4+ T cells responding to IRIV are antigen experienced. In addition, supernatants of IRIV stimulated PBMC cultures favoured maturation of dendritic cells, as demonstrated by upregulation of HLA-ABC, CD86 and CD83.

Both, influenza virosomes admixed with peptides or encapsulating peptides significantly enhanced specific CTL induction, as detected by multimer staining and cytotoxicity assays. CTL induction experiments in presence of irradiated CD4+ T cells indicated that IRIV CTL adjuvance required CD4+ T cell activation. In addition, transwell cultures pointed to a key role of cytokines in IRIV mediated CTL adjuvance.

In contrast to empty IRIV, FCIRIV with encapsulated peptides were characterized by CD4+ T cell independent adjuvant potential, possibly attributable to influenza virosome delivery capacities.

Taken together, our results demonstrate that influenza virosomes are endowed with the capacity to enhance HLA class I restricted CTL induction in vitro. Importantly, this could be demonstrated not only for the highly immunogenic IM_{58-66} epitope, but also for the melanoma associated epitopes L_{27}Melan-A/Mart-1_{26-35} and Melan-A/Mart-1_{27-35}.

Moreover, CTL induced by L_{27}Melan-A/Mart-1_{26-35} encapsulated in FCIRIV were capable of recognizing and lysing tumor cells that constitutively express the Melan-A/Mart-1 antigen. These in vitro findings encourage further evaluation of influenza virosome CTL adjuvance in vivo.

INTRODUCTION

The identification of the first tumor associated antigen (TAA) in 1991 [1] has represented the starting point for the performance of clinical trials aiming to activate the adaptive immune system against various kinds of tumors by expanding TAA specific cytotoxic T lymphocytes (CTL).

In this regard it should be emphasized that the development of „T cell vaccines“ is still in its infancy as compared to the majority of existing vaccines which mainly act through the humoral arm of the immune sytem. Especially chronic viral infections like HIV and HCV and tumors have raised wide attention regarding the possibility of generating specific cellular immune responses. The forte of activated specific CTL may be represented by their ability to kill infected cells or tumor cells, possibly resulting in reduced spread of the infectious agent or regression of tumors, respectively. In contrast to humoral responses the induction of CTL responses is dependent on histocompatibility antigens which are highly polymorphic. Thus, responsiveness among different individuals might display a high variability, depending on the antigen and the individual HLA class I phenotype.

Major limitations of T cell vaccines, as reported for viral infections, are the ability of pathogens to escape T cell response by mutating target epitopes and the potential for T cells to become exhausted by high levels of persisting antigen [2]. Moreover, priming of CTL requires, in addition to TCR recognition of the epitope-HLA complex, a costimulatory signal provided by the APC. Provision of both signals is crucial, as absence of costimulation usually results in anergy of specific CTL.

Technically, the development of T cell vaccines is also challenged by difficulties in monitoring CTL responses eventually induced. Surrogate markers for efficacy and protection are frequently unclear [2] and in vitro assays require short- to medium-term stimulation in vitro.

Regarding induction of TAA specific CTL, it should be noted that tumors are part of the „self“ and therefore are likely to be tolerated by the immune system.

Administration of TAA derived peptides demands formulations that, in the best case provide protection from enzymatic degradation, access to antigen presenting cells and enhancement of peptide induced CTL response. Considering the latter, there are very few adjuvants approved for human use, all with proven efficacy in humoral immune responses.

Our group has performed a phase I/II clinical trial in melanoma by using a heterologous vaccination protocol [3]. HLA-A0201 restricted epitopes of the TAA Melan-A/Mart-1 (27-35), gp100 (280-288) and tyrosinase (1-9) were administered subcutaneously either as

minigenes encoded together with costimulatory molecules by an inactivated, nonreplicating recombinant vaccinia virus (TAA - rVV) or as synthetic peptides [3]. In addition, GM-CSF was administered as supporting cytokine. The heterologous protocol was chosen to minimize immune responses against rVV that may hamper the capacity of the recombinant virus to infect antigen presenting cells. Monitoring of TAA specific CTL frequency in peripheral blood demonstrated an increase upon TAA-rVV administration which was not sustained upon peptide administration.

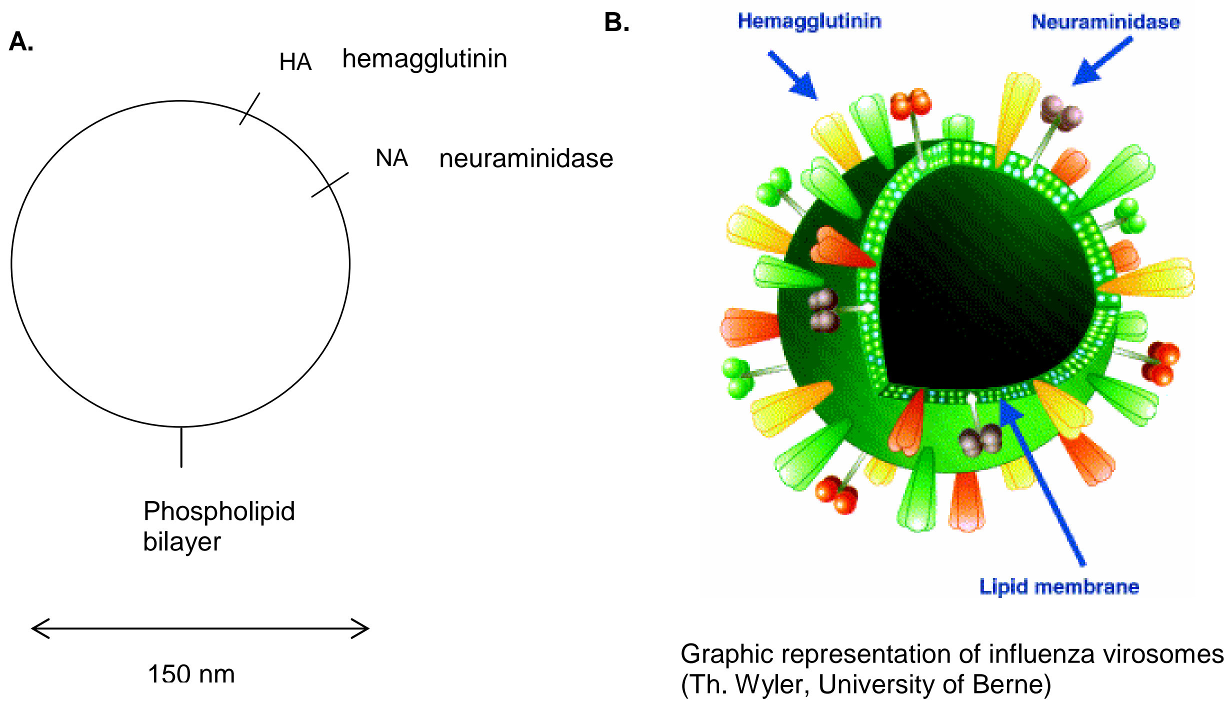
The decrease of TAA specific CTL frequency upon peptide administration may be due to low stability and poor immunogenicity of synthetic peptides as such. Furthermore, at difference with the TAA-rVV formulation, HLA class I restricted peptides per se do not provide CD4+ T cell help.

This pattern initiated the search for adjuvants appropriately enhancing CTL responses induced by synthetic peptide formulations.

We focused on Immunopotentiating Reconstituted Influenza Virosomes (IRIV), one of the very few adjuvants approved for human use beside aluminium salts and MF-59 (microfluidized detergent stabilized oil in water emulsion) [4].

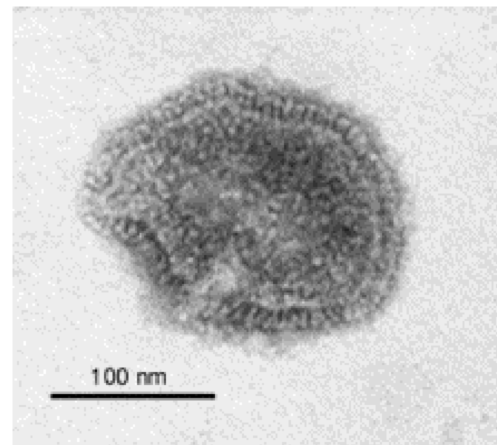
Influenza virosomes in general were first described by Almeida et al. in 1975 [5].

IRIV, produced by Pevion Biotech Ltd. (Berne, Switzerland), are used as adjuvant in hepatitis A vaccination and as subunit vaccine in influenza vaccination. They are spherical 150nm sized particles, consisting of a phospholipid bilayer in which influenza virus derived hemagglutinin (HA) and neuraminidase (NA) are intercalated. Basically, these particles mimick structurally and functionally the envelope of influenza virus (Fig. Introduction 1, panels A, B and C).



Graphic representation of influenza virosomes (Th. Wyler, University of Berne)

C.



Electron microscopy of influenza virosomes (L. Bungener et al. *Vaccine 20, 2002)*

Fig. Introduction1. Schematic and electron microscopical presentation of influenza virosomes.

Regarding functional properties, influenza virus derived HA plays a key role in virosome uptake by APC through receptor mediated endocytosis and in intracellular fusion of the virosome with the endosomal membrane [6]. Moreover, it is a highly immunogenic antigen derived from a widespread and frequently occuring pathogen. Finally, the spherical structure of virosomes may be suitable to encapsulate peptides and protect them from enzymatic degradation. Whereas IRIV have been demonstrated to enhance humoral responses in hepatitis A vaccination [7-9], little is known on their adjuvant capacity as related to CTL responses. In studies on hepatitis C it has been shown that IRIV containing HLA class I restricted HCV core peptides can prime CTL from peripheral blood mononuclear cells of HCV^- healthy blood donors in vitro [10]. These primed CTL were capable of recognizing and lysing HCV infected target cells, but no major adjuvance was observed as compared to priming with peptides alone. However, in a more recent study in vivo adjuvance in the induction of HCV core peptide specific CTL could be demonstrated in mice by using chimeric IRIV containing the HCV core 132 peptide [11]. Moreover, in vivo administration of influenza virosomes encapsulating a murine CTL epitope of the influenza virus nucleoprotein (NP) was shown to enhance the induction of a class I MHC-restricted CTL response against influenza-infected cells as compared to administration of soluble peptides [12]. This adjuvant effect has been shown to require the membrane fusion activity of influenza virosomes, as fusion-inactivated virosomes and NP-peptide mixed with empty virosomes did not induce CTL activity. Recently, influenza virosomes have been demonstrated to enhance CTL induction against virosome-encapsulated ovalbumin (OVA) in mice as well [13].

In summary, Influenza virosome CTL adjuvance has been demonstrated in mice using formulations including encapsulated peptides or proteins.

The goal of this work was to characterize IRIV elicited immune responses and to evaluate IRIV adjuvant capacity in relation to CTL induction. Unlike previous studies we addressed immunological effects of IRIV per se and investigated TAA specific CTL responses in human cell cultures.

Regarding TAA, MAGE-1, detected in 1991 in melanoma, was the first gene reported to encode a human tumor antigen recognized by T cells [1]. One year later, the first HLA class I restricted epitope, a nonapeptide encoded by MAGE-1, was characterized [14]. Since then, identification and characterization of novel HLA class I and HLA class II restricted TAA has rapidly evolved. According to their tissue distribution, TAA are classified in cancer-testis antigens, differentiation antigens, widely occurring, overexpressed TAA

and unique and shared tumor-specific antigens [15]. All melanoma associated epitopes used in our clinical trial, Melan-A/ Mart-$1_{27\text{-}35}$, $gp100_{280\text{-}288}$ and $tyrosinase_{1\text{-}9}$ are derived from differentiation antigens. These TAA are shared between tumors and the normal tissue from which the tumor arose. Most are detectable in melanomas and normal melanocytes [15].

Among the epitopes used in our clinical trial, Melan-A/Mart-$1_{27\text{-}35}$ is the most and tyrosinase the least immunogenic. In this work we focused on Melan-A/Mart-1 specific CTL induction, using the nonapeptide Melan-A/Mart-$1_{27\text{-}35}$ and the more immunogenic decapeptide analog L_{27}Melan-A/Mart-$1_{26\text{-}35}$ as epitopes. Considering the variability of Melan-A/Mart-1 specific CTL precursor frequencies among healthy donors, we first investigated IRIV CTL adjuvance in relation to influenza $matrix_{58\text{-}66}$ specific CTL induction. As influenza virus is a frequently occurring and widespread pathogen, $IM_{58\text{-}66}$ specific CTL may be present in PBMC of most HLA-A0201+ healthy donors. Thus, CTL induction experiments were performed focusing on $IM_{58\text{-}66}$, Melan-A/Mart-$1_{27\text{-}35}$ and L_{27}Melan-A/ Mart-$1_{26\text{-}35}$ HLA-A0201 restricted epitopes.

IRIV CTL adjuvance was evaluated using two different formulations, both produced by Pevion Biotech Ltd.: Empty IRIV admixed with soluble peptides and chimeric IRIV (CIRIV) encapsulating peptides. The step from empty IRIV to peptide encapsulating CIRIV required an elegant circumventing of the low peptide encapsulation efficiency (0.1-2%) of IRIV per se [11]. As liposomes display a much higher peptide encapsulation efficiency (15-20%), peptides were first encapsulated in liposomes. In parallel empty CIRIV including hemagglutinin derived from two influenza virus strains (X-31 and A/Sing) were produced. Finally, these empty CIRIV were fused with peptide encapsulating liposomes at acidic pH and18℃, a temperature at which only the X-31 deriv ed HA is active and suffices for the fusion process. This procedure resulted in peptide encapsulation into CIRIV with native HA derived from A/Sing influenza virus and inactivated HA derived from X-31 influenza virus (Peptide-FCIRIV: Peptide encapsulated into CIRIV fused with liposomes).

Before evaluating IRIV CTL adjuvance we aimed at characterizing immune responses elicited in vitro by IRIV per se, in absence of peptides. Here we addressed cell proliferation, cytokine profile and IRIV effects on antigen presenting cells in PBMC cultures of healthy donors. Then, we tested IRIV mediated CTL adjuvance in relation to $IM_{58\text{-}66}$ and Melan-A/Mart-$1_{27\text{-}35}$ using empty IRIV admixed with peptides as compared to peptides alone. Finally, FCIRIV adjuvance was evaluated, using FCIRIV encapsulating L_{27}Melan-A/Mart-$1_{26\text{-}35}$ as compared to non-encapsulated L_{27}Melan-A/Mart-$1_{26\text{-}35}$ peptide in

solution. As FCIRIV differ from IRIV in their hemagglutinin and lipid content we evaluated in parallel, same as for IRIV, immune responses elicited by this formulation in absence of encapsulated peptides.

MATERIALS AND METHODS

1. Influenza virosome formulations

IRIV (Immunopotentiating Reconstituted Influenza Virosomes)
CIRIV (Chimeric Immunopotentiating Reconstituted Influenza Virosomes)
FCIRIV (CIRIV fused to empty liposomes)
Mart-FCIRIV (FCIRIV with encapsulated L_{27}Melan-A/Mart-$1_{26\text{-}35}$)

(All from Pevion Biotech Ltd., Bern, Switzerland)

1.1 Preparation of IRIV

Egg phosphatidylcholine (PC, 32 mg), (Lipoid GmbH,Ludwigshafen, Germany) and phosphatidylethanolamine (PE, 8 mg), (R. Berchtold, Biochemisches Labor, Bern, Switzerland) were dissolved in 2.66 ml of PBS containing 100mM octaethyleneglycol (OEG) (Fluka Chemicals,Switzerland), (PBS-OEG). The influenza A/Singapore hemagglutinin was purified as described previously [16]. A solution containing 2mg hemagglutinin was centrifuged for 30 min at 100,000 × *g* and the pellet was dissolved in 1.33 ml of PBS-OEG. The phospholipids and the hemagglutinin-solution were mixed and sonicated for 1 min. This mixture was then centrifuged for 1 h at 100,000 × *g* and the supernatant sterile filtered (0.22 μ). Detergent was removed by using SM Bio-Beads (BioRad, Hercules, PA). Control liposomes (L) were similarly produced, in the absence of influenza virus components.

1.2 Preparation of CIRIV

Chimeric virosomes with hemagglutinin (HA) from the X-31 and the A/Sing Influenza strain, respectively, were prepared by the methods described previously [11,17,18]. Briefly, 32 mg egg PC and 8 mg PE were dissolved in 2 ml of PBS (10.4 μmol/ml PC; 2.7 μmol/ml PE), 100 mM OEG (PBS/OEG). 4 mg HA of each influenza virus was centrifuged at 100,000 x *g* for 1 h at 4°C and the pellet was dissolved in 2 m l of PBS/OEG. The detergent solubilised phospholipids and viruses were mixed and sonicated for 1 min. This mixture was centrifuged at 100,000 x *g* for 1 h at 20°C and the supernatant was sterile filtered (0.22 μm). Virosomes were then formed by detergent removal using 1.24 g of wet SM2 Bio-

Beads for 1 h at room temperature with shaking and three times for 30 min with 0.62 g of SM2 Bio-Beads each.

1.3 Preparation of liposomes encapsulating peptides

25.4 µmol (19.5 mg) PC and 11.5 µmol (8.2 mg) DPPG (molar ratio 70:30) were dissolved in methanol/chloroform (2:1). The solvent was removed by a rotary evaporator (Rotavapor R-205, Büchi Labortechnik, Switzerland) at 40℃ at a gradual vacuum of 30-10 kPa. The dried lipid film was hydrated with 250 µl PBS containing 0.4 mg L_{27}Melan-A/Mart-1_{26-35} peptide to be encapsulated. Several identical preparations were pooled for extrusion. The liposome suspension was extruded four times through polycarbonate membranes (Nucleopore Track-Etch membrane, 0.2 µm, Whatman, UK) with a 1.5 ml Lipex Extruder (Northern Lipids, Canada). Size determination of extruded liposomes was performed by light scattering using a Zetasizer 1000HS instrument (Malvern Instruments, UK).

1.4 Preparation of Mart-FCIRIV

CIRIVs (290 µl in PBS, approx. 2.9 mg phospholipid) were incubated with 160 µl (approx. 17 mg phospholipid) of PC/DPPG extruded liposomes (0.2 µm diameter) containing the L_{27}Melan-A/Mart-1_{26-35} peptide at 18℃ in PBS under constant stirring. To trigger fusion the pH was adjusted to 5.0 ± 0.2 with 1 M HCl. After incubation for 20 min, the mixture was neutralised with 1 M NaOH to a pH of 7.4 ± 0.2 and fusion products were extruded five times through polycarbonate membranes (Nucleopore Track-Etch membrane, 0.2 µm) with a 1.5 ml Lipex Extruder (Northern Lipids, Canada).

Hemagglutinin content of all influenza virosome formulations ranged between
0.5 mg/ml and 2 mg/ml.

L_{27}Melan-A/Mart-1_{26-35} stock concentration of the Mart-FCIRIV formulation was
100 µg/ml.

2. HLA-A0201 restricted peptides	Sequence
IM_{58-66} (Neosystem, Strasbourg, France)	GILGFVFTL
Melan-A/Mart-1_{27-35} (Neosystem, Strasbourg, France)	AAGIGILTV
L_{27}Melan-A/Mart-1_{26-35} (Bachem AG, Bubendorf, Switzerland)	ELAGIGILTV

3. Cell culture

3.1 Isolation of PBMC

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood by gradient centrifugation according to standard methods.

3.2 Culture of PBMC

PBMC were cultured in:

RPMI 1640 (with L-Glutamine, GIBCO) supplemented with

Kanamycin (100µg/ml)
Hepes buffer 10mM
Sodium pyruvate MEM 1mM
Glutamax 1mM
MEM Non essential amino acids

All from GIBCO Paisley, Scotland, thereafter referred to as complete medium (CM)

5% human serum (HS, Blutspendezentrum, University Hospital Basel, Switzerland)

3.3 Isolation of PBMC cell subsets

Isolation of PBMC cell subsets was perfomed by magnetic cell separation (Miltenyi Biotech, Bergisch Gladbach, Germany) according to producers‘ protocols.

3.4 Generation of monocyte derived immature dendritic cells (iDC)

CD14+ cells were isolated from peripheral blood of healthy donors and cultured for 5 to 7 days in DC-medium in 6 well plates (1 x 10^6 to 1.5 x 10^6 cells per well). DC medium drives the differentiation from CD14+ cells to immature dendritic cells (iDC) and was prepared as follows:

RPMI 1640 (with L-Glutamine) supplemented with

Kanamycin (100μg/ml)
Sodium pyruvate MEM 1mM
Glutamax 1mM
MEM Non essential amino acids

All from GIBCO Paisley, Scotland

10% fetal calf serum (GIBCO)
0.004% (v/v) β-mercaptoethanol
IL-4 (1000 U/ml, courtesy of Dr. Lanzavecchia, Bellinzona, Switzerland)
50 ng/ml GM-CSF (Novartis, Basel, Switzerland).

4. Assays

4.1 Proliferation assays

Cells were cultured in CM 5% HS in 96 well flat bottom plates (Becton Dickinson, Le Pont de Claix, France) at 2 x 10^5 cells/ well. On day 5 (antigenic stimulation of peripheral blood cells) or 2 (mitogenic stimulation) ^{3}H-thymidine (Amersham, Little Chalfont, UK) was added at 1μCi per well. After a further incubation for 18 hours, cells were harvested and tracer incorporation was measured by beta counting. Finally, cell proliferation was expressed as ^{3}H-thymidine incorporation in counts per minutes (cpm).

4.2 Phenotyping by flow cytometry

Cells were washed in PBS and 2µl fluorescent labeled (FITC or PE) antibodies (BD Biosciences Pharmingen) were added to each sample. Fluorescent labeled mouse immunoglobulin isotype controls were used to exclude unspecific background staining. Following incubation for 30 minutes on ice in the dark, cells were washed twice, resuspended in 200µl PBS and acquired by a flow-cytometer (FACScalibur) equipped with Cell Quest software (Becton Dickinson, San Diego, CA).

4.3 CTL induction

CD14- cells were cocultured with iDC (CD14- cells : iDC ratio ranged from 5:1 to 20:1) in presence of the HLA class I restricted peptide (IM_{58-66} : 1-2 µg/ml, Melan-A/Mart-1_{27-35}: 10µg/ml, L_{27}Melan-A/Mart-1_{26-35}: 0.25–2µg/ml final concentration) with or without influenza virosomes. In case of IM_{58-66} CTL induction was evaluated 6 to 8 days after setup without IL-2 supplementation and without restimulation.
For Melan-A/Mart-1_{27-35} and L_{27}Melan-A/Mart-1_{26-35} CTL induction was evaluated after IL-2 supplementations and one restimulation with irradiated peptide pulsed APC. IL-2 supplementations were usually performed at 10-20 units/ ml on days 4, 5 and 6 and at 100 units/ml on days 7 and 10. Restimulation was usually performed on day 7 as follows: autologous iDC or CD14+ cells were incubated for 2-3 hours at 37°C in presence of 10 µg peptide/ml. After incubation cells were irradiated (CD14+ cells: 3500 rad, iDC: 2500 rad), washed and added to the assay cultures.

Evaluation of CTL induction was performed by multimer staining and/or ^{51}Cr release cytotoxicity assays. Regarding IM_{58-66}, limiting dilution analysis of CTL precursor frequency was also performed (see below).

4.3.1 Multimer staining
Cells were washed once in PBS and supernatants discarded.
Following addition of 1µl PE labeled pentamers (Proimmune, Oxford, UK), samples were incubated for 10-20 min. at room temperature in the dark. After one wash in PBS 2µl FITC

labeled anti-CD8 were added to each sample and all samples were incubated for 30 min. on ice in the dark.

Following two washes in PBS, cells were resuspended in 200µl PBS and acquired by a flow-cytometer (FACScalibur) equipped with Cell Quests software (Becton Dickinson, San Diego, CA).

CTL induction was evaluated by quantification of the percentages of tetramer/pentamer positive CD8+ cells within the whole CD8+ cell population.

When tetramers were used, the staining procedure was performed in one step:
After wash, tetramers PE and anti-CD8 FITC were added simultaneously to each sample and all samples were incubated for 45 minutes at 4°C in the dark.

4.3.2 Cytotoxicity assays

Target cells (NA-8 cells, T2 cells or HBL cells) were washed in PBS and resuspended in 0.2ml complete medium supplemented with 10% FCS (RPMI 10% FCS). Following ^{51}Cr pulsing (100µCi per sample, 1 hour at 37°C) target cells were wa shed twice in PBS, resuspended in RPMI 10% FCS and preincubated with the target or control peptide (2 hours at 37°C in the waterbath, 10 µg peptide/ml). After incubation cells were washed once in PBS and resuspended in RPMI 10% FCS.

During preincubation with peptides effector cells were plated in 96 well round bottom plates (Becton Dickinson, Le Pont de Claix, France). At least 20 min. before addition of target cells 100'000 K562 cells per well were added to effector cells in a volume of 50µl each. Target cells (1000 per well in a volume of 50µl each) were then added. Plates were centrifuged to provide cell : cell contact between target and effector cells and incubated at 37°C for 4 hours. After incubation supernatants fro m each well were transferred into corresponding wells of Luma plates (Perkin Elmer, Boston, MA). Dried Luma plates were read by a microplate scintillation and luminescence counter.

Percentage of specific lysis was evaluated by the following formula:
% cytotoxicity = sample value – spont value / max value – spont value x 100
spont value: value of spontaneous release
max value: value of maximal release

4.3.3 Limiting dilution assays

CD8+ cells were cocultured in 96 well round bottom plates (Becton Dickinson, Le Pont de Claix, France) with irradiated CD8- cells pulsed with individual peptides.
CD8+ cells were plated in different cell numbers as follows: columns 1-4 (32 wells): maximal CD8+ cell number per well ranging between 5000 and 10000; columns 5-8 (32 wells): ½ maximal CD8+ cell number per well ranging between 2500 and 5000; columns 9-12 (32 wells): ¼ maximal CD8+ cell number per well ranging between 1250 and 2500. CD8- cells were plated in constant numbers (70'000 per well) into each well. Final volume of cell suspension per well was 200µl. Antigenic formulations were added and the plates incubated at 37°C, 5% CO_2. Further procedures included IL-2 supplementation (20 units/ml) on day 3, restimulation with antigenic peptide and second IL-2 supplementation (20 units/ml) on day 7, a third IL-2 supplementation (100 units/ml) on day 10 and cytotoxicity assay on day 15. Cytotoxicity assays were performed by splitting each well in two for assays with specific or control peptides, respectively. Epitope specific CTL precursor frequency was measured by evaluating numbers of positive wells (displaying at least 12% specific cytotoxicity) according to the Poisson's formula.

4.4 Cytokine gene expression assays

PBMC were harvested at different times of culture and total RNA was extracted by using an RNeasy Mini Kit (Qiagen).
For conventional PCR, total RNA was reverse transcribed as follows: 2µg RNA, 2µl oligo d (T) and RNAse free water were mixed in a total volume of 24 µl and incubated at 65 °C for 10 minutes in the waterbath. Samples were then immediately put on ice and supplemented with a mix of 2µl dNTP (2.5 mM), 4µl DTT (100mM, Gibco BRL), 8µl 5x first strand buffer (Gibco BRL) and 2µl M-MLV reverse transcriptase (200 U/ml, Gibco BRL). Samples were incubated at 37°C for 90 minutes in the waterbath. Subsequently the M-MLV reverse transcriptase was inactivated by heating the samples at 94 °C for 5 minutes and cDNA samples were stored at –70°C.

PCRs were performed as follows:

Primary denaturation of the templates by 10 min heating at 95°C

Amplification cycles included the following protocoll:
40 sec denaturation at 94℃, 40 sec annealing at 62 ℃, 1 min extension at 72℃.

Final extension was performed by 15 min heating at 72℃.

Primers [19]:

β-actin	Sense primer: Antisense primer:	TGACGGGGTCACCCACACTGTGCCCATCTA CTAGAAGCATTGCGGTGGACGATGGAGGG
IL-2	Sense primer: Antisense primer:	ATGTACAGGATGCAACTCCTGTCTT GTCAGTGTTGAGATGATGCTTTGAC
IL-4	Sense primer: Antisense primer:	ATGGGTCTCACCTCCCAACTGCT CGAACACTTTGAATATTTCTCTCTCAT
IL-5	Sense primer: Antisense primer:	GCTTCTGCATTTGAGTTTGCTAGCT TGGCCGTCAATGTATTTCTTTATTAAG
IL-10	Sense primer: Antisense primer:	AAGGCATGCACAGCTCAGCACT TCCTAGAGTCTATAGAGTCGCCA
TNF-α	Sense primer: Antisense primer:	ATGAGCACTGAAAGCATGATCCGG GCAATGATCCCAAAGTAGACCTGCCC
IFN-γ	Sense primer: Antisense primer:	ATGAAATATACAAGTTATATCTTGGCTTT GATGCTCTTCGACCTCGAAACAGCAT
GM-CSF	Sense primer: Antisense primer:	ACACTGCTGAGATGAATGAAACAGTAG TGGACTGGCTCCCAGCAGTCAAAGGGGATG

In case of CIRIV and FCIRIV formulations expression of IFN-γ and IL-4 genes was addressed by one step real time PCR. Briefly, ΔC_T [C_T (gene of interest) – C_T (reference gene GAPDH)] was calculated for each sample and reference sample. $\Delta\Delta C_T$ [ΔC_T (reference sample) – ΔC_T (sample)] was evaluated, and relative quantification was calculated as $2^{-\Delta\Delta C_T}$. The results were expressed as n-fold difference relative to the reference sample.Real-Time qPCR were performed in Thermofast® 96 well plates (Abgene, Epsom, UK), using the TaqMan® One Step PCR Master Mix Reagents Kit (Applied Biosystems, Forster City, CA) and the ABI prim™ 7700 sequence detection system (Applied Biosystems, Forster City, CA).
Stage1: 2 min. at 50℃, stage2: 10 min. at 95℃, st age3: 15 sec. at 95℃ followed by 1 min. at 60℃ (repeated 45 times). Normalization of sample was performed using GAPDH as reference gene.

Primers and Probes:

GAPDH [20]: Fwd ATG GGG AAG GTG AAG GTC G
Rev TAA AAG CAG CCC TGG TGA CC
Probe FAM-CGC CCA ATA CGA CCA AAT CCG TTG AC-TAMRA

IFN-γ [21]: Fwd AGC TCT GCA TCG TTT TGG GTT
Rev GTT CCA TTA TCC GCT ACA TCT GAA
Probe FAM-TCT TGG CTG TTA CTG CCA GGA CCC A-TAMRA

IL-4 [22]: Fwd CCA CGG ACA CAA GTG CGA TA
Rev CCC TGC AGA AGG TTT CCT TCT
Probe TCTGTGCACCGAGTTGACCGTAACAGAC

IL-6: Fwd CAG CCC TGA GAA AGG AGA CAT G
Rev GGT TCA GGT TGT TTT CTG CCA
Probe AGT AAC ATG TGT GAA AGC AGC AAA GAG GCA C-TAMRA

Quantification of cytokine gene expression was calculated by using a reference sample for comparison of gene expression in experimental samples.
IL-6 gene expression was addressed by real time PCR following separately performed reverse transcription, using the TaqMan® Universal PCR Master Mix , No AmpErase® UNG (Applied Biosystems, Forster City, CA).

4.5 Cytokine secretion assays

Supernatants of PBMC cultures were harvested at different times of culture and cytokine concentrations analysed by standard ELISA assays. Either BD OptEIA ™ ELISA Sets (Becton Dickinson, Franklin Lakes, NJ) or reagents from BD Pharmingen (BD Pharmingen, San Diego, CA) were used according to company's descriptions. Data were analyzed using Softmax software (Molecular Devices Corporation, Menlo Park, CA).

4.6 General remarks

Data displayed are usually representative for at least two independently performed experiments, except those indicated as preliminary and those displayed in Fig. 3. Standard deviations of all cytotoxicity assays and real time PCR assays were below 10% and are not displayed in the figures.

RESULTS

PART 1: STUDIES ON EMPTY IRIV

I. Immune responses elicited by IRIV in PBMC

To characterize immune responses elicited by IRIV in vitro we addressed cell proliferation, cytokine gene expression and secretion as well as effects on antigen presentation.

1. IRIV induce antigen specific proliferation of CD4+CD45RO+ cells

PBMC from healthy donors were cultured in the presence of IRIV at different concentrations and proliferation was measured as ^{3}H-thymidine incorporation after 6 days incubation. Upon IRIV stimulation cell proliferation could be observed in PBMC cultures from all (n = 10) donors tested. One representative experiment is presented in Fig. 1, A . The extent of ^{3}H-thymidine incorporation was variable in cultures from different donors but no PBMC proliferation was detectable in cultures performed in the presence of control liposomes (L) devoid of viral proteins.
To address the identity of proliferating cells proliferation assays were performed with purified CD4+ T cells or CD8+ T cells cocultured with autologous irradiated PBMC in presence and absence of IRIV.
As shown in Fig. 1, B CD4+ T cells but not CD8+ T cells proliferated in presence of IRIV. Further dissection of CD4+ cells into CD45RA+ and CD45RO+ cells indicated that CD4CD45RO+ T cells represented the main cell population responding to IRIV stimulation (Fig. 2, B). The CD45RO+ phenotype is characteristic for memory T cells and therefore these data indicate that IRIV induced cell stimulation is of antigenic nature. This observation is corroborated by proliferation assays performed with cord blood mononuclear cell cultures in presence of IRIV or conventional mitogens. As shown in Fig. 2, A both mitogens PHA and Con A induced marked cell proliferation. In contrast, IRIV only induced a marginal ^{3}H-thymidine incorporation in naive cells, similar to that detectable in cultures performed in the presence of control L.

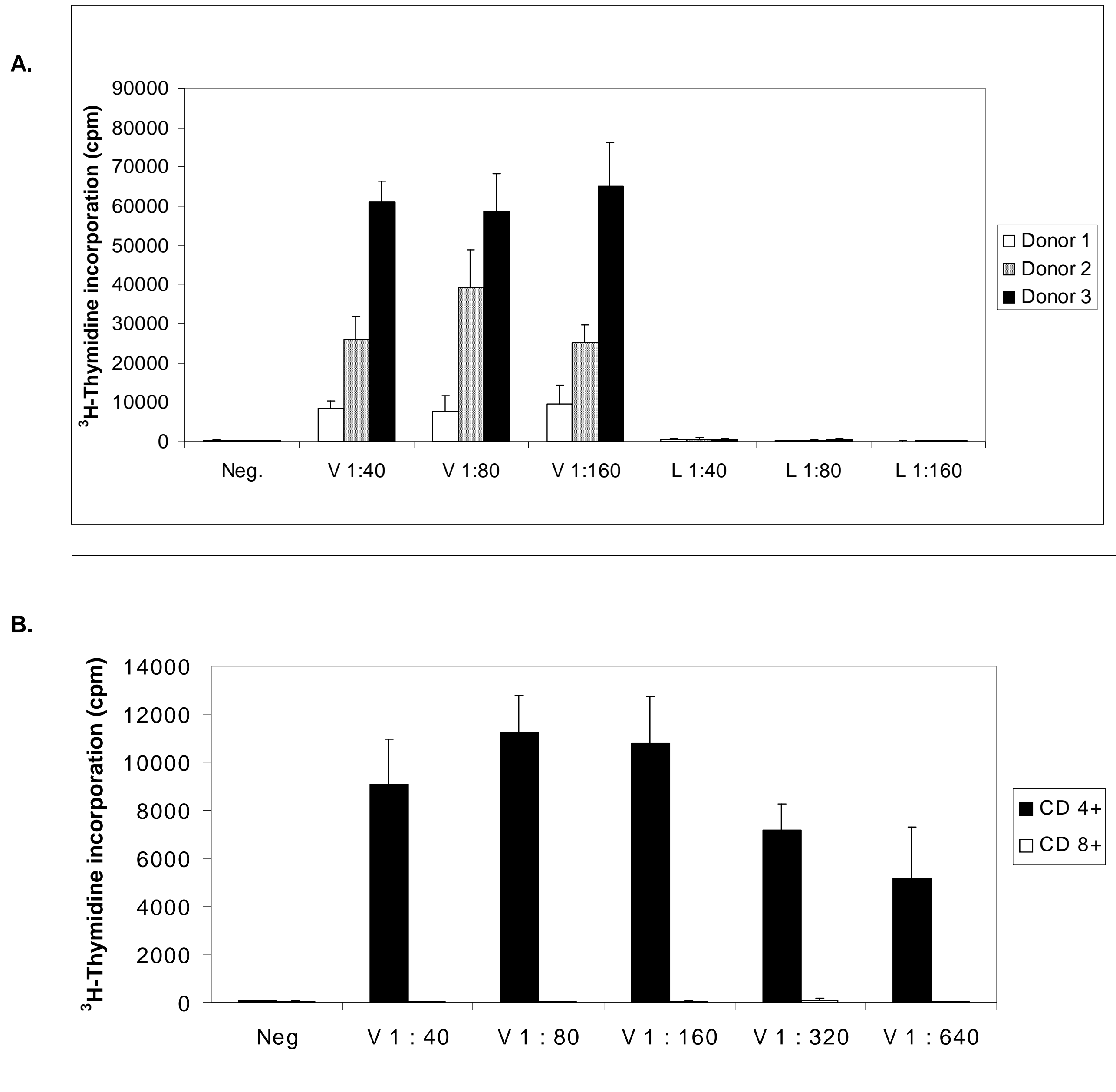
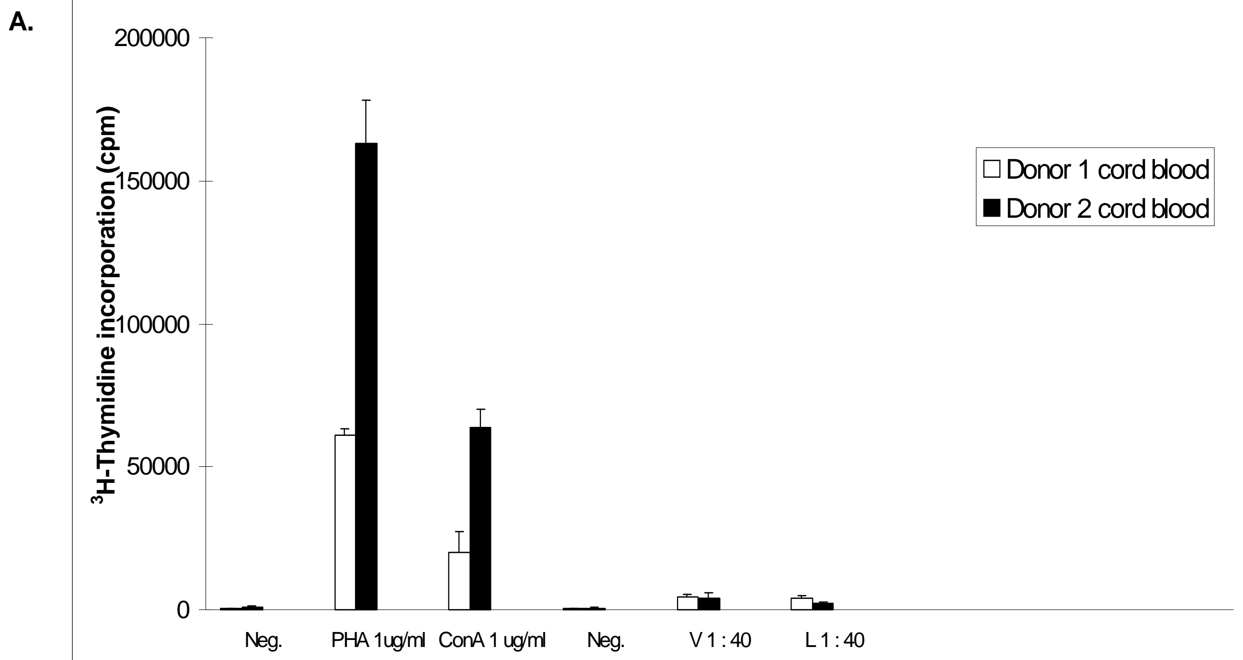


Fig. 1

IRIV induce cell proliferation in PBMC cultures and CD4+ T cells were identified as proliferative responders. Panel A: PBMC from healthy donors (n=3) were cultured in the absence of stimuli (Neg), in the presence of IRIV (V) and in the presence of control liposomes (L) at the indicated dilutions. Proliferation was measured on day six of culture by ^{3}H-thymidine incorporation. Panel B: Purified CD4+ or CD8+ cells were cocultured with autologous irradiated PBMC in the absence of stimuli (Neg) and in the presence of IRIV (V) at the indicated concentrations. Proliferation was measured on day six of culture by ^{3}H-thymidine incorporation.

A.



B.

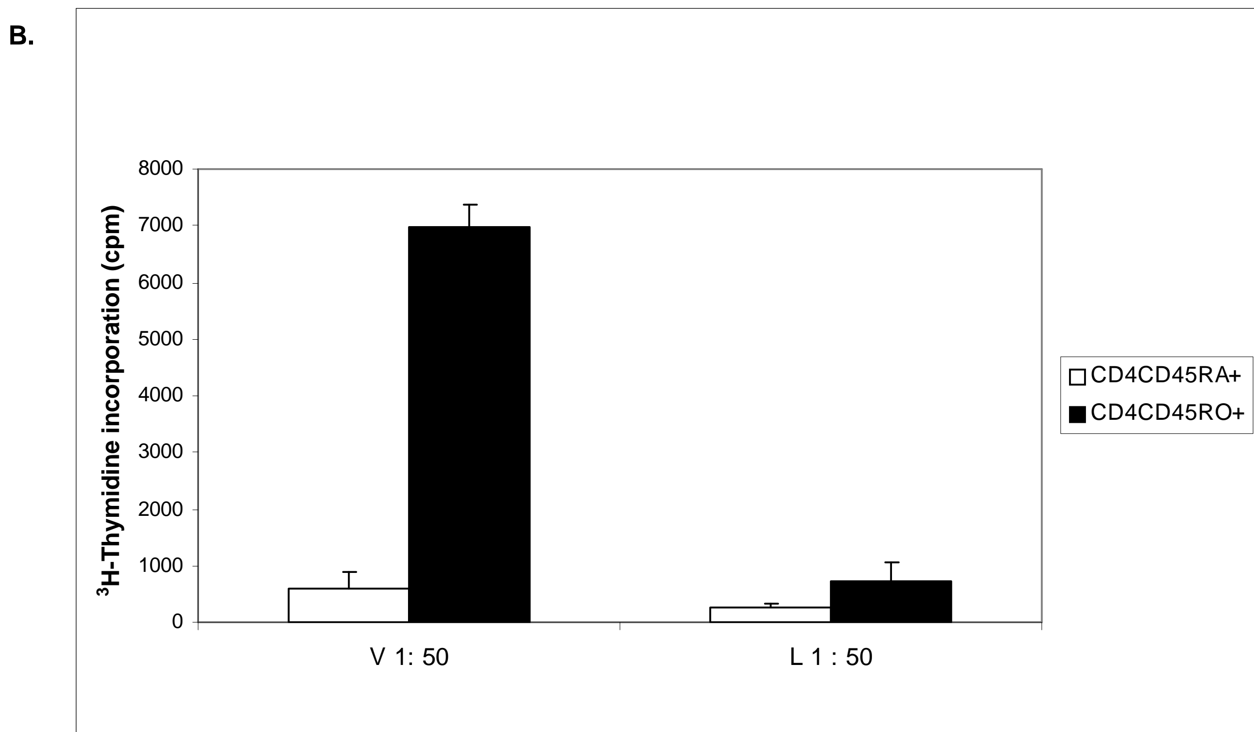


Fig. 2

IRIV induce antigen specific proliferation of CD4+CD45RO+ cells. Panel A: cord blood mononuclear cells from two donors were cultured in the absence of stimuli (Neg) or in the presence of PHA, ConA, IRIV (V) or liposomes (L) at the indicated concentrations. Proliferation was measured on day three of culture for PHA and ConA stimulated cultures and on day six for IRIV and L stimulated cultures by ^{3}H-thymidine incorporation. Panel B: Purified CD4+CD45RA+ cells and CD4+CD45RO+ cells were isolated from PBMC of one healthy donor and cocultured with autologous irradiated PBMC in the presence of IRIV (V) or liposomes (L) at the indicated concentration. Proliferation was measured on day six of culture by ^{3}H-thymidine incorporation.

To further verify antigen dependence of IRIV induced cell proliferation we addressed CD4+ T cell proliferation in presence and absence of APC. As shown in Fig. 3, IRIV induced marginal cell proliferation in absence of APC, whereas major cell proliferation was observed only in presence of APC after 6 days of culture (panel B). In contrast, PHA, used as mitogen positive control, induced strong proliferation of CD4+ cells in absence of APC (panel A), measured after 3 days of culture. No cell proliferation could be observed in absence of any stimuli (Neg).

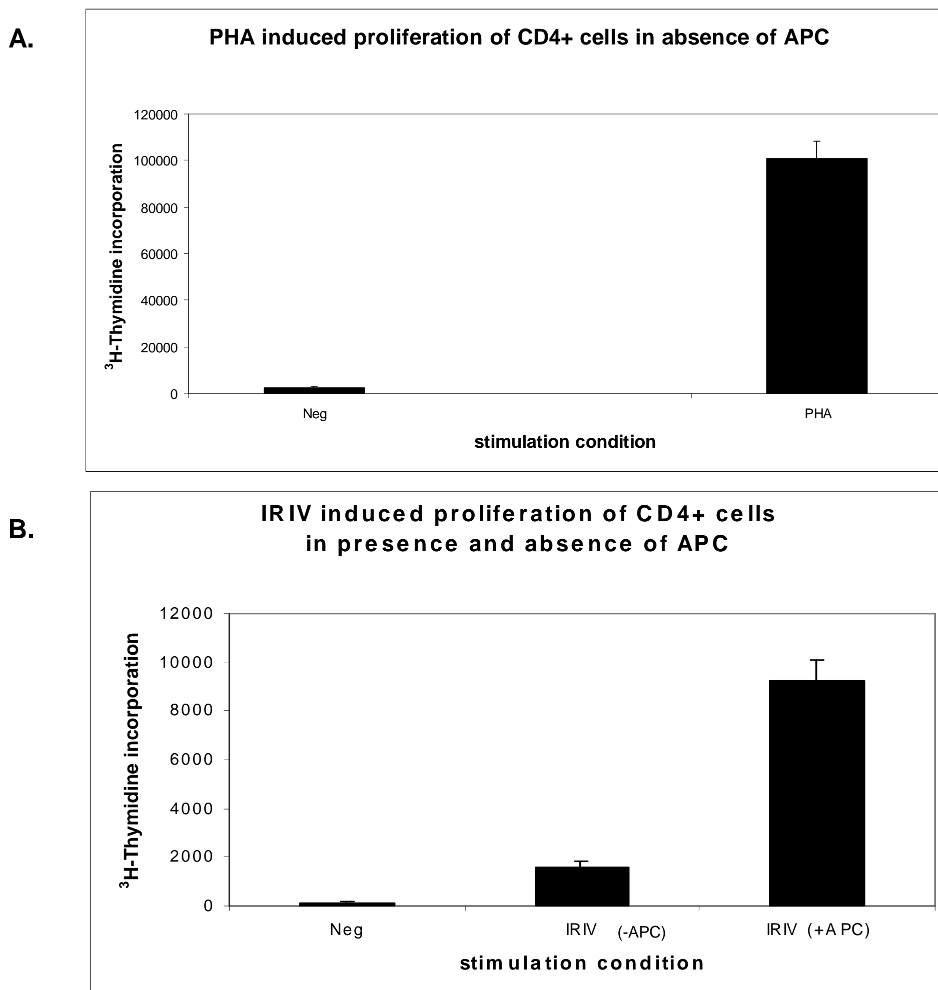


Fig. 3

CD4+ cell proliferation in absence and presence of APC, induced by IRIV.
CD4+ cells of one healthy donor's PBMC were cultured in presence or absence of autologous CD14+ cells in 96well flat bottom plates. Following incubation with either IRIV (1:160 diluted) or PHA (1µg/ml), mitogen induced cell proliferation was measured on day 3 (panel A), IRIV induced cell proliferation on day 6 (panel B) by ^{3}H-thymidine incorporation.
IRIV (-APC): IRIV stimulated CD4+ cell cultures in absence of APC (CD14+ cells).
IRIV (+APC): IRIV stimulated CD4+/CD14+ cell cocultures

2. IRIV induce typical T helper 1 cytokine gene expression and secretion profiles

PBMC from healthy donors were cultured in presence or absence of IRIV. On day 1 and 2 cells and supernatants were harvested. RT- PCR with cytokine specific primers demonstrated expression of IFN-γ, GM-CSF, TNF-α and IL-2 genes in PBMC upon IRIV stimulation (Fig. 4) whereas no expression of IL-4, IL-5 and IL-10 genes could be observed. IFN-γ gene expression could be observed on day 2, but not on day 1 whereas expression of GM-CSF, TNF-α and IL-2 could be observed on day 1 and 2 of culture. ELISA assays performed with supernatants harvested on day 1, 2 and 4 of culture demonstrated increased secretion of IFN-γ, GM-CSF, TNF-α, but not of IL-4 in PBMC upon IRIV stimulation (Fig. 5 A-D). IFN-γ concentration in supernatants of IRIV stimulated PBMC cultures increased after day 2 of culture, whereas TNF-α concentration was at its peak on day 1 and decreased with time. GM-CSF concentration in supernatants of IRIV stimulated PBMC did not vary much within 4 days of culture.

These results demonstrate an IRIV induced cytokine expression pattern in PBMC culture characteristic of a T helper 1 immune response. The different kinetics of cytokine gene expression and secretion suggest that TNF-α and GM-CSF expression represent „early events“ whereas IFN-γ expression represents a later event of IRIV induced lymphocyte activation.

Another indication suggesting the notion of an IRIV induced T helper 1 response results from the quantification of CD4+ T cells expressing CXCR3, a chemokine receptor characteristic for inflammatory and T helper 1 responses [23]. Healthy donor‘s PBMC cultured in presence of IRIV displayed 56% CD4+CXCR3+ T cells within the CD4+ T cell population (Fig. 6, C) on day six of culture whereas PBMC cultured in presence of liposomes or in the absence of any formulation displayed 41% and 40% CD4+CXCR3+ T cells within the CD4+ T cell population, respectively. Thus, IRIV stimulation of PBMC induced an increase of CD4+ T cells expressing CXCR3.

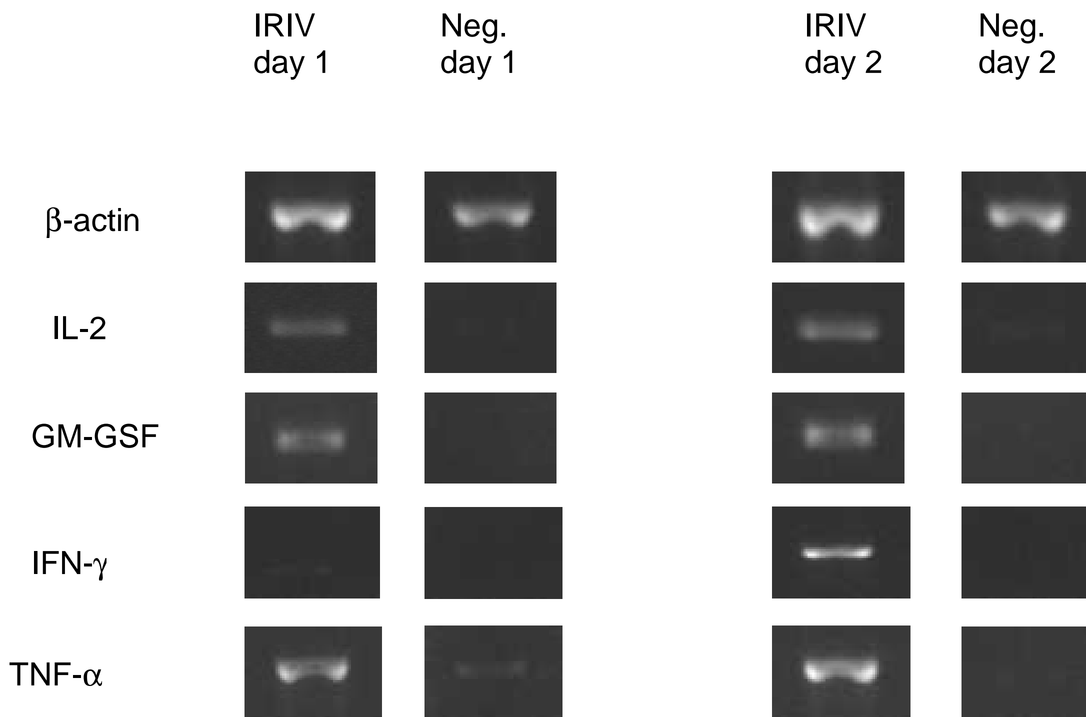
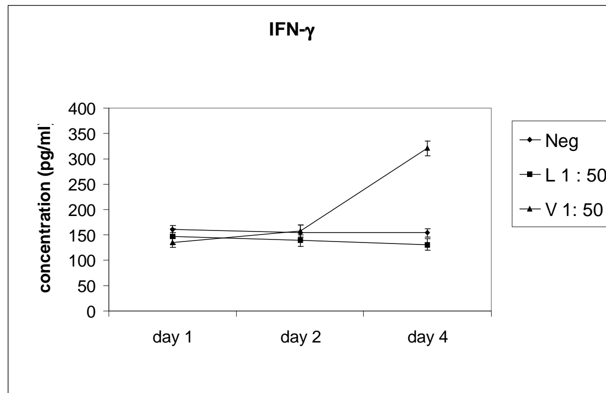


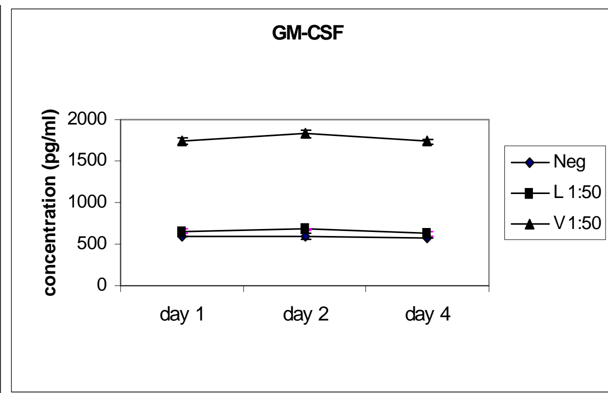
Fig. 4

Cytokine gene expression in IRIV stimulated PBMC. PBMC were cultured in the presence or absence of IRIV. On day one and two of culture, cells were harvested and total cellular RNA was extracted and reverse transcribed. The cDNAs thus obtained were tested in RT-PCR assays in the presence of primers specific for the indicated cytokine genes.

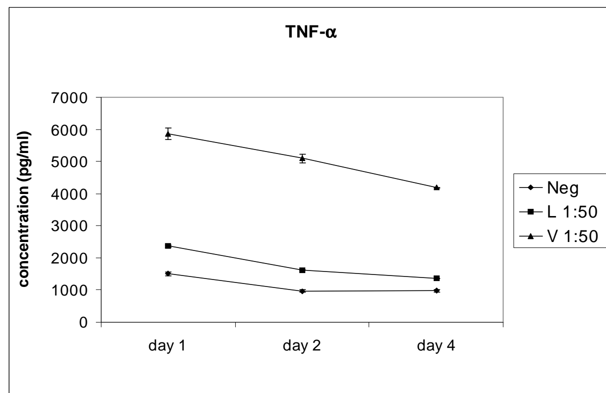
A



B



C



D

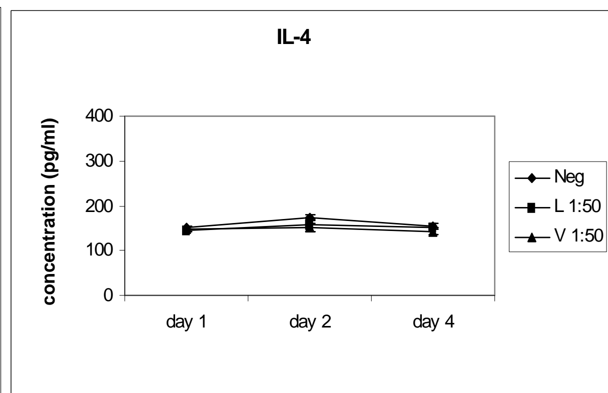


Fig. 5

Cytokine secretion in IRIV stimulated PBMC. PBMC from a healthy donor were cultured in the absence of stimuli (Neg) or in the presence of IRIV (V, 1:50 diluted) or control liposomes (L, 1:50 diluted). On day one, two and four, supernatants were harvested and the concentrations of IFN-γ (Panel A), GM-CSF (Panel B), TNF-α (Panel C) and IL-4 (Panel D) were determined by ELISA.

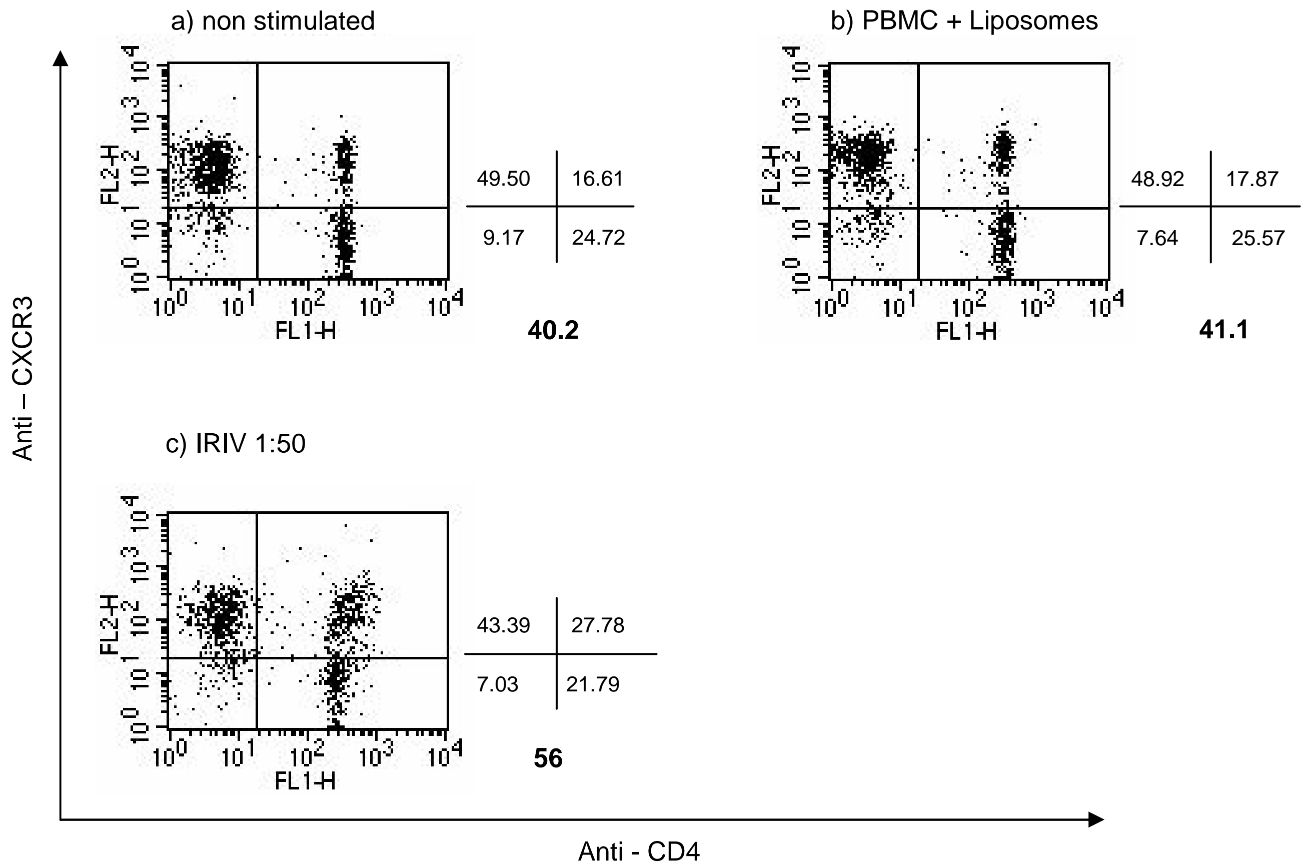


Fig. 6

Increased percentages of CXCR3+CD4+ T cells in IRIV stimulated PBMC. PBMC from a healthy donor were cultured in the absence of stimuli (panel a), in the presence of liposomes (1:50 final dilution, panel b) or IRIV (1:50 final dilution, panel c). After 6 days of culture, cells were phenotyped for the expression of CXCR3 and CD4 by PE and FITC labeled mAbs, respectively. Numbers indicate percentages of cells within each quadrant.

In bold, percentages of CXCR3+ cells within the CD4+ cell population are shown.

2. IRIV induce secretion of chemokines

Then, we addressed secretion of chemokines, important factors for the recruitment of immunocompetent cells.

We evaluated secretion of IP-10 (CXCL10), MIG (CXCL9) and Rantes (CCL5) in IRIV stimulated PBMC cultures by ELISA assays. All three chemokines were present in higher concentrations in IRIV (V) stimulated PBMC cultures as opposed to PBMC cultured in presence of liposomes (L) or in absence of any stimuli (Neg) (Fig. 7). IP-10 (panel B) and MIG (panel A) concentrations in IRIV stimulated PBMC cultures increased over time reaching levels of 8900 pg/ml (V day 4) as compared to 1000 pg/ml (L, Neg) and 5100 pg/ml (V day 5) as compared to 1300 pg/ml (L, Neg) respectively. Rantes (panel C) concentrations in IRIV stimulated PBMC cultures were higher than 10000 pg/ml on day 1 and 2 and decreased to 6600 pg/ml on day 4. A decrease in Rantes concentrations over time was also observed in PBMC cultures performed in presence of liposomes (L: 5500 pg/ml day 1 to 630 pg/ml day 4) and in absence of any stimuli (Neg: 2400 pg/ml day 1 to 340 pg/ml day 4). The upregulation of IP-10, MIG and Rantes upon IRIV stimulation is intriguing, as these chemokines are involved in the recruitment of T lymphocytes [24-26]. In particular, MIG and IP-10 interact with CXCR3 [27], which has been shown to be expressed by CD4+ T cells responding to IRIV. The expression of these two chemokines is inducible by IFN-γ [27] which is also upregulated in IRIV stimulated PBMC.

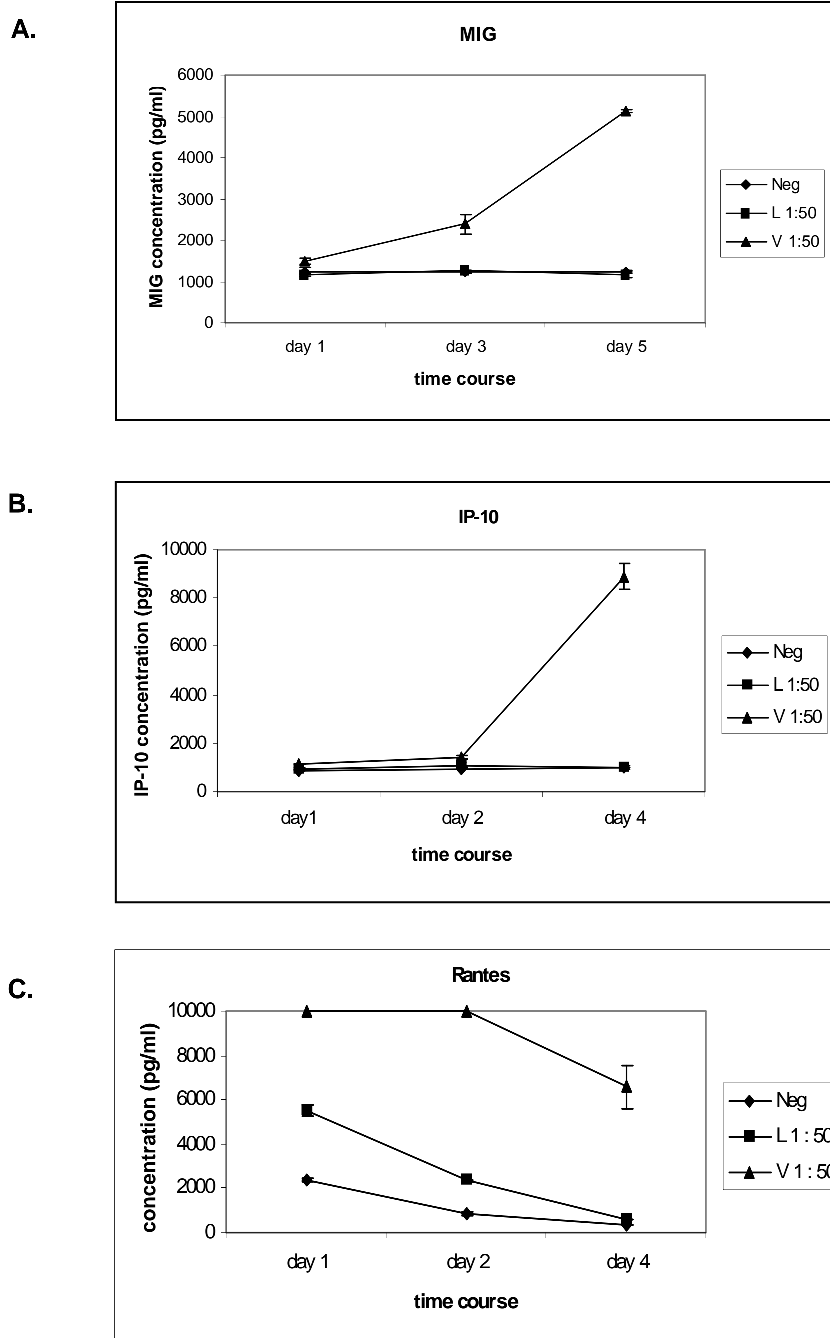


Fig. 7

Chemokine secretion in IRIV stimulated PBMC. PBMC from a healthy donor were cultured in the absence of stimuli (Neg) or in the presence of IRIV (V, 1:50 diluted) or control liposomes (L, 1:50 diluted). At the indicated incubation times, supernatants were harvested and the concentrations of MIG (Panel A), IP-10 (Panel B) and Rantes (Panel C) were determined by ELISA.

3. IRIV effects on antigen presenting cells

We then tested whether IRIV could directly or indirectly induce maturation of dendritic cells, highly professional antigen presenting cells. Immature dendritic cells (iDC) were incubated in presence or absence of IRIV and phenotyped after 24 and 48 hours for the surface expression of CD83, CD86 and HLA-ABC, molecules known to be increasingly expressed on mature dendritic cells (mDC) [28,29]. There was no major difference in expression of these maturation markers between iDC incubated in presence of IRIV and iDC incubated in absence of IRIV. However, when iDC were incubated with culture supernatants, expression of CD83, CD86 and HLA-ABC was upregulated on iDC incubated with supernatants from IRIV stimulated PBMC but not on iDC incubated with supernatants from non-stimulated PBMC (Fig. 8). These results demonstrate that IRIV do not directly induce maturation of dendritic cells but they induce secretion of cytokines in PBMC cultures that favour dendritic cell maturation.

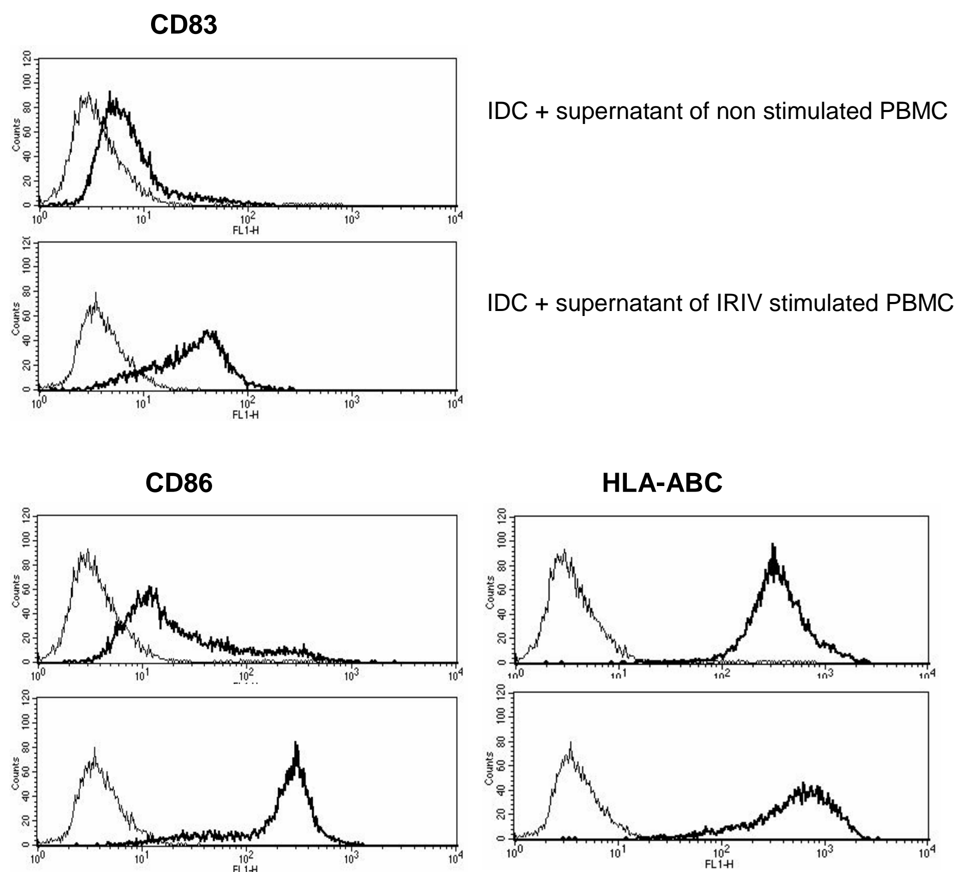


Fig. 8
Supernatants derived from IRIV stimulated PBMC induce upregulation of maturation markers on dendritic cells. Immature dendritic cells (iDC) were cultured in presence of supernatants derived from IRIV stimulated PBMC (lower histograms) or in presence of supernatants derived from non stimulated PBMC (upper histograms). After 48 hours cells were phenotyped for surface expression of CD83, CD86 and HLA-ABC as indicated. Supernatants were added at a final 1:2 dilution.

II. IRIV adjuvance in CTL induction

To evaluate IRIV adjuvance in CTL induction, cells were cultured in presence of HLA class I restricted epitopes with or without IRIV. Expansion of CTL specific for individual epitopes was evaluated by anti-CD8 FITC/ HLA-A0201/epitope tetramer PE double staining or, additionally, by limiting dilution assays addressing CTL precursors (CTLp) frequency.
HLA class-A201 restricted epitopes from Influenza matrix (IM_{58-66}) and from the tumor associated antigen Melan-A/Mart-1 (Melan-A/Mart-1_{27-35}) were used throughout the study.

1. IRIV adjuvance in IM_{58-66} specific CTL induction

As IM_{58-66} is a highly immunogenic HLA-A0201 restricted epitope from the widespread influenza virus, its use facilitates short time CTL induction experiments without restimulation extended to a large range of donors. Thus, CTL induction experiments were first performed as related to IM_{58-66}.
As shown in Fig. 9 culture in presence of IRIV and IM_{58-66} strongly enhanced IM_{58-66} specific CTL induction (7.6% IM_{58-66} specific CTL within the CD8+ T cell population, panel c) as compared to culture in presence of liposomes and IM_{58-66} (0.369%, panel b) or in presence of IM_{58-66} alone (0.179%, panel a), as evaluated on day 7 of culture by anti-CD8 FITC/ IM_{58-66} tetramer PE double staining. Limiting dilution analysis of CTLp demonstrated that in cultures stimulated with IM_{58-66} and IRIV, 1/22.000 CD8+ T cells specifically recognized the target peptide (panel d) whereas no cytotoxicity was detectable in cultures stimulated with IM_{58-66} alone. In PBMC cultures stimulated with
IM_{58-66} and liposomes, 1/84 wells showed evidence of specific cytotoxic activity, a frequency below the threshold evaluable by Poisson distribution. Taken together these results indicate that IRIV enhance the induction of functional IM_{58-66} specific CTL.

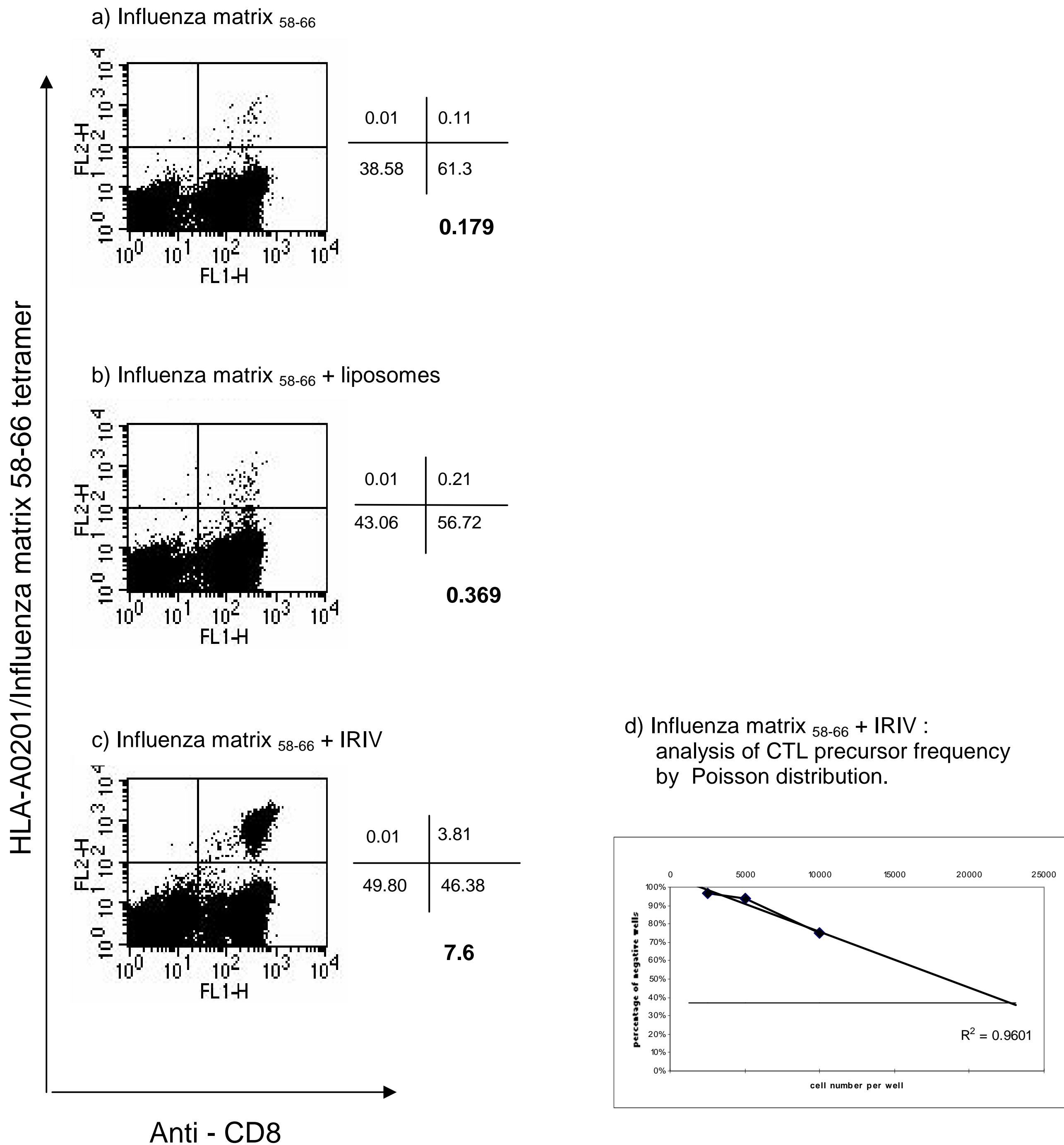


Fig. 9
IRIV adjuvance on CTL induction. PBMC from a healthy donor were cultured in the presence of IM_{58-66} (a), IM_{58-66} and control liposomes (b) or IM_{58-66} and IRIV (c). After a 7 days culture, percentages of IM_{58-66} specific CTL within cultured cells were quantified by HLA-A0201/IM_{58-66} PE tetramer staining (fluorescence 2) and anti CD8 FITC staining (fluorescence 1). CTL precursor frequencies detected in IM_{58-66} and IRIV stimulated cultures within the same experiment are shown in panel d.

Bold numbers in panels a), b) and c) present percentages of tetramer positive cells within the CD8+ cell population.

2. IRIV adjuvance in Mart-1/ Melan-A $_{27\text{-}35}$ specific CTL induction

Enhancement of CTL induction is a major goal of cancer immunotherapy. Thus, we addressed whether IRIV adjuvance could be also observed in CTL induction specific for the tumor associated differentiation HLA-A0201 restricted epitope Melan-A/Mart-$1_{27\text{-}35}$. CD14- cells from healthy donors were cocultured with autologous iDC in presence of Melan-A/ Mart-$1_{27\text{-}35}$, in presence of Melan-A/ Mart-$1_{27\text{-}35}$ and liposomes and in presence of Melan-A/Mart-$1_{27\text{-}35}$ and IRIV. After one restimulation with Melan-A/Mart-$1_{27\text{-}35}$ pulsed iDC and further culture in presence of IL-2, cells were stained with anti-CD8 FITC and Melan-A/Mart-$1_{27\text{-}35}$ tetramers PE on day 13. As shown in Fig 10, IRIV enhanced Melan-A/ Mart-$1_{27\text{-}35}$ specific CTL induction as demonstrated by a higher percentage of Melan-A/Mart-$1_{27\text{-}35}$ specific CTL within CD8+ T cells (11.38%, panel c)) in comparison with cell culture in presence of Melan-A/Mart-$1_{27\text{-}35}$ and liposomes (1.1%, panel b)) or Melan-A/ Mart-$1_{27\text{-}35}$ alone (1.56%, panel a)). Thus, IRIV adjuvance was also observed in the induction of CTL specific for a tumor associated self epitope which is less immunogenic than the non self $IM_{58\text{-}66}$ epitope.

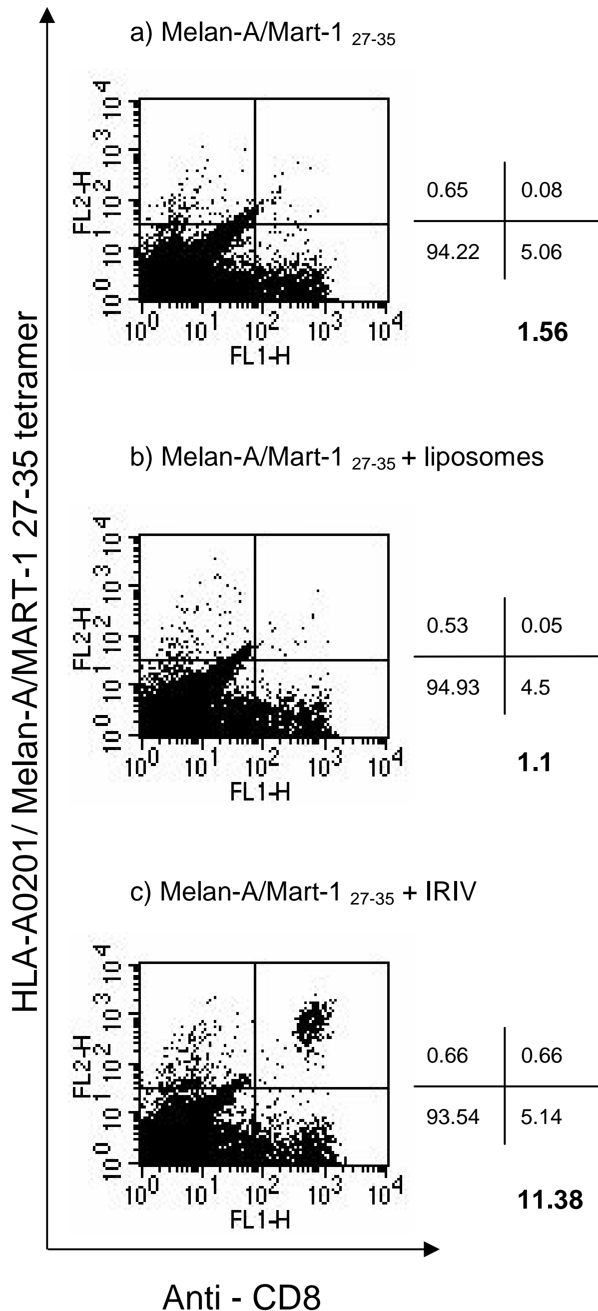


Fig. 10
Adjuvant effects of IRIV in the induction of tumour associated antigen specific CTL. CD14 negative cells from PBMC of a healthy donor were cocultured with autologous iDC in the presence of Melan-A/Mart-1_{27-35}, alone (a) or supplemented with either control liposomes (b) or IRIV (1:50, c). On day seven of culture, cells were restimulated with Melan-A/MART-1_{27-35} pulsed iDC and cultured for six further days (see material and methods). On day 13 cells were stained with FITC conjugated anti-CD8 and PE conjugated HLA-A0201/Melan-A/MART-1_{27-35} tetramers. Numbers represent percentages of cells within each quadrant. In bold, percentages of tetramer positive cells within the CD8+ cell population are shown.

3. IRIV adjuvance in CTL induction is based on CD4+ T cell activation

As previously described, proliferation experiments demonstrated that IRIV induce CD4+ T cell activation and expansion. We then asked whether CD4+ T cells capable to proliferate are required for IRIV mediated CTL adjuvance. To address this issue we cocultured CD8+ T cells, CD14+ cells and either irradiated or non-irradiated CD4+ T cells in presence of IM_{58-66} with or without IRIV. After one restimulation with irradiated IM_{58-66} pulsed CD14+ cells and further culture in presence of IL-2, cocultures were stained with anti-CD8 FITC and IM_{58-66} tetramers PE on day 13. As shown in Fig. 11, IRIV adjuvance could be observed in cocultures performed with non-irradiated CD4+ T cells (12.89% IM_{58-66} specific CTL within CD8+ T cells in presence of IM_{58-66} and IRIV as compared to 1.06% in presence of IM_{58-66} alone) but not in cocultures performed with irradiated CD4+ T cells (0.46% IM_{58-66} specific CTL within CD8+ T cells in presence of IM_{58-66} and IRIV as compared to 0.68% in presence of IM_{58-66} alone). These results demonstrate that IRIV CTL adjuvance is mediated through CD4+ T cell activation and expansion.

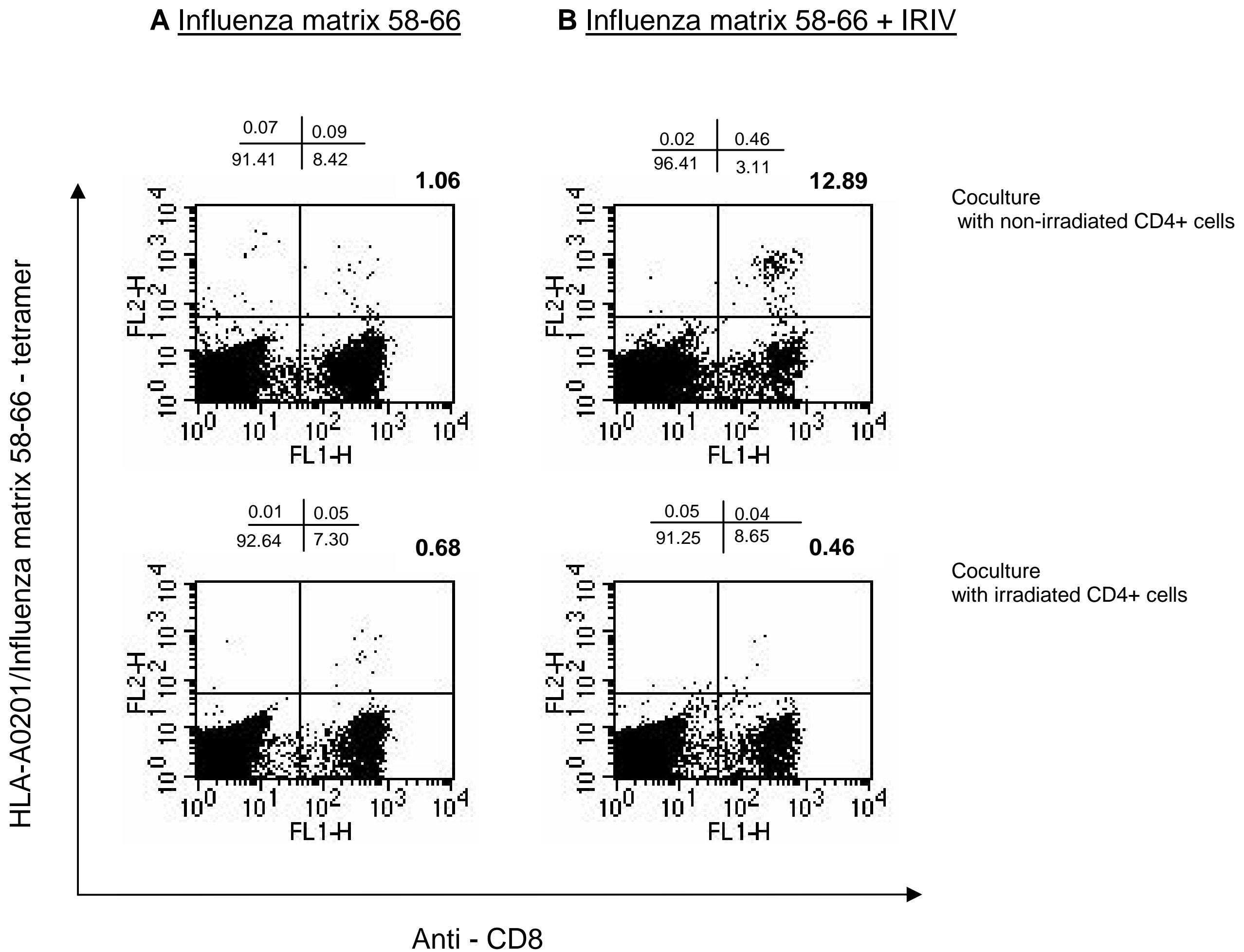


Fig. 11
IRIV mediated adjuvance in CTL induction requires CD4+ T cells.
CD8+ and CD14+ cells were cultured in the presence of autologous intact or irradiated CD4+ cells. These cultures were stimulated with influenza matrix $_{58\text{-}66}$ (1µg/ml) alone (A) or supplemented with IRIV (1:50) (B). After seven days of incubation both cocultures were restimulated with irradiated influenza matrix$_{58\text{-}66}$ pulsed CD14+ cells and cultured for six further days in the presence of IL-2. Six days after restimulation cultures were stained with HLA-A0201/Influenza matrix $_{58\text{-}66}$ PE specific tetramers and anti CD8 FITC mAbs.
Numbers represent percentages of cells within each quadrant. In bold, percentages of tetramer positive cells within the CD8+ cell population are shown.

4. Cytokines may play a major role in IRIV mediated CTL adjuvance

(preliminary results)

Induction of T helper 1 cytokines in IRIV stimulated PBMC cultures suggests that soluble factors may be key players in IRIV CTL adjuvance. However, cell : cell contact dependent interactions (e.g. CD40 : CD40L) could provide a major contribution as well [30-33]. To address this issue we performed CTL induction experiments using 24 transwell plates with a membrane (0.1 μm pore size, corning costar) that allows diffusion of soluble factors but not cell : cell contact between upper and lower wells. Briefly, CD4+ T cells were cocultured with iDC in lower wells and CD8+ T cells with iDC in upper wells, all cells isolated/generated from blood of the same donor. Cocultures of lower and upper wells were each performed either in presence of IM_{58-66} alone or in presence of IM_{58-66} and IRIV. In parallel, cocultures were performed in control wells, allowing cell : cell contact dependent and cell : cell contact independent interactions. As shown in Fig.12 there was no major difference in CTL induction between transwell cocultures (panel D: 7.7% IM_{58-66} specific CTL within CD8+ T cells) and control cocultures (panel C: 8.6%) performed in the presence of IM_{58-66} and IRIV. CTL induction in presence of IM_{58-66} alone was lower, as compared to CTL induction in presence of IM_{58-66} and IRIV, in both, cocultures in transwells (panel C: 3.1% IM_{58-66} specific CTL within CD8+ T cells) and in control wells (panel A: 1.3%). A possible explanation for the higher percentage of IM_{58-66} specific CTL in transwell cocultures as compared to control cocultures could be represented by the absence of CD4+CD25+ T regulatory cell : cell contact dependent suppression in transwell cocultures [34].

Taken together, these results suggest that cell : cell contact dependent interactions do not play a major role in IRIV CTL adjuvance in vitro whereas soluble factors appear to provide a major contribution.

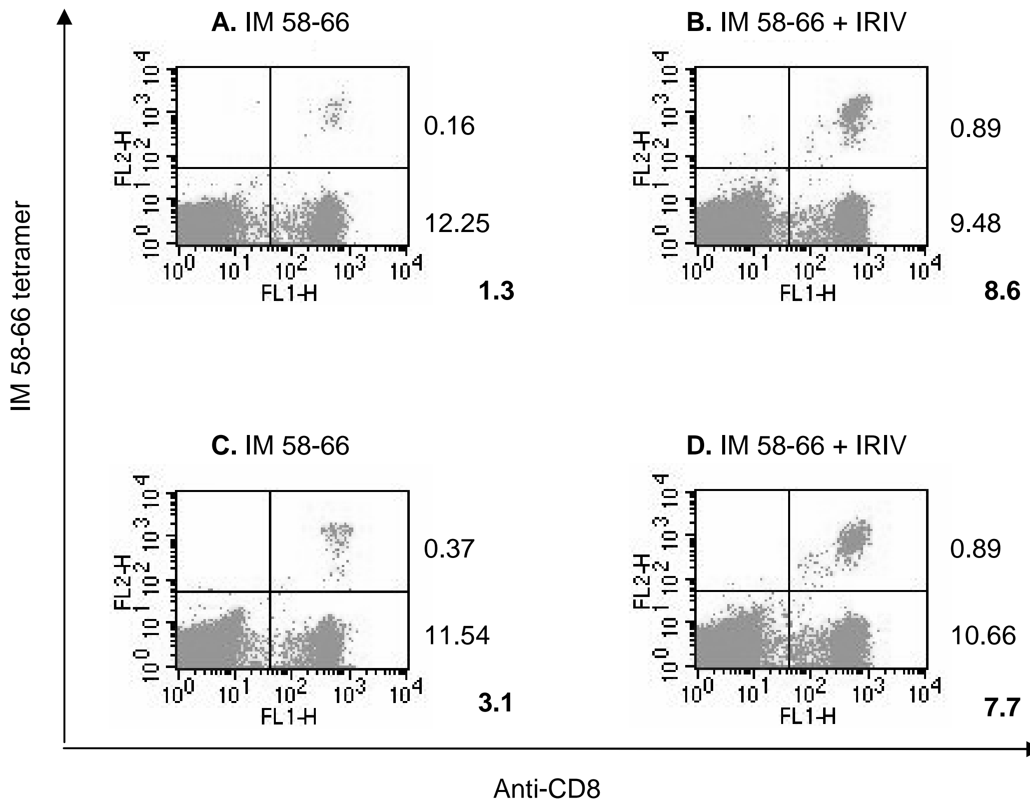


Fig. 12
Major role of soluble factors in IRIV mediated CTL adjuvance.
IDC, CD4+ cells and CD8+ cells were cocultured in transwells (panels C + D, lower wells: iDC and CD4+ cells, upper wells: iDC and CD8+ cells) or control wells (panels A + B) in presence of IM_{58-66} (1µg/ml) or IM_{58-66} (1µg/ml) and IRIV (1:150). On day 7 cells were phenotyped for the expression of CD8 and IM_{58-66} specific TCR by anti-CD8 FITC/ IM_{58-66} tetramer PE double staining. Numbers display percentages of tetramer positive (upper right) and of tetramer negative (lower right) cells, in bold percentages of tetramer positive CD8+ cells within the CD8+ cell population are shown.

PART 2: STUDIES ON PEPTIDE CONTAINING INFLUENZA VIROSOMES

III. Characterization and CTL adjuvance of HLA class I restricted peptide containing influenza virosomes

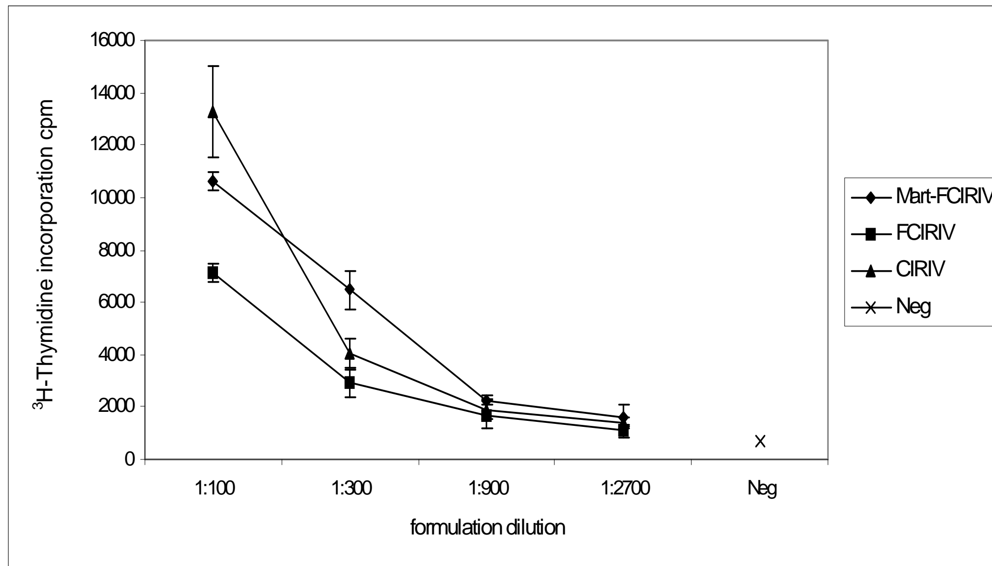
CTL induction experiments described previously were performed by using soluble HLA class I restricted peptides, either added to cultures as such or admixed with empty IRIV. IRIV CTL adjuvance observed in these experiments was solely due to IRIV immunogenic properties and independent of IRIV delivery capacities. However, another approach to formulate the immunogenic epitope is to encapsulate it in influenza virosomes. Such a formulation would provide, in addition to IRIV own immunogenic properties, delivery of the epitope into the cytosol [6]. Moreover, the encapsulated epitope may be protected from enzymatic degradation by serum or cell surface associated peptidases, whereas the non-encapsulated epitope, depending on its structure, is sensitive to degradation.
These considerations urged the production of an influenza virosome formulation containing encapsulated L_{27}Melan-A/Mart-$1_{26\text{-}35}$ peptide, an analog of the Melan-A/Mart-$1_{26\text{-}35}$ epitope with higher immunogenicity [35]. The production of this formulation was performed by Pevion Biotech Ltd. and required the overcoming of the poor encapsulation efficiency by IRIV per se [11]. To address this problem, the advantage of liposomes' high encapsulation efficiency was combined with the fusion activity of hemagglutinin derived from two influenza virus strains (X-31 and A/Sing). Briefly, so called chimeric IRIV (CIRIV) were produced by inclusion of hemagglutinins derived from influenza virus X-31 and from influenza virus A/Sing in the production process. At acidic pH and 18℃ temperature, these CIRIV were fused with liposomes containing the L_{27}Melan-A/Mart-$1_{26\text{-}35}$ peptide. The fusion process under these conditions is mediated by X-31 hemagglutinin, as A/Sing hemagglutinin is active at 37℃ but not 18℃. This fusion step finally results in L_{27}Melan-A/Mart-$1_{26\text{-}35}$ encapsulating fused CIRIV (Mart-FCIRIV) with conformationally modified and fusion incompetent X-31 hemagglutinin and still native and fusion competent A/Sing hemagglutinin.
The production of peptide containing FCIRIV leads to an influenza virosome formulation that, in addition to encapsulation of peptides, is different from IRIV regarding lipid content (higher) and hemagglutinin content (hemagglutinin derived from two different influenza virus strains). For this reason we first evaluated the immune responses elicited by the virosomal part of peptide containing FCIRIV. As for IRIV we addressed proliferation of

CD4+ T cells, cytokine expression of IFN-γ and IL-4, as well as quantification of CD4+ cells expressing CXCR3. This evaluation should clarify whether peptide encapsulating FCIRIV induce, like IRIV, CD4+ T cell proliferation with a T helper 1 profile. In proliferation assays and cytokine expression studies also intermediated stages in the production of peptide encapsulating FCIRIV were assayed (CIRIV, FCIRIV: CIRIV fused with empty liposomes). In parallel, CTL adjuvance of peptide encapsulating FCIRIV was addressed, using L_{27}Melan-A/Mart-$1_{26\text{-}35}$ encapsulated in FCIRIV (Mart-FCIRIV) as priming formulation.

1. Mart-FCIRIV induce CD4+ T cell proliferation

CD4+ and CD14+ cells isolated from peripheral blood of healthy donors were cultured in the absence of stimuli or in the presence of empty chimeric IRIV (CIRIV), empty chimeric IRIV fused with empty liposomes (FCIRIV), or chimeric IRIV fused to liposomes containing the Leu27 $_{26\text{-}35}$ epitope from Melan-A/MART-1 melanoma associated antigen (Mart-FCIRIV) at different dilutions. CIRIV and FCIRIV represent intermediate stages of the production of FCIRIV containing HLA class I restricted peptides.
Upon a six days culture significant proliferation was induced by all preparations in all donors tested, the highest ^{3}H-thymidine incorporation being usually detectable upon stimulation of PBMC with CIRIV. As previously described for IRIV, cells from different donors showed wide differences in their responsiveness to influenza virosome preparations. Data reported in figure 13, panels A and B are representative of results obtained by using cells from weak and strong responders, respectively.

A



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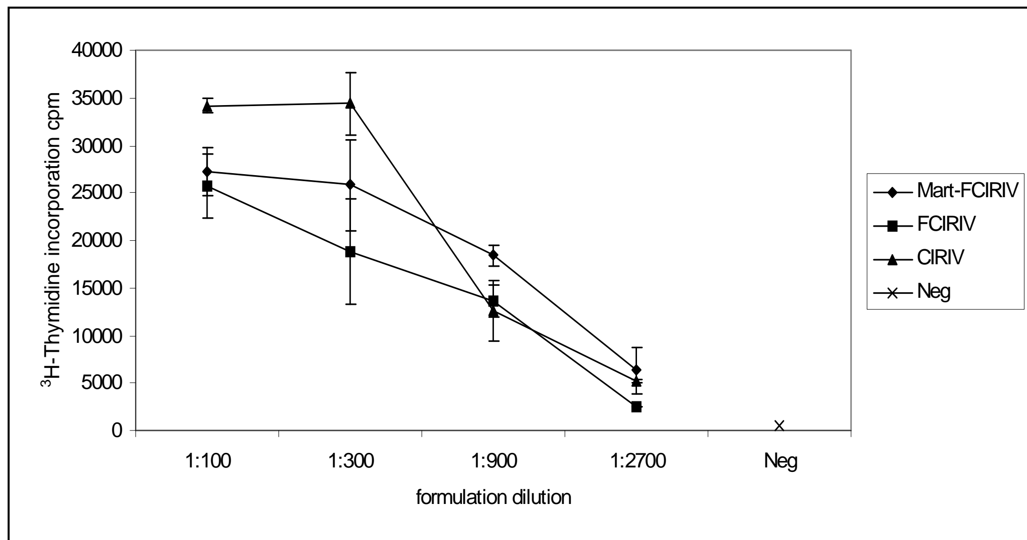


Fig. 13. CD4+ T cell proliferation induced by different virosome formulations. CD4+ and CD14+ cells isolated from healthy donors‘ blood were cocultured in the absence of stimulation (Neg) and in presence of Mart-FCIRIV, empty CIRIV fused with empty liposomes (FCIRIV) and empty CIRIV (CIRIV). On day 5 of culture cells were pulsed with ^{3}H-thymidine for 18 hours and then proliferation was measured as ^{3}H-thymidine incorporation. Panel A and B represent results of cell cultures from two healthy donors.

2. Mart-FCIRIV induce gene expression and secretion of cytokines consistent with a T helper 1 profile.

To verify whether the observed CD4+ T cell response was T helper 1 (Th1) or T helper 2 (Th2) in nature, we then addressed IFN-γ and IL-4 cytokine gene expression upon stimulation of CD4+/CD14+ cell cocultures with CIRIV, FCIRIV and Mart-FCIRIV or in absence of stimulation. Cells were harvested on day 1 and 2 of culture, total RNA was extracted and specific transcripts were quantified by real time PCR. IFN-γ gene expression was not detectable in cells from unstimulated cultures. In contrast, in cells stimulated with different concentrations of CIRIV, FCIRIV or Mart-FCIRIV a high expression of IFN-γ gene was observed on days 1-2 of culture. Figure 14, panels A and C reports data from two different donors. On the other hand IL-4 gene expression was undetectable in all culture conditions tested.

IFN-γ and IL-4 secretion was then tested by ELISA in supernatants harvested on day 2 from CD4+/CD14+ cell cultures performed in the presence of the different influenza virosome formulations. IFN-γ was found to be produced to different extents by CIRIV, FCIRIV or Mart-FCIRIV stimulated but not control cultures from different donors (Figure 13, panels B and D). Instead, IL-4 was undetectable in supernatants from all cultures.

3. Stimulation by Mart-FCIRIV results in increased percentages of CXCR3+ CD4+ cells.

To further validate the T helper 1 nature of virosome induced CD4+ T cell responses we comparatively addressed the percentage of CD4+ cells expressing CXCR3 in presence of Mart-FCIRIV, in presence of liposomes (L) or in absence of any stimuli (Neg). CD4+ and iDC from healthy donors were cocultured in presence of different dilutions of either reagent or in the absence of stimulation. On day six of culture CD4+ cells were tested by flow cytometry for CXCR3 expression. Data reported in figure 15, show that stimulation with Mart-FCIRIV (panels A and B) resulted in increased percentages of CD4+ cells expressing CXCR3 as compared to culture in presence of liposomes (panels C and D) and culture in absence of any stimulation (panel E).

Taken together, these results indicate that Mart-FCIRIV, same as IRIV, possess a high capacity to induce CD4+ T cell responses characterized by a T helper 1 profile.

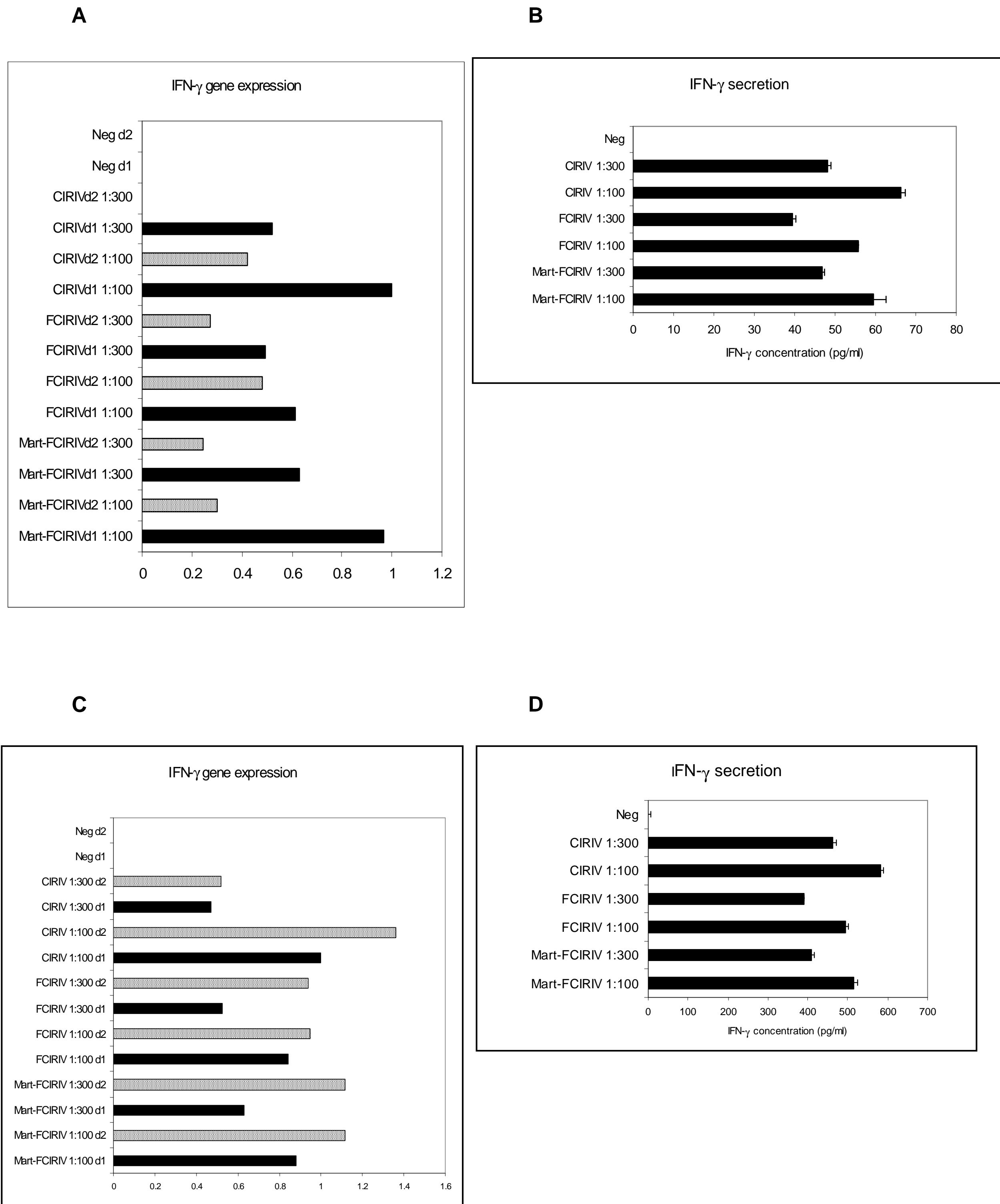


Fig. 14. IFN-γ gene expression (panels A and C) and secretion (panels B and D) in Mart – FCIRIV stimulated CD4+ / CD14+ cell cocultures. CD4+ T cells and CD14+ cells were isolated from healthy donor's blood and cocultured in presence of Mart – FCIRIV), empty CIRIV fused with empty liposomes (FCIRIV), empty CIRIV (CIRIV) and in absence of any stimuli (Neg) at indicated dilutions. Cells were harvested on day 1 (d1, black bars) and on day 2 (d2, hatched). Total RNA was extracted from each sample and IFN-γ (panels A, C) gene expression analysed by real time PCR.

IFN-γ protein concentrations were determined in supernatants harvested on day 2 by cytokine specific ELISA (panels B and D). IL-4 gene expression and secretion was also addressed, but was not detectable neither in real time PCR nor in ELISA. Results of cell cultures from two donors (panels A and B and panels C and D respectively) are presented.

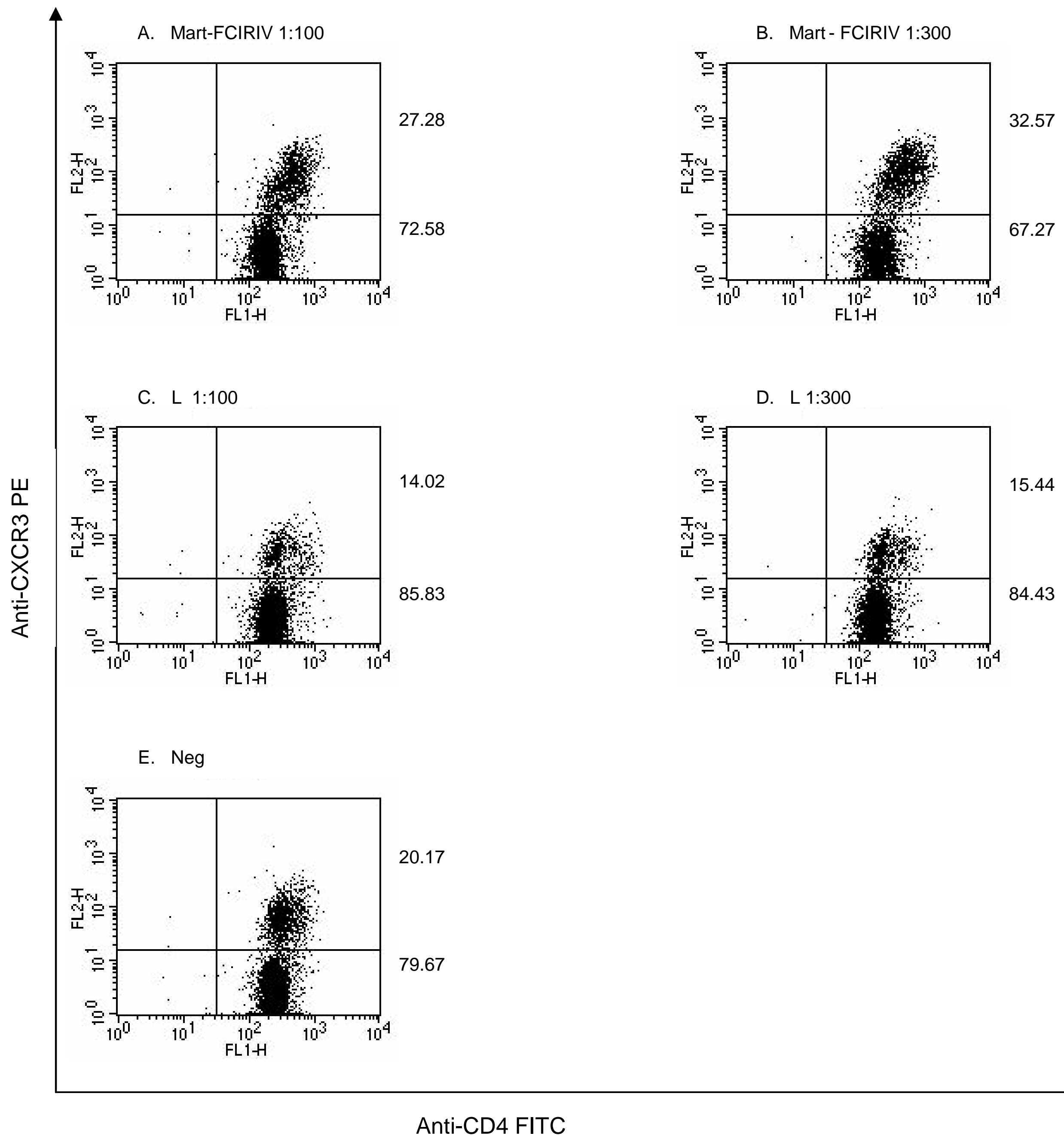


Fig.15. Mart – FCIRIV increase the percentage of CXCR3 expressing CD4+ cells within the CD4+ T cell population. CD4+ T cells were cocultured with autologous iDC in presence of Mart– FCIRIV (panel A: 1:100 and panel B: 1:300), Liposomes (L, panel C: 1:100 and panel D: 1:300) and in absence of any stimuli (Neg, panel E). On day six of culture cells were phenotyped for the expression of CD4 and CXCR3 by flow cytometry.
Dot plots are representative of data from two different experiments. Percentages of CXCR3+CD4+ cells and CXCR3-CD4+ cells are shown.

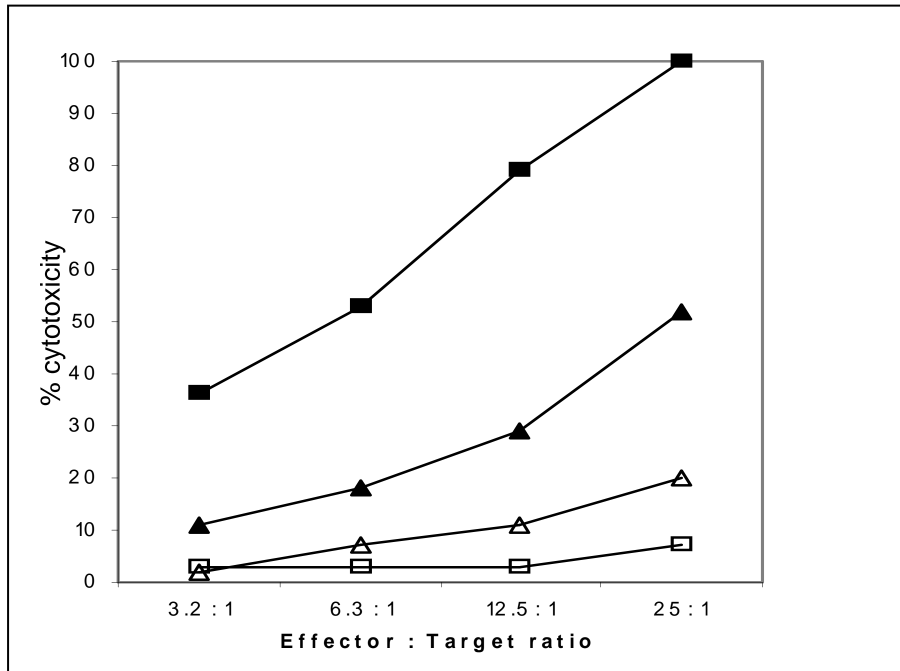
4. Mart-FCIRIV adjuvance in L_{27}Melan-A/Mart-1_{26-35} specific CTL induction

Mart-FCIRIV adjuvance in L_{27}Melan-A/Mart-1_{26-35} specific CTL induction was assessed by cytotoxicity assays and pentamer staining. First, cytotoxicity assays were performed by using peptide pulsed HLA-A0201+ target cells that do not express Melan-A/Mart-1 gene. In a second step, to evaluate the capacity of induced L_{27}Melan-A/Mart-1_{26-35} specific CTL to recognize the naturally expressed epitope and to lyse Melan-A/Mart-1 expressing tumor cells, cytotoxicity assays were performed with tumor target cells that express both, HLA-A0201 and Melan-A/Mart-1 gene. Third, CD4+ T cell independent adjuvance of Mart-FCIRIV was addressed by CTL induction experiments in the absence of CD4+ T cells.

A. Mart-FCIRIV induce L_{27}Melan-A/Mart-1_{26-35} specific CTL

Peripheral blood CD14- cells from healthy donors were cocultured with autologous immature dendritic cells (iDC) in presence of L_{27}Melan-A/Mart-1_{26-35} in solution or encapsulated in FCIRIV. After IL-2 supplementations (see materials and methods) cells were phenotyped and restimulated with peptide pulsed iDC on day 6-7 of culture. Additional phenotypes and cytotoxicity assays were performed between days 13 to 15 after further IL-2 supplementations. HLA-A0201+ T2 cells exogenously pulsed with synthetic epitopes were used as target cells. In the presence of limiting amounts of peptide (0.1-1 µg/ml), a significantly improved CTL induction was observed when synthetic epitopes were encapsulated into FCIRIV as compared to soluble reagents. Representative results obtained by using cells from two different donors are shown in Fig. 16 (panels A and B). HLA-A0201/L_{27}Melan-A/Mart-1_{26-35} pentamer staining data were also consistent with a higher immunogenicity of the peptide included in virosomes as compared to the soluble reagent (Figure 17). Occasionally, however, discrepancies between cytotoxic capacity and tetramer staining were also observed, as previously described [3].

A



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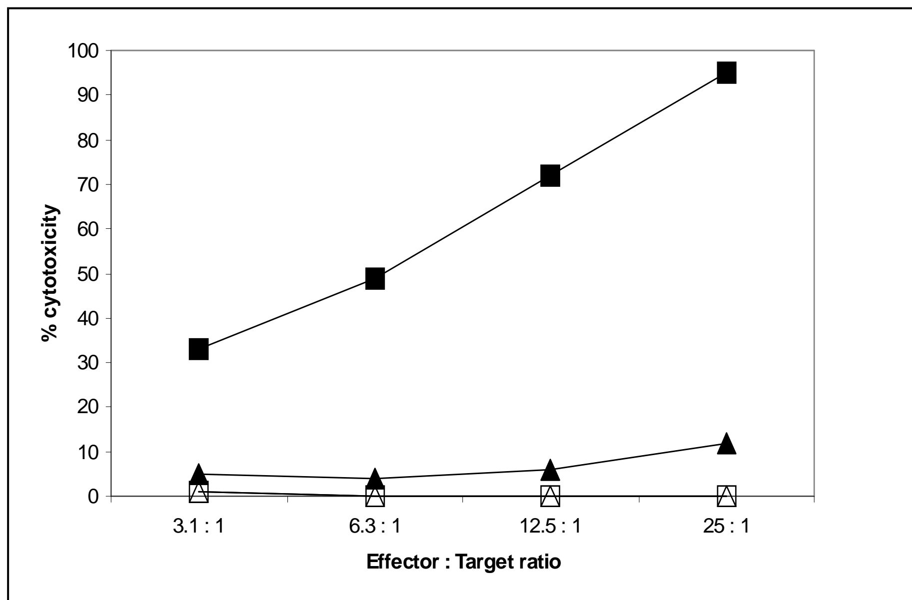


Fig. 16. Mart–FCIRIV mediated induction of CTL specific for L_{27}Melan-A/Mart-1 $_{26\text{-}35}$.
CD8+ cells and CD4+ cells were isolated from peripheral blood of two healthy donors and cocultured together with autologous immature dendritic cells (iDC) in presence of L_{27}Melan-A/ Mart-1$_{26\text{-}35}$ and of Mart-FCIRIV (stock: 100µg peptide/ml) at 0.25µg peptide/ml. IL-2 supplementation was performed as described in Materials and Methods. On day 7 or 8 cell cultures were restimulated with peptide pulsed irradiated iDC in presence of IL-2. Cytotoxicity assays were performed on day 16 of culture. Cytotoxicity assay results refer to cultures from two donors (panels A and B) with control peptide pulsed (open symbols) and L_{27}Melan-A/Mart-1 $_{26\text{-}35}$ pulsed (filled symbols), HLA0201+ target cells in presence of L_{27} Mart-1/Melan-A $_{26\text{-}35}$ (triangles) or Mart–FCIRIV (squares) primed CD8+/CD4+/iDC cocultures.

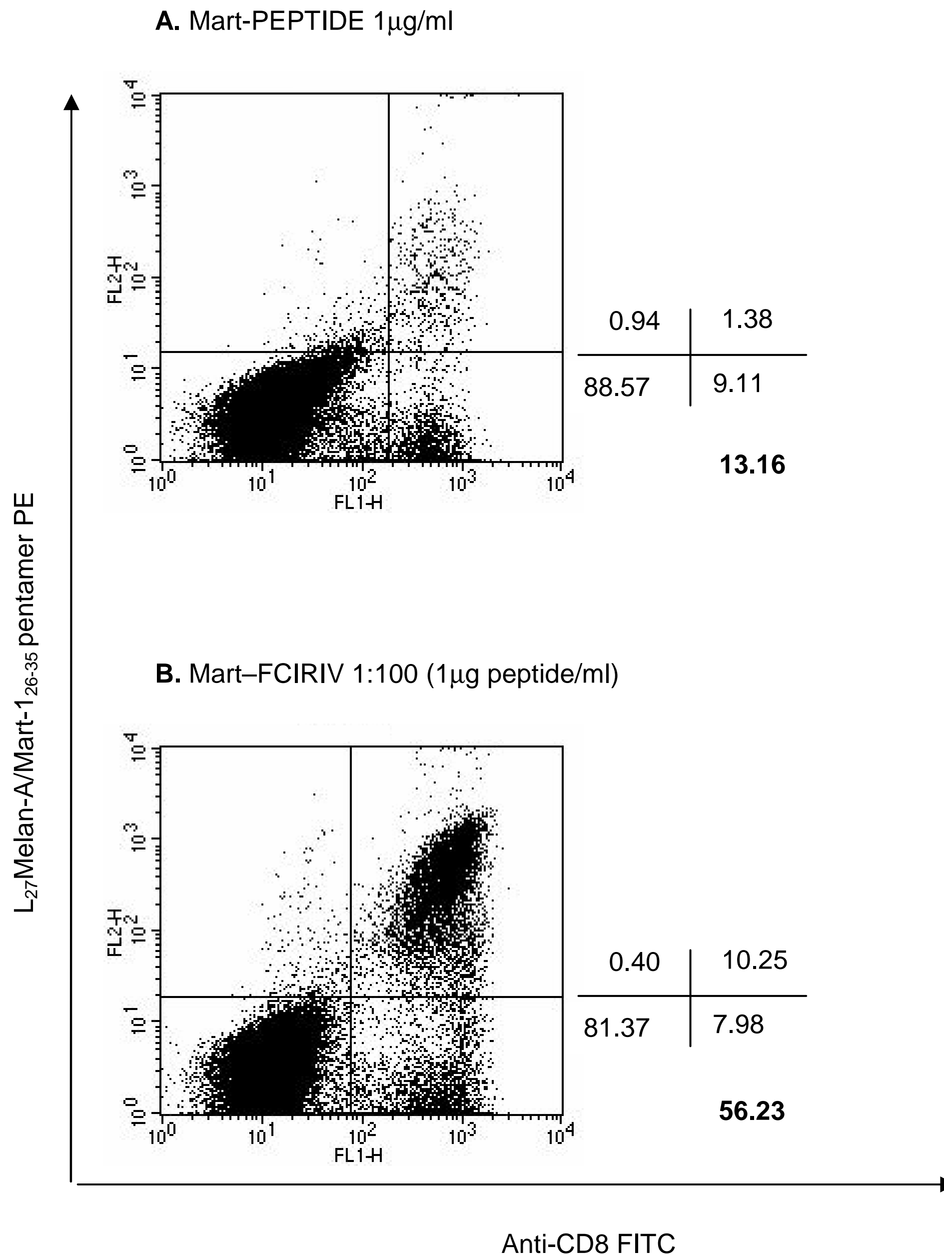


Fig. 17. Mart-FCIRIV increase the percentage of L_{27}Melan-A/Mart-$1_{26\text{-}35}$ specific CTL as compared to L_{27}Melan-A/Mart-$1_{26\text{-}35}$ in solution. CD14- cells were isolated from healthy donor's blood and cocultured with autologous iDC in presence of 1µg L_{27}Melan-A/Mart-$1_{26\text{-}35}$ / ml formulated either as soluble peptide as such (Mart-PEPTIDE, panel A) or as encapsulated peptide in FCIRIV (Mart-FCIRIV, panel B). After IL-2 supplementations and one restimulation with peptide pulsed iDC (see materials and methods) cell cultures were phenotyped for the expression of CD8 and TCR specific to L_{27}Melan-A/ Mart-$1_{26\text{-}35}$ by anti-CD8 FITC / L_{27}Melan-A/ Mart-$1_{26\text{-}35}$ pentamer PE double staining. Numbers represent percentages of cells within each quadrant.

In bold, percentages of pentamer positive cells within the CD8+ cell population are presented.

B. L_{27}Melan-A/Mart-$1_{26\text{-}35}$ specific CTL induced by Mart-FCIRIV are able to lyse HLA-0201+ melanoma cells expressing Melan-A/Mart-1

Peptide specific cytotoxicity demonstrated that CTL induced by Mart-FCIRIV recognized exogenously pulsed HLA-A0201+ target cells.
We then addressed recognition of the naturally expressed Melan-A/Mart-1 TAA by performing cytotoxicity assays using, as targets, HLA-A0201+ HBL melanoma cells constitutively expressing the Melan-A/Mart-1 gene.
CD14- cells from two donors were cocultured with autologous iDC in the presence of antigenic peptide in solution or contained into influenza virosomes. Cytotoxicity assays showed that CTL from either donor (figure 18, panel A and B) induced by Mart-FCIRIV were indeed capable of recognizing the native Melan-A/Mart-1 epitope, as expressed by HLA-A0201+ HBL melanoma cells. Most importantly, cytotoxic activities were in both cases significantly higher than those mediated by L_{27}Melan-A/Mart-$1_{26\text{-}35}$ specific CTL induced by soluble peptides.

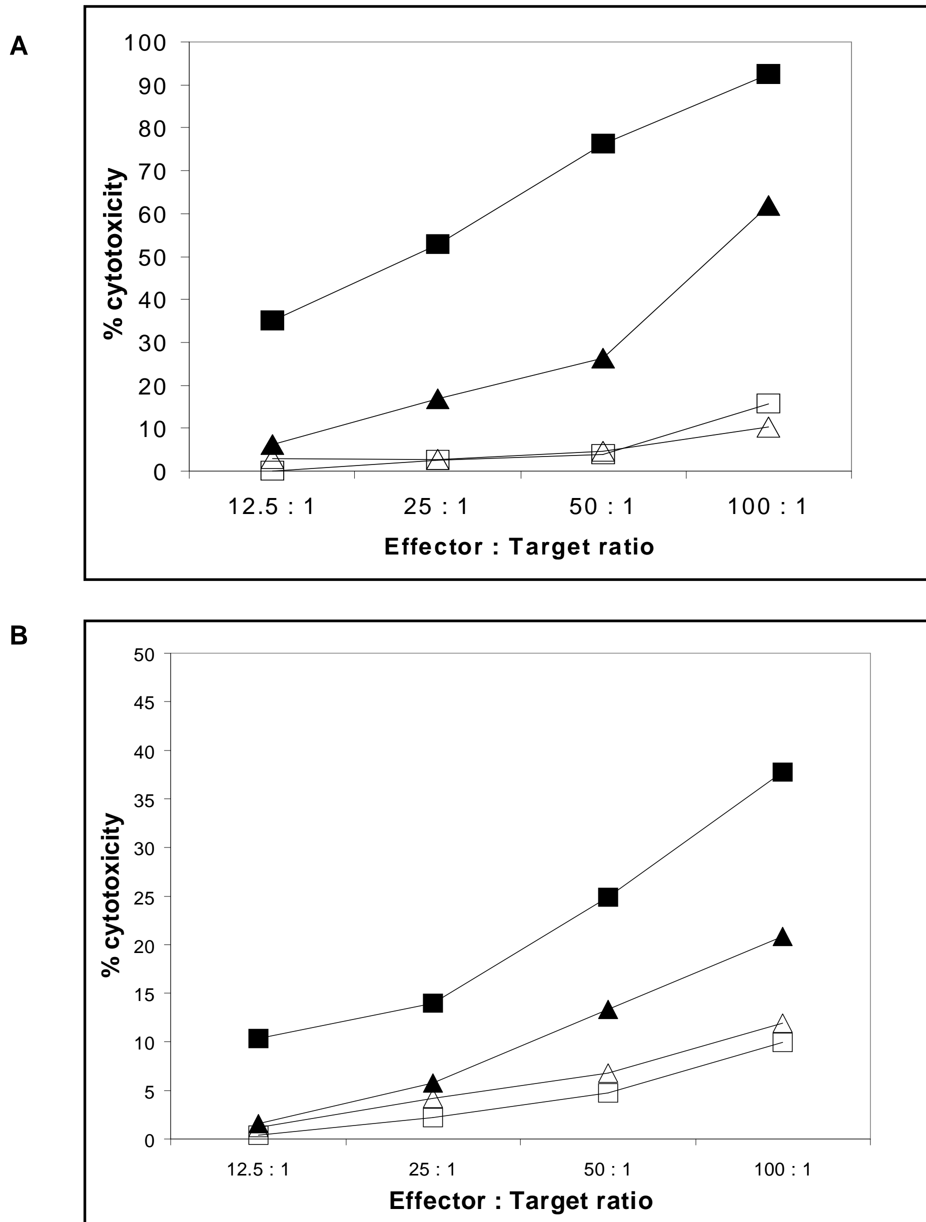


Fig. 18. Cytotoxicity of Mart-FCIRIV stimulated effector cells against HLA-A0201+ HBL tumor cells expressing Melan-A/Mart-1. CD14- cells were isolated from blood of two healthy donors (panels A and B) and cocultured with autologous immature dendritic cells (iDC) in presence of L_{27}Melan-A/ Mart-$1_{26\text{-}35}$ in solution (triangles) and of Mart-FCIRIV (squares) at 1µg peptide/ml concentration.
IL-2 supplementation was performed as described in Materials and Methods. On day 6 (panel A) or 7 (panel B) cell cultures were restimulated with peptide pulsed irradiated autologous iDC in presence of IL-2. Cytotoxicity assays were performed, using the Melan-A/Mart-1 expressing HLA-A0201+ tumor cell line HBL (filled symbols) and the Melan-A/Mart-1 negative, HLA-A0201+ NA-8 cell line (open symbols, negative control) as target cells.

C. CD4+ T-cell independent CTL adjuvance of Mart-FCIRIV

Taken together, these data indicate that Mart-FCIRIV are highly immunogenic, possibly due to their capacity to stimulate specific CD4+ T-cell responses, in accordance with our study on IRIV mediated CTL adjuvance. However, work by other groups indicates that CD4 T-cell independent adjuvance may also be related to the capacity of virosomes to efficiently deliver antigens into APC cytosol [12,36]. This property results from receptor mediated endocytosis and fusion of influenza virosomes with endosomes, both mechanisms dependent on viral hemagglutinin [37-39]. To address the issue of CD4+ T cell independent virosome adjuvance, we stimulated purified CD8+ T cells with L_{27}Melan-A/Mart-1_{26-35} peptide encapsulated in FCIRIV (Mart-FCIRIV) or in solution, in the absence of CD4+ cells.

Both, pentamer staining (Fig. 20) and cytotoxicity assays (Fig. 19) clearly indicated that stimulation of CD8+ cells with Mart-FCIRIV in the absence of CD4+ T-cells also powerfully promoted the induction of antigen specific CTL, at low (0.25-0.75 µg/ml) peptide concentrations.

These results demonstrate that peptide containing influenza virosomes, in addition to CD4+ T cell activation, mediate CD4+ T cell independent CTL adjuvance.

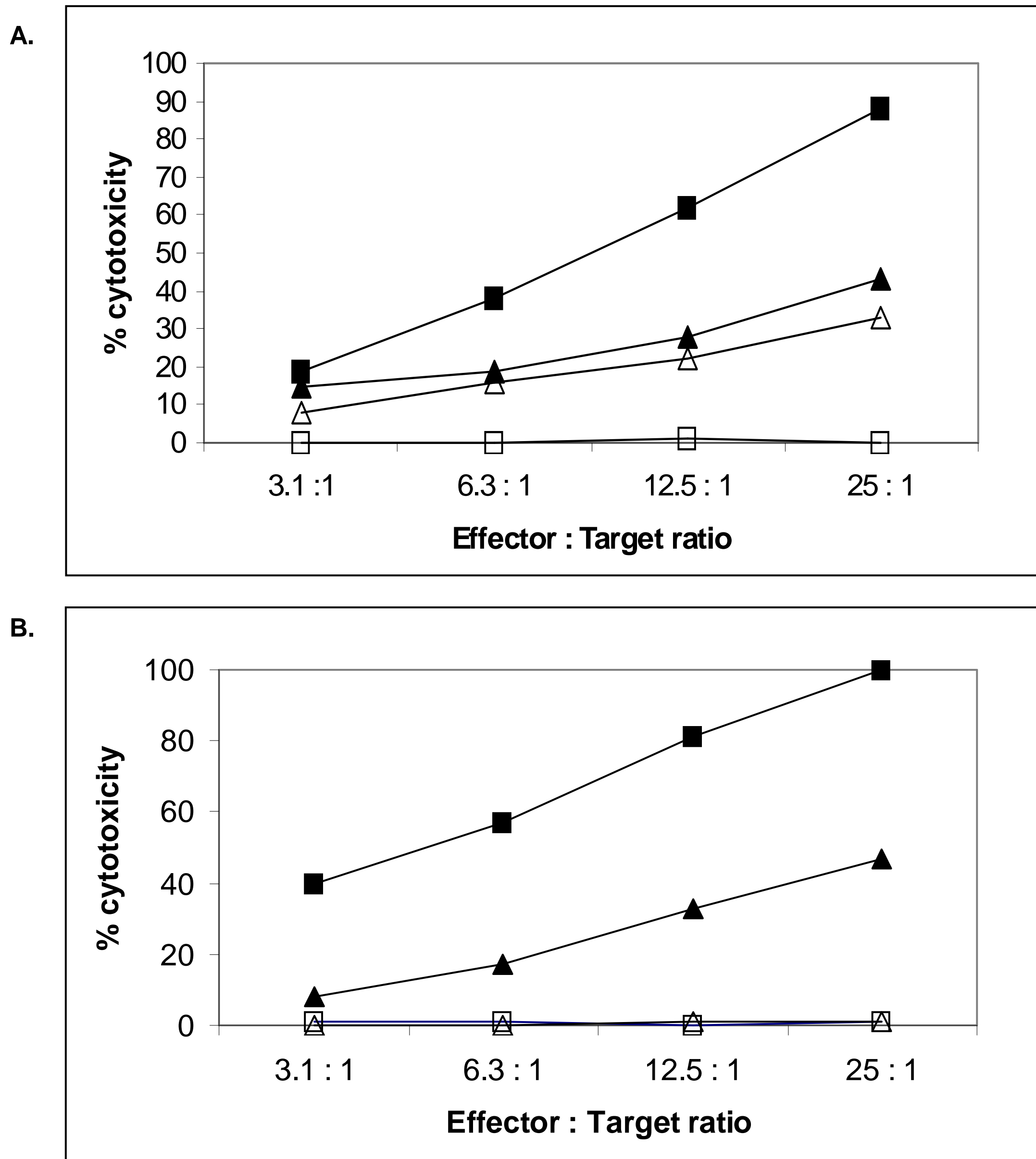


Fig. 19. Mart-FCIRIV enhanced CD4+ T cell independent L_{27}Melan-A/Mart-$1_{26\text{-}35}$ specific CTL induction. CD8+ T cells were isolated from peripheral blood of a healthy donor and cocultured with autologous iDC in presence of L_{27}Melan-A/Mart-$1_{26\text{-}35}$ in solution (triangles) or Mart–FCIRIV (squares). After IL-2 supplementation (see Material and Methods) and one restimulation with irradiated monocytes cytotoxicity assays were performed using control peptide pulsed (open symbols) or L_{27}Melan-A/Mart-$1_{26\text{-}35}$ pulsed (filled symbols) HLA-A0201+ target cells. Panels A and B display CTL cytotoxicity induced by each formulation corresponding to 0.25µg peptide/ml (panel A) and 0.75µg peptide/ml (panel B) assayed on day 16. CTL induction experiments with CD8+ T cells from additional cultures from two different donors provided similar results.

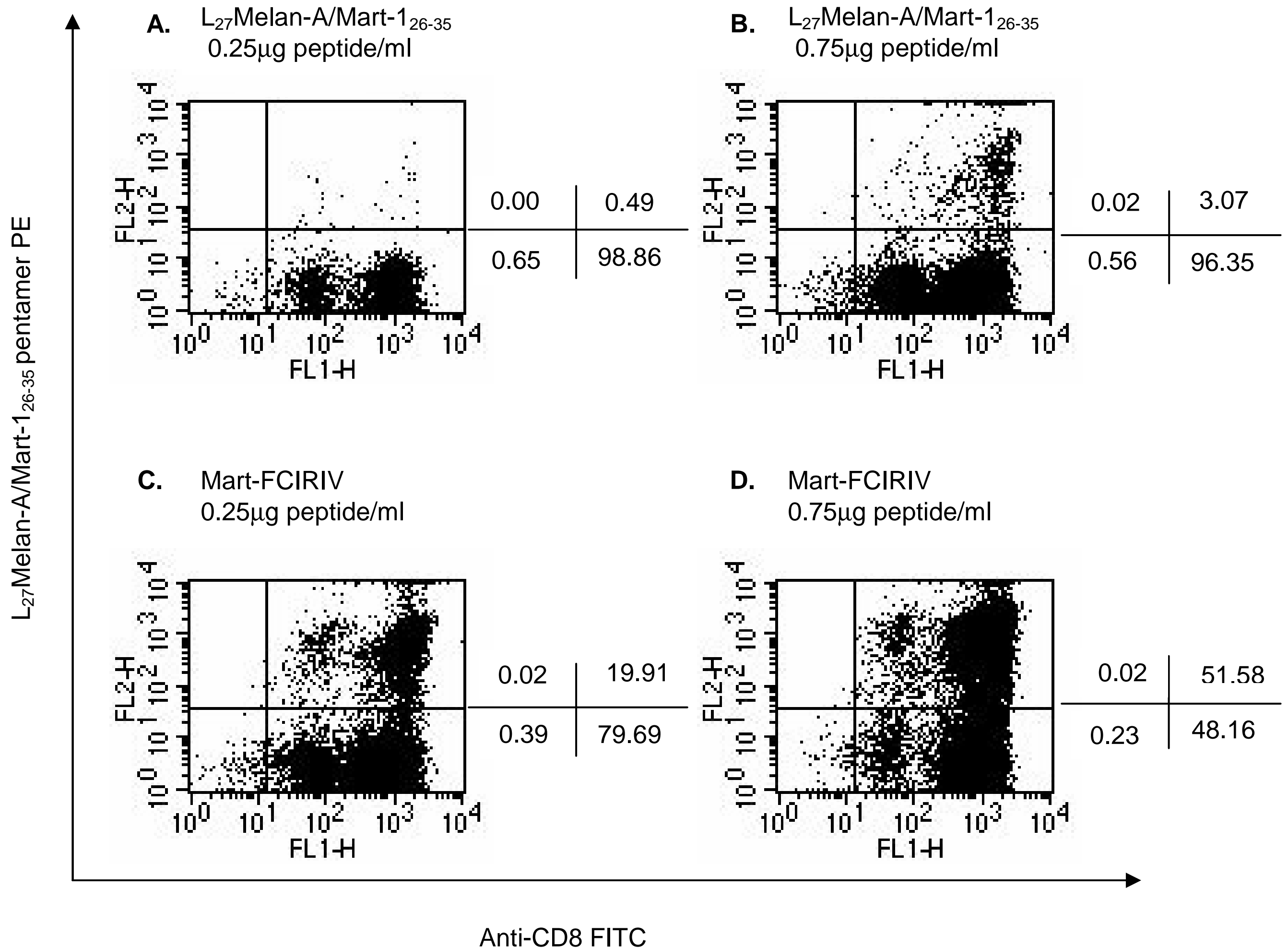


Fig. 20. Enhanced L_{27}Melan-A/Mart-$1_{26\text{-}35}$ specific CTL induction by Mart-FCIRIV in absence of CD4+ T cells, as demonstrated by pentamer staining. Peripheral CD8+ T cells from one healthy donor were cocultured with autologous iDC in presence of L_{27}Melan-A/Mart-$1_{26\text{-}35}$ or Mart-FCIRIV. After IL-2 supplementation (see Material and Methods) and one restimulation with irradiated monocytes cells were phenotyped for the expression of CD8 and L_{27}Melan-A/Mart-$1_{26\text{-}35}$ specific TCR by anti-CD8 FITC/ L_{27}Melan-A/Mart-$1_{26\text{-}35}$ pentamer PE double staining. Panels A , B, C and D display results of cultures induced by each formulation corresponding to 0.25µg peptide/ml (panels A and C) and 0.75µg peptide/ml (panels B and D) assayed on day 13. CTL induction experiments with CD8+ T cells from two additional donors provided similar results. Numbers indicate percentages of cells within each quadrant.

PART 3: INFLUENZA VIROSOMES AND CD4+CD25+ T REGULATORY CELLS

Effects of influenza virosomes on CD4+CD25+ T regulatory cell mediated suppression of immune responses were also addressed. CD4+CD25+ T regulatory cells have been described to play a key role in maintaining peripheral tolerance to self-antigens [40,41]. Moreover, indications that CD4+CD25+ T regulatory cells represent a hindrance to antitumor immune responses are increasingly reported [42,43]. Although underlying mechanisms of CD4+CD25+ T regulatory cell mediated suppression are still not clarified, it is mostly accepted that CD4+CD25+ T regulatory cells are activated through TCR triggering, and following activation, act in an unspecific way [44,45] .
We addressed CIRIV CTL adjuvance in presence of different CD4+ T cell subsets as shown in Fig. 21. IM_{58-66} peptide admixed with CIRIV resulted in higher induction of IM_{58-66} specific CTL (8% of CD8+ cells, panel B) as compared to IM_{58-66} peptide alone (5.66%, panel A) in presence of bulk CD4+ T cells.
In presence of purified CD4+CD25+ T regulatory cells induction of IM_{58-66} specific CTL by either formulation was low (0.64% without CIRIV, panel C; 0.48% with CIRIV, panel D) and no CIRIV adjuvance could be observed. In presence of purified CD4+CD25- cells induction of IM_{58-66} specific CTL was equal between the two formulations (9.39%, panel F and 9.8%, panel E, respectively) and similar to CTL induction by IM_{58-66} admixed with CIRIV in presence of bulk CD4+ T cells (8%, panel B). These preliminary results suggest that CIRIV provide adjuvance in presence of the whole CD4+ T cell population, inclusive CD4+CD25+ T regulatory cells. Recent studies reported overcoming of CD4+CD25+ T regulatory cell mediated immunosuppression by toll like receptor ligands [46-48]. Investigations on underlying mechanisms suggested an important role of IL-6 in rendering CD4+CD25- cells unresponsive to CD4+CD25+ T regulatory cells [47]. Thus, we tested whether influenza virosomes induce IL-6 gene expression in PBMC cultures. As shown in Fig. 22, IL-6 gene expression was higher in PBMC cultured in presence of IRIV or PHA than in PBMC cultured without any stimuli, as demonstrated in PBMC cultures from two donors (panels A and B). Comparing these results between the two different PBMC cultures, IL-6 gene expression followed different kinetics. In one case IRIV induced IL-6 gene expression reached its maximum after 29 hours of stimulation (grey bars, panel A), in the second case after 15 hours of stimulation (black bars, panel B). IL-6 gene expression was at highest in presence of PHA. In each case PHA induced IL-6 gene expression reached its maximum after 15 hours (black bars) of stimulation.

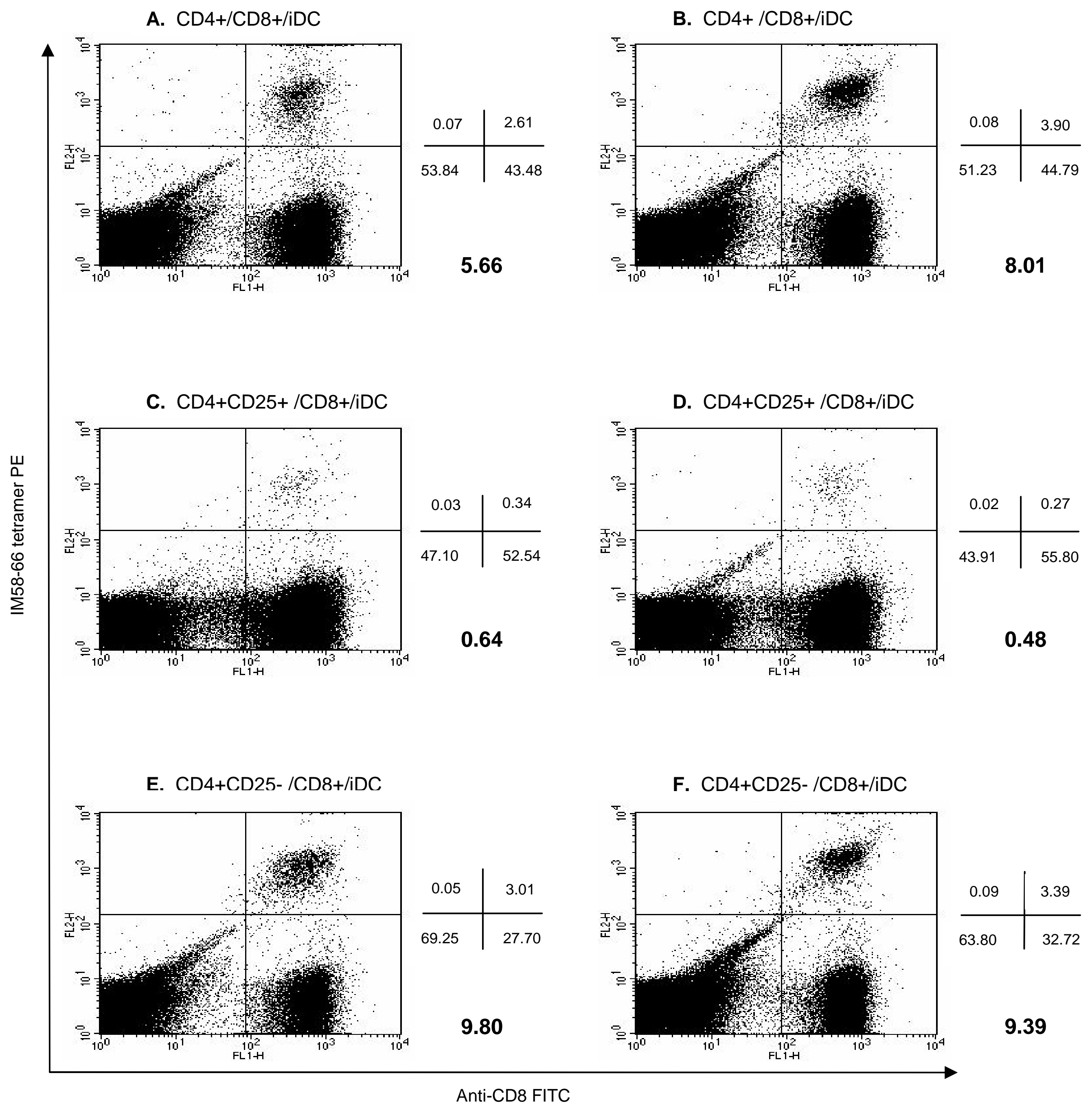
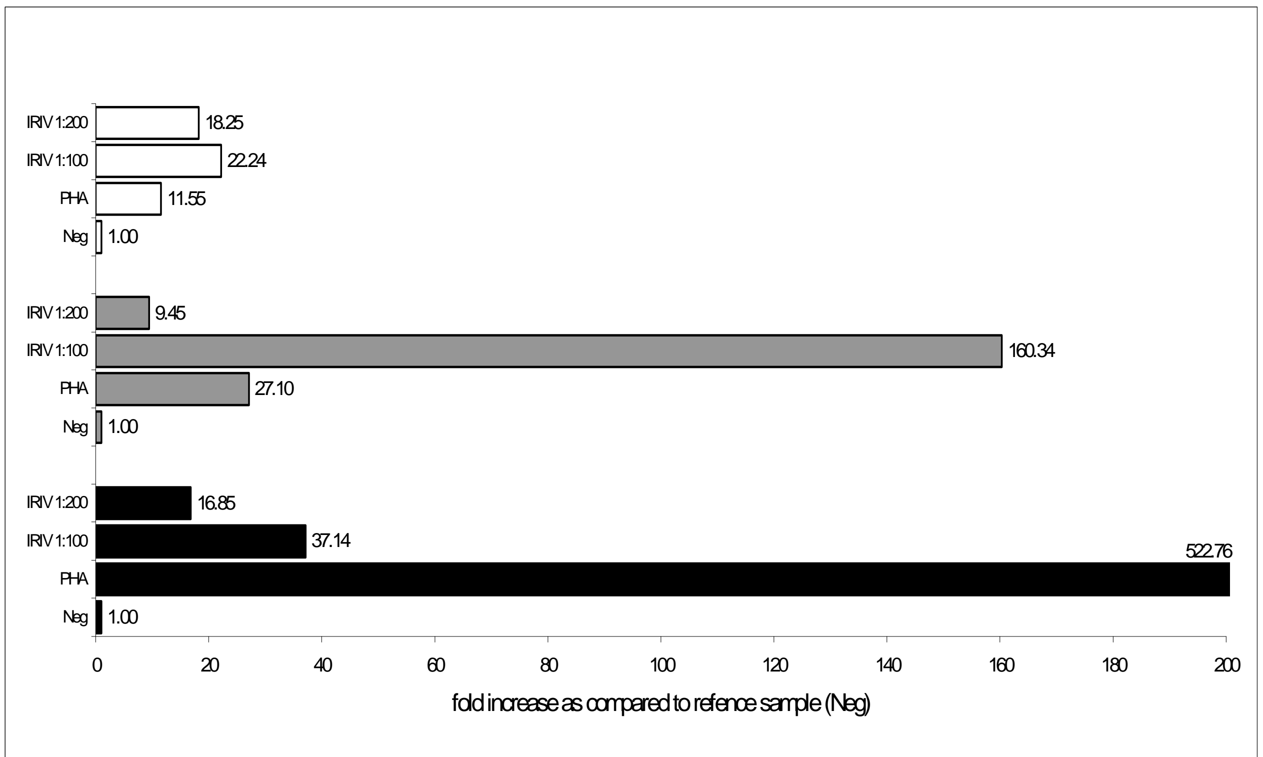


Fig. 21. CIRIV CTL adjuvance in presence of different CD4+ T cell subsets. CD8+ cells were cocultured with iDC and either CD4+ cells (Fig. Panels A and B) or CD4+ CD25+ T regulatory cells (Fig. Panels C and D) or CD4+CD25- cells (Fig. Panels E and F). These cocultures were performed in presence of IM_{58-66} (1µg/ml) alone (Fig. panels A, C, E) and in presence of IM_{58-66} (1µg/ml) admixed with CIRIV 1:200 (Fig. Panels B, D, F). On day 7 of culture cells were phenotyped by anti-CD8 FITC/ IM_{58-66} tetramer PE double staining. Numbers represent percentages of cells within each quadrant, in bold percentages of pentamer positive CD8+ cells within the CD8+ cell population are shown.

A.



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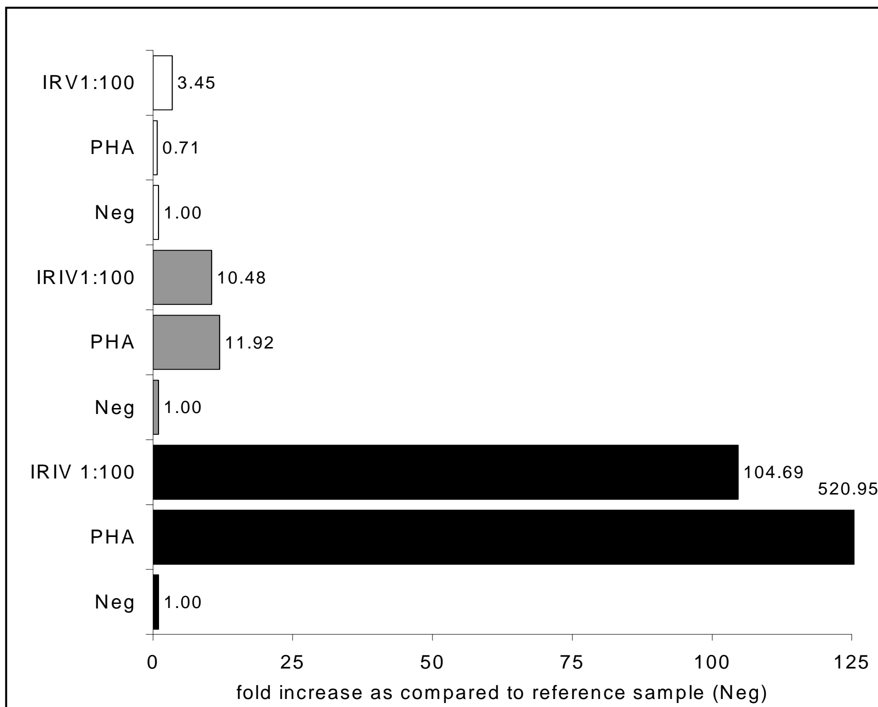

Fig. 22. IRIV induce IL-6 gene expression in PBMC cultures. PBMC from two donors (panels A and B) were cultured in presence of IRIV (diluted 1:100 and 1:200 panel A, 1:100 panel B), in presence of PHA (1μg/ml) and in absence of any stimuli (Neg). After 15 (black), 29 (grey) and 48 (white) hours cells were harvested. Following RNA extraction and reverse transcription, gene expression of IL-6 was measured by real time PCR.

DISCUSSION

The goal of this thesis work was to characterise immune responses elicited by immunopotentiating reconstituted influenza virosomes (IRIV) and to evaluate influenza virosome adjuvance in relation to induction of HLA class I restricted cytotoxic T lymphocytes (CTL). The relevance of these objectives is underlined by the low number of adjuvants approved for human use and the general need of adjuvants that enhance cellular immune responses against antigens from intracellular pathogens and tumors [2,49]. This work may also contribute to the evaluation of influenza virosomes as adjuvants for future clinical trials, aiming to enhance melanoma associated antigen specific CTL responses. We addressed these objectives by testing different formulations, all produced by Pevion Biotech Ltd.: IRIV, IRIV admixed with HLA class I restricted peptides (either IM_{58-66} or Melan-A/Mart-1_{27-35}), chimeric IRIV (CIRIV), chimeric IRIV fused to empty liposomes (FCIRIV) and FCIRIV encapsulating the HLA class I restricted L_{27}Melan-A/Mart-1_{26-35} peptide. All experiments were performed in vitro by using peripheral blood mononuclear cells (PBMC) from healthy donors.

IRIV and FCIRIV induced a T helper 1 like CD4+ T cell proliferation characterized by IFN-γ expression and increased frequencies of CD4+ T cells expressing CXCR3. The extent of these proliferative responses displayed a marked variability between different donors, but all healthy donors responded. Further dissection into CD45RA+ and CD45RO+ cell subsets demonstrated that proliferating cells were CD4+CD45RO+ cells. In addition, no major cell proliferation could be observed upon IRIV supplementation to cell cultures depleted of antigen presenting cells nor to cord blood mononuclear cell cultures. Taken together, these results demonstrate that influenza virosomes induce antigen triggered proliferation of CD4+ T cells displaying a memory phenotype. Although identity of the proliferation inducing antigen(s) has not been formally addressed, HLA class II restricted hemagglutinin epitopes presumably provide the major contribution, as influenza virus derived neuraminidase is only marginally present on influenza virosomes. Moreover, liposomes devoid of viral proteins did not induce cell proliferation.

In addition to IFN-γ, IRIV induced expression of TNF-α, GM-CSF and the chemokines MIG (CXCL9), Rantes (CCL5) and IP-10 (CXCL10). MIG, Rantes and IP-10 are reported to attract mainly T lymphocytes [24,25]. Interestingly, MIG and IP-10 share as receptor CXCR3 [27], which is also expressed on IRIV and Mart-FCIRIV stimulated CD4+ T cells. Although the functional relevance of these chemokines has not been addressed in our studies, this pattern suggests that they may play an important role in the recruitment of

T lymphocytes, thus facilitating IRIV elicited immune responses.
Regarding IRIV effects on immature dendritic cells, no upregulation of surface maturation markers could be observed in the absence of other cell types. This finding is consistent with a previous study on influenza virus and subunit vaccines in human cell cultures [50]. On the other hand, one study on influenza virosomes in murine cell cultures demonstrated that OVA encapsulating influenza virosomes may directly induce maturation of dendritic cells .Whereas no direct induction of dendritic cell maturation could be observed in our hands, supernatants derived from IRIV stimulated PBMC cultures favoured maturation of dendritic cells as demonstrated by increased surface expression of HLA- class I molecules, CD86 costimulatory molecules and CD83. These results suggest that IRIV induce expression and secretion of cytokines in PBMC cultures that favour dendritic cell maturation. Maturation of dendritic cells has been shown to correlate with improved antigen presentation and costimulation [51]. These findings are consistent with increased expression of IFN-γ, TNF-α and GM-CSF in IRIV stimulated PBMC cultures, as these cytokines have been reported to play a role in development, differentiation and maturation of APC [52-54]. The capacity of IRIV and FCIRIV to elicite CD4+ T cell proliferation, a T helper 1 characteristic cytokine profile, and to promote antigen presentation may be relevant for the enhancement of HLA class I restricted CTL induction.
Indeed, tetramer/pentamer staining and cytotoxicity assays demonstrated enhanced induction of CTL specific for IM_{58-66}, Melan-A/Mart-1_{27-35} and L_{27}Melan-A/Mart-1_{26-35} using IRIV admixed with peptides or FCIRIV encapsulating peptides as compared to peptides admixed with liposomes or peptides alone. Taken together, these findings show a marked influenza virosome mediated CTL adjuvance in vitro. Importantly, this could be demonstrated not only in relation to the IM_{58-66} epitope but also in relation to the less immunogenic melanoma associated Melan-A/Mart-1_{27-35} epitope. Quantification of specific CTL, as addressed by multimer staining, suggests that stimulation with IRIV admixed with peptides results in at least five to ten times higher numbers of specific CTL as compared to liposomes admixed with peptides and to peptides in absence of any other stimuli.
The finding of IRIV mediated in vitro CTL adjuvance prompted the investigation on underlying mechanisms. Given the formulation of IRIV admixed with peptides, the observed IRIV CTL adjuvance is likely to be solely attributable to IRIV immunogenic properties and independent of IRIV delivery capacities.
As our studies on IRIV showed immune responses in the absence of antigenic peptides and demonstrated proliferation of antigen experienced CD4+ T cells, we addressed the importance of CD4+ T cell help in IRIV mediated CTL adjuvance. Irradiation of CD4+ T

cells resulted in abrogation of IRIV mediated CTL adjuvance as demonstrated by tetramer staining addressing $IM_{58\text{-}66}$ specific CTL induction. This observation demonstrates that IRIV mediated CTL adjuvance acts through CD4+ T cell activation.

With regard to CD4+ T cell help in CTL generation, at least two mechanisms have been described. One is acting through APC activation by cell : cell contact dependent CD40 Ligand – CD40 interaction between activated CD4+ T cells and APC, the other by CD4+ T cell mediated cytokine secretion, directly favouring expansion of activated CTL. Finally, also a CD40-independent but cell : cell contact dependent pathway has been suggested [55]. In our system, preliminary results of transwell experiments demonstrated no difference in IRIV enhanced CTL induction in the presence or absence of contact between CD4+ T cell/ APC and CD8+ T cell/ APC cocultures. As only physical cell : cell contact was inhibited, but not diffusion of soluble factors, this observation suggests that cytokines play a key role in IRIV mediated CTL adjuvance. Again, the cytokines addressed in this work, might all contribute to IRIV mediated CTL adjuvance, in that they favour APC maturation (TNF-α, IFN-γ, GM-CSF) or, in the case of IL-2, act as growth factors directly on activated lymphocytes.

Obviously, the requirement of antigen triggered CD4+ T cell activation and the absence of direct induced APC maturation suggest that IRIV CTL adjuvance is driven by adaptive and not innate immune responses, e.g. through toll like receptor ligand triggering [4]. Moreover, the non-responsiveness of naive T lymphocytes indicates that IRIV CTL adjuvance depends on previous exposures to or vaccinations against influenza virus. Finally, these prerequisites also point to the immunocompetence of vaccinees. As CD4+ T cell memory against influenza is usually developed during childhood, use of IRIV as CTL adjuvant in cancer immunotherapy should not be hampered from this point of view. In contrast, defective immunocompetence may be observed in advanced tumors.

In this regard it should be noted that tumor induced immunosuppression increases with tumor size and stage, leaving a time window for early treatment, following surgical tumor resection. In relation to infectious diseases, IRIV CTL adjuvance may be at least in part abrogated in AIDS patients due to HIV infection and subsequent destruction of CD4+ T cells.

Overall, our results suggest that IRIV induce adaptive CD4+ T cell responses which, presumably through cytokines, enhance adaptive CD8+ T cell responses induced by the HLA class I restricted epitope of interest.

Given the possibility to combine influenza virosome immunogenic capacities with their delivery capacities, we tested L_{27}Melan-A/Mart-$1_{26\text{-}35}$ specific CTL induction by FCIRIV

containing L_{27}Melan-A/Mart-1_{26-35} peptides. This formulation, as IRIV, was produced by Pevion Biotech Ltd. Regarding delivery capacities, influenza virosomes have been shown to be uptaken by APC through receptor mediated endocytosis and to fuse with the endosomal membrane, both processes triggered by influenza virus derived hemagglutinin [6]. Virosome fusion with the endosomal membrane hypothetically results in release of encapsulated content into the cytosol [6]. This intracellular delivery of HLA class I restricted epitopes is quite different from the exogenous application of peptides as such and may be important for HLA class I presentation.

Indeed, fusion competence of influenza virosomes encapsulating a murine CTL epitope of the influenza virus nucleoprotein (NP) has been shown to be required in vivo for efficient induction of a class I MHC-restricted CTL response against influenza-infected cells [12]. Enhancement of L_{27}Melan-A/Mart-1_{26-35} specific CTL induction in presence of Mart-FCIRIV as compared to L_{27}Melan-A/Mart-1_{26-35} peptides in solution could be demonstrated by cytotoxicity assays and pentamer staining. Importantly, this adjuvance could be observed in cytotoxicity assays using as target cells HBL, HLA-A0201+ tumor cells that constitutively express Melan-A/Mart-1. This observation demonstrates that CTL primed by Mart-FCIRIV are able to recognize the native antigen, and, upon recognition, to lyse tumor cells.

In a minority of cases, pentamer staining did not correlate with cytotoxicity assay results. Such discrepancies in general have been described previously [3]. In such cases we rather relied on cytotoxicity assay results as they are functionally representative for influenza virosome CTL adjuvant capacities. Regarding the cases without discrepancies, pentamer staining results suggest that CTL adjuvance displayed by FCIRIV encapsulating peptides lies approximately in the same range as described for IRIV admixed with peptides. However, in contrast to IRIV admixed with peptides, Mart-FCIRIV displayed remarkable CTL adjuvance in absence of CD4+ T cells as demonstrated by pentamer staining. This CD4+ T cell independent CTL adjuvance is likely attributable to influenza virosome delivery capacities. A second explanation might suggest direct effects of Mart-FCIRIV on APC. Such direct effects however would be independent of APC maturation, given the fact that Mart-FCIRIV were unable to induce upregulation of maturation markers on APC within 24 hours of culture.

A third explanation for the mechanism of CD4+ T cell independent adjuvance of FCIRIV might be represented by their capacity to protect the encapsulated peptide from enzymatic degradation by serum or cell associated peptidases. In our system this is rather unlikely as L_{27}Melan-A/Mart-1_{26-35}, our model peptide, is relatively resistant to enzymatic degradation [56].

In addition, the in vitro cell culture system provides immediate access of peptides to APC in conditions with low percentages of human serum.

The observation of both, T helper 1 CD4+ T cell activation and CD4+ T cell independent CTL adjuvance by Mart-FCIRIV suggests an advantage of this formulation as compared to IRIV admixed with peptides. This advantage, if related to influenza virosome delivery capacities, might result in a lower amount of peptides required to induce a strong CTL response. The advantage may become more important in conditions where memory CD4+ T cells specific to influenza virosome antigens are in short supply or suppressed. Further, peptide protection from enzymatic degradation could be of major relevance in vivo. Given the route of subcutaneous administration, Langerhans cells are most probably the first APC that would uptake the administered antigen. However, far higher numbers of fibroblasts are present. In vitro, fibroblasts have been shown to be able to degrade antigenic peptides [57].

Our in vitro studies have demonstrated remarkable CTL adjuvance of two influenza virosome formulations as related to the HLA class I restricted epitopes IM_{58-66} and Melan-A/Mart-1_{27-35} in case of IRIV and L_{27}Melan-A/Mart-1_{26-35} in case of Mart-FCIRIV. These epitopes have not been addressed by other experimental studies on influenza virosome formulations, which were mostly performed in animal models. The relevance of our data, in the context of future clinical trials, is represented by the use of human cell cultures. The drawback, of all in vitro studies, is most obviously represented by the lack of the cellular complexity present in vivo.

On the other hand, CTL adjuvance in vivo has been proven for influenza virosomes encapsulating an epitope of the Influenza virus nucleoprotein [12], chimeric influenza virosomes encapsulating the HCV core 132 peptide [11] and influenza virosomes encapsulating OVA protein [13].

Regarding underlying mechanisms of Influenza virosome CTL adjuvance, it is of interest to address influenza virosome effects on CD4+CD25+ T regulatory cell mediated suppression as well. These cells have been reported to play an important role in maintenance of peripheral tolerance to self antigens [40,41]-. As differentiation tumor associated antigens are self antigens, efficient induction of antitumor immune responses might be hampered by CD4+CD25+ T regulatory cells. A few studies have described a overcoming of CD4+CD25+ T regulatory cell mediated suppression by toll like receptor ligands in murine models [46-48]. IL-6 has been proposed to render CD4+CD25- cells unresponsive to CD4+CD25+ T regulatory cells [47]. The preliminary data of our study

indicate that influenza virosomes induce IL-6 gene expression in PBMC cultures. Furthermore, in vitro IRIV mediated CTL adjuvance related to IM_{58-66} in the presence of the whole CD4+ T cell population reached the same levels of CTL expansion detectable in CD4+CD25+ T regulatory cell depleted CD4+ T cell population (CD4+CD25- cells). In presence of a CD4+ T cell population consisting only of CD4+CD25+ T regulatory cells no CTL induction could be observed with either formulation. These results suggest the possibility that mechanisms underlying IRIV mediated CTL adjuvance might include effects counteracting physiological CD4+CD25+ T regulatory cell mediated immunosuppression.

The evaluation of a pharmaceutical product requires not only efficacy but also toxicity studies. In this regard, the use of peptides encapsulated in FCIRIV or IRIV admixed with peptides in future clinical trials is likely to provide the regulatory advantage that IRIV are already approved as adjuvants for hepatitis A vaccination and as subunit vaccine for influenza vaccination.

In summary, this work demonstrates that two different influenza virosome formulations provide CTL adjuvance in relation to IM_{58-66} and HLA class I restricted epitopes of the tumor associated antigen Melan-A/Mart-1. These results encourage further evaluation of influenza virosome formulations in vivo, addressing induction of CTL specific for melanoma associated antigens and therapeutic efficacy against melanoma.
Influenza virosome formulations encapsulating HLA class I restricted peptides would have the advantage of both, T helper 1 CD4+ T cell activation and CD4+ T cell independent CTL adjuvance, presumably due to intracellular delivery and protection of peptides from degradation.
Finally, although not in our scope, the observation of IRIV IM_{58-66} CTL adjuvance might be important with regard to vaccination against influenza. Influenza virosomes in combination with the influenza HLA class I restricted epitope IM_{58-66} might provide both, humoral and CTL responses against influenza viruses. In addition, given the fact that HA and NA are highly variable antigens of influenza virus [58-60], a more conserved epitope might be of high relevance in case of lack of memory to newly evolved HA or NA variants.

References

1. van der Bruggen P., Traversari C, Chomez P, Lurquin C, De Plaen E, Van den EB, Knuth A, Boon T: A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 254:1643-1647, 1991.

2. Robinson HL, Amara RR: T cell vaccines for microbial infections. Nat Med 11:S25-S32, 2005.

3. Zajac P, Oertli D, Marti W, Adamina M, Bolli M, Guller U, Noppen C, Padovan E, Schultz-Thater E, Heberer M, Spagnoli G: Phase I/II clinical trial of a nonreplicative vaccinia virus expressing multiple HLA-A0201-restricted tumor-associated epitopes and costimulatory molecules in metastatic melanoma patients. Hum Gene Ther 14:1497-1510, 2003.

4. Pashine A, Valiante NM, Ulmer JB: Targeting the innate immune response with improved vaccine adjuvants. Nat Med 11:S63-S68, 2005.

5. Almeida JD, Edwards DC, Brand CM, Heath TD: Formation of virosomes from influenza subunits and liposomes. Lancet 2:899-901, 1975.

6. Bungener L, Serre K, Bijl L, Leserman L, Wilschut J, Daemen T, Machy P: Virosome-mediated delivery of protein antigens to dendritic cells. Vaccine 20:2287-2295, 2002.

7. Gluck R: Adjuvant activity of immunopotentiating reconstituted influenza virosomes (IRIVs). Vaccine 17:1782-1787, 1999.

8. Holzer BR, Hatz C, Schmidt-Sissolak D, Gluck R, Althaus B, Egger M: Immunogenicity and adverse effects of inactivated virosome versus alum-adsorbed hepatitis A vaccine: a randomized controlled trial. Vaccine 14:982-986, 1996.

9. Loutan L, Bovier P, Althaus B, Gluck R: Inactivated virosome hepatitis A vaccine. Lancet 343:322-324, 1994.

10. Hunziker IP, Grabscheid B, Zurbriggen R, Gluck R, Pichler WJ, Cerny A: In vitro studies of core peptide-bearing immunopotentiating reconstituted influenza virosomes as a non-live prototype vaccine against hepatitis C virus. Int Immunol 14:615-626, 2002.

11. Amacker M, Engler O, Kammer AR, Vadrucci S, Oberholzer D, Cerny A, Zurbriggen R: Peptide-loaded chimeric influenza virosomes for efficient in vivo induction of cytotoxic T cells. Int Immunol 2005.

12. Arkema A, Huckriede A, Schoen P, Wilschut J, Daemen T: Induction of cytotoxic T lymphocyte activity by fusion-active peptide-containing virosomes. Vaccine 18:1327-1333, 2000.

13. Bungener L, Huckriede A, de Mare A, Vries-Idema J, Wilschut J, Daemen T: Virosome-mediated delivery of protein antigens in vivo: efficient induction of class I MHC-restricted cytotoxic T lymphocyte activity. Vaccine 23:1232-1241, 2005.

14. Traversari C, van der BP, Luescher IF, Lurquin C, Chomez P, Van Pel A, De Plaen E, Amar-Costesec A, Boon T: A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. J Exp Med 176:1453-1457, 1992.

15. Novellino L, Castelli C, Parmiani G: A listing of human tumor antigens recognized by T cells: March 2004 update. Cancer Immunol Immunother 54:187-207, 2005.

16. Skehel JJ, Schild GC: The polypeptide composition of influenza A viruses. Virology 44:396-408, 1971.

17. Zurbriggen R, Novak-Hofer I, Seelig A, Gluck R: IRIV-adjuvanted hepatitis A vaccine: in vivo absorption and biophysical characterization. Prog Lipid Res 39:3-18, 2000.

18. Bron R, Ortiz A, Dijkstra J, Stegmann T, Wilschut J: Preparation, properties, and applications of reconstituted influenza virus envelopes (virosomes). Methods Enzymol 220:313-331, 1993.

19. Filgueira L, Zuber M, Juretic A, Luscher U, Caetano V, Harder F, Garotta G, Heberer M, Spagnoli GC: Differential effects of interleukin-2 and CD3 triggering on cytokine gene transcription and secretion in cultured tumor infiltrating lymphocytes. Cell Immunol 150:205-218, 1993.

20. Martin I, Jakob M, Schafer D, Dick W, Spagnoli G, Heberer M: Quantitative analysis of gene expression in human articular cartilage from normal and osteoarthritic joints. Osteoarthritis Cartilage 9:112-118, 2001.

21. Kammula US, Lee KH, Riker AI, Wang E, Ohnmacht GA, Rosenberg SA, Marincola FM: Functional analysis of antigen-specific T lymphocytes by serial measurement of gene expression in peripheral blood mononuclear cells and tumor specimens. J Immunol 163:6867-6875, 1999.

22. Overbergh L, Giulietti A, Valckx D, Decallonne R, Bouillon R, Mathieu C: The use of real-time reverse transcriptase PCR for the quantification of cytokine gene expression. J Biomol Tech 14:33-43, 2003.

23. Sallusto F, Lenig D, Mackay CR, Lanzavecchia A: Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. J Exp Med 187:875-883, 1998.

24. Huang H, Xiang J: Synergistic effect of lymphotactin and interferon gamma-inducible protein-10 transgene expression in T-cell localization and adoptive T-cell therapy of tumors. Int J Cancer 109:817-825, 2004.

25. Arai K, Liu ZX, Lane T, Dennert G: IP-10 and Mig facilitate accumulation of T cells in the virus-infected liver. Cell Immunol 219:48-56, 2002.

26. Apolinario A, Majano PL, Alvarez-Perez E, Saez A, Lozano C, Vargas J, Garcia-Monzon C: Increased expression of T cell chemokines and their receptors in chronic hepatitis C: relationship with the histological activity of liver disease. Am J Gastroenterol 97:2861-2870, 2002.

27. Fernandes JL, Mamoni RL, Orford JL, Garcia C, Selwyn AP, Coelho OR, Blotta MH: Increased Th1 activity in patients with coronary artery disease. Cytokine 26:131-137, 2004.

28. Nabeshima S, Murata M, Hamada M, Chong Y, Yamaji K, Hayashi J: Maturation of monocyte-derived dendritic cells by Hochu-ekki-to, a traditional Japanese herbal medicine. Int Immunopharmacol 4:37-45, 2004.

29. Lechmann M, Zinser E, Golka A, Steinkasserer A: Role of CD83 in the immunomodulation of dendritic cells. Int Arch Allergy Immunol 129:113-118, 2002.

30. Ridge JP, Di Rosa F, Matzinger P: A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature 393:474-478, 1998.

31. Toes RE, Schoenberger SP, van der Voort EI, Offringa R, Melief CJ: CD40-CD40Ligand interactions and their role in cytotoxic T lymphocyte priming and anti-tumor immunity. Semin Immunol 10:443-448, 1998.

32. Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ: T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature 393:480-483, 1998.

33. Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath WR: Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature 393:478-480, 1998.

34. Trzonkowski P, Szmit E, Mysliwska J, Dobyszuk A, Mysliwski A: CD4+CD25+ T regulatory cells inhibit cytotoxic activity of T CD8+ and NK lymphocytes in the direct cell-to-cell interaction. Clin Immunol 112:258-267, 2004.

35. Valmori D, Fonteneau JF, Lizana CM, Gervois N, Lienard D, Rimoldi D, Jongeneel V, Jotereau F, Cerottini JC, Romero P: Enhanced generation of specific tumor-reactive CTL in vitro by selected Melan-A/MART-1 immunodominant peptide analogues. J Immunol 160:1750-1758, 1998.

36. Bungener L, Idema J, ter Veer W, Huckriede A, Daemen T, Wilschut J: Virosomes in vaccine development: induction of cytotoxic T lymphocyte activity with virosome-encapsulated protein antigens. J Liposome Res 12:155-163, 2002.

37. White J, Helenius A, Gething MJ: Haemagglutinin of influenza virus expressed from a cloned gene promotes membrane fusion. Nature 300:658-659, 1982.

38. White J, Kartenbeck J, Helenius A: Membrane fusion activity of influenza virus. EMBO J 1:217-222, 1982.

39. Cross KJ, Burleigh LM, Steinhauer DA: Mechanisms of cell entry by influenza virus. Expert Rev Mol Med 2001:1-18, 2001.

40. Fehervari Z, Sakaguchi S: CD4+ Tregs and immune control. J Clin Invest 114:1209-1217, 2004.

41. Fehervari Z, Sakaguchi S: A paragon of self-tolerance: CD25+CD4+ regulatory T cells and the control of immune responses. Arthritis Res Ther 6:19-25, 2004.

42. Terabe M, Berzofsky JA: Immunoregulatory T cells in tumor immunity. Curr Opin Immunol 16:157-162, 2004.

43. Antony PA, Piccirillo CA, Akpinarli A, Finkelstein SE, Speiss PJ, Surman DR, Palmer DC, Chan CC, Klebanoff CA, Overwijk WW, Rosenberg SA, Restifo NP: CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells. J Immunol 174:2591-2601, 2005.

44. Shevach EM: CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol 2:389-400, 2002.

45. Shevach EM, McHugh RS, Piccirillo CA, Thornton AM: Control of T-cell activation by CD4+ CD25+ suppressor T cells. Immunol Rev 182:58-67, 2001.

46. Pasare C, Medzhitov R: Toll-like receptors: balancing host resistance with immune tolerance. Curr Opin Immunol 15:677-682, 2003.

47. Pasare C, Medzhitov R: Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science 299:1033-1036, 2003.

48. Yang Y, Huang CT, Huang X, Pardoll DM: Persistent Toll-like receptor signals are required for reversal of regulatory T cell-mediated CD8 tolerance. Nat Immunol 5:508-515, 2004.

49. Petrovsky N, Aguilar JC: Vaccine adjuvants: current state and future trends. Immunol Cell Biol 82:488-496, 2004.

50. Saurwein-Teissl M, Zisterer K, Schmitt TL, Gluck R, Cryz S, Grubeck-Loebenstein B: Whole virus influenza vaccine activates dendritic cells (DC) and stimulates cytokine production by peripheral blood mononuclear cells (PBMC) while subunit vaccines support T cell proliferation. Clin Exp Immunol 114:271-276, 1998.

51. Avigan D: Dendritic cells: development, function and potential use for cancer immunotherapy. Blood Rev 13:51-64, 1999.

52. Hertz CJ, Kiertscher SM, Godowski PJ, Bouis DA, Norgard MV, Roth MD, Modlin RL: Microbial lipopeptides stimulate dendritic cell maturation via Toll-like receptor 2. J Immunol 166:2444-2450, 2001.

53. Pan J, Zhang M, Wang J, Wang Q, Xia D, Sun W, Zhang L, Yu H, Liu Y, Cao X: Interferon-gamma is an autocrine mediator for dendritic cell maturation. Immunol Lett 94:141-151, 2004.

54. Cella M, Sallusto F, Lanzavecchia A: Origin, maturation and antigen presenting function of dendritic cells. Curr Opin Immunol 9:10-16, 1997.

55. Lu Z, Yuan L, Zhou X, Sotomayor E, Levitsky HI, Pardoll DM: CD40-independent pathways of T cell help for priming of CD8(+) cytotoxic T lymphocytes. J Exp Med 191:541-550, 2000.

56. Blanchet JS, Valmori D, Dufau I, Ayyoub M, Nguyen C, Guillaume P, Monsarrat B, Cerottini JC, Romero P, Gairin JE: A new generation of Melan-A/MART-1 peptides that fulfill both increased immunogenicity and high resistance to biodegradation: implication for molecular anti-melanoma immunotherapy. J Immunol 167:5852-5861, 2001.

57. Albo F, Cavazza A, Giardina B, Marini M, Roda LG, Schumacher R, Spagnoli GC: Degradation of the tumor antigen epitope gp100(280-288) by fibroblast-associated enzymes abolishes specific immunorecognition. Biochim Biophys Acta 1671:59-69, 2004.

58. Smirnov YA, Gitelman AK, Govorkova EA, Lipatov AS, Kaverin NV: Influenza H5 virus escape mutants: immune protection and antibody production in mice. Virus Res 99:205-208, 2004.

59. Kilbourne ED: What are the prospects for a universal influenza vaccine? Nat Med 5:1119-1120, 1999.

60. Treanor J: Influenza vaccine--outmaneuvering antigenic shift and drift. N Engl J Med 350:218-220, 2004.

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