Influenza vaccination in immunosuppressed and healthy individuals

✦✦✦

DC-induced regulatory NK cell-function

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosphisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von Stefanie Fritz (geb. Hamm)

aus Weil am Rhein, DE

Basel, 2011

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
auf Antrag der Professoren:

Christoph Hess, Ed Palmer und Antonius Rolink

Basel, den 22.02.2011

Prof. Dr. Martin Spiess
Dekan

General Summary

Infection with the human immunodeficiency virus (HIV) leads to immunosuppression, mainly through depletion of CD4+ T cells. Anti-retroviral therapy (ART) inhibits virus replication and thereby promotes recovery of T cell numbers and reconstitution of cellular immunity. Importantly, ART improves control of opportunistic infections, increases quality of life and restores life expectancy of HIV-infected individuals [1, 2].

We assessed how HIV-infected individuals compare to HIV-negative individuals in terms of mounting an influenza-vaccine specific humoral and cellular immune response. We were able to show that the influenza-specific IgM- but not the IgG response was absent in HIV-infected individuals with low CD4+ T cell counts. Moreover, expansion of IFN-γ secreting CD4+ T cells was impaired. These findings point out the importance of building B cell memory while immunological competence is maintained.

Fingolimod-treated individuals have –similar to HIV-infected untreated individuals– low CD4+ T cell counts in their peripheral circulation. In the second section of the thesis we characterized cellular and humoral immune responses to influenza-vaccine in fingolimod-treated patients (diagnosed with multiple sclerosis), and in untreated healthy controls. Intriguingly, vaccine-triggered T cells accumulated normally in blood of fingolimod-treated individuals, despite reduced peripheral T cell counts. Concentrations of anti-influenza A/B IgM and IgG also increased similarly in both groups. These results indicate that fingolimod-treated individuals can mount vaccine-specific adaptive immune responses comparable to healthy controls.

Natural killer cells (NK cells) have the ability to link the innate to the adaptive immune system. We determined the Killer-cell immunoglobulin-like receptor (KIR) genotype in two cohorts of healthy and one cohort of HIV-infected individuals, and related genotype and influenza-vaccine induced adaptive immune response(s). These experiments uncovered enhanced vaccine-specific CD4+ T cell-immunity to be induced in HIV-negative but not HIV-positive individuals bearing the KIR 2DS1/3DS1 genes.

Following up on our observation that NK cell-receptor gene polymorphisms impact adaptive immune responses we lastly tested the hypothesis that NK cells may directly impact the interaction between dendritic cells (DCs) and T cells. Assessing this interaction *in vitro* we were able to demonstrate that LPS-matured DCs, but not immature DCs, have the capacity to induce immunosuppressive reactivity in autologous NK cells.

General Introduction

The acquired immunodeficiency syndrome (AIDS) is caused by the human immunodeficiency virus (HIV) [3, 4]. Although CD4+ T cells represent the primary target for the virus, other lymphocyte populations are affected as well. Apart from a polyclonal B cell dysfunction, the virus induces B cell hyperactivation, which results in a hypergammaglobulinemia. While evidence that HIV can infect B cells *in vivo* is lacking, it has been shown that HIV interacts directly with B cells through CD21. This association is mediated through complement [5]. The factors causing hyperactivity of B cells remain largely unknown. Studies that focused on that issue indicate that the following cytokines/pathway may be involved: Interferon-α (IFN-α) [6], tumor necrosis factor-α (TNF-α) [7], IL-6 [8], IL-10 [9], CD40 Ligand (CD40L) [9] and B-cell-activating factor (BAFF) [10]. *In vivo*, infection with HIV modifies circulating B cell subpopulations. Of note, early HIV-specific B cell responses are directed against non-neutralizing epitopes of the viral envelope. Later during infection, when B cells secrete neutralizing antibodies, the rapidly diversifying virus is already in a winning margin [11].

The key feature of a progressing HIV-infection is reflected in the decline of CD4+ T cell counts. The CD4+ T cell counts represent an important parameter to monitor the immune status of infected patients. Antiretroviral therapy (ART) is highly efficient in suppressing viral replication and leads to recovery of CD4+ T cell counts [12].

Infection with influenza remains a threat for immunocompromised patients like HIV-infected individuals. Therefore, the “Center for Disease Control and Prevention (CDC)” recommends yearly vaccination against influenza.

Influenza viruses can be distinguished according to their subtypes of the surface molecules, hemagglutinin (HA) and neuraminidase (NA). HA and NA are highly variable epitopes, able to undergo genetic and antigenic variation in order to escape immune response. Neutralizing antibodies can provide immediate protection, whereas the clearance is finally mediated by the cellular immune response [13]. Protection can be mediated either by CD8+ T cells or appropriate antibodies as various experiments with mice showed. Mice lacking both are not able to survive an infection [14]. In line with these findings, mice with severe combined immunodeficiency succumb to influenza infections, but can be rescued by injection of a dose containing an HA-specific monoclonal antibody [15].

Vaccination e.g. with a trivalent inactivated vaccine leads to a protective antibody response which peaks 2-3 weeks postvaccination. The most prominent antibody class after influenza vaccination is IgG (particularly IgG1) with lower concentrations of IgM and IgA [16]. Antibodies against influenza are predominantly directed against the highly variable HA and NA, whereas epitopes recognized from cytotoxic T cells are present on nucleoprotein (NP), or the RNA polymerase basic protein 2 (PB2) and polymerase acidic protein (PA).

Antibodies directed against HA or NA prevent the entrance and release of the virion, respectively, whereas activated CD8+ T cells release cytokines with antiviral activity like IFN-γ or tumour-necrosis factor (TNF) [13].

The role played by CD4+ T cells in influenza infection has been neglected for a long period because of early findings that mice lacking CD4+ T cells were still able to clear the virus. Even the antibody titer seemed to be comparable [17].

The important role for CD8+ T cells in fighting influenza infection has been demonstrated during infection in mice with deficiency in humoral immunity [18, 19]. However, despite the protective role of CD8+ T cells, memory CD8+ T cells generated following vaccination were only mildly protective [20, 21].

Influenza-specific CD4+ T cells are generated from both, natural infection and vaccination. A very recent finding demonstrated that HA-specific CD4+ T cells were able to mediate protection from influenza infection when CD4+ T cells were transferred in lymphocyte deficient RAG-mice. This was dependent on IFN-γ production because antibody-mediated neutralization of IFN-γ abrogated the protective effect [22].

In the **first section** of the thesis we investigated humoral and cellular immune responses after a virosome-based vaccination in HIV-infected patients with low and high CD4+ T cell counts and in HIV-negative controls. Using a virosome-based vaccine as the source of antigen in the ELISpot assays provided an opportunity to further investigate the “helping” role of CD4+ T cells.

We were also interested in influenza-specific IgG and IgM to further discriminate the “helping” role of CD4+ T cells: When antigen activates B cells as well as T cells, T cells provide “help” for the B cells to induce isotype switching and somatic mutation. But B cells can also be activated without T cell help. Antigens, which have repetitive structures, e.g. like polysaccharides, or are polyclonal B cell activators, can induce B cell responses independent of T cell help [23]. IgM is either produced by “natural” B-1 cells or as antigen-induced IgM by conventional (B-2) cells [24].

How influenza triggers B cell response is incompletely understood. Rothaeusler et al. could identify that follicular B cells rapidly induced extrafollicular foci to produce HA-specific antibodies. Interestingly, increased CD4+ T cell help enhanced the magnitude of extrafollicular responses [25].

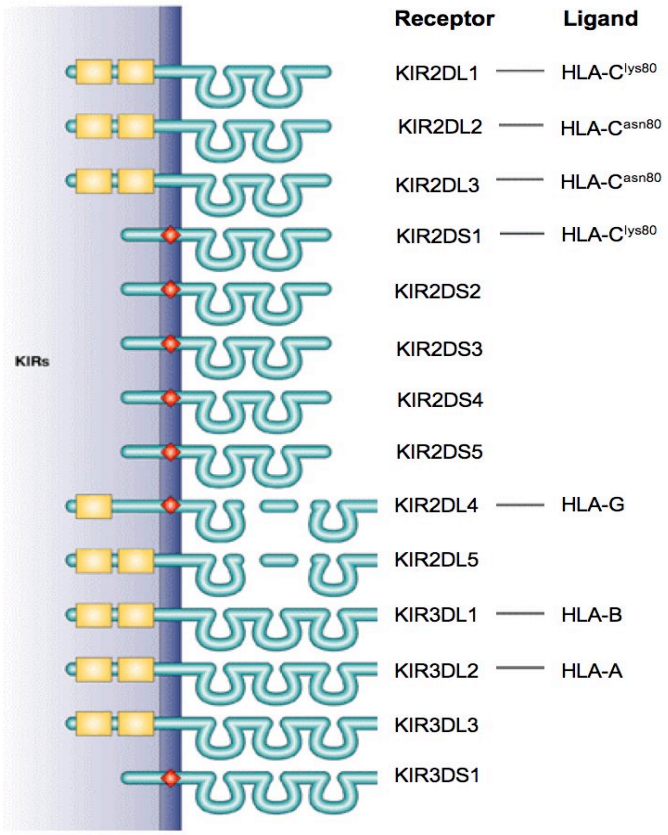
Multiple sclerosis [26] is a relatively common disease for which has been documented that autoimmune T cells are directed against antigens in the central nervous system (CNS). The auto-immune inflammation is predominantly mediated by CD4+ T cells, but CD8+ T cells also enter the inflamed tissue [27]. The drug FTY720 (fingolimod) is an analog of the sphingosine-1-phosphate (S1P) and interacts with S1P receptors. FTY720 has shown clinical and radiological efficiency in patients with MS. FTY720 differentially downregulate $S1P_1$ surface expression in T cells, and since T cells migrate out of lymph nodes along a S1P gradient, the drug inhibits T cell egress from lymph nodes [28, 29]. As a consequence, naïve T cells and central memory T cells remain in the lymph node and CCR7 negative T cells are the abundant T cell population in peripheral blood. In **Section 2** of the thesis we investigated how FTY720 treatment impacts the immune response to influenza vaccination**.**

Natural killer cells (NK cells) comprise 5-10% of peripheral blood lymphocytes and belong to the first line of defense [30]. NK cells mediate cytotoxic immune responses without prior sensitization –hence their name. On their cell surface, NK cells express activating and inhibiting receptors. Killer-cell immunoglobulin-like receptors (KIR) belong to a receptor family of activating and inhibiting receptors [31]. Inhibitory KIR signal through an ITIM [32, 33], while activating KIR recruit the DAP-12 molecule [34]. In the past years it has been revealed that 15 distinct KIR gene loci exist (including two pseudogenes KIR2DP1 and KIR3DP1). The KIR nomenclature is based on the number of extracellular immunoglobulin domains, called 2D or 3D, and the presence of either a long (L) or a short (S) intracellular domain (**Fig. 1**). Major ligands for KIR are MHC class I molecules. Among these, HLA-C molecules can be subgrouped into class C1 and C2 molecules, where MHC class C1 holds an asparagine at position 80 and is a functional ligand for KIR2DL2 and KIR2DL3, class C2 a lysine at position 80 and acts as ligand for KIR2DL1. KIR3DL1 and KIR3DS1 have specificity for HLA-A and -B alleles with a polymorphic sequence at position 80 to 83 (HLA-Bw4 epitope) [35, 36]. The ligands of activating KIR remain largely unknown. Several studies have demonstrated that KIR2DS1 and its counterpart KIR2DL1 have ligand specificities for C2 group molecules [37]. KIR-genotypes can be grouped into haplotype A with only KIR2DL4 as activating KIR, and haplotype B which contains at least another activating KIR (KIR2DS1/2/3/5 or KIR3DS1) [36]. In almost all individuals four framework genes are found, namely KIR2DL4, KIR3DL2, KIR3DL3, and KIR3DP1. The distributions of gene frequencies of the KIR genes in various populations are listed in **table 1**.

In the past few years a large number of KIR-genotype–disease phenotype association studies have been conducted. A seminal study from Martin et al. indicated that individuals carrying the KIR3DS1 allele in conjunction with HLA class I alleles from HLA-Bw4 that encode an isoleucine at position 80 (HLA-BW4-80I) have a slowed progression towards AIDS [38]. Several studies have

since described that HLA-Bw4 is associated with enhanced protection from HIV-progression [26, 39]. Apart from HIV, KIR also play a role in various other infections: Khakoo et al. demonstrated that patients that cleared hepatitis C infection carried an increased frequency of the inhibitory receptor KIR2DL3 in combination with HLA-C^{Asn80} [40]. Stern et al. related activating KIR with an improved resistance to CMV reactivation in kidney-transplant recipients [41]. Functional studies of KIR –on the other hand– are still sparse: Alter et al. were able to demonstrate how KIR3DS1+ NK cells showed cell contact-dependent inhibition of HIV in cells expressing HLA-Bw4-80I [42]. Ahlenstiel et al. used an *in vitro*-infection model for influenza-virus and detected that NK cells from HLA-C1 homozygous individuals showed a stronger degranulation and cytokine secretion upon infection compared to individuals homozygous for HLA-C2 [43]. In **Section 3** of this thesis we assessed in a prospective genotype-phenotype association study how distinct KIR/HLA compound genotypes relate to the development of an adaptive influenza-vaccine induced immune response.

Figure 1. Killer-cell immunoglobulin-like receptors (KIR) and their ligands.



Modified from Parham et al. *Nature Reviews Immmunology*. 2003.

Table 1 Comparison of observed KIR frequencies (carriers) and estimated KIR gene frequencies with HLA-C2 group frequency in the Iranian, other Asian populations and an English Caucasian cohort

Population	KIR gene	2DL1	2DL2	2DL3	2DL5	3DL1	2DS1	2DS2	2DS3	2DS4	2DS5	3DS1	2DP1	KIRAA genotype%	HLA-C2 group %
Caucasoid[a] (n=584)	Carrier frequency (%)	96.2	52.9	89.7	55.5	94.4	43.6	53.4	29.5	94.3	36.3	44.4	96.2	27.6	32.6
	Estimated gene frequency	.83	.31	.72	.33	.78	.25	.31	.15	.78	.21	.26	.83		
Palestine[b] (n=105)	Carrier frequency %	97	62	85	63	88	44	64	37	88	27	39	ND	23	24
	Estimated gene frequency	.83	.38	.61	.39	.65	.25	.40	.21	.65	.14	.22			
Lebanese[b] (n=120)	Carrier frequency (%)	99	60	88	ND	96	41	59	38	95	31	36	ND	26	31
	Estimated gene frequency	.91	.36	.66		.80	.23	.36	.21	.78	.17	.20			
Jonobi[a] (n=100)	Carrier frequency (%)	97	54	85	62	93	42	57	42	93	36	37	97	26	57
	Estimated gene frequency	.83	.32	.61	.38	.74	.24	.34	.24	.74	.20	.21	.83		
Azerbaijani[a] (n=100)	Carrier frequency (%)	99	56	84	64	97	49	58	42	97	38	47	99	23	44
	Estimated gene frequency	.91	.34	.60	.39	.83	.29	.35	.24	.83	.21	.27	.91		
Karachi S. Asians[c] (n=78)	Carrier frequency (%)	90	67	91	78	81	60	69	45	72	48	56	ND	11.5	
	Estimated gene frequency	.68	.42	.70	.53	.56	.37	.45	.26	.47	.28	.34			
Parsis[d] (n=145)	Carrier frequency (%)	99.3	62.8	81.9	71.0	87.4	62.8	62.8	53.8	86.1	51.0	62.2	99.3	24.1	47.4
	Estimated gene frequency	.92	.39	.58	.46	.65	.39	.39	.32	.63	.30	.39	.92		
N Indians[e] (n=72)	Carrier frequency (%)	87.5	79.2	65.3	79.2	87.5	54.2	62.5	43.1	80.6	47.2	38.9	ND	5.6	31.3
	Estimated gene frequency	.65	.54	.41	.54	.65	.32	.39	.25	.56	.73	.22			

[a] Populations genotyped by the authors

[b] Norman et al. 2001, 2002, Mahfouz et al. 2006

[c] Norman et al. 2002

[d] Kulkarni et al. 2008

[e] Rajalingam et al. 2002. (Estimated gene frequency from GF $= 1 - \sqrt{1 - f}$ where f is the carrier frequency)

From Hiby et al. *Immunogenetics*. 2010

Dendritic cells (DC) were discovered in 1973 as antigen-presenting cells (APC), and they have subsequently been shown to have a powerful ability to initiate immune responses [44-46]. Activated DCs home from peripheral tissues into the T cell zones of secondary lymphoid organs [47]. The DC lifespan is limited, with a half-life ranging between 1.5 and 2.9 days [48]. Upon activation, mature DCs up-regulate genes that permit initiation of an adaptive immune response [49]. On the other hand, DC can promote tolerance by presenting antigens from dying cells without co-stimulation of T cells [50]. Of importance, DCs are functional and phenotypically diverse [51], and several DC subpopulations exist, including plasmacytoid dendritic cells and conventional dendritic cells. Both conventional DCs and plasmacytoid DCs can be found in the thymus, spleen, lymph node and Peyer`s patch, whereas in the skin and liver only conventional DCs are present [52]. It is thought that myeloid progenitors in the bone marrow give rise to macrophage-DC progenitors, which further differentiate into monocytes and DC progenitors [53]. DC progenitors than likely give rise to conventional DCs and plasmacytoid DCs [54]. Due to limited numbers of DCs circulating in blood, *in vitro* generated DCs are often used for human studies. The prevalent method is to generate monocyte-derived DCs (moDCs) by cultivating monocytes in the presence of Interleukin-4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF).

Innate recognition of a microorganism is mediated via pathogen recognition receptors (PRRs). These PRRs comprise Toll-like receptors (TLRs) and NOD-like receptors [55-57]. The expression of TLRs varies between the DC types and subtypes.

The interaction of DCs with NK cells has been a recent focus of considerable research [58]. DC-derived cytokines such as IL-2, IL-12, IL-15 and IL-18 can prime NK cell function [59, 60]. In addition, NK cells can also be activated through direct cell-cell contact. For example INF-α treatment of DCs leads to upregulation of the NKG2D ligands MICA/B and subsequently to activation of NK cells [61]. Recent work has convincingly shown that NK cells can influence adaptive immune responses [62-66]. One study has, for example, demonstrated that infection of NK cell-depleted mice with T. gondii showed impaired CD4+ T cell responses [67]. In **Section 4** of the thesis we investigated whether NK cells may be differentially activated by immature vs. mature monocyte-derived DCs.

References

1. Autran B, Carcelain G, Li TS, Blanc C, Mathez D, Tubiana R*, et al.* **Positive effects of combined antiretroviral therapy on CD4+ T cell homeostasis and function in advanced HIV disease**. *Science* 1997,277:112-116.
2. Hogg RS, O'Shaughnessy MV, Gataric N, Yip B, Craib K, Schechter MT, Montaner JS. **Decline in deaths from AIDS due to new antiretrovirals**. *Lancet* 1997,349:1294.
3. Barre-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J*, et al.* **Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS)**. *Science* 1983,220:868-871.
4. Lane HC, Masur H, Edgar LC, Whalen G, Rook AH, Fauci AS. **Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome**. *N Engl J Med* 1983,309:453-458.
5. Moir S, Malaspina A, Li Y, Chun TW, Lowe T, Adelsberger J*, et al.* **B cells of HIV-1-infected patients bind virions through CD21-complement interactions and transmit infectious virus to activated T cells**. *J Exp Med* 2000,192:637-646.
6. Mandl JN, Barry AP, Vanderford TH, Kozyr N, Chavan R, Klucking S*, et al.* **Divergent TLR7 and TLR9 signaling and type I interferon production distinguish pathogenic and nonpathogenic AIDS virus infections**. *Nat Med* 2008,14:1077-1087.
7. Rieckmann P, Poli G, Fox CH, Kehrl JH, Fauci AS. **Recombinant gp120 specifically enhances tumor necrosis factor-alpha production and Ig secretion in B lymphocytes from HIV-infected individuals but not from seronegative donors**. *J Immunol* 1991,147:2922-2927.
8. Weimer R, Zipperle S, Daniel V, Zimmermann R, Schimpf K, Opelz G. **HIV-induced IL-6/IL-10 dysregulation of CD4 cells is associated with defective B cell help and autoantibody formation against CD4 cells**. *Clin Exp Immunol* 1998,111:20-29.
9. Muller F, Aukrust P, Nordoy I, Froland SS. **Possible role of interleukin-10 (IL-10) and CD40 ligand expression in the pathogenesis of hypergammaglobulinemia in human immunodeficiency virus infection: modulation of IL-10 and Ig production after intravenous Ig infusion**. *Blood* 1998,92:3721-3729.
10. He B, Qiao X, Klasse PJ, Chiu A, Chadburn A, Knowles DM*, et al.* **HIV-1 envelope triggers polyclonal Ig class switch recombination through a CD40-independent mechanism involving BAFF and C-type lectin receptors**. *J Immunol* 2006,176:3931-3941.
11. Moore JP, Cao Y, Qing L, Sattentau QJ, Pyati J, Koduri R*, et al.* **Primary isolates of human immunodeficiency virus type 1 are relatively resistant to neutralization by**

monoclonal antibodies to gp120, and their neutralization is not predicted by studies with monomeric gp120. *J Virol* 1995,69:101-109.

12. Clerici M. **Immunomodulants for the treatment of HIV infection: the search goes on**. *Expert Opin Investig Drugs* 2006,15:197-200.
13. Subbarao K, Joseph T. **Scientific barriers to developing vaccines against avian influenza viruses**. *Nat Rev Immunol* 2007,7:267-278.
14. Doherty PC, Turner SJ, Webby RG, Thomas PG. **Influenza and the challenge for immunology**. *Nat Immunol* 2006,7:449-455.
15. Gerhard W. **The role of the antibody response in influenza virus infection**. *Curr Top Microbiol Immunol* 2001,260:171-190.
16. Cox RJ, Brokstad KA, Ogra P. **Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines**. *Scand J Immunol* 2004,59:1-15.
17. Allan W, Tabi Z, Cleary A, Doherty PC. **Cellular events in the lymph node and lung of mice with influenza. Consequences of depleting CD4+ T cells**. *J Immunol* 1990,144:3980-3986.
18. Graham MB, Braciale TJ. **Resistance to and recovery from lethal influenza virus infection in B lymphocyte-deficient mice**. *J Exp Med* 1997,186:2063-2068.
19. Epstein SL, Lo CY, Misplon JA, Bennink JR. **Mechanism of protective immunity against influenza virus infection in mice without antibodies**. *J Immunol* 1998,160:322-327.
20. Andrew ME, Coupar BE. **Efficacy of influenza haemagglutinin and nucleoprotein as protective antigens against influenza virus infection in mice**. *Scand J Immunol* 1988,28:81-85.
21. Taylor J, Weinberg R, Kawaoka Y, Webster RG, Paoletti E. **Protective immunity against avian influenza induced by a fowlpox virus recombinant**. *Vaccine* 1988,6:504-508.
22. Teijaro JR, Verhoeven D, Page CA, Turner D, Farber DL. **Memory CD4 T cells direct protective responses to influenza virus in the lungs through helper-independent mechanisms**. *J Virol* 2010,84:9217-9226.
23. Bachmann MF, Zinkernagel RM. **Neutralizing antiviral B cell responses**. *Annu Rev Immunol* 1997,15:235-270.
24. Baumgarth N. **A two-phase model of B-cell activation**. *Immunol Rev* 2000,176:171-180.
25. Rothaeusler K, Baumgarth N. **B-cell fate decisions following influenza virus infection**. *Eur J Immunol* 2010,40:366-377.

26. Flores-Villanueva PO, Yunis EJ, Delgado JC, Vittinghoff E, Buchbinder S, Leung JY*, et al.* **Control of HIV-1 viremia and protection from AIDS are associated with HLA-Bw4 homozygosity**. *Proc Natl Acad Sci U S A* 2001,98:5140-5145.
27. Goverman J. **Autoimmune T cell responses in the central nervous system**. *Nat Rev Immunol* 2009,9:393-407.
28. Baumruker T, Billich A, Brinkmann V. **FTY720, an immunomodulatory sphingolipid mimetic: translation of a novel mechanism into clinical benefit in multiple sclerosis**. *Expert Opin Investig Drugs* 2007,16:283-289.
29. Martini S, Peters H, Bohler T, Budde K. **Current perspectives on FTY720**. *Expert Opin Investig Drugs* 2007,16:505-518.
30. Caligiuri MA. **Human natural killer cells**. *Blood* 2008,112:461-469.
31. Zamai L, Ponti C, Mirandola P, Gobbi G, Papa S, Galeotti L*, et al.* **NK cells and cancer**. *J Immunol* 2007,178:4011-4016.
32. Burshtyn DN, Scharenberg AM, Wagtmann N, Rajagopalan S, Berrada K, Yi T*, et al.* **Recruitment of tyrosine phosphatase HCP by the killer cell inhibitor receptor**. *Immunity* 1996,4:77-85.
33. Campbell KS, Dessing M, Lopez-Botet M, Cella M, Colonna M. **Tyrosine phosphorylation of a human killer inhibitory receptor recruits protein tyrosine phosphatase 1C**. *J Exp Med* 1996,184:93-100.
34. Lanier LL. **NK cell receptors**. *Annu Rev Immunol* 1998,16:359-393.
35. Parham P. **MHC class I molecules and KIRs in human history, health and survival**. *Nat Rev Immunol* 2005,5:201-214.
36. Middleton D, Gonzelez F. **The extensive polymorphism of KIR genes**. *Immunology* 2010,129:8-19.
37. Stewart CA, Laugier-Anfossi F, Vely F, Saulquin X, Riedmuller J, Tisserant A*, et al.* **Recognition of peptide-MHC class I complexes by activating killer immunoglobulin-like receptors**. *Proc Natl Acad Sci U S A* 2005,102:13224-13229.
38. Martin MP, Gao X, Lee JH, Nelson GW, Detels R, Goedert JJ*, et al.* **Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS**. *Nat Genet* 2002,31:429-434.
39. Carrington M, O'Brien SJ. **The influence of HLA genotype on AIDS**. *Annu Rev Med* 2003,54:535-551.
40. Khakoo SI, Thio CL, Martin MP, Brooks CR, Gao X, Astemborski J*, et al.* **HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection**. *Science* 2004,305:872-874.

41. Stern M, Elsasser H, Honger G, Steiger J, Schaub S, Hess C. **The number of activating KIR genes inversely correlates with the rate of CMV infection/reactivation in kidney transplant recipients**. *Am J Transplant* 2008,8:1312-1317.
42. Alter G, Martin MP, Teigen N, Carr WH, Suscovich TJ, Schneidewind A*, et al.* **Differential natural killer cell-mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes**. *J Exp Med* 2007,204:3027-3036.
43. Ahlenstiel G, Martin MP, Gao X, Carrington M, Rehermann B. **Distinct KIR/HLA compound genotypes affect the kinetics of human antiviral natural killer cell responses**. *J Clin Invest* 2008,118:1017-1026.
44. Steinman RM, Witmer MD. **Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice**. *Proc Natl Acad Sci U S A* 1978,75:5132-5136.
45. Steinman RM, Cohn ZA. **Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution**. *J Exp Med* 1973,137:1142-1162.
46. Steinman RM. **The dendritic cell system and its role in immunogenicity**. *Annu Rev Immunol* 1991,9:271-296.
47. Randolph GJ, Ochando J, Partida-Sanchez S. **Migration of dendritic cell subsets and their precursors**. *Annu Rev Immunol* 2008,26:293-316.
48. Kamath AT, Pooley J, O'Keeffe MA, Vremec D, Zhan Y, Lew AM*, et al.* **The development, maturation, and turnover rate of mouse spleen dendritic cell populations**. *J Immunol* 2000,165:6762-6770.
49. Zanoni I, Ostuni R, Capuano G, Collini M, Caccia M, Ronchi AE*, et al.* **CD14 regulates the dendritic cell life cycle after LPS exposure through NFAT activation**. *Nature* 2009,460:264-268.
50. Banchereau J, Steinman RM. **Dendritic cells and the control of immunity**. *Nature* 1998,392:245-252.
51. Shortman K, Liu YJ. **Mouse and human dendritic cell subtypes**. *Nat Rev Immunol* 2002,2:151-161.
52. Ardavin C. **Origin, precursors and differentiation of mouse dendritic cells**. *Nat Rev Immunol* 2003,3:582-590.
53. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. **Development of monocytes, macrophages, and dendritic cells**. *Science* 2010,327:656-661.
54. Liu K, Nussenzweig MC. **Origin and development of dendritic cells**. *Immunol Rev* 2010,234:45-54.
55. Iwasaki A, Medzhitov R. **Toll-like receptor control of the adaptive immune responses**. *Nat Immunol* 2004,5:987-995.

56. Marshak-Rothstein A. **Toll-like receptors in systemic autoimmune disease**. *Nat Rev Immunol* 2006,6:823-835.

57. Granucci F, Zanoni I, Ricciardi-Castagnoli P. **Central role of dendritic cells in the regulation and deregulation of immune responses**. *Cell Mol Life Sci* 2008,65:1683-1697.

58. Fehniger TA, Cooper MA, Nuovo GJ, Cella M, Facchetti F, Colonna M, Caligiuri MA. **CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity**. *Blood* 2003,101:3052-3057.

59. Degli-Esposti MA, Smyth MJ. **Close encounters of different kinds: dendritic cells and NK cells take centre stage**. *Nat Rev Immunol* 2005,5:112-124.

60. Feau S, Facchinetti V, Granucci F, Citterio S, Jarrossay D, Seresini S*, et al.* **Dendritic cell-derived IL-2 production is regulated by IL-15 in humans and in mice**. *Blood* 2005,105:697-702.

61. Jinushi M, Takehara T, Kanto T, Tatsumi T, Groh V, Spies T*, et al.* **Critical role of MHC class I-related chain A and B expression on IFN-alpha-stimulated dendritic cells in NK cell activation: impairment in chronic hepatitis C virus infection**. *J Immunol* 2003,170:1249-1256.

62. Raulet DH. **Interplay of natural killer cells and their receptors with the adaptive immune response**. *Nat Immunol* 2004,5:996-1002.

63. Zingoni A, Sornasse T, Cocks BG, Tanaka Y, Santoni A, Lanier LL. **NK cell regulation of T cell-mediated responses**. *Mol Immunol* 2005,42:451-454.

64. Yokoyama WM. **Specific and non-specific natural killer cell responses to viral infection**. *Adv Exp Med Biol* 2005,560:57-61.

65. Lunemann A, Lunemann JD, Munz C. **Regulatory NK-cell functions in inflammation and autoimmunity**. *Mol Med* 2009,15:352-358.

66. Paust S, Senman B, von Andrian UH. **Adaptive immune responses mediated by natural killer cells**. *Immunol Rev* 2010,235:286-296.

67. Goldszmid RS, Bafica A, Jankovic D, Feng CG, Caspar P, Winkler-Pickett R*, et al.* **TAP-1 indirectly regulates CD4+ T cell priming in Toxoplasma gondii infection by controlling NK cell IFN-gamma production**. *J Exp Med* 2007,204:2591-2602.

Section 1: Virosomal influenza-vaccine induced immunity in HIV-infected individuals with high versus low CD4+ T cell counts; clues towards a rational vaccination strategy

Stefanie Fritz, Erik Mossdorf, Bojana Durovic, Gabriela Zenhaeusern, Anna Conen, Ingrid Steffen, Manuel Battegay, Reto Nüesch, Christoph Hess.

Abstract

In a prospective influenza-vaccination trial we show that HIV-infected individuals with CD4+ T cell counts <350/µL were distinct from HIV-infected individuals with >350 CD4+ T cell counts/µL, and from HIV-negative individuals, in that an influenza-specific IgM-response was absent and expansion of IFNγ-secreting CD4+ T cells was impaired. By contrast, IgG-responses were induced in all study-groups. These data suggest that establishing broad influenza-specific (IgG) B cell memory prior to severe immunodeficiency is important.

Introduction

Antibody affinity maturation, isotype class-switch and B cell memory formation all depend on adequate CD4+ T cell help [1, 2]. By contrast, maintenance of established B cell memory needs little or no T cell help in order to persist [3, 4]. In HIV-infection T cell help is progressively lost as CD4+ T cell counts decline [5]. Seasonal influenza vaccination is advised for HIV-infected individuals [6]. Whether, based on specific immunological assessments, subgroups of HIV-infected individuals should be vaccinated with priority has not been assessed.

How in HIV-infected individuals virosomal influenza antigen –known to trigger both cellular and humoral immunity [7]– is capable of inducing influenza-specific IgM and IgG, and how this antibody-production relates to total CD4+ T cell counts and influenza-specific CD4+ T cell function is unknown.

Patients and Methods

In a prospective observational clinical study we characterized vaccine-specific immunity in 24 HIV-negative, and in 31 HIV-infected individuals. Study participants were recruited and followed-up at the University Hospital Basel during the vaccination season 2007/2008. Inclusion criteria were: age >18 years, and in HIV-infected individuals anti-retroviral therapy (ART) since >3 months and HIV viral load <200 copies/µL). Exclusion criteria were: a febrile illness, allergies to compounds of the vaccine, any vaccination within 30 days of inclusion and/or during follow-up, concomitant or planed medication with steroids/other immunosuppressive drugs, malignant disease, and pregnancy. The study was IRB approved and written informed consent was obtained from all study participants.

All participants were vaccinated with a trivalent virosomal vaccine (Inflexal V, Berna Biotech, Basel, Switzerland). Blood was collected immediately prior to vaccination, and at day 7 (=follow-up 1 [FU1]), 14 [=FU2] and 28 [=FU3]) post-vaccination. Influenza-specific IgM and IgG were quantified using a commercially available kit (Genzyme Virotech, Ruesselsheim, Germany), antibody-quantifications expressed as mean optical density values from duplicate measurements. The frequency of CD4+ T cells secreting IFNγ was measured using ELISpot assays as previously described [8], using Inflexal (Berna Biotech, Bern, Switzerland) as the source of antigen. Peak post-vaccination frequencies are shown. Wilcoxon matched paired test was performed to compare frequencies of influenza-specific CD4+ T cells and influenza-specific IgG and IgM between differing time-points.

Results

Table 1. Patient characteristics

Supplementary Table 1. Characteristics of the study populations

Patient Characteristics	HIV-negative	HIV-infected CD4+ T cells > 350/µL	HIV-infected CD4+ T cells < 350/µL
Study Participants (n)	24	22	9
Age - median (range)	34 (22-76)	45 (21-65)	46 (30-64)
Male gender - no. (%)	13 (54%)	16 (73%)	7 (78%)
CDC Staging			
AIDS	n.a.	62%	89%
Therapy			
Days on ART before vaccination - median (range)	n.a.	2.364 (513-5.377)	287 (70-5.257)
Lymphocyte Counts - Lymphocytes x10E9/L			
Baseline- median (range)	> 500	545 (364-941)	200 (40-326)
Day 84 post-vaccination - median (range)	> 500	520 (371-972)	246 (56-467)
Day 168 post-vaccination	n.a.	565 (324-1.031)	287 (17-456)
Viral Loads - Copies/mL			
Baseline	n.a.	< 200	< 200
Baseline VL <40 copies/ml	n.a.	71%	67%
Day 168 post-vaccination	n.a.	<200	<200*
Day 168 VL <40 copies/ml	n.a.	81%	78%

n.a.= not applicable
ART = anti-retroviral therapy
* one exception due to non-adherence of ART (VL=498)

Clinical characteristics of the study population are summarized in **Table 1**. In HIV-negative individuals median levels of influenza-specific IgM (FU1-3) and IgG (FU2&3) significantly increased as compared to pre-vaccination levels (**Figure 1A**, left and medium panel). The median frequency of vaccine-induced CD4+ T cells also significantly increased, with a rise observed in 22/24 (92%) individuals (**Figure 1A**, right panel). In HIV-infected participants with preserved CD4+ T cell counts (>350/µL of blood) median levels of influenza-specific IgM (FU1-3) and IgG (FU1-3) also significantly increased as compared to pre-vaccination levels (**Figure 1B**, left and medium panel). However, while the median frequency of vaccine-induced CD4+ T cells rose significantly, an increase was observed in only 14/22 individuals (64%) (**Figure 1B**, right panel).

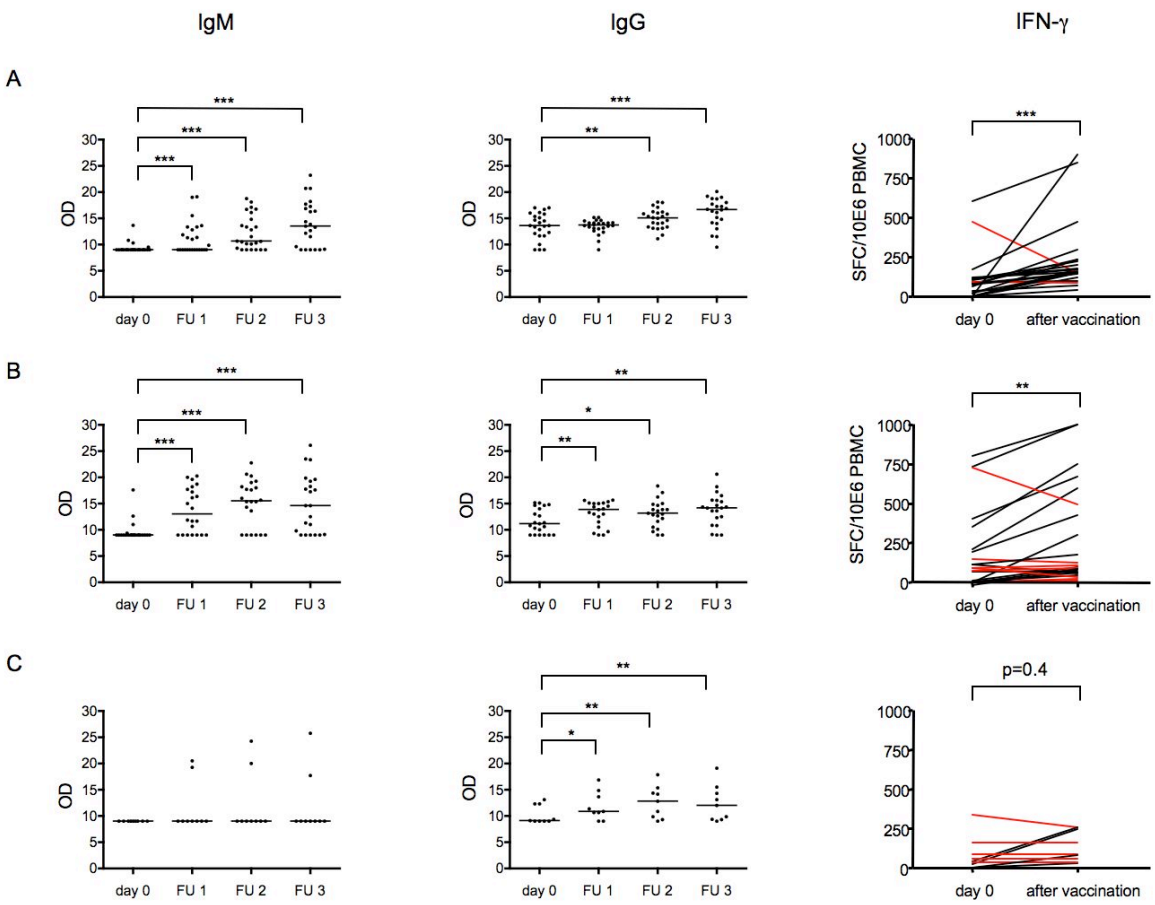


Figure 1. Humoral and cellular immune response in HIV-negative and HIV-infected individuals vaccinated with virosomal influenza-antigen. Humoral and cellular immune response in HIV-negative and HIV-infected individuals vaccinated with virosomal influenza-antigen. Levels of influenza-specific IgM (left panel), influenza-specific IgG (medium panel), and the frequency of influenza-specific CD4+ T cells (right panel) is shown in HIV-negative study participants **(A)**, HIV-infected study participants with >350 CD4+ T cells/µL **(B)**, and in HIV-infected study participants with <350 CD4+ T cells/µL **(C)**. Dots indicate antibody-measurements in individual study participants (horizontal bars indicate median levels); pre- and post-vaccination frequencies of influenza-specific CD4+ T cells are linked with black lines in case of expanding responses, with red lines in case of contracting responses. FU1-3 = days 7, 14 and 28 post-vaccination. Dots present values from each participant at indicated time points. *, Values of $p < 0.05$; ** a p value of < 0.01 and ***, a p value of < 0.001 compared with baseline.

In contrast to HIV-negative and HIV-infected individuals with preserved CD4+ T cell counts, in HIV-infected study participants with <350 CD4+ T cell counts/µL no significant increase in median influenza-specific IgM levels was detected at any follow-up time-point, with only 2/9 participants responding at all (**Figure 1C**, left panel). However, a significant increase in median levels of post-vaccination influenza-specific IgG was observed (**Figure 1C**, medium panel). No significant increase in the median frequency of vaccine-induced CD4+ T cells was detected in the group of individuals with low CD4+ T cell counts, with a response induced in only 4/9 individuals (44%)

(**Figure 1C**, right panel). Of note, time on ART in 'IgM responders' with low CD4+ T cell counts tended to be longer, in 'IgM non-responders' with high CD4+ T cell counts shorter than among their respective intra-population controls. No such trend was observed comparing CDC disease stage, nadir CD4+ T cell counts and virological suppression (data not shown).

Discussion

The inability of most HIV-infected individuals with low CD4+ T cell counts to produce detectable amounts of influenza-specific IgM was unexpected. A possible explanation for the observed lack of IgM-production could be an intrinsic defect of B cells, which has been demonstrated in patients with viremia and –although to a much lesser extent– also in patients with undetectable viral load [8, 10]. By contrast, vaccine-induced IgG increased in all study groups, including the one with low CD4+ T cell counts. Given the lack of IgM-response in HIV-infected individuals with low CD4+ T cell counts, increasing levels of IgG in this study group most likely reflects a memory response. This finding is important and provides an immunological rational supporting the recommendation of annual influenza vaccinations *throughout* the course of HIV-infection, permitting the buildup of a broad and long-lasting B cell memory when immunological competence is still maintained. The clinical importance of such memory responses has been impressively underscored during the recent influenza H1N1 pandemic, where children –presumably due to a lack of sero-protection from cross-reactive antibodies induced by previous contact with influenza antigen– were most severely affected [11].

Limitations of our study are the lack of qualitative assessments of the humoral and the cellular vaccine-specific immune response, patient diversity, and the fact that the study was underpowered to evaluate clinical endpoints such as protection from influenza infection. However, the read-outs used did permit the capture of decreasing vaccine-inducible cellular responsiveness, and they uncovered a likely dependency of the vaccine-response on B cell memory in advanced HIV infection. These preliminary data should trigger future research aiming at understanding the molecular basis of the observed lack of IgM-production, and they lend support to strictly enacting annual influenza-vaccination *in all* HIV-infected individuals regardless of their CD4+ T cell count.

References

1. Grewal IS, Flavell RA. **CD40 and CD154 in cell-mediated immunity**. *Annu Rev Immunol* 1998,16:111-135.
2. Parker DC. **T cell-dependent B cell activation**. *Annu Rev Immunol* 1993,11:331-360.
3. Vieira P, Rajewsky K. **Persistence of memory B cells in mice deprived of T cell help**. *Int Immunol* 1990,2:487-494.
4. Hebeis BJ, Klenovsek K, Rohwer P, Ritter U, Schneider A, Mach M, Winkler TH. **Activation of virus-specific memory B cells in the absence of T cell help**. *J Exp Med* 2004,199:593-602.
5. Margolick JB, Munoz A, Donnenberg AD, Park LP, Galai N, Giorgi JV*, et al.* **Failure of T-cell homeostasis preceding AIDS in HIV-1 infection. The Multicenter AIDS Cohort Study**. *Nat Med* 1995,1:674-680.
6. Harper SA, Fukuda K, Uyeki TM, Cox NJ, Bridges CB. **Prevention and control of influenza. Recommendations of the Advisory Committee on Immunization Practices (ACIP)**. *MMWR Recomm Rep* 2005,54:1-40.
7. Huckriede A, Bungener L, Stegmann T, Daemen T, Medema J, Palache AM, Wilschut J. **The virosome concept for influenza vaccines**. *Vaccine* 2005,23 Suppl 1:S26-38.
8. Moir S, Ho J, Malaspina A, Wang W, DiPoto AC, O'Shea MA*, et al.* **Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals**. *J Exp Med* 2008,205:1797-1805.
9. Gasser O, Bihl FK, Wolbers M, Loggi E, Steffen I, Hirsch HH*, et al.* **HIV patients developing primary CNS lymphoma lack EBV-specific CD4+ T cell function irrespective of absolute CD4+ T cell counts**. *PLoS Med* 2007,4:e96.
10. Moir S, Fauci AS. **B cells in HIV infection and disease**. *Nat Rev Immunol* 2009,9:235-245.
11. Bansal S, Pourbohloul B, Grenfell B, Meyers LA. **The shifting demographic landscape of influenza**. *PLoS Curr Influenza* 2009:RRN1047.

Section 2: Antigen-specific adaptive immune responses in fingolimod-treated MS patients [1]

Matthias Mehling, Stefanie Fritz, Patricia Hilbert, Bojana Durovic, Dominik Eichin, Olivier Gasser, Jens Kuhle, Thomas Klimkait, Raja Lindberg, Ludwig Kappos, Christoph Hess.

Abstract

T cells exit secondary lymphoid organs along a sphingosine1-phosphate (S1P)-gradient and, accordingly, are reduced in blood upon fingolimod-mediated S1P-receptor (S1PR)-blockade. Serving as a model of adaptive immunity we characterized cellular and humoral immune responses to influenza-vaccine in fingolimod-treated patients with multiple sclerosis and in untreated healthy controls. Although the mode of action of fingolimod might predict reduced immunity, vaccine-triggered T cells accumulated normally in blood despite efficient S1PR-blockade. Concentrations of anti-influenza A/B IgM and IgG also increased similarly in both groups. These results indicate that fingolimod-treated individuals can mount vaccine-specific adaptive immune responses comparable to healthy controls.

---

[1] As second author of this report that has been published at the *Annals of Neurology*, Stefanie Fritz was involved in collecting samples, performing ELISpot assays, and in analyzing and interpretation of data.

Introduction

In multiple sclerosis (MS) lesions lymphocytes mediate inflammation, demyelination, and axonal damage [1]. Lymphocytes that express the chemokine receptor CCR7 are able to migrate from peripheral tissues –such as the central nervous system (CNS)– to secondary lymphoid organs (SLO) such as lymph nodes, whereas cells not expressing CCR7 (CCR7-negative) do not re-circulate to SLO on a regular basis. In order to egress from SLO to the peripheral blood circulation cells migrate along a sphingosine 1-phosphate (S1P) gradient [2].

The oral S1P receptor (S1PR)-agonist fingolimod –which has shown efficacy in the treatment of MS [3-5] – blocks this egress, thereby reducing peripheral lymphocyte counts, in a dose-dependent manner, to 25-40% of baseline values [3, 6-8]. As a consequence, CCR7-negative cells represent the major T cell population circulating in the blood of fingolimod-treated patients. Despite severe lymphopenia only few infectious complications were observed in fingolimod-treated patients with MS. However, a case of fatal disseminated varicella zoster infection and a case of herpes simplex virus type 1 encephalitis nonetheless raise concern with regards to the immunological competence vis-à-vis viral pathogens in fingolimod-exposed individuals [5, 9]

So far only animal data are available on the effect of S1PR-blockade on adaptive immune responses following viral antigen exposure. In simian human immunodeficiency virus (SHIV)-infected rhesus macaques treatment with fingolimod did not result in deviations from the natural pattern of viral control [10]. Treatment with fingolimod also had no effect on the disease course and T cell exhaustion in mice infected with lymphocytic choriomeningitis virus (LCMV) [11]. In contrast, treatment with fingolimod lead to a significant reduction of influenza-antigen specific CD8+ T cells in lungs of animals infected with influenza [12].

Fundamental in this context, yet never experimentally addressed in humans, is how blocking S1PR impacts on the presence of bulk versus recently antigen-activated T cells in the peripheral circulation. Here we sought to define in a prospective observational study the effect of fingolimod-mediated S1PR-blockade on the development of antigen-specific immune responses in patients with MS.

Patients and Methods

Study subjects and procedures

We conducted an open-label, observational, prospective study to assess the adaptive immune response induced by influenza-vaccine in fingolimod-treated patients with MS and in healthy controls (HC). The trial was conducted during the influenza-vaccination periods 2008/2009 and 2009/2010. The institutional review board of Basel approved the study. After written informed consent, blood samples from study subjects were obtained before and 7, 14 and 28 days after seasonal influenza-vaccination with Mutagrip® (Sanofi Pasteur SA, Lyon). Inclusion criteria for patients were definite relapsing MS, treatment with fingolimod (either 0.5mg/d or 1.25mg/d), and age ≥18 and ≤65 years. Inclusion criteria for healthy controls were absence of chronic disease and age ≥18 and ≤65 years. Exclusion criteria for patients and controls were known hypersensitivity to the vaccine under investigation, fever at time of planned vaccination, influenza vaccination <180 days before recruitment into the study, treatment with immunoglobulins or exogenous blood products within 90 days before recruitment into the study, simultaneous medication with steroids or immunomodulators / immunosuppressants other than fingolimod, and pregnancy.

Flow cytometry

T cells were analyzed for expression of CD3, CD4 and CD8 using a CyAn cytometer (DakoCytomation, Glostrup, Denmark) according to standard procedures. The following antibodies were used: anti-human CD4 phycoerythrin (PE)-cyanin5 (Cy5) (S3.5), anti-human CD8 PE (MEM-31) and anti-human CD3 FITC (MEM-57). As isotype controls served IgG2a-FITC (713), IgG2a-PE-Cy5 (upc-10), IgG2a-PE (upc-10) (all from Immunotools, Friesoythe, Germany).

Enzyme linked immuno spot assay

Enzyme linked immuno-spot (ELISpot) was done as described previously [13] with the modification that we used with Inflexal® (Berna Biotech, Bern, Switzerland) as source of antigen (year adjusted). Enzyme linked immuno-spot (ELISpot): ELISpot plates (MSIPS4510, Millipore AG, Volketswil, Switzerland) were coated with 2 µg/mL of anti-interferon gamma (IFN-γ) mAb 1-D1K (Mabtech, Nacka Strand, Sweden) overnight. In each well 200.000 peripheral blood mononuclear cells (PBMC) were added in R10 (RPMI 1640 containing 10% heat inactivated Fetal Bovine Serum [FBS], 50 U/mL penicillin and 50 µg/mL Streptomycin [all from GIBCO™, LuBioScience GmbH, Luzern, Switzerland]) (final volume 130 µL/well). All measurements were performed in duplicates. Inflexal® was used as source of antigen (year adjusted) at a final

concentration of 14 µg/mL for each peptide, phytohemagglutinin (PHA) (1.8 µg/mL; REMEL, Oxoid AG, Basel, Switzerland) served as a positive control. Plates were incubated for 16 hours at 37°C with 5% CO2, washed with PBS (phosphate-buffered saline) and blocked with PBS 1% FBS. After washing, plates were incubated with 100 µL anti-IFN-γ mAb (1:200) coupled with alkaline phosphatase (7-B6-1-ALP, Mabtech) for 2 hours at room temperature. Spots were developed with HistoMark RED phosphatase system (KPL, Gaithersburg, Maryland, USA) and counted with the ELISpot Reader System (CSR01, AID GmbH, Strassberg, Germany) using the ELISpot 3.5 software (AID GmbH). 50 spot forming cells (SFC)/10e6 PBMC were defined as cut-off for a positive antigen-specific response. EBV- and CMV-specific ELISpot responses were assessed in the presence or absence of phosphorylated fingolimod, using EBV- or CMV-infected cell lysates (Virusys, http://www. virusys.com) at a final concentration of 10 µg/mL as the source of antigen.

Virus specific antigen presentation assay

EBV-specific T cell responses were characterized in the presence or absence of fingolimod as described previously [14]: Bulk PBMC from healthy EBV positive donors were used to generate EBV-transformed B cell clones. $1x10^5$ autologous CD3+ T cells were isolated with MACS bead technology (Miltenyi Biotec, Bergisch Gladbach, Germany). T cells were then cultured with $5x10^4$ paraformaldehyde-fixed EBV-transformed B cells in 96 well plates in LCM-10 medium (supplemented with 200 U/mL IL-2 (Roche, Basel, Switzerland) in the presence of phosphorylated or unphosphorylated fingolimod (10 ng/mL each). or medium alone. On day 3 fresh medium alone or medium containing phosphorylated or unphosphorylated fingolimod supplemented with 200 U/mL IL-2 was added. By day 7 cultures contained no B cells as assessed by CD19 staining and FACScan analysis (data not shown).

Anti-influenza A and anti-influenza B enzyme-linked immunosorbent assay

Concentrations (given as virotech [VE] units/mL) of IgM and IgG anti-influenza A and anti-influenza B were determined using a quantitative enzyme-linked immunosorbent assay (ELISA) according to the manufacturer (Genzyme Virotech, Ruesselsheim, Germany). As recommended by the manufacturer, seroprotection was defined as an anti-influenza A/B IgG-concentration of ≥10 VE/mL.

Statistical analyses

Data were tested for normality with the Shapiro-Wilk test and Levene's test was used to assess the equality of variances. Mann-Whitney test was performed in case of non-normality and/or

differing variance among study-groups. Data with normal distribution were assessed by paired Student's two-sided t-test. Values of $p<0.05$ were considered to be statistically significant.

Results

Characteristics of the study population are summarized in **Table 1** (upper part). Rates of local injection site reactions and general tolerability of the vaccine, as monitored by clinical assessments and a patient diary, were comparable in fingolimod-treated patients and HC (**Table 1**, lower part). Flow cytometric analyses revealed a reduction of mean lymphocyte counts in fingolimod-treated patients by 64% compared to the lower limit of the reference range (CD4+ by 76-83%, CD8+ by 42-63%) (**Figure 1A**), an observation that is in line with our previous findings [6]. The frequency of T cells producing IFN-γ in response to influenza-antigen was assessed by ELISpot.

Before vaccination, frequencies of influenza-specific IFN-γ secreting T cells were comparable in fingolimod-treated patients and HC, as was the number of individuals with no detectable influenza-specific response. By day 7 post-vaccination frequencies significantly increased in both groups and reached comparable levels (**Figure 1B**). Numbers of influenza-specific T cells remained increased and comparable until day 14 post-vaccination in both study groups. By day 28 post-vaccination frequencies of IFN-γ secreting cells contracted to pre-vaccination levels in both groups.

No individual mounting a very high frequency of influenza-specific cells was contained in the fingolimod-group –a finding which, however, did not reach statistical significance. No statistical significant correlation between lymphocyte counts and vaccine-specific immune responses was found in HC or fingolimod-treated individuals (data not shown). To assess whether fingolimod alters antigen-specific triggering of circulating virus-specific T cells or their in vitro induction, the effect of active (i.e. phosphorylated) fingolimod on *ex vivo* triggering and on *in vitro* expansion of antigen-specific T cells was assessed. In presence versus absence of fingolimod (in its active, i.e. phosphorylated form) no differences were detected in either experimental system (**Figure 1C and D**).

To investigate how S1PR-blockade influences antibody responses we quantified in these same patients and HC influenza-specific IgM and IgG antibody production by ELISA. Pre-vaccination levels of anti-influenza A and anti-influenza B IgM were comparably low in fingolimod-treated patients and HC. Following vaccination, concentrations of IgM anti-influenza A and anti-influenza B increased significantly and comparably in both study groups, and remained increased at comparable levels until day 28 post vaccination (**Figure 2A**/**B**).

Table 1

	healthy controls	**MS fingolimod**
	baseline characteristics	
n	18	14
median age (years) [range]	37 [19-46]	44 [31-60]
female/male	6/12	8/6
median disease duration (years) [range]	N.A.	12.3 [3-20]
median EDSS [range]	N.A.	2.6 [1.0-4.0]
median therapy duration (months) [range]	N.A.	36 [7-42]
fingolimod dosage 0.5mg/1.25mg	N.A.	6/8
	tolerability of vaccine / incidence of influenza-like illness	
injection-site reactions day 0-3 post vaccination	12/18 (66%)	7/14 (50%)
general symptoms day 0-3 post vaccination	2/18 (11%)	4/14 (29%)
MS relapses	N.A.	0/14 (0%)
incidence of influenza-like illness	2/18 (11%)	2/14 (14%)

Table 1. Characteristics of the study population (upper part) and tolerability of influenza vaccination and incidence of influenza-like illnesses (lower part).
Abbreviations: fingolimod-treated patients with multiple sclerosis (MS fingolimod), not applicable (N.A.), Expanded Disability Status Scale (EDSS)

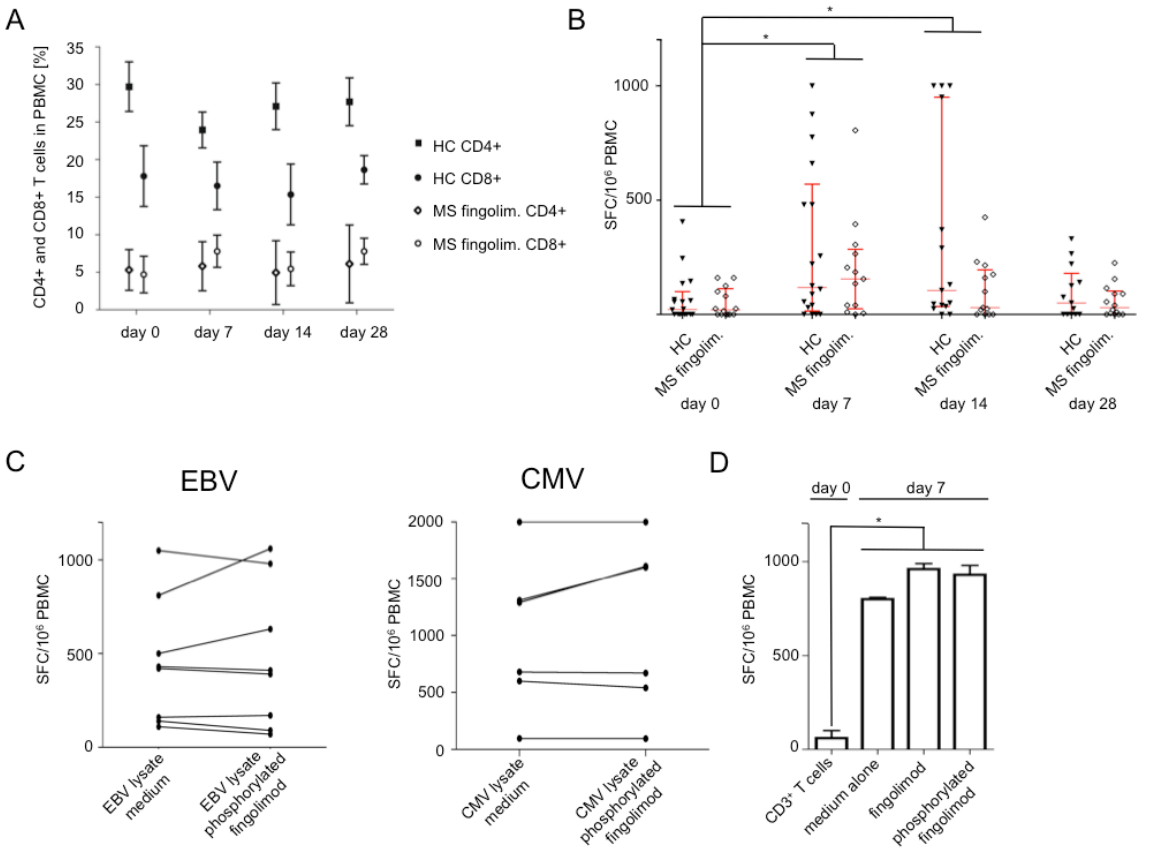


Figure 1. Lymphocyte counts, cellular immune response after influenza-vaccination in fingolimod-treated patients vs. healthy controls, and virus-specific T cell responses in the presence or absence of fingolimod. **(A)** Mean lymphocyte counts in fingolimod-treated patients with MS and in healthy controls (HC) as assessed by flow cytometry (± standard error of the mean). **(B)** The frequency of influenza-specific cells in fingolimod-treated patients with MS (MS fingolim.) and healthy controls (HC) as detected by spot forming cells (SFC) in equal amounts of peripheral blood mononuclear cells (PBMC) is shown before (day 0) and at day 7, 14 and 28 after influenza vaccination. **(C)** *Ex vivo* assessment of Epstein-Barr virus (EBV)- and cytomegalovirus (CMV)-specific $CD4^+$ T cell-dependent IFN-γ production as detected by spot forming cells (SFC) in equal amounts of peripheral blood mononuclear cells (PBMC) from HC in the absence (EBV lysate medium) or presence of phosphorylated (active) fingolimod (EBV lysate phosphorylated fingolimod). **(D)** Frequencies of EBV-specific T cells before (day 0, CD3+ T cells) and after expansion of IFN-γ secreting cells (day 7) by autologous EBV-transformed B cell clones in the absence (medium alone) and presence of fingolimod and phosphorylated fingolimod.

* indicates $p<0.05$

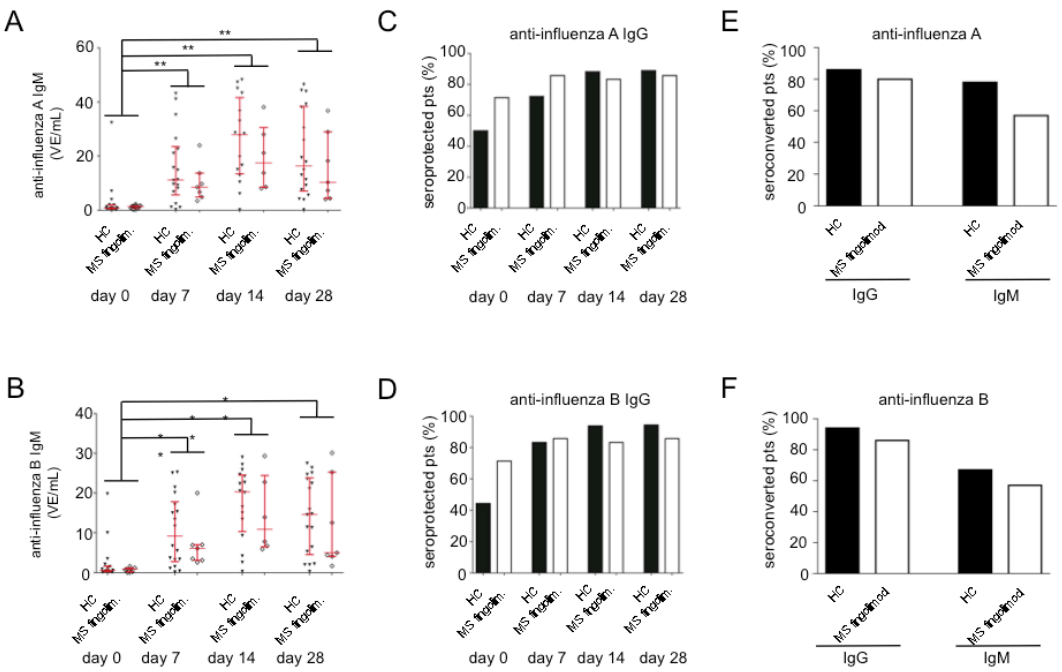


Figure 2. Antibody-response after influenza-vaccination in fingolimod-treated patients and in healthy controls. The concentration of IgM anti-influenza A (**panel A**) and anti-influenza B (**panel B**) is shown as detected before (day 0) and at day 7, 14 and 28 after influenza vaccination in fingolimod-treated patients with MS (MS fingolim.) and healthy controls (HC). The percentage of patients fulfilling IgG sero-protection criteria for influenza A (**panel C**) and influenza B (**panel D**) is shown before (day 0) and at day 7, 14 and 28 after influenza vaccination in fingolimod-treated patients with MS (MS fingolim.) and healthy controls (HC). The percentage of initially sero-negative patients converting to seroprotection for influenza A (**panel E**) and influenza B (**panel F**) following vaccination (day 7-28). ** indicates p<0.001

Before vaccination, 71% of the fingolimod-treated patients and 50% of the HC fulfilled the predefined sero-protection criteria (IgG ≥10 VE/mL) for influenza A (p=0.41), 71% of the fingolimod-treated patients and 44% of the HC for influenza B (p=0.38), indicating previous contact with antigen from these viruses in a substantial proportion of study participants (**Figure 2C**/**D**). At day 7 after vaccination the proportion of individuals fulfilling sero-protection criteria was comparably increased in both fingolimod-treated patients and HC (influenza A: p=0.64, influenza B: p=0.53), and remained increased at days 14 and 28 post vaccination in both groups (day 14 and 28: influenza A and B: p=1.0).

The proportion of individuals converting from undetectable to protective antibody levels was also similar in fingolimod-treated patients and HC (**Figure 2E/F**). Thus, the vaccine-specific production of IgM and, more importantly, IgG in fingolimod-treated individuals was not impaired when compared to levels in HC.

Discussion

The key observation of this study was that fingolimod-treated patients with MS –despite severe peripheral lymphopenia– could mount a vaccine-specific adaptive immune response that is comparable to the response observed in healthy controls. Finding a similar post-vaccination frequency of influenza specific peripheral blood T cells in fingolimod-treated patients and HC –in spite of fingolimod-mediated lymphopenia– was unexpected. This observation indicates that in humans lymphocyte-egress from SLO is controlled differentially between lymph-node homing T cells interacting with cognate antigen, as opposed to T cells screening for –but not interacting with– cognate antigen. Recent animal data are in line with such a model [15].

An impaired antibody-response in fingolimod treated individuals is a concern, as the drug directly impacts on germinal center reactions and B cell migration [16, 17]. Again, the vaccine-specific production of IgM and IgG in fingolimod treated individuals was, however, not detectably impaired when compared to levels in healthy controls, a finding in line with some [18], but not all [17] data obtained in animal models.

Our study has limitations, both from an immunological and from a clinical point-of-view. The vaccination model we used does not take into account the complexity brought by an influenza-infection or any other virus infection, and our study was underpowered to evaluate clinical endpoints such as protection from influenza infection. Likewise, our experiments detecting unchanged EBV-specific immune responses *in vitro* cannot directly be extrapolated to indicate intact immune control of other virus infections (e.g. herpes viruses) in fingolimod-treated individuals. However, the data serve as definite proof-of-principle demonstrating that blocking S1P-dependent lymphocyte migration in humans does not hinder the appearance of antigen-activated T cells in the peripheral circulation, nor does it affect the antibody response quantitatively. Clinical conditions with T cell lymphopenia comparable to the one induced by fingolimod (HIV-infection, myelotoxic chemotherapy) are associated with a high risk for opportunistic infections [19, 20]. Our data indicate that fingolimod-treated patients in principle can mount a virus-specific immune response. It remains unclear, however, to what extent these findings in the context of vaccine-responses allow extrapolation to immunological competence vis-à-vis infectious pathogens. The molecular basis of the observed bypass of S1PR-dependent SLO-egress by vaccine-triggered T cells in humans remains to be determined. For clinicians these data are informative when weighing the grade of immunosuppression inflicted on individuals treated with fingolimod, and they permit a more rational interpretation of infectious complications if they occur.

References

1. Goverman J. **Autoimmune T cell responses in the central nervous system.** *Nat Rev Immunol* 2009.
2. Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V*, et al.* **Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1.** *Nature* 2004,427:355-360.
3. Kappos L, Antel J, Comi G, Montalban X, O'Connor P, Polman CH*, et al.* **Oral fingolimod (FTY720) for relapsing multiple sclerosis.** *N Engl J Med* 2006,355:1124-1140.
4. Kappos L, Radue EW, O'Connor P, Polman C, Hohlfeld R, Calabresi P*, et al.* **A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis.** *N Engl J Med* 2010,362:387-401.
5. Cohen JA, Barkhof F, Comi G, Hartung HP, Khatri BO, Montalban X*, et al.* **Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis.** *N Engl J Med* 2010,362:402-415.
6. Mehling M, Brinkmann V, Antel J, Bar-Or A, Goebels N, Vedrine C*, et al.* **FTY720 therapy exerts differential effects on T cell subsets in multiple sclerosis.** *Neurology* 2008,71:1261-1267.
7. Vaessen LM, van Besouw NM, Mol WM, Ijzermans JN, Weimar W. **FTY720 treatment of kidney transplant patients: a differential effect on B cells, naive T cells, memory T cells and NK cells.** *Transpl Immunol* 2006,15:281-288.
8. Mehling M, Lindberg R, Raulf F, Kuhle J, Hess C, Kappos L, Brinkmann V. **Th17 central memory T cells are reduced by FTY720 in patients with multiple sclerosis.** *Neurology* 2010,75:403-410.
9. Garber K. **Infections cast cloud over Novartis' MS therapy.** *Nat Biotechnol* 2008,26:844-845.
10. Kersh EN, Luo W, Adams DR, Mitchell J, Garcia-Lerma JG, Butera S*, et al.* **Evaluation of the lymphocyte trafficking drug FTY720 in SHIVSF162P3-infected rhesus macaques.** *J Antimicrob Chemother* 2009,63:758-762.
11. Walsh KB, Marsolais D, Welch MJ, Rosen H, Oldstone MB. **Treatment with a sphingosine analog does not alter the outcome of a persistent virus infection.** *Virology* 2010,397:260-269.
12. McGill J, Legge KL. **Cutting edge: contribution of lung-resident T cell proliferation to the overall magnitude of the antigen-specific CD8 T cell response in the lungs following murine influenza virus infection.** *J Immunol* 2009,183:4177-4181.

13. Gasser O, Bihl FK, Wolbers M, Loggi E, Steffen I, Hirsch HH*, et al.* **HIV patients developing primary CNS lymphoma lack EBV-specific CD4+ T cell function irrespective of absolute CD4+ T cell counts.** *PLoS Med* 2007,4:e96.
14. Hill AB, Lee SP, Haurum JS, Murray N, Yao QY, Rowe M*, et al.* **Class I major histocompatibility complex-restricted cytotoxic T lymphocytes specific for Epstein-Barr virus (EBV)-transformed B lymphoblastoid cell lines against which they were raised.** *J Exp Med* 1995,181:2221-2228.
15. Pham TH, Okada T, Matloubian M, Lo CG, Cyster JG. **S1P1 receptor signaling overrides retention mediated by G alpha i-coupled receptors to promote T cell egress.** *Immunity* 2008,28:122-133.
16. Cinamon G, Zachariah MA, Lam OM, Foss FW, Jr., Cyster JG. **Follicular shuttling of marginal zone B cells facilitates antigen transport.** *Nat Immunol* 2008,9:54-62.
17. Han S, Zhang X, Wang G, Guan H, Garcia G, Li P*, et al.* **FTY720 suppresses humoral immunity by inhibiting germinal center reaction.** *Blood* 2004,104:4129-4133.
18. Pinschewer DD, Ochsenbein AF, Odermatt B, Brinkmann V, Hengartner H, Zinkernagel RM. **FTY720 immunosuppression impairs effector T cell peripheral homing without affecting induction, expansion, and memory.** *J Immunol* 2000,164:5761-5770.
19. Masur H, Ognibene FP, Yarchoan R, Shelhamer JH, Baird BF, Travis W*, et al.* **CD4 counts as predictors of opportunistic pneumonias in human immunodeficiency virus (HIV) infection.** *Ann Intern Med* 1989,111:223-231.
20. Mackall CL. **T-cell immunodeficiency following cytotoxic antineoplastic therapy: a review.** *Stem Cells* 2000,18:10-18.

Section 3: In absence of HIV-infection KIR 2DS1 and 3DS1 genes are associated with enhanced CD4+ T cell-responses to influenza-vaccine

Stefanie Fritz, Erik Mossdorf, Gabriela Zenhaeusern, Matthias Mehling, Bojana Durovic, Patricia Hilbert, Anna Conen, Ludwig Kappos, Manuel Battegay, Martin Stern, Reto Nüesch, Christoph Hess.

Abstract

Here we monitored influenza-specific CD4+ T cell-responses (IFN-γ secretion) before and after influenza vaccination and assessed KIR and HLA genotypes in HIV-negative (n=41) and HIV-infected individuals (n=21) in a prospective clinical trial.

Day 7 post-vaccination expansion of influenza-specific CD4+ T cells was observed in HIV-negative and HIV-infected individuals. Only in HIV-negative study participants expansion of influenza-specific CD4+ T cells was maintained two weeks post-vaccination. Four weeks post-vaccination contraction was complete in all study-groups. Compound KIR/HLA genotypes were not related to the increase of influenza-specific CD4+ T cells. However, in HIV-negative study participants (exploratory [n=23] and validation [n=18] cohort) a genotype including activating KIR 2DS1/3DS1 was associated with a larger day 7 post-vaccination increase of influenza-specific CD4+ T cells. This association was not present in HIV-infected individuals.

This is the first human study that prospectively links KIR-immunogenetics with the magnitude of an antigen-specific T cell-response. In HIV-infection this association was not observed.

Introduction

Killer cell immunoglobulin-like receptors (KIR) represent an important family of innate immune-receptors that define cellular reactivity via inhibitory and activating signals [1]. KIR are encoded by highly polymorphic genes, and the KIR gene complex is polygenic with varying numbers of inhibitory and activating receptors [2]. The principal known ligands of KIR are HLA molecules [3,4]. In humans, establishing the functional role of activating KIR in mediating anti-viral effector function has proven difficult, and direct evidence for their implication in viral control is lacking [5]. However, genetic association studies have related specific KIR genes and KIR/HLA compound genotypes with the clinical outcome after various types of viral infection [6-8].

KIR are expressed on natural killer (NK) cells –representing a key cellular component of the innate immune system participating in early responses against infected or transformed cells– as well as on subsets of T cells [9-11]. Virus-induced down-modulation of ligands for inhibitory murine KIR-analogues on infected cells, as well as cognate KIR-mediated recognition of infected cells, has been shown to contribute to NK cell mediated protection from infection [12].

The importance of NK cells in controlling HIV-replication is suggested by immunogenetic association-studies that related a KIR3DS1/HLA Bw4-I80 compound-genotype with slower progression to AIDS as compared to cohorts defined by a genotype including only one or none of these alleles [13]. In line with these data, KIR3DS1-expressing NK cells better suppress viral replication *in vitro* in the presence of Bw4-I80-positive HIV-infected CD4+ T cells [14]. Intriguingly, infection with HIV is also associated with significant changes in the phenotype of circulating NK cells which –in turn– relates to a progressive loss of NK cell-function [15].

In humans, data directly linking the outcome of adaptive immune responses with innate immune mechanisms are lacking. Here we conducted a prospective influenza vaccine trial comparing HIV-negative and HIV-infected individuals, permitting us to longitudinally monitor influenza vaccine specific CD4+ T cell-responses in individuals with defined KIR and HLA genotypes.

Materials and methods

Study design, inclusion/exclusion criteria, sampling-scheme and primary read-outs

We conducted a prospective observational study in HIV-negative and chronically HIV-infected individuals vaccinated with standard, year-adjusted influenza-vaccine preparations. Study participants were recruited and followed-up at the University Hospital in Basel. Inclusion criteria were: age >18 years, CD4+ T cell counts >350/µL, anti-retroviral therapy (ART) since >3 months (as applicable) and HIV viral load <200 copies/mL (as applicable). Exclusion criteria were: a febrile illness, allergies to compounds of the vaccine, any vaccination within 30 days of inclusion and/or during follow-up, concomitant or planed medication with steroids/other immunosuppressive drugs, malignant disease, and pregnancy. The study was IRB approved and written informed consent was obtained from all study participants. Blood was collected immediately prior to vaccination and at day 7, 14 and 28 post-vaccination. The study was open to recruit 25 HIV-negative and 25 HIV-infected individuals into exploratory and validation cohorts. The primary readouts were 'frequency of influenza vaccine-specific CD4+ T cells', quantified at each follow-up time-point, and 'KIR genotype'. The frequency of influenza vaccine-specific CD4+ T cells was compared among HIV-negative and HIV-infected study participants sub-grouped according to their KIR genotype. KIR/HLA compound genotypes were included in the analysis as applicable. Genotyping assays were performed blinded for corresponding functional CD4+ T cell analyses. In order to minimize the risk of false positive findings, statistically significant differences in the frequency of influenza vaccine-specific CD4+ T cells between subgroups required re-confirmation in an independent similar-sized cohort and using an alternative vaccination-product (year adjusted). The exploratory study groups ('07/'08) were vaccinated with a trivalent virosomal vaccine (Inflexal V, Berna Biotech, Basel, Switzerland), the validation cohort with a trivalent split vaccine (Mutagrip®, Sanofi Pasteur MSD, Baar, Switzerland).

Isolation of peripheral blood mononuclear cells and T cell-depletion

Peripheral blood mononuclear cells (PBMC) were prepared by centrifugation through a density gradient on Lymphoprep (Axis-Shield, Oslo, Norway) and were kept in liquid nitrogen until use. After thawing, PBMC were depleted from CD4+ or CD8+ T cells, or NK cells as indicated, using magnetic-beads according to the manufacturer's protocol (Miltenyi Biotec, Bergisch-Gladbach, Germany). Depletion efficiency was assessed by flow cytometry and was always >97% (data not shown).

ELISpot assay

ELISpot plates (MSIPS4510, Millipore AG, Volketswil, Switzerland) were coated with 2 µg/mL of anti- IFN γ mAb 1-D1K (Mabtech, Nacka Strand, Sweden) overnight. In each well 200.000 cells

in R10 (RPMI 1640 containing 10% heat inactivated Fetal Bovine Serum (FBS), 50 U/mL penicillin and 50 µg/mL Streptomycin (all from GIBCO™, LuBioScience GmbH, Luzern, Switzerland) were added in duplicates (final volume 130 µL/well). Inflexal V (Berna Biotech) was used as source of antigen (year adjusted) at a final concentration of 14 µg/mL for each peptide, Phytohemagglutinin (PHA) (1.8 µg/mL; REMEL, Oxoid AG, Basel, Switzerland) served as a positive control. Plates were incubated for 16 h at 37°C with 5% CO2, washed with PBS (phosphate-buffered saline) and blocked with PBS 1% FBS. After washing, plates were incubated with 100 µL anti-IFN-γ mAb (1:200) coupled with alkaline phosphatase (7-B6-1-ALP, Mabtech) for 2 hours at room temperature. Spots were developed with HistoMark RED phosphatase system (KPL, Gaithersburg, Maryland, USA) and counted with the ELISpot Reader System (CSR01, AID GmbH, Strassberg, Germany) using the ELISpot 3.5 software (AID GmbH).

Genotyping

KIR genotypes were determined by multiplex PCR, followed by a reverse sequence-specific oligonucleotide method according to the manufacturer's instructions (Onelambda Inc., Canoga Park, Ca, USA). KIR genes analyzed included 2DL1, 2DL2, 2DL3, 2DL5, 3DL1, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, and 3DS1. The framework KIR genes 2DL4, 3DL2, 3DL3, and the pseudogenes 2DP1 and 3DP1 were not analyzed. Presence of KIR2DS1, KIR2DS2 and KIR3DS1 was confirmed by SSP PCR according to a published protocol [23]. HLA genes were analyzed by high-resolution (four digit) sequence based genotyping (Histogenetics, New York, NY, USA).

Statistical methods

The KIR genotype was compared in the three cohorts by Pearson's chi square test. Mann-Whitney U test and Wilcoxon's signed rank tests were performed to compare frequencies of influenza specific CD4+ T cells. Evolution of influenza specific CD4+ T cells over time was analyzed by multivariable linear regression including as covariates the baseline influenza specific CD4+ T cell-count, presence of KIR2DS1/KIR3DS1 genes, HIV carrier status, and the interaction term of KIR2DS1/KIR3DS1 and HIV. The impact of the compound KIR/HLA genotype was assessed by multivariable linear regression including as covariates the presence of a KIR gene, its HLA ligand, and the interaction term of KIR and HLA ligand. Two sided P values <0.05 were considered statistically significant.

Results

Characteristics of the study populations

Twenty-three HIV-negative (median age 34 years [range 22 to 76 years], 43% female) and 21 HIV-infected study-participants (median age 45 years [range 21 to 65 years], 19% female) were recruited into an exploration cohort investigating immune responses of these populations.

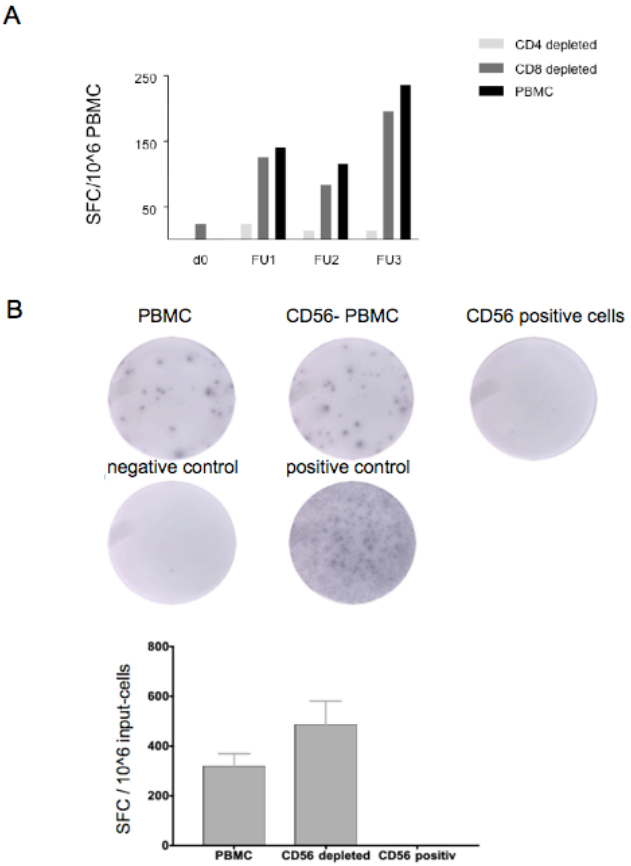


Figure 1.

(A) Vaccination-induced influenza-specific IFN-γ secretion of bulk and T cell-depleted PBMC. Depletion of CD4+ T cells in PBMC samples reduced the frequency of spot-forming cells to background-level. By contrast, depletion of CD8+ T cells had no effect on the frequency of spot-forming cells. A representative series of experiments is shown, analogous controls were included in all ELISpot assays.
(B) NK cells do not contribute to influenza-vaccine specific IFN-γ secretion. A representative experiment is shown: Influenza-vaccine was used as the source of antigen, the frequency of IFN-γ secreting cells determined by ELISpot analysis. Comparing IFN-γ secretion in CD56 depleted PBMC and CD56 expressing cells indicates that the CD56 negative fraction is the source of IFN-γ. The increase in SFC in the CD56 depleted fraction reflects the increased proportion of CD4+ T cells in CD56 depleted PBMC (data not shown). Mean ± SD of triplicates are shown.

The trial took place during the vaccination season '07/'08 (**Table 1**). A validation cohort of HIV-negative subjects was vaccinated '08/'09 (n=18, median age 37 years [range 19 to 46 years], 33% female). All study participants adhered to the study protocol and sampling was obtained for all predefined time points. In none of the vaccinated individuals significant adverse reactions or non-vaccine related clinically relevant problems occurred.

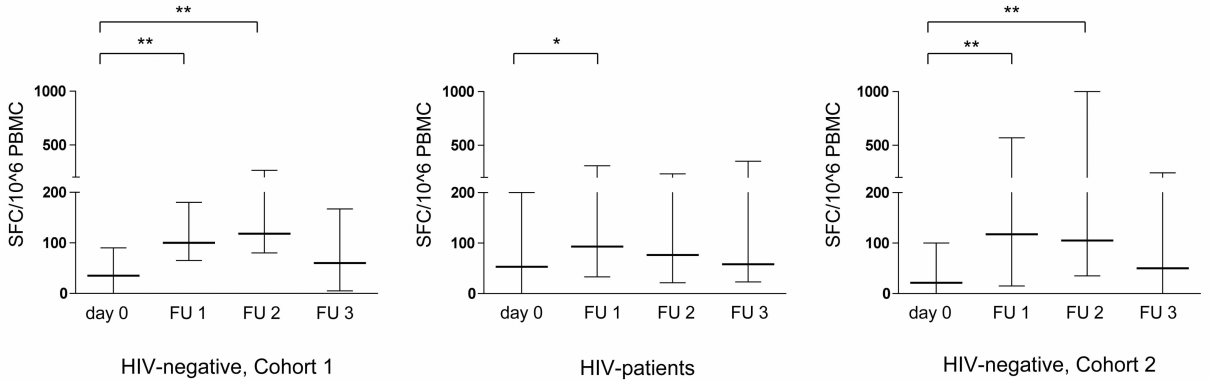


Figure 2. Frequency of influenza-specific CD4+ T cells before and after vaccination. Early after vaccination (FU 1 = day 7 post-vaccination) the median frequency of IFN-γ secreting CD4+ T cells significantly increased in all three cohorts (HIV-negative Cohort 1 [n=23], HIV-infected Cohort [n=21], and HIV-negative Cohort 2 [n=18]). Two weeks after vaccination (FU 2) a significant expansion of influenza-specific CD4+ T cells was seen only HIV-negative participants. Four weeks after vaccination influenza-specific CD4+ T cells were contracted to pre-vaccination levels in all three cohorts. * $p<0.05$; ** $p<0.01$

Table 1. Characteristics of study populations.

Patient Characteristics	HIV-negative Cohort 1	HIV-infected	HIV-negative Cohort 2
Study Participants (n)	23	21	18
Age - median (range)	34 (22-76)	45 (21-65)	37 (19-46)
Male gender - no. (%)	13 (57%)	17 (81%)	12 (67%)
Lymphocyte Counts - Lymphocytes x10^9/L			
Baseline- median (range)	>500	545 (364-941)	>500
Day 84 post-vaccination - median (range)	>500	520 (371-972)	>500
Viral Loads - Copies/mL			
Baseline	n.a.	<200	n.a.
Day 84 post-vaccination	n.a.	<200	n.a.
Therapy			
ART (> 3 months)	n.a.	100%	n.a.
Study year	2007/2008	2007/2008	2008/2009
Vaccine-Type	Inflexal®	Inflexal®	Mutagrip®

n.a. = not applicable

ART = anti-retroviral therapy

Influenza specific CD4+ T cell-response

The frequency of antigen-specific cells was quantified in ELISpot assays using a trivalent virosomal influenza vaccine (Inflexal®) as the source of antigen. To define the cells contributing to the secretion of IFN-γ, aliquots of all samples were depleted of either CD4+ or CD8+ lymphocytes prior to performing ELISpot assays. These control experiments confirmed that *in vitro* influenza vaccine almost exclusively triggers CD4+ and not CD8+ T cell-responses (**Figure 1a**). To exclude the possibility that NK cells are contributing to the IFN-γ secretion we depleted samples from NK cells, which had no effect on the frequency of IFN-γ secreting cells (**Figure 1b**).

Before vaccination the median frequency of IFN-γ secreting CD4+ T cells was comparable in HIV-negative and HIV-infected individuals. After vaccination the frequency significantly increased and reached comparable levels day 7 post-vaccination in both the HIV-negative and HIV-infected cohorts. By contrast, 14 days after vaccination only in HIV-negative individuals the frequency of influenza-specific IFN-γ secreting CD4+ T cells remained increased. By twenty-eight days after vaccination the frequency of IFN-γ secreting CD4+ T cells contracted to pre-vaccination levels in both HIV-negative and HIV-infected individuals (**Figure 2**).

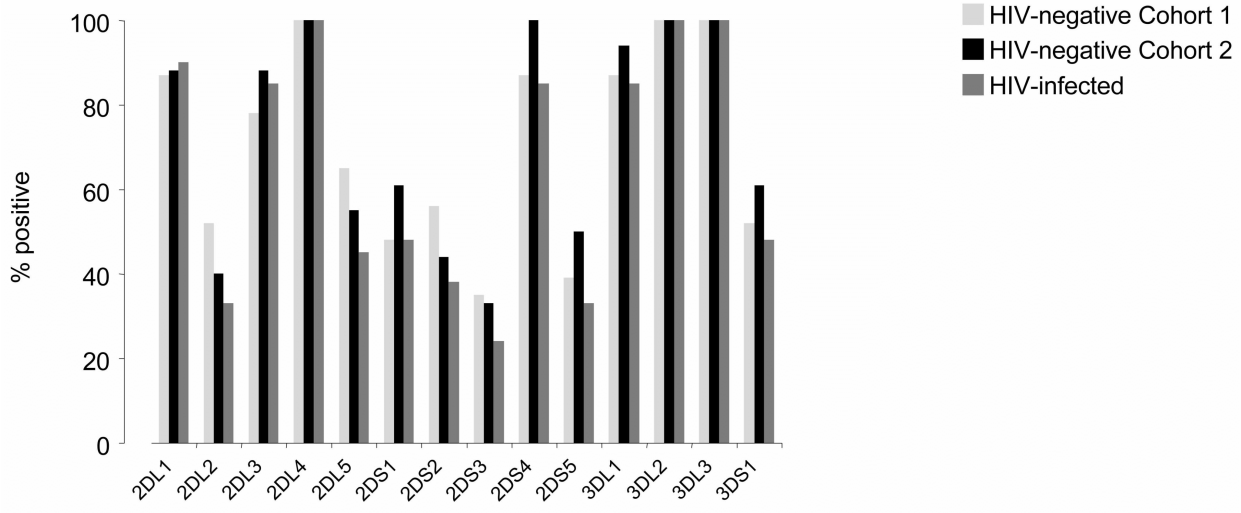


Figure 3. Distribution of KIR genes among study participants. KIR genotypes in HIV-negative study participants (Cohort 1 [n=23], and Cohort 2 [n=18]) and in HIV-infected study participants (n=21) were similar (p>0.2 for all comparisons).

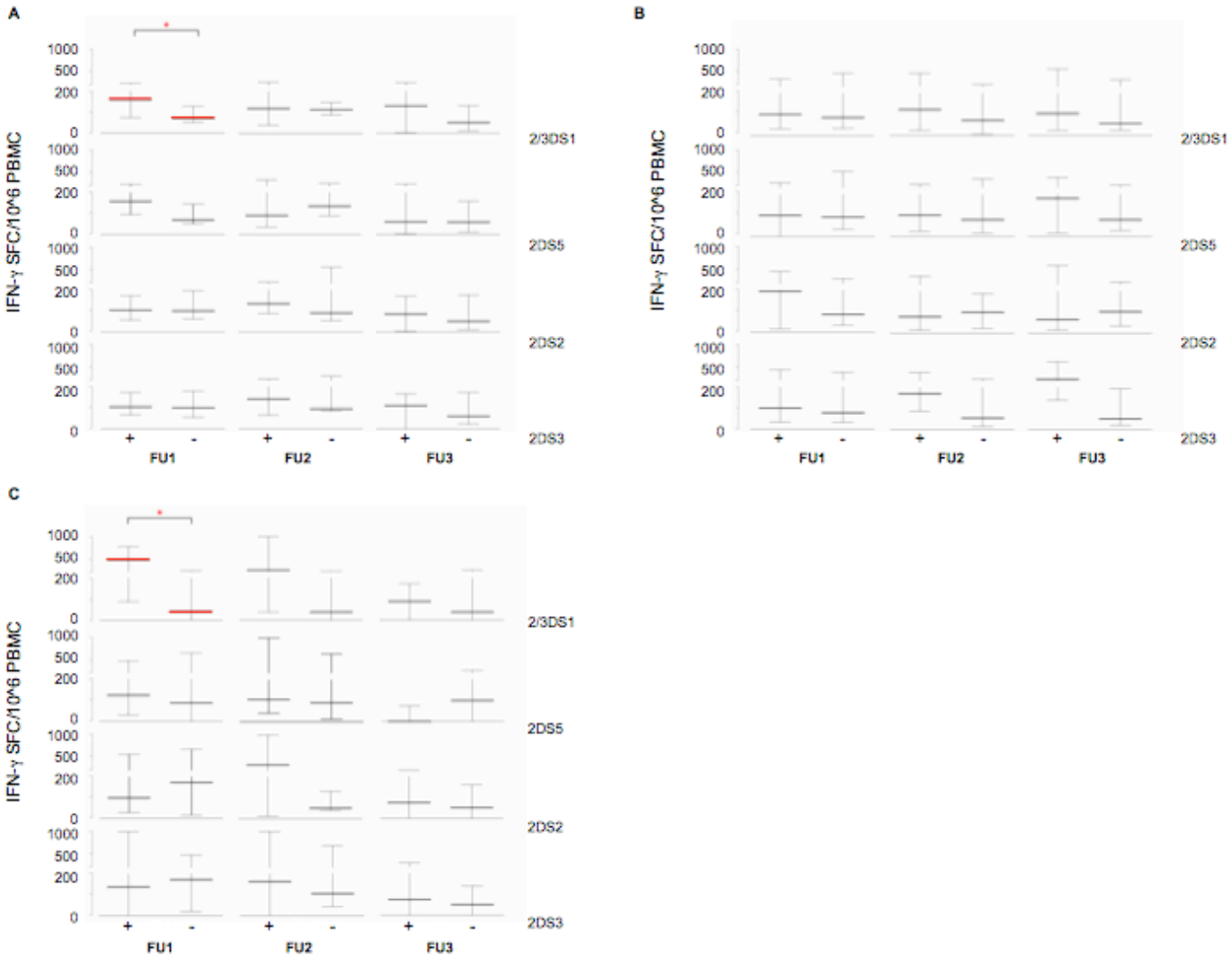


Figure 4. Relation of KIR genotype and vaccination-induced influenza-specific CD4+ T cell-response. **(A)** In HIV-negative individuals (Cohort 1 [n=23]) the presence of genes encoding for the activating receptors KIR2DS1 and KIR3DS1 was associated with a higher frequency of influenza-specific CD4+ T cells 7 days after vaccination. No such association was detected for the activating receptors KIR2DS5, KIR2DS2 and KIR2DS3. **(B)** In HIV-infected individuals (n=21) genes encoding for activating KIR did not relate to the expansion of influenza-specific CD4+ T cells. **(C)** In a validation cohort (HIV-negative individuals; Cohort 2 [n=18]) the association between the presence of the genes encoding for the activating receptors KIR2DS1 and KIR3DS1 and the frequency of influenza-specific CD4+ T cells day 7 after vaccination was confirmed.

KIR / HLA genotypes

KIR genotyping was performed sequentially, first in the exploratory study populations including HIV-negative and HIV-infected individuals, followed by the confirmatory cohort including HIV-negative individuals only (**Figure 3**). KIR gene distribution was similar in the three groups ($p>0.2$ for all comparisons). In line with the reported strong linkage disequilibrium between KIR2DS1 and KIR3DS1, presence of these two genes was concordant in all individuals tested with the exception of one healthy donor in the validation cohort that carried KIR2DS1 but not KIR3DS1 [16]. For reasons of simplicity KIR2DS1 and KIR3DS1 are grouped together throughout the manuscript.

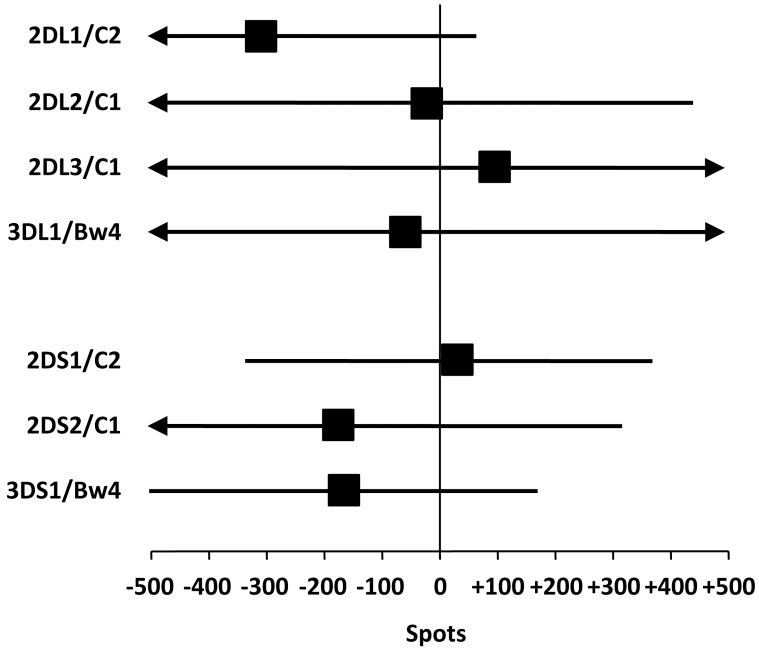


Figure 5. Relation of KIR/HLA compound genotype and vaccination-induced influenza-specific CD4+ T cell-response. The presence of compound KIR/HLA genotypes of known relevance was not associated with the frequency of influenza-specific CD4+ T cells 7 days after vaccination. Shown is the mean effect (square) and 95% confidence interval (horizontal line) of the combined presence of KIR and cognate HLA ligand on ELISpot frequency as estimated by multivariable regression in the combined cohorts of HIV-negative study participants.

Associations between 'CD4+ T cell-response–KIR genotype' and 'CD4+ T cell-response –KIR/HLA compound genotype'

KIR genes and the median frequencies of influenza-specific CD4+ T cells were related for each time-point in the exploratory cohorts (HIV-negative Cohort 1 and HIV-infected Cohort). In HIV-negative study participants, a genotype including KIR2DS1/KIR3DS1 was significantly associated with a higher count of influenza specific CD4+ T cells one week after vaccination (**Figure 4A**). None of the other KIR genes were associated with differing changes in the frequencies of vaccine specific CD4+ T cells (**Figure 4A**). In HIV-infected individuals no link between a genotype including KIR2DS1/KIR3DS1 or any other KIR genes was detected for any time point (**Figure 4B**).

To minimize the risk of describing chance associations we re-examined and confirmed the observed association between a genotype including KIR2DS1/KIR3DS1 and early post-vaccine frequencies of influenza-specific CD4+ T cells in an independent HIV-negative validation cohort (HIV-negative Cohort 2, n=18). To avoid vaccine-type specific confounders a different type of vaccine (split vaccine) was used in the validation cohort (**Figure 4C**).

Multivariable linear regression analysis was used to model the influenza-specific CD4+ T cell-response over time. This analysis confirmed a significant increase in IFN-γ producing CD4+ T cells that was confined to KIR2DS1/KIR3DS1 carrying individuals (**Table 2**). Importantly, interaction analysis between HIV and KIR2DS1/KIR3DS1 carrier status resulted in a p-value of 0.01, indicating that HIV significantly modulates the effect of KIR2DS1/KIR3DS1 on the influenza specific immune response (**Table 2**). No significant differences or interactions were noted for any time point other than day 7 post-vaccination.

In analogy, also KIR/HLA compound genotypes of established relevance were assessed (2DL1/C2, 2DL2/C1, 2DL3/C1, 3DL1/Bw4, 2DS1/C2, 2DS2/C1, 3DS1/Bw4) [7, 17, 18]. None of these compound genotypes had a measurable impact on the frequency of the vaccine-specific CD4+ T cell-response in the HIV-negative cohorts (Cohort 1 and 2 combined) (**Figure 5**). In the HIV-infected cohort, the comparatively small patient population and skewing in HLA/KIR combinations ($n \leq 2$ for double-negative individuals in all 7 compound genotypes) precluded analysis of the effect of the compound HLA/KIR genotype.

Table 2. Statistical analyses.

	Baseline	**1 Week**	P vs Baseline	P between groups	**2 Weeks**	P vs Baseline	P between groups	**4 Weeks**	P vs Baseline	P between groups
HIV 2DS1 -	64 (0-159)	85 (35-381)	0.14	} 0.32	70 (6-216)	0.68	} 0.8	57 (27-316)	0.59	} 0.81
HIV 2DS1+	53 (0-340)	100 (30-380)	0.04	} 0.01	123 (25-496)	0.11	} 0.43	103 (23-595)	0.16	} 0.67
HD 2DS1-	35 (0-95)	65 (40-128)	0.25	} 0.002	95 (68-149)	0.01	} 0.1	50 (0-150)	0.17	} 0.56
HD 2DS1+	29 (0-80)	169 (84-525)	<0.001		112 (39-575)	0.001		0.07	0.07	

Median SFC/10^6 PBMC and interquartile ranges are given

Discussion

Innate immune responses are important for protecting the host early after infection, i.e. during the time when adaptive responses are evolving.

Functional assays permitting to directly assess the features of innate immune-receptors belonging to the activating KIR-family remain elusive. However, genetic association studies represent an important tool to gain insight into their biological roles. Here we investigated in a human influenza-vaccination trial how KIR genotypes and KIR/HLA compound genotypes relate to the frequency of vaccine-induced CD4+ T cells. The key findings of our study were that (i) a genotype including KIR2DS1/KIR3DS1 is associated with enhanced vaccine-induced CD4+ T cell-responses, and (ii) in HIV-infected individuals the relation between this KIR genotype and vaccine-induced CD4+ T cell-responses is dissociated. No influence on vaccine-induced CD4+ T cell-responses by KIR/HLA compound genotypes was detected.

In a number of immunogenetic association-studies combinations of KIR variants and HLA genes have been linked to the clinical course and/or outcome of human infections, including those with hepatitis C virus, HIV, human papillomavirus and cytomegalovirus [6, 8, 19-21]. Whether in these infections KIR impact on the course of disease directly or indirectly –e.g. by influencing adaptive immune responses– remains unknown. Taking advantage of an experimental system that permits monitoring the expansion- and contraction-phase of an adaptive immune response, our data for the first time link KIR immunogenetics and the dynamics of adaptive immunity, suggesting an effect of KIR-triggered reactivities on the adaptive response in humans. Thus, designing adjuvants able to mobilize KIR2DS1/KIR3DS1 expressing cells into the site of influenza vaccination may be an intriguing approach to enhance immunogenicity.

In many of the study participants influenza vaccine-specific CD4+ T cells were detectable at low frequencies before vaccination already, reflecting prior contact with influenza antigen through vaccination or infection (shared antigens). Thus –in the experimental setting applied here– no distinction between *de novo* induction of CD4+ T cell-responses and re-expansion of memory CD4+ T cells is made, and similar expansion was observed in both groups (data not shown). It follows that the suggested KIR-dependent 'help' for CD4+ T cells likely applies to re-expansion of preformed memory and *de novo* priming alike, a finding that adds a novel facet to our understanding of the interaction between innate and adaptive immunity.

In HIV infection NK cell-function is deregulated [15, 22]. Intriguingly, even under virological control and vis-à-vis relatively preserved CD4+ T cell-counts we here observed that cellular reactivities associated with KIR in HIV-negative individuals are not linked in the context of HIV-infection.

In summary, studying a model situation that allows associating innate immunogenetics with dynamic changes in adaptive cellular responses, our data differentially associate

KIR2DS1/KIR3DS1 and the adaptive CD4+ T cell response triggered by influenza vaccination in healthy vs. HIV-infected individuals. From a clinical perspective it will be interesting to study whether the level of protection from influenza vaccine relates to the here-defined KIR genotype and, inversely, whether one could safely vaccinate genetically defined 'responders' with less antigen, which in turn might permit more efficient handling of limited resources.

References

1. Parham P. **MHC class I molecules and KIRs in human history, health and survival.** *Nat. Rev. Immunol.* 2005; 5: 201-14.
2. Long EO, Burshtyn DN, Clark WP, Peruzzi M, Rajagopalan S, Rojo S, *et al.* **Killer cell inhibitory receptors: diversity, specificity, and function.** *Immunol. Rev;* 1997. **155**: 135-44.
3. Ljunggren HG, Karre K. **In search of the 'missing self': MHC molecules and NK cell recognition.** *Immunol. Today* 1990; 11: 237-44.
4. Lanier LL, **NK cell recognition.** *Annu. Rev. Immunol.* 2005; **23**: 225-74.
5. Khakoo SI, Carrington M. **KIR and disease: a model system or system of models?** *Immunol. Rev.* 2006; 214: 186-201.
6. Kulkarni S, Martin MP, Carrington M. **The Yin and Yang of HLA and KIR in human disease.** *Semin. Immunol.* 2008; 20: 343-52.
7. Carrington M, Martin MP, van Bergen J. **KIR-HLA intercourse in HIV disease.** *Trends Microbiol.* 2008. 16; 620-7.
8. Stern M, Elsasser H, Hoenger G, Steiger J, Schaub S, Hess C. **The number of activating KIR genes inversely correlates with the rate of CMV infection/reactivation in kidney transplant recipients.** *Am. J. Transplant.* 2008; 8: 1312-7.
9. Trinchieri G. **Biology of natural killer cells.** *Adv. Immunol.* 1989; 47: 187-376.
10. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. **Functions of natural killer cells.** *Nat. Immunol.* 2008; 9: 503-10.
11. Vivier E, Anfossi N. **Inhibitory NK-cell receptors on T cells: witness of the past, actors of the future.** *Nat. Rev. Immunol.* 2004; 4. 190-8.
12. Lanier LL. **Evolutionary struggles between NK cells and viruses.** *Nat. Rev. Immunol.* 2008; 8: 259-68.
13. Martin MP, Gao X, Lee JH, Nelson GW, Detels R, Goedert JJ, *et al.* **Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS.** *Nat. Genet.* 2002; 31: 429-34.
14. Alter G, Martin MP, Teigen N, Carr WH, Suscovich TJ, Schneidewind A, *et al.* **Differential natural killer cell-mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes.** *J. Exp. Med.* 2007; 204: 3027-36.
15. Alter G, Teigen N, Davis BT, Addo MM, Suscovich TJ, Waring MT, *et al.* **Sequential deregulation of NK cell subset distribution and function starting in acute HIV-1 infection.** Blood 2005; 106: 3366-9.

16. Martin MP, Single RM, Wilson MJ, Trowsdale J, Carrington M. **KIR haplotypes defined by segregation analysis in 59 Centre d'Etude Polymorphisme Humain (CEPH) families.** Immunogenetics 2008; 60: 767-74.
17. Stewart CA, Laugier-Anfossi F, Vely F, Saulquin X, Riedmuller J, Tisserant A, *et al.* **Recognition of peptide-MHC class I complexes by activating killer immunoglobulin-like receptors.** Proc. Natl. Acad. Sci. U S A 2005; 102:13224-9.
18. Williams AP, Bateman AR, Khakoo SI. **Hanging in the balance. KIR and their role in disease.** Mol. Interv. 2005; 5: 226-40.
19. Khakoo SI, Thio CL, Martin MP, Brooks CR, Gao X, Astemborski J, *et al.* **HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection.** Science 2004; 305: 872-4.
20. de Arias AE, Haworth SE, Belli LS, Burra P, Pinzello G, Vangeli M, e*t al.* **Killer cell immunoglobulin-like receptor genotype and killer cell immunoglobulin-like receptor-human leukocyte antigen C ligand compatibility affect the severity of hepatitis C virus recurrence after liver transplantation.** Liver Transpl. 2009; 15: 390-9.
21. Carrington M, Wang S, Martin MP, Gao X, Schiffman M, Cheng J, *et al.* **Hierarchy of resistance to cervical neoplasia mediated by combinations of killer immunoglobulin-like receptor and human leukocyte antigen loci.** J. Exp. Med. 2005. 201; 1069-75.
22. Mavilio D, Lombardo G, Kinter A, Fogli M, La Sala A, Ortolano SA, *et al.* **Characterization of the defective interaction between a subset of natural killer cells and dendritic cells in HIV-1 infection.** J. Exp. Med. 2006; 203: 2339-50.
23. Vilches C, Castano J, Gomez-Lozano N, Estefania E. **Facilitation of KIR genotyping by a PCR-SSP method that amplifies short DNA fragments.** Tissue Antigens 2007; 70: 415-22.

Section 4: Mature dendritic cells trigger anti-proliferative activity in NK cells

Stefanie Fritz, Bojana Durovic, Gabriela Zenhaeusern, Nikolai Hodel, Johannes Nemeth, Olivier Gasser, Christoph Hess.

Abstract

Interaction of dendritic cells (DCs) and natural killer (NK) cells has been demonstrated to impact the outcome of adaptive immune responses in various models.

Here we show that mature monocyte-derived DCs (moDCs) selectively induce regulatory/anti-proliferative activity in autologous NK cells. Specifically, LPS-matured moDCs, but not immature moDCs, induced a population of IL-10 secreting NK cells. Functionally, in the presence of autologous NK cells, allo T cell-proliferation induced by mature moDCs was significantly reduced. This NK cell-derived capacity to inhibit allo T cell-proliferation was transferable by supernatant from NK cells triggered by mature moDCs.

Anti-proliferative NK cell-reactivity induced by mature moDCs may participate in the timely ending of DC-induced T cell expansion, a hypothesis that requires testing using *in vivo* models.

Introduction

Immature dendritic cells (DCs) have high endocytic activity and low T cell activation potential, constantly sampling their environment for pathogens. Upon receiving danger signals, DCs 'mature' by increase MHC-expression and co-stimulatory molecules, now functioning as initiators of T cell immune responses and regulators of immune tolerance [1-3].

Natural killer (NK) cells represent a key effector-component of the innate immune system, able to produce cytokines and lyse cells without prior sensitization [4]. They compose approximately 10-15% of circulating lymphocytes [5].

NK cells and DCs have been shown to interact at various stages during induction of an immune response. In peripheral tissues, DC-derived soluble factors, as well as direct cell–cell interactions, can prime NK cells [6-8]. NK cells in turn can influence DC maturation and T cell polarization, as e.g. shown in a model where NK cell depleted mice infected with T. gondii showed impaired CD4+ T cell responses and enhanced susceptibility to infection [9]. In addition to DC-priming, NK cells can also kill autologous DCs both in non-lymphoid as well as in secondary lymphoid tissue, thereby 'editing' the nature of downstream adaptive (auto)immune responses [10, 11].

Although the biology of immature vs. mature DCs is fundamentally different, it remains unknown whether these DC maturation-subsets *differentially trigger NK cells to secrete factors influencing T cell responsiveness*. To begin to address this important issue we here assessed the effect of immature vs. mature monocyte-derived DCs (moDCs) on the frequency of NK cells producing IFN-γ and IL-10, and on the capacity of NK cells and NK cell-derived soluble factors to influence moDC-mediated allo T cell-proliferation.

Materials and Methods

Isolation, culture and generation of moDCs

MoDCs were derived from moncytes isolated from fresh buffy coats. Isolation of PBMC was performed by diluting a buffy coat 1/1 (v/v) with PBS (phosphate-buffered saline) prior to layering over Lymphoprep (Axis-Shield, Oslo, Norway). PBMC were washed and cultured in R10 (RPMI 1640 containing 10% heat inactivated Fetal Bovine Serum (FBS), 50 U/mL penicillin and 50 µg/mL Streptomycin (all from GIBCO™, LuBioScience GmbH, Luzern, Switzerland) for 3h at 37°C in 75mL flasks. Aliquots of the cells were not added to the flasks, but frozen and stored at minus 80°C for subsequent isolation of NK cells. After incubation of PBMC for 3h, non-adherent cells were removed by washing twice with R10. The remaining cells were cultured in complete medium supplemented with 50 ng/mL GM-CSF and 50 ng/mL IL-4 (ImmunoTools). On day 5, non-adherent immature moDCs were harvested, purified by Lymphoprep, plated in 6-well plates (1x10^5 cells/mL) and fractions were stimulated with LPS (10 ng/mL: LPS ^{low}; 100 ng/mL: LPS ^{high}) as indicated.

Isolation of autologous NK cells

On day 6, frozen PBMC were thawn and washed twice. After an incubation period of 2h at 37°C, NK cells were isolated by negative isolation using magnetic-beads according to manufacturer`s protocol (Miltenyi Biotec, Bergisch-Gladbach, Germany). Purity was assessed by flow cytometry ($CD3^{neg}$, CD16+ and CD56+) and was always >98% (data not shown).

FACScan analysis

Antibodies recognizing CD3 (SK7), CD11c (S-HCL-3), CD14 (MOP9), CD16 (NKP15), CD56 (B159), HLA-ABC (G46-2.6), HLA-DR (L243) and appropriate isotype controls all from BD Biosciences (BD Pharmingen, Allschwill, Switzerland) were used. Data were acquired on a FACS Calibur flow cytometer and analyzed with the FlowJo 8.8.6 software (Tree Star, Inc., Ashland, Oregon, USA).

Dual-color ELISpot assay

ELISpot plates (MSIPS4510, Millipore AG, Volketswil, Switzerland) were coated with 2 µg/mL of anti- IFN γ mAb 1-D1K and anti-human interleukin-10 (IL-10) mAb (9D7) (Mabtech, Nacka Strand, Sweden) overnight. In each well 5x10^4 paraformaldehyde-fixed moDCs as target cells and 5x10^4 NK cells as effector cells in R10 were added in duplicates. Plates were incubated for

16 h at 37°C with 5% CO2, washed with PBS and blocked with PBS 1% FBS. After washing, plates were incubated with 100 µL anti-IFN-γ mAb (1:200) coupled with alkaline phosphatase (7-B6-1-ALP, Mabtech) and biotinylated anti-human IL-10 mAB (1:2000) (12G8-Biotin) for 2 hours, followed by incubation with streptavidin-horseradish peroxidase (1:2000) (Mabtech) for 45min. Spots were developed with HistoMark® RED phosphatase system and HistoMark® TrueBlue peroxidase system (KPL, Gaithersburg, Maryland, USA) and counted with the ELISpot Reader System (CSR01, AID GmbH, Strassberg, Germany) using the ELISpot 3.5 software (AID GmbH).

Proliferation assay

To assess proliferation, moDCs and autologous NK cells were co-cultured in 96-well plates. After overnight incubation, 5x10^4 allogeneic CD3+ T cells stained with CFSE were added (representing the effector/target ratio as optimized for the ELISpot assay). Recombinant human IL-2 (Proleukin, Roche, Basel, Switzerland) was supplemented at a final concentration of 250 U/mL and proliferation of allogeneic T cells was semi-quantified after 4 days by flow cytometry.

Statistical analyses

Normally distributed data were analyzed by Student`s t-test, non-normally distributed data by Mann-Whitney U test. Paired analyses were performed as appropriate. All tests were performed using Prism4 software (GraphPad Software, Inc. San Diego, California, USA). P values <0.05 were considered statistically significant.

Results

Mature moDCs trigger a subset of NK cells to secrete IL-10

First we assessed secretion of IFN-γ and IL-10 by NK cells exposed to immature and mature autologous moDCs. IFN-γ and IL-10 were chosen as prototypic indicators of pro- (IFN-γ) and anti-inflammatory/regulatory (IL-10) immune responses, respectively. Specifically, we incubated NK cells with autologous immature moDCs and LPS-stimulated ('matured') moDCs overnight. LPS-induced maturation of moDCs was controlled for by assessing cell-surface expression of markers of mature moDCs, such as HLA class I, CD80 and CD86 (data not shown) [12, 13]. All experiments have been performed using moDCs from various donors. Our focus was on studying the effect of direct moDC–NK cell interactions under non-changing conditions and in absence of DC-derived factors. Therefore, prior to co-culture experiments, moDCs were mildly fixed with paraformaldehyde [14]. NK cells induced to secrete IFN-γ and/or IL-10 through contact with moDCs were enumerated using dual color ELISpot technology.

In these experiments only minimal induction of IFN-γ was observed either after incubation of NK cells with immature or mature moDCs. Intriguingly however, mature –i.e. LPS-activated moDCs– but not immature moDCs induced a population of NK cells to secrete IL-10 (**Fig. 1**). These data indicated that a subset of NK cells could be triggered to secrete the regulatory/anti-inflammatory cytokine IL-10 when interacting with LPS-matured autologous moDCs.

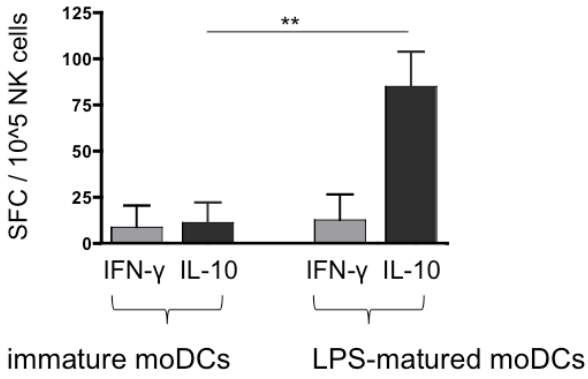


Figure 1. NK cells are capable to recognize LPS-activated DCs. NK cells were mixed with autologous moDCs (stimulated [DCs plus LPS] or non-stimulated [immature DC; iDC]), which were fixed using a mild paraformaldehyde (PAF) (0,2%) solution to abolish cytokine production. Frequencies of IFN-γ and IL-10 secreting NK cells were measured by dual color ELISpot assay. NK cells did not produce IFN-γ vis-à-vis autologous iDCs, but maturation of moDCs induced IL-10 secretion in a subset of NK cells. Results represent mean ± SD of four experiments. **p<0.005

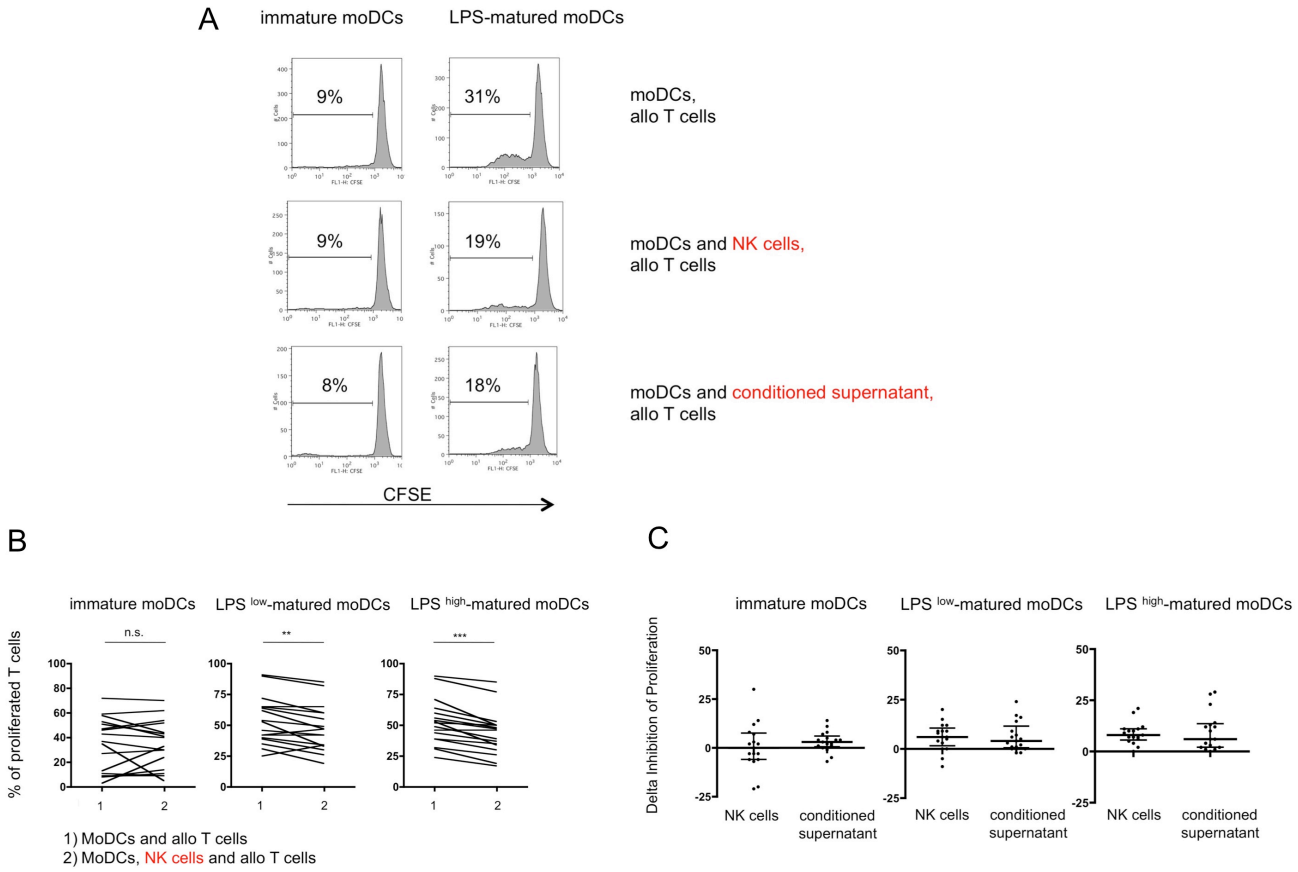


Figure 2. LPS matured moDCs induce autologous NK cells to secrete anti-proliferative factors. MoDCs were either left untreated (immature moDCs), or matured with LPS (LPS-matured moDCs). Cytokine production of moDCs was abolished using mild paraformaldehyde-fixation. Immature and LPS-matured MoDC-subsets were then cultured with autologous NK cells overnight. Subsequently, allogeneic T cells (plus IL-2) were added, and proliferation of allogeneic T cells was measured after 4 days of co-culture. **(A)** Allogeneic T cell-proliferation was more efficiently induced by LPS-matured than by immature moDCs *(upper panel)*. Allogeneic T cell-proliferation –as induced by LPS-matured moDCs– was suppressed when LPS-matured moDCs were incubated with autologous NK cells prior to the addition of allo T cells *(upper vs. middle panel)*. This inhibitory effect was transferable using conditioned culture medium from LPS-matured moDCs incubated with autologous NK cells *(lower panel)*. **(B)** MoDCs matured with either a low or a high concentration of LPS, but not immature moDCs, induced inhibitory NK cell-reactivity. However, moDCs matured with a low LPS-concentration induced inhibitory NK cell-reactivity less consistently than moDCs matured with a higher concentration of LPS *(middle vs. right panel)* (n=17). **(C)** Supernatant from co-culture experiments of moDCs and autologous NK cells (n=17) consistently reproduced the NK cell-mediated effect on allo T cell-proliferation. n.s. = not significant; ** p<0.005; *** p<0.001.

Inhibition of allogeneic T cell-proliferation

To examine the functional impact of moDC-induced regulatory NK cell activity –as exemplified by the secretion of IL-10– we next assessed their effect on allo T cell-proliferation. To that end, NK cells and mildly fixed autologous moDCs –either immature or LPS-matured– were co-cultured for 24h prior to adding allogeneic T cells (moDC:NK cell:allo T cell ratio = 1:1:1 ; established as optimal ratio in exploratory experiments). Proliferation of T cells was then assessed after 4 days of allo-stimulation.

Using this experimental system, immature moDCs induced proliferation in ~10% of allogeneic T cells, LPS-matured moDCs in ~30% of allogeneic T cells (**Fig. 2A**, upper row). Co-incubating NK cells and immature moDCs had no effect on allo T cell-proliferation. By contrast, co-incubation of LPS-matured moDCs with NK cells reduced allo T cell-proliferation by approximately half (reduction from ~30% to ~20% against ~10% baseline allo-proliferation) (**Fig. 2A**, middle row). Reduced allo-proliferation was not rescued by adding IL-2 (data not shown). To define whether cell contact is required to inhibit the observed allo T cell-proliferation, the anti-proliferative capacity of supernatant from co-cultures of fixed immature and LPS-matured moDCs with NK cells was assessed. While supernatant from NK cells co-cultured with immature moDCs had no impact on allo T cell-proliferation, allo-proliferation was again reduced by approximately half using supernatant from NK cells co-cultured with LPS-matured moDCs (**Fig. 2A**, lower row).

Of note, while recombinant IL-10 at high concentrations was also able to suppress allo T cell-proliferation, no relevant impact was observed when adding IL-10 at concentrations detected in the supernatant from NK cells activated by LPS-matured moDCs (data not shown). Blocking of IL-10, using blocking antibodies, also had no effect (data not shown).

Results from all 17 allo T cell-proliferation experiments are summarized in **Figure 2B**. Overall, LPS-matured moDCs (either LPS ^{low} or LPS ^{high}) induced stronger allo T cell-proliferation than immature moDCs ($p<0.001$). Co-culturing autologous NK cells with immature moDCs had no significant effect on their capacity to induce allo-proliferation (**Fig. 2B**, left panel). By contrast, allo-proliferation was significantly reduced when adding autologous NK cells to LPS-matured moDCs (either LPS ^{low} or LPS ^{high}) (**Fig. 2B**, middle and right panel). Both *magnitude* and *consistency* of the effect of LPS-matured moDCs on NK cell-mediated inhibition of allo T cell-proliferation were dependent on the dose of LPS used to mature moDCs (*magnitude:* median inhibition mediated by NK cells incubated with (i) immature, (ii) LPS ^{low} and (iii) LPS ^{high} stimulated moDCs; -1%, 15% and 18%; *consistency:* effect observed in 8/17, 13/17 and 16/17 experiments, respectively). Comparing the effect on allo T cell-proliferation of 'NK cell–allo T cell co-incubation' vs. 'NK cell-conditioned supernatant' across all 17 experiments confirmed that the observed effect was dependent on one or several NK cell-derived soluble factor(s) (**Fig. 2C**).

Discussion

To function efficiently, T cell immunity depends on the induction of an appropriately sized and polarized effector population *during a defined window of time*. While many of the signals/factors defining T cell polarization have been well defined, less is known on mechanisms regulating the duration during which T cell immunity can and should be induced [15, 16]. Regulatory/anti-proliferative reactivity of NK cells induced by mature moDCs –as observed in the current study– may reflect a mechanism *limiting in time* the period during which T cell immunity can be triggered. In the model studied here –where moDCs were mildly fixed using paraformaldehyde– induction of immunosuppressive NK cell reactivity by moDCs strictly depended on cell–cell contact. This system permitted us to study the effect on NK cells of stable, non-changing moDC populations. Our 'reductionist' approach permitted us to define the fact that anti-proliferative properties are selectively induced in NK cells upon interaction with LPS-matured moDCs. This approach is, however, limited by the fact that *in vivo*, DC subsets interact with NK cells in a dynamic setting, in changing environments, and are responding to these interactions by secreting cytokines, changes in cell-surface phenotype, etc. The here presented data thus can only be viewed as a *prototypic* NK cell reactivity inducible by mature moDCs. Testing its immunological *in vivo* relevance now requires models that take into account the dynamics of an immune response.

References

1. Coquerelle C, Moser M. **DC subsets in positive and negative regulation of immunity**. *Immunol Rev* 2010,234:317-334.
2. Banchereau J, Steinman RM. **Dendritic cells and the control of immunity**. *Nature* 1998,392:245-252.
3. Steinman RM, Banchereau J. **Taking dendritic cells into medicine**. *Nature* 2007,449:419-426.
4. Cooper MA, Fehniger TA, Caligiuri MA. **The biology of human natural killer-cell subsets**. *Trends Immunol* 2001,22:633-640.
5. Caligiuri MA. **Human natural killer cells**. *Blood* 2008,112:461-469.
6. Ferlazzo G, Munz C. **Dendritic cell interactions with NK cells from different tissues**. *J Clin Immunol* 2009,29:265-273.
7. Piccioli D, Sbrana S, Melandri E, Valiante NM. **Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells**. *J Exp Med* 2002,195:335-341.
8. Lucas M, Schachterle W, Oberle K, Aichele P, Diefenbach A. **Dendritic cells prime natural killer cells by trans-presenting interleukin 15**. *Immunity* 2007,26:503-517.
9. Goldszmid RS, Bafica A, Jankovic D, Feng CG, Caspar P, Winkler-Pickett R*, et al.* **TAP-1 indirectly regulates CD4+ T cell priming in Toxoplasma gondii infection by controlling NK cell IFN-gamma production**. *J Exp Med* 2007,204:2591-2602.
10. Moretta L, Ferlazzo G, Bottino C, Vitale M, Pende D, Mingari MC, Moretta A. **Effector and regulatory events during natural killer-dendritic cell interactions**. *Immunol Rev* 2006,214:219-228.
11. Lunemann A, Lunemann JD, Munz C. **Regulatory NK-cell functions in inflammation and autoimmunity**. *Mol Med* 2009,15:352-358.
12. Jeras M, Bergant M, Repnik U. **In vitro preparation and functional assessment of human monocyte-derived dendritic cells-potential antigen-specific modulators of in vivo immune responses**. *Transpl Immunol* 2005,14:231-244.
13. Moutaftsi M, Mehl AM, Borysiewicz LK, Tabi Z. **Human cytomegalovirus inhibits maturation and impairs function of monocyte-derived dendritic cells**. *Blood* 2002,99:2913-2921.
14. Zenhaeusern G, Gasser O, Saleh L, Villard J, Tiercy JM, Hess C. **Investigation of alloreactive NK cells in mixed lymphocyte reactions using paraformaldehyde-silenced target cells**. *J Immunol Methods* 2007,321:196-199.
15. van Lier RA, ten Berge IJ, Gamadia LE. **Human CD8(+) T-cell differentiation in response to viruses**. *Nat Rev Immunol* 2003,3:931-939.

16. Ahmed R, Bevan MJ, Reiner SL, Fearon DT. **The precursors of memory: models and controversies**. *Nat Rev Immunol* 2009,9:662-668.

Future perspectives:

The immune response induced by influenza-vaccination (Part I)

Virosome-based vaccines (VBV) –as used in the vaccination studies described in this thesis– promote antigen presentation both via MHC class I as well as MHC class II [1,2]. In general, administration of VBV leads to a good antibody response as it mimics natural viral infections. Why some individuals do not generate protecting antibodies after VBV remains unknown.

As an immediate follow-up to the studies presented in this thesis-work it will now be interesting to use hemagglutinin-derived peptide loaded tetramers to further characterize the influenza-specific population induced by vaccination and to use proliferation/re-expansion assays to begin to understand the potential of the influenza VBV to induce distinct memory T cell populations [3].

Establishing cell lines expressing KIR2DS1 or KIR3DS1

The best-described model of NK cells fighting viral infections is the murine cytomegalovirus (MCMV) model. In humans, functional evidence on the protective role of KIR remains sparse. Alter et al. performed one of the first *in vitro* functional NK cell studies. In their work they were able to provide functional evidence with regards to the protective role of KIR3DS1+ and HLA-Bw4-80I in progressing AIDS. Another recently published study providing functional KIR data demonstrated that KIR2DS1 recognizes the C2 molecule with a 50% lower avidity than its counterpart KIR2DL1 [4]. Little is known regarding clonal expansion of NK cells bearing activating receptors in human infection. Alter et al. reported a specific expansion of KIR3DS1+ and KIR3DL1+ NK cells during acute HIV-1 infection in the presence of HLA-Bw4-80I [5]. This finding is in line with a dramatic expansion of a Ly49H-bearing NK cells in the model of MCMV [6].

Aiming to functionally assess the observed association between KIR 2DS1 and 3DS1 genotype and outcome after influenza vaccination we decided to establish single KIR expressing cell lines. To that end we chose NK-92 and YT cells to perform stable transfections. NK-92 is a human natural killer lymphoma cell line, which is devoid of KIR and grows in the presence of IL-2. YT is human T/NK cell leukemia cell line which is also devoid of KIR and furthermore of CD3, and has been reported to be easier to be transfected than NK-92. Once these cell lines are stably transfected with KIR2DS1 and KIR3DS1, respectively, the following experiments will be performed: Transfected cells expressing KIR 2DS1 and 3DS1, respectively, will be incubated with either the virosome-based vaccine or hemagglutinin and neuraminidase. Additionally, target cells, which are positive for different genotypes, for example C2 molecule or HLA-Bw4-80I, will be added for co-incubation. Binding will be assessed by flow cytometry and confocal microscopy. Since NK cells express a number of activating/inhibiting receptors, this reductionist approach may

shed light on the function of these specific KIR. We also aim to functionally characterize KIR–KIR ligand interactions using cell lysis assays and measurements of induced cytokine production.

New insights into the immune response after virosome-based vaccines (Part II)

Characterizing the subsets that are induced after vaccination and that appear in the peripheral blood circulation in fingolimod-treated individuals will require experiments similar as those outlined above (*"The immune response induced by influenza-vaccination (Part I)"*). Specifically in fingolimod-treated individuals it will be interesting to determine the mechanism(s) allowing peptide-triggered T cells to egress from the lymph nodes despite the inhibiting effect of fingolimod. To that end chemotaxis assays using S1P as well as other T cell chemoattractants will be performed. Another unresolved issue is whether the quality of the antibody response in fingolimod-treated individuals is also unchanged. Preliminary experiments analyzing the avidity of influenza-specific antibodies indicate that no correlation exists between the quantity and quality (i.e. avidity) in fingolimod-treated individuals and healthy controls (D. Eichin et al., unpublished observations).

Towards defining the soluble, NK cell-derived T cell-inhibitory molecules

In a series of experiments we have already started to investigate what soluble factors might confer the observed NK cell-derived T cell inhibitory effect ('blocking signal' in the summary sketch below; Figure 1). We initially focused on the two anti-inflammatory cytokines IL-10 and TGF-β1. While indeed allo-proliferation of T cells could be reduced in the presence of IL-10 as well as in the presence of TGF-β1, cytokine concentrations released by mature moDC-triggered NK cells were much lower than the ones required to inhibit allo T cell proliferation. Neutralizing IL-10 and TGF-β1 using neutralizing antibodies in our experimental system also had no impact. The search for the inhibitory factor(s) induced in NK cells by mature moDC is ongoing. Towards defining the molecular interaction that is inducing the observed inhibitory activity in NK cells, blocking experiments (e.g. blocking HLA – KIR interactions) will be performed.

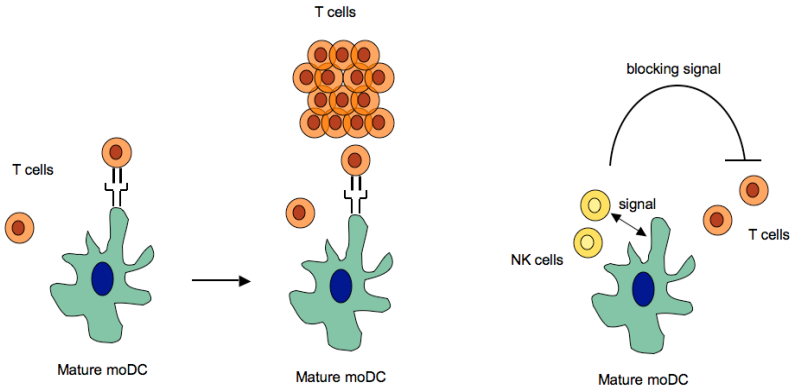


Figure 1 Model of a potential interaction between NK cells and mature moDC.

References

1. Souza AR, Braga JA, de Paiva TM, Loggetto SR, Azevedo RS, Weckx LY. **Immunogenicity and tolerability of a virosome influenza vaccine compared to split influenza vaccine in patients with sickle cell anemia**. *Vaccine*,28:1117-1120.
2. Herzog C, Hartmann K, Kunzi V, Kursteiner O, Mischler R, Lazar H, Gluck R. **Eleven years of Inflexal V-a virosomal adjuvanted influenza vaccine**. *Vaccine* 2009,27:4381-4387.
3. Cusick MF, Wang S, Eckels DD. **In vitro responses to avian influenza H5 by human CD4 T cells**. *J Immunol* 2009,183:6432-6441.
4. Moesta AK, Graef T, Abi-Rached L, Older Aguilar AM, Guethlein LA, Parham P. **Humans differ from other hominids in lacking an activating NK cell receptor that recognizes the C1 epitope of MHC class I**. *J Immunol*,185:4233-4237.
5. Alter G, Rihn S, Walter K, Nolting A, Martin M, Rosenberg ES*, et al.* **HLA class I subtype-dependent expansion of KIR3DS1+ and KIR3DL1+ NK cells during acute human immunodeficiency virus type 1 infection**. *J Virol* 2009,83:6798-6805.
6. Dokun AO, Kim S, Smith HR, Kang HS, Chu DT, Yokoyama WM. **Specific and nonspecific NK cell activation during virus infection**. *Nat Immunol* 2001,2:951-956.

Acknowledgements

Zum Abschluss möchte ich besonders Prof. Christoph Hess für seine Betreuung und fachliche sowie menschliche Unterstützung während meiner Dissertation vielmals danken. Ich bin ihm sehr dankbar, dass er mir die Möglichkeit gegeben hat, so vielfältige und interessante Aspekte der Immunologie zu untersuchen.

Ausserdem möchte ich meinem gesamten Dissertationskomitee bestehend aus den Professoren Christoph Hess, Antonius Rolink und Ed Palmer vielmals für ihre ausgezeichnete Betreuung danken.
Prof. Jürg Schifferli bin ich für die anregenden Ideen und Diskussionen während unserer gemeinsamen Meetings ebenfalls sehr dankbar.

Besonders Bojana Durovic und Gabriela Zenhäusern waren mir ebenfalls eine sehr große Hilfe und haben mich durch die gesamte Zeit sehr unterstützt. Gabi hat mich am Anfang eingewiesen – Bojana und ich haben zum gleichen Zeitpunkt angefangen und konnten die Freuden und Sorgen einer Dissertation aufrichtig teilen. Zudem waren wir nicht nur im Labor ein sehr gutes Team, sondern haben eine sehr schöne Freundschaft aufgebaut.

Mit Olivier Gasser habe ich dieses Jahr ein Projekt angefangen und sehr viel Hilfe und Anleitung erhalten. Er ist ein sehr wichtiger Koordinator im Labor und wir konnten immerwährend mit Fragen zu ihm kommen. Zudem möchte ich mich bei Gideon Hönger, Patrick Gubser, Johannes Nemeth, Dominik Eichin, Martin Stern, Nikolai Hodel, Matthias Mehling und Glenn Bantug für die Zeit bedanken, in der ich mich fachlich aber auch persönlich weiterentwickeln konnte.

Ich möchte mich insgesamt bei unserem Labor 301 bedanken. Es hat immerwährend eine sehr freundliche und hilfsbereite Atmosphäre geherrscht. Bei Meetings konnten wir durch interessante Diskussionen neue Ansätzen entdecken und zudem verbindet uns alle ein sehr freundschaftliches Verhältnis, das sich auch bei privaten Anlässen immer wieder zeigt.

Ebenfalls möchte ich Michaj Rajski, Reto Ritschard und Brigitte Vogel danken, mit denen wir uns anfangs im vierten Stock das Labor geteilt haben.

Zu größtem Dank bin ich meinem Mann Thilo Fritz sowie meiner Schwester Ann-Cathrine und meinen Eltern Ingrid und Hans-Jörg Hamm verpflichtet. Sie haben mich jahrelang liebevoll während meines Weges unterstützt. Zudem bin ich meinen Eltern sehr dankbar, dass sie mir die Möglichkeit gegeben haben, mein Wunsch-Studium auszuüben.

Zuletzt möchte ich meinen sehr guten Freundinen Nadine, Andrea und Alexandra für ihre herzliche Unterstützung danken.

Stefanie Fritz
Gartenstrasse 23a
DE-79576 Weil am Rhein
Phone: +49 7621 66 59 51
Email: stefanie.fritz@unibas.ch

Personal Information

Birthday: 10.01.1981
Place of Birth: Lörrach (Germany)
Personal status: married, born Hamm

Education

Master of Science, Biology

University of Freiburg, Germany, Division of Rheumatology and Clinical Immunology, University Medical Center Freiburg

"The development of plasmablasts in CVID-patients"
Supervisor: PD Dr. Hermann Eibel, group leader

Publications

Stefanie Fritz, Erik Mossdorf, Bojana Durovic, Gabriela Zenhaeusern, Anna Conen, Ingrid Steffen, Manuel Battegay, Reto Nüesch, Christoph Hess. Virosomal influenza-vaccine induced immunity in HIV-infected individuals with high versus low CD4+ T cell counts: clues towards a rational vaccination strategy. *AIDS*, 2010 Sep 10;24(14):2287-9.

Stefanie Fritz, Erik Mossdorf, Gabriela Zenhaeusern, Matthias Mehling, Bojana Durovic, Patricia Hilbert, Anna Conen, Ludwig Kappos, Manuel Battegay, Martin Stern, Reto Nüesch, Christoph Hess. In absence of HIV infection KIR 2DS1 and 3DS1 genes are associated with enhanced CD4+ T cell response to influenza-vaccine. *Submitted.*

Matthias Mehling, **Stefanie Fritz**, Patricia Hilbert, Jens Kuhle, Thomas Klimkait, Raja P. Lindberg, Ludwig Kappos, Christoph Hess. Cellular and humoral influenza vaccine-specific immune responses are maintained in patients with multiple sclerosis treated with FTY 720. *Annals of Neurology*, 2011 Feb;69(2):408-13)

Bojana Durovic, **Stefanie Fritz**, Gabriela Zenhaeusern, Stefan Dirnhofer, Christoph Hess. Rapidly proliferating EBV-transformed B cells skew CD8+ T cells towards inefficiency. *Submitted*

Stefanie Fritz, Bojana Durovic, Gabriela Zenhaeusern, Johannes Nehmeth, Nikolai Hodel, Olivier Gasser, Christoph Hess. Mature Dendritic cells trigger natural killer cells to release T cell-inhibitory factors. *In preparation.*

Presentations

PhD student meeting, Wolfsberg, Switzerland, March 30th-April 1st, 2009

"Virosome-based influenza-vaccine induced immunity in HIV-infected individuals with high versus low CD4+ T cell counts"

Stefanie Hamm, Erik Mossdorf, Bojana Durovic, Gabriela Zenhaeusern, Anna Conen, Ingrid Steffen, Manuel Battegay, Reto Nüesch and Christoph Hess

Posters

NK cell symposium, Freiburg, Germany, November 4th-6th, 2009

"In absence of HIV-infection KIR 2DS1 and 3DS1 link NK cell and T cell immunity"

Stefanie Fritz, Erik Mossdorf, Gariela Zenhaeusern, Martin Stern, Reto Nüesch and Christoph Hess

International Experience

Semester 2003/2004: La Réunion