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TESI DI DOTTORATO DI RICERCA

MULTIPARAMETRIC ANALYSIS OF IMMUNE-THERAPEUTIC MODEL FOR HCV ASSOCIATED B CELL NON-HODGKIN'S LYMPHOMA

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INDEX

ABSTRACT

Hepatitis C virus (HCV) is one of the major risk factors for chronic hepatitis, which may progress to cirrhosis and hepatocellular carcinoma, as well as for type II mixed cryoglobulinemia (MC), which may further evolve into an overt B-cell non-Hodgkin's lymphoma (NHL).

In particular, it has been previously shown that B-cell receptor (BCR) repertoire, expressed by clonal B-cells involved in HCV-associated NHL, is constrained to a limited number of variable heavy (VH)- and light (VL)-chain genes. Among these, the VK3-20 light chain idiotype has been selected as a target for passive as well as active immunization strategy.

Here, we present the results of a multiparametric analysis of the innate and early adaptive immune response induced after *ex vivo* stimulation of human peripheral blood mononuclear cells (PBMCs) with the recombinant VK3-20 protein. This objective has been pursued performing gene expression profiling analysis as well as multiparameter flow cytometry and multiplex analyses of cytokines.

1. INTRODUCTION

1.1. HCV

Hepatitis C virus (HCV) is an enveloped, positive-strand RNA virus of the Flaviviridae family. The viral genome consists of a 9.6 kb RNA molecule, containing a large open-reading frame flanked by 5' and 3' non-translated regions (NTRs) [1].

The viral proteins are translated as a large precursor polyprotein, which undergoes a complex series of cleavage events catalysed by host and viral factors to generate the individual mature HCV proteins [2].

The virus structural proteins include the core (C) protein and the envelope glycoproteins E1 and E2, the latter being responsible for host-cell receptors binding [3].

The non-structural proteins include the small membrane protein p7 and the NS2, NS3, NS4A, NS4B, NS5A and NS5B proteins, which coordinate the intracellular processes of the virus life cycle (Fig. 1) [3].

In particular, the NS2 protein and the N-terminal region of NS3 represent the NS2-NS3 protease, which catalyses a single cleavage between NS2 and NS3 [4].

Once released from NS2, NS3 acts a multifunctional protein, with a N-terminal serine protease domain for the release of the remaining non-structural proteins and a C-terminal RNA helicase/NTPase domain [5].

The NS4A protein acts as a co-factor for the NS3, while the hydrophobic membrane protein NS4B works together with the viral replicase [6].

NS5A is a hydrophilic membrane-associated protein and NS5B represents the viral RNA-dependent RNA polymerase [7,8].

An additional small protein, called F (for frame shift protein), is generated by alternative reading frame within the core gene [9,10].

1.2. HCV genetic diversity and replication

The study of HCV life cycle and replication has been hampered by the lack of a cell culture system supporting high-level HCV replication [11].

As an RNA virus, HCV is characterized by a high nucleotide mutation rate which is reflected in a significant global genetic diversity. Indeed, HCV is currently divided into six major genotypes, with

numerous subtypes and quasispecies within each infected individual [12,13]. The identification of a seventh genotype has been recently proposed in patients from central Africa [12]. HCV genome is known to contain both highly conserved and highly variable regions and the genetic variability of the virus is known to affect liver cell metabolism as well as the outcome of IFN therapy [14].

Indeed, the liver is the major site of HCV replication, however, evidence exists for extrahepatic reservoirs, including peripheral blood cells, epithelial cells in the gut and the central nervous system [13,15,16].

HCV particles bind several host-cell receptors, including the CD81 tetraspannin [17,18], the human scavenger receptor SR-BI [19], claudin-1 (CLDN-1) [20] and occludin (OCLN) [21], as well as glycosaminoglycans [22] and the low-density lipoprotein receptor (LDL-R) [23].

The fusion of viral and cellular membranes leads to the intra-cytoplasmatic release of the single-stranded, positive-sense RNA genome, which, acts as messenger RNA (mRNA) driving the translation of the viral proteins, as well as, template for RNA replication to generate new copies of genomic RNA to be incorporated in the newly formed virus particles, which, in turn, leave the cell through the secretory pathway [11].

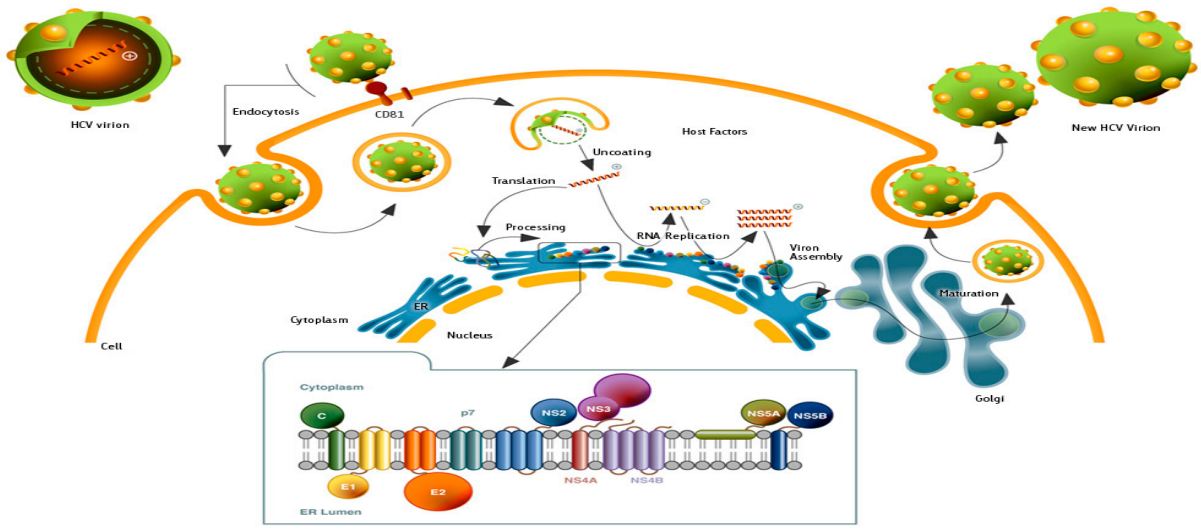

Fig. 1. Schematic representation of the replication cycle of HCV.

1.3. HCV chronic infection

HCV infection evolves to a chronic hepatitis in >70% of cases, which may progress to cirrhosis in about 10-20% of cases and further to hepatocellular carcinoma (HCC) in 1-5% of cirrhotic patients [24]. Host factors as well as, environmental and viral factors are known to play an important role

in determining progression of chronic hepatitis C to liver cirrhosis and HCC, a process that frequently takes several decades [24].

The molecular mechanisms underlying HCC development involve several indirect mechanisms including the interplay between chronic inflammation, steatosis, fibrosis, oxidative stress and their pathological consequences [24].

Indeed, HCC development is associated with multistep processes including the occurrence of somatic mutations, loss of tumor suppressor genes and possibly the activation or over-expression of proto-oncogenes [24].

More recently, the virus has been identified as one of the major risk factors for type II mixed cryoglobulinemia (MC), an autoimmune disease characterized by the clonal expansion of rheumatoid factor-expressing B cells, that may evolve into an overt B cell non-Hodgkin's lymphoma (NHL) in about 10% of MC patients (Fig. 2) [25-29].

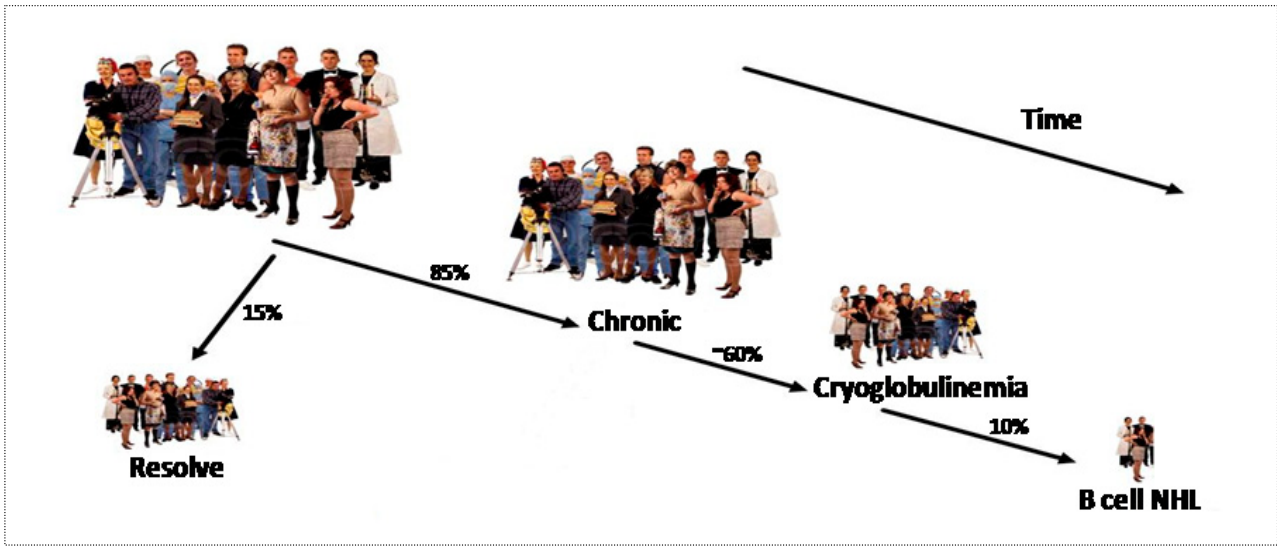

Fig. 2. HCV chronic infection is the leading cause of distinct malignant disorders.

Several studies have contributed to establish the causative role of HCV infection in the etiopathogenesis of MC, showing the presence of the viral RNA and/or anti-HCV antibodies in a range of 70 to 100% of MC [30-32]. Furthermore, the clinical evolution of MC is closely linked to the natural history of the underlying HCV chronic infection [33,34].

The most accredited pathogenetic mechanism of MC during HCV chronic infection is the persistent immune stimulation sustained by viral proteins which, in turn, may result in production of cross-reactive autoantibodies, including cryoglobulins [35,36].

Chronic B cell stimulation by HCV epitopes may produce the expansion of B cell subpopulations with dominant genetic characteristics. In particular, the interaction between HCV E2 protein and CD81 molecule, an almost ubiquitous tetraspannin present on B cell surface, has been postulated to induce a sustained polyclonal stimulation of B cell compartment [17]. Furthermore, the t(14,18) translocation observed in 85% of the patients affected by HCV-related type II MC might lead to abnormally elevated expression of Bcl-2 protein with consequent inhibition of apoptosis and increased B cell survival [37]. This multistep process may ultimately lead to the B cell NHL as late complication of the MC syndrome [33,38].

The clonality of expanded B cells can be defined by the analysis of the antigen-binding region (so called idiotype, Id) of the immunoglobulin (Ig) produced by the B cell clone and expressed on its surface as B cell receptor (BCR). According to the variety of Ids identified, the lymphoproliferative disorder may be sustained by mono-, oligo- or polyclonal B cells. It has been previously demonstrated that the BCR repertoire expressed by clonal B cells involved in HCV-associated NHL is not random, with V1-69, V3-7, V4-59 variable heavy (VH)- and still more variable k (VK)3-20 and VK3-15 light (VL)-chain genes being the most represented (Tab. 1) [39-41].

Table 1. Ig chain usage by B cell neoplastic clones in HCV+ subjects.

No.	Histologic classification	H-chain			L-chain	
1	Lymphoplasmacytoid	V1–2	D2–15	J4	nd	
2	Extranodal marginal zone	V1–2	D3–3	J4	nd	
3	Lymphoplasmacytoid	V1–69	D3–9	J4	V3–20	Jk2
4	Extranodal marginal zone	V1–69	D3–22	J2	V3–20	Jk2
5	Follicle center, follicular	V1–69	D3–22	J4	V3–20	Jk1
6	Small lymphocytic	V1–69	D3–22	J4	V3–20	Jk2
7	Lymphoplasmacytoid	V1–69	D3–22	J5	V3–20	Jk2
8	Nodal marginal zone	V1–69	D3–22	J4	nd	
9	Nodal marginal zone	V1–69	D3–22	J4	V3D–20	nd
10	Nodal marginal zone	V1–69	D3–22	J4	V5	nd
11	Lymphoplasmacytoid	V1–69	D5–12	J4	V3–20	Jk3
12	Lymphoplasmacytoid	V1–69	D6–6	J4	V3–20	Jk2
13	Lymphoplasmacytoid	V1–69	D6–6	J3	V3–20	Jk1
14	Lymphoplasmacytoid	V1–69	nd		nd	
15	Lymphoplasmacytoid	V1–69	nd		nd	
16	Lymphoplasmacytoid	V1–69	nd		nd	
17	Lymphoplasmacytoid	V1–69	nd		nd	
18	Extranodal marginal zone	V3–7	D3–16	J3	nd	
19	Diffuse large cell	V3–7	D3–22	J3	V3–15	Jk1
20	Diffuse large cell	V3–7	D5–24	J3	V3–15	Jk1
21	Diffuse large cell	V3–7	D6–6	J4	V2	nd
22	Lymphoplasmacytoid	V3–23	D2–2	J5	nd	
23	Diffuse large cell	V3–23	D3–9	J4	V1–5	Jk2
24	Diffuse large cell	V3–23	D3–22	J4	V3–20	Jk2
25	Nodal marginal zone	V3–30	D7–27	J4	V2	nd
26	Extranodal marginal zone	V3–30.5	D6–13	J4	V3–15	Jk1
27	Diffuse large cell	V3–48	D3–22	J5	V2	nd
28	Lymphoplasmacytoid	V3–48	D6–13	J6	V4–1	Jk4
29	Mantle cell	V3–48	D6–19	J5	nd	
30	Nodal marginal zone	V4–30.4	nd	J6	nd	
31	Lymphoplasmacytoid	V4–34	D4–11	J2	V3D–11	Jk3
32	Diffuse large cell	V4–34	D5–18/D5–5	J4	V1–17	Jk1
33	Small lymphocytic	V4–59	D2–15	J2	V3–20	Jk1
34	Extranodal marginal zone	V4–59	D2–15	J2/J5	V3–20	Jk1

These data suggest a model of antigen-driven origin for these lymphoproliferative disorders with the recognition of a limited number of HCV antigens [41,42].

1.4. Idiotype as vaccine antigen

The mentioned constrained heterogeneity of Ids strongly suggests the possibility of targeting one or few idiotypes to hit and eliminate the B cell clone sustaining the HCV-associated NHL.

One strategy is to generate idiotype-specific monoclonal antibodies (MAbs) to be employed in a selective passive immunization and, in this regard, highly reactive MAbs specific for VK3-20 idiotype of a subject with HCV infection and type II MC associated NHL have been generated [43].

The alternative strategy is to use the idiotype as a vaccine molecule in order to elicit an active humoral/cellular immune response as preventive and/or therapeutic approach against the expansion of the B cell clone sustaining the HCV-associated NHL [44-46].

Indeed, idiotype vaccines have already proven to be effective in inducing specific immune responses which are able to kill in vivo follicular lymphoma cells that had survived pre-vaccine chemotherapy [47,48], ultimately resulting in prolonged survival of responding patients [49].

Several ongoing Phase I/II clinical trials for the treatment of tumors of hematological origin are, indeed, based on idiotype vaccines.

In particular, a Phase I clinical trial is currently ongoing to test the safety of autologous recombinant idiotype vaccine for the treatment of patients with relapsed or transformed follicular lymphoma (ClinicalTrials.gov Identifier: NCT01022255).

Moreover, a Phase II clinical trial is currently recruiting participants to test the efficacy of a specific idiotype vaccine (conjugated to keyhole limpet hemocyanin (KLH) plus granulocyte macrophage colony stimulating factor (GM-CSF)) in preventing relapse of follicular lymphoma in responding patients (ClinicalTrials.gov Identifier: NCT00530140).

Finally, a randomized Phase II clinical trial is ongoing to evaluate the outcome of a combination strategy based on infusion of CD3/CD28 autologous T cells, primed ex vivo with Id-KLH, and vaccine administration in patients with multiple myeloma (ClinicalTrials.gov Identifier: NCT01426828).

Nevertheless, the broader use of such idiotype vaccines has been hampered by the fact that autologous Ids are patient-specific so that vaccines must be individually prepared for each patient.

Yet, a higher frequency of idiotype sharing exists among patients with HCV-associated NHL, allowing the use of limited Ids for vaccine therapy, thus, overcoming the issue of complex and time-consuming approach needed for vaccines production.

1.5. Systems biology in vaccine development

In the general context of vaccinology the need for clear immunological markers to predict and evaluate the immunogenicity of vaccines and to optimize vaccine formulation critically exemplifies the usefulness of systems biology approaches.

Indeed, the experimental paradigm of systems biology is to look at a biological system as a whole, evaluating interactions among biological elements and their relationship with the environment.

Importantly, there are two major approaches to systems biology: a top-down view in which potential permutations predicted by experimentally formed knowledge are speculated with a deductive approach; conversely, and inductive approach is often applied in clinical research following a bottom up analysis in which the global picture associated with a specific biological occurrence is depicted using high throughput technology and the reasons for occurrence are then hypothesized using a reverse engineering approach [50].

In this perspective, it has been shown that multivariate and multiparametric analyses can predict the innate and early adaptive immune response induced by a vaccine molecule in human monocyte-derived dendritic cells (MDDCs), as well as whole peripheral blood mononuclear cells (PBMCs), using *ex vivo* experimental setting [51-53].

This systems biology approach involves high-throughput technologies such as global gene expression profiling, multiplex analysis of cytokines and chemokines, as well as multiparameter flow cytometry, combined with computational modeling [54-58].

Such approach provides detailed level of investigation to analyze the network of interactions between a vaccine molecule and the innate and adaptive immune response [59,60].

Indeed, systems biology approaches have been increasingly applied to oncology [61-63], autoimmunity [64], infections [65] and, more recently, to vaccinology [51,52].

2. AIM OF THE STUDY

In the present study, we performed a multiparametric analysis of the innate and early adaptive immune response induced after *ex vivo* stimulation of human PBMCs with VK3-20 light chain protein, the idiotype most frequently identified on B cell clones sustaining the HCV-associated type II MC and NHL.

This objective has been pursued using freshly isolated circulating human PBMCs, on which we performed gene expression profiling analysis as well as multiparameter flow cytometry and multiplex analysis of cytokines.

A preliminary study of the innate and early adaptive immune signature induced by recombinant VK3-20 protein was performed on whole PBMCs, as well as MDDCs from 15 enrolled subjects, including 10 HCV seropositive subjects and 5 healthy controls [66].

Subsequently, the effect of a conjugated form of VK3-20 (i.e. VK3-20-KLH) protein on immune response was studied on whole PBMCs isolated from a second cohort of subjects, including 10 HCV seropositive subjects and 5 healthy controls (Petrizzo et al., submitted)**.**

Finally, to further investigate the effect of VK3-20 protein *ex vivo* "vaccination" on whole PBMCs after 24h or 6 days incubation, the gene expression profile of samples from 5 HCV seropositive subjects and 4 healthy controls was examined. In parallel, molecular detection of immune signature induced by VK3-20 protein stimulation after 24h or 6 days incubation was performed (Petrizzo et al., submitted)**.**

The main results and implications of the present study are summarized and discussed in the context of current knowledge.

3. RESULTS

3.1. Preliminary study of immune signature induced by recombinant VK3-20 protein

Fifteen subjects were enrolled for preliminary study of immune signature induced by VK3-20 protein. Ten subjects were HCV seropositive, of which four were diagnosed with NHL and one showed a type II MC. In addition, five healthy subjects were enrolled as controls.

Freshly derived PBMCs and immature MDDCs were obtained from healthy HCV-negative subjects and were incubated with 1.5 µg/ml, 5 µg/ml or 15 µg/ml of the recombinant VK3-20 protein. After 16h stimulation, the expression of surface maturation/activation markers, such as CD83, CD86 and HLA-DR was examined. The results showed a trend of up-regulation of all markers in $CD14^+$ monocyte population as well as $CD11c^+$ myeloid DCs (mDCs) or $CD123^+$ plasmacytoid DCs (pDCs) [66] (Fig. 3). Furthermore, MDDCs showed patterns of activation comparable to circulating mDCs and pDCs [66].

Given the very similar results observed in MDDCs and in PBMCs, subsequent analyses on samples from the enrolled HCV seropositive subjects were performed on circulating monocytes, mDCs and pDCs and the VK3-20-induced expression of the markers was evaluated in terms of mean fluorescence index (MFI).

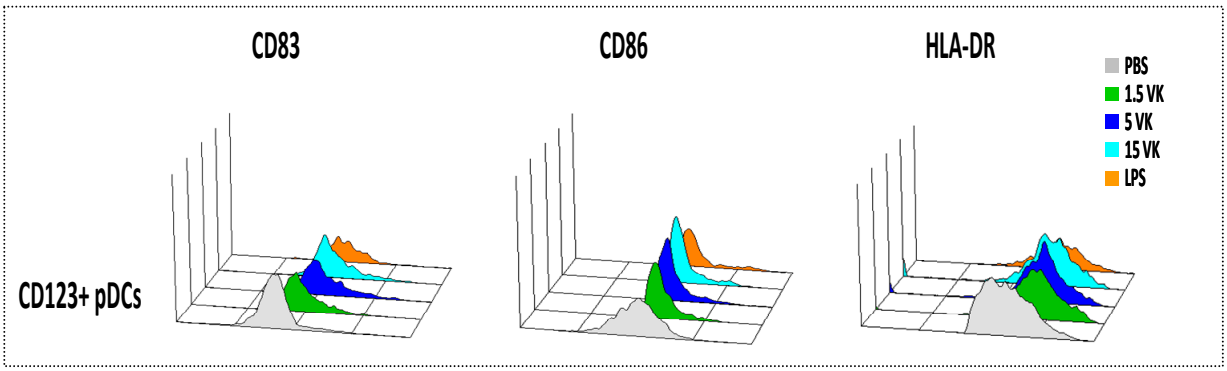

Fig. 3. Expression of surface markers on ex vivo activated cells from a healthy control. PBMCs were incubated with increasing doses of VK3-20 protein for 16h. The expression of CD83, CD86 and HLADR was analysed by FACScalibur flow cytometer in $CD14^+$ monocytes, $CD123^+$ pDCs and $CD11c^+$ mDCs. Data analysis was carried out with WinMDI2.8 Software. One representative experiment is shown.

The stimulation with VK3-20 protein induced a trend of increased expression of the activation/maturation markers in all circulating cells from the HCV seropositive subjects, although the most evident and consistent pattern was observed in the $CD11c^+$ mDC and/or $CD123^+$ pDC cells (Fig. 4) [66].

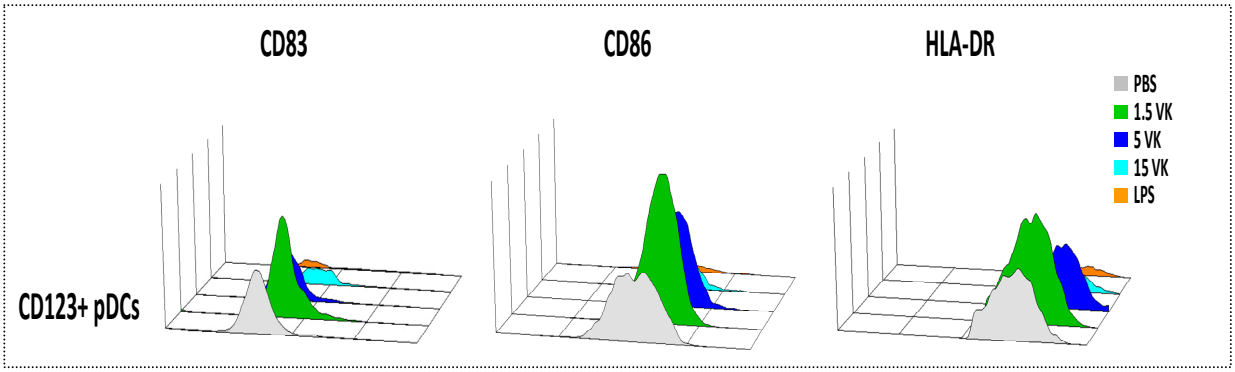

Fig. 4. Expression of surface markers on ex vivo activated cells from a HCV positive subject. PBMCs were incubated with increasing doses of VK3-20 protein for 16h. The expression of CD83, CD86 and HLADR was analysed by FACScalibur flow cytometer in $CD14^+$ monocytes, $CD123^+$ pDCs and $CD11c^+$ mDCs. Data analysis was carried out with WinMDI2.8 Software. One representative experiment is shown.

Moreover, the levels of IL-2, gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), IL-6, IL-4 and IL-10 were assessed in the supernatant of PBMCs stimulated with the recombinant VK3-20 protein.

Our results indicate that VK3-20 protein stimulation does not induce any increase in the production of IL-2 and IFN-γ [66]. On the contrary, VK3-20 induced a significant production of IL-4, IL-6, IL-10 and TNF-α in PBMCs from both healthy controls and HCV seropositive subjects, with the highest levels observed in the samples treated with the highest concentration of VK3- 20 protein (15 μg/ml) (p-value < 0.05) (Fig. 5-6) [66].

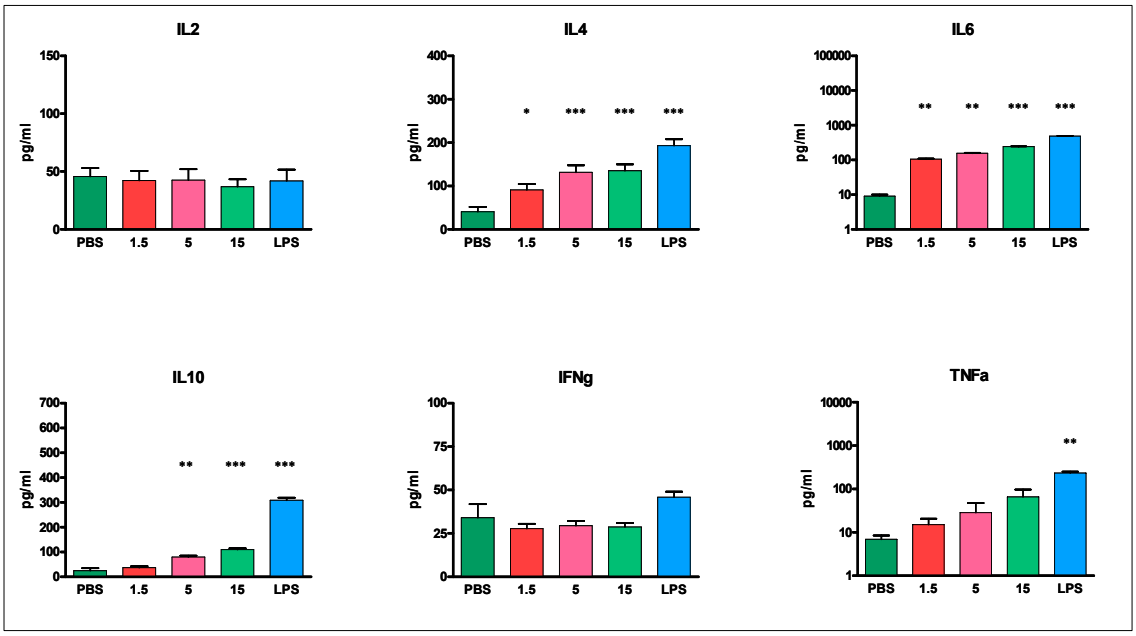

Fig. 5. Cytokines analysis in supernatants of PBMCs from control subjects induced by the indicated concentrations of VK3-20 and LPS. * P < 0.05, ** P < 0.01, *** P < 0.001.

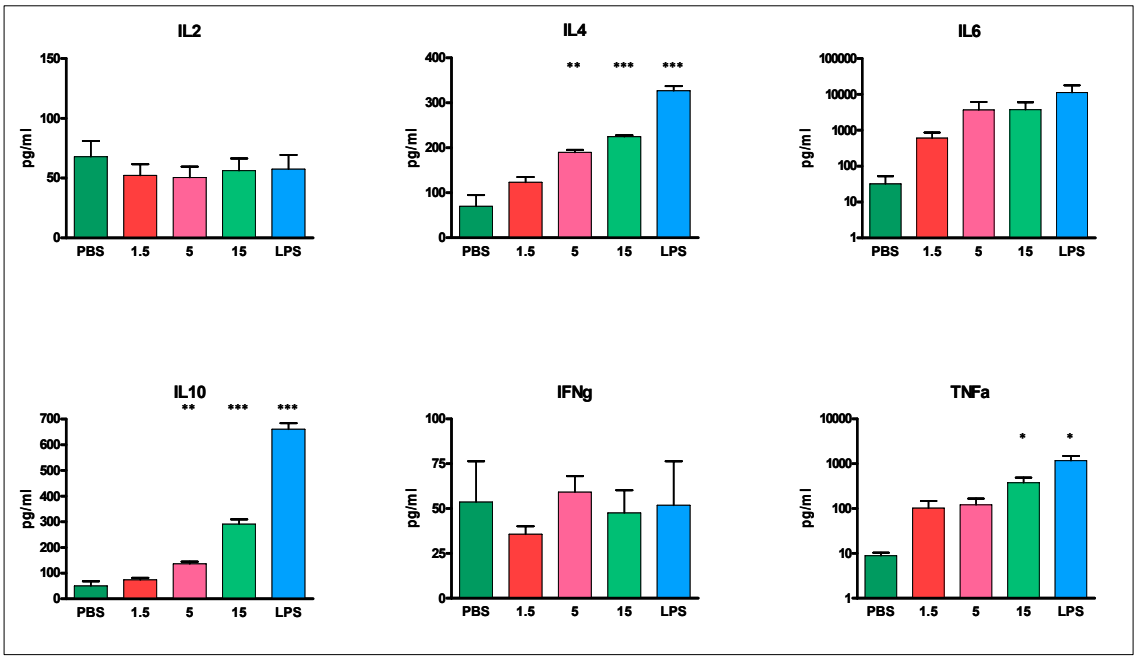

Fig. 6. Cytokines analysis in supernatants of PBMCs from HCV seropositive subjects induced by the indicated concentrations of VK3-20 and LPS. * P < 0.05, ** P < 0.01, *** P < 0.001.

3.2. Second cohort of subjects included in the study

Fifteen subjects were enrolled for the analysis of immune signature induced by a conjugated form of VK3-20 protein and for gene expression profiling analysis. Ten subjects were HCV-seropositive, of whom six were males and four were females, with known blood levels of cryoglobulins. Five healthy subjects were enrolled as controls, matched for age and life style. Clinical parameters of enrolled subjects are described in Table 2.

Table 2. Clinical parameters of enrolled subjects.

SAMPLE CODE	SEX	HCV RNA (IU/ml)	CRYOCRIT %	RF TEST (IU/ml)	WAALER-ROSE TEST	NHL
S.R.	F	NEG.	NEG.	NEG.	NEG.	NEG.
A.I.	F	NEG.	NEG.	NEG.	NEG.	NEG.
S.F.	M	NEG.	NEG.	NEG.	NEG.	NEG.
C.R.	F	NEG.	NEG.	NEG.	NEG.	NEG.
A.C.	F	NEG.	NEG.	NEG.	NEG.	NEG.
N.L.	M	1900	1.50	31.2	POS.	NEG.
M.M.L.	F	629000	0.5	127	POS.	NEG.
F.M.	F	500000	0.1	3.9	NEG.	NEG.
P.M.	F	700000	2	607	POS.	NEG.
V.A.	M	3000000	0.5	7.7	NEG.	NEG.
B.E.	M	9000	1.1	670	POS.	NEG.
D.N.	M	1130000	0.3	122.00	POS.	NEG.
L.M.R.	M	5850000	0.8	114	POS.	NEG.
D.B.A.	M	2340000	0.6	112	POS.	NEG.
B.D.	F	HCV RNA neg./HIV RNA pos.	0.2	12	NEG.	NEG.

3.3. Immune signature induced by a conjugated form of VK3-20 protein

Our previous study showed that VK3-20 induced higher levels of specific Th2 cytokines in PBMCs from HCV seropositive subjects compared to healthy controls [66], reflecting the prevalent Th2 status induced by the established HCV infection, as previously extensively reported [67-69].
Subsequently, we decided to evaluate whether a conjugated form of VK3-20 protein, i.e. VK3-20-KLH, would induce a different pattern of immunological effect on circulating antigen presenting cells.

Indeed, the immune-potentiating effect of KLH carrier protein on tumor antigens has, already, been tested in therapeutic vaccine strategies for a variety of cancers, including non-Hodgkin's lymphoma, melanoma, breast and bladder cancer [70-74].

In this framework, PBMCs from 10 HCV seropositive subjects and 5 healthy controls were pulsed with 15µg/ml VK3-20 protein or VK3-20-KLH conjugate. After 16h stimulation, cells were stained for phenotypic analysis by flow cytometry and the expression of surface maturation/activation markers, such as CD40, CD80, CD83, CD86 and HLA-DR on monocytes and $CD11c^+$ mDCs was evaluated as MFI (Fig. 7 and 8).

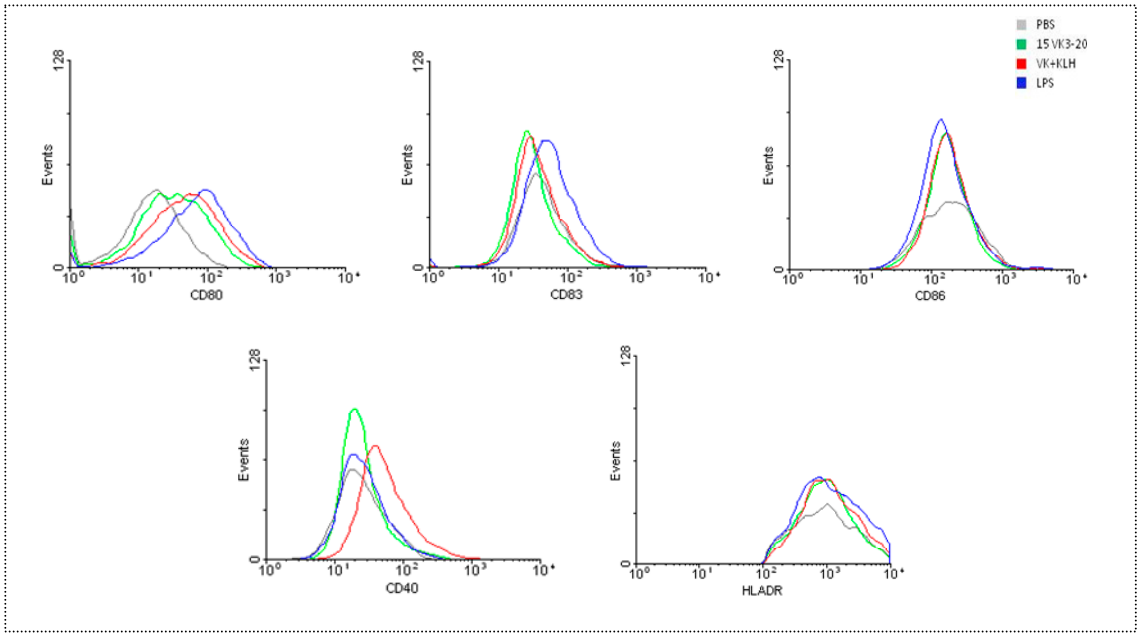

Fig. 7. Expression of surface markers on ex vivo activated cells from a healthy control. PBMCs were incubated with VK3-20 or VK3-20-KLH protein for 16h. The expression of CD80, CD83, CD86, CD40 and HLADR was analysed by FACScalibur flow cytometer in $CD14^+$ monocytes and $CD11c^+$ mDCs. Data analysis was carried out with WinMDI2.8 Software. One representative experiment is shown.

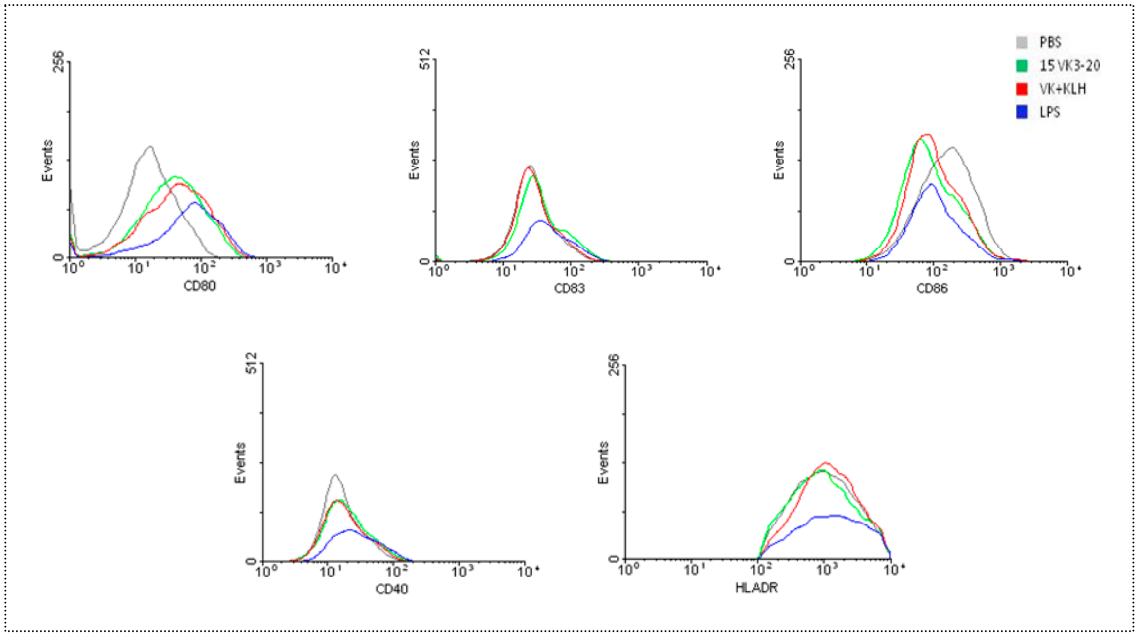

Fig. 8. Expression of surface markers on ex vivo activated cells from a HCV positive subject. PBMCs were incubated with VK3-20 or VK3-20-KLH protein for 16h**.** The expression of CD80, CD83, CD86, CD40 and HLADR was analysed by FACScalibur flow cytometer in $CD14^+$ monocytes and $CD11c^+$ mDCs. Data analysis was carried out with WinMDI2.8 Software. One representative experiment is shown.

The basal expression of the markers was largely comparable between HCV- and HCV+ subjects with no significant differences, with a trend of higher expression in cell populations from healthy controls (Fig. 9). The only exception was represented by basal CD83 expression, which showed a trend of higher expression in cells from HCV+ subjects (Fig. 9)

The expression of activation markers and costimulatory molecules was largely comparable between healthy controls and HCV seropositive subjects, with minor differences between VK3-20 and VK3-20-KLH treatment (Fig. 10-11). However, the stimulation with VK3-20-KLH conjugate induced a higher expression of CD80 and CD40 (p-value < 0.05) in PBMCs from healthy controls (Fig. 7 and 10).

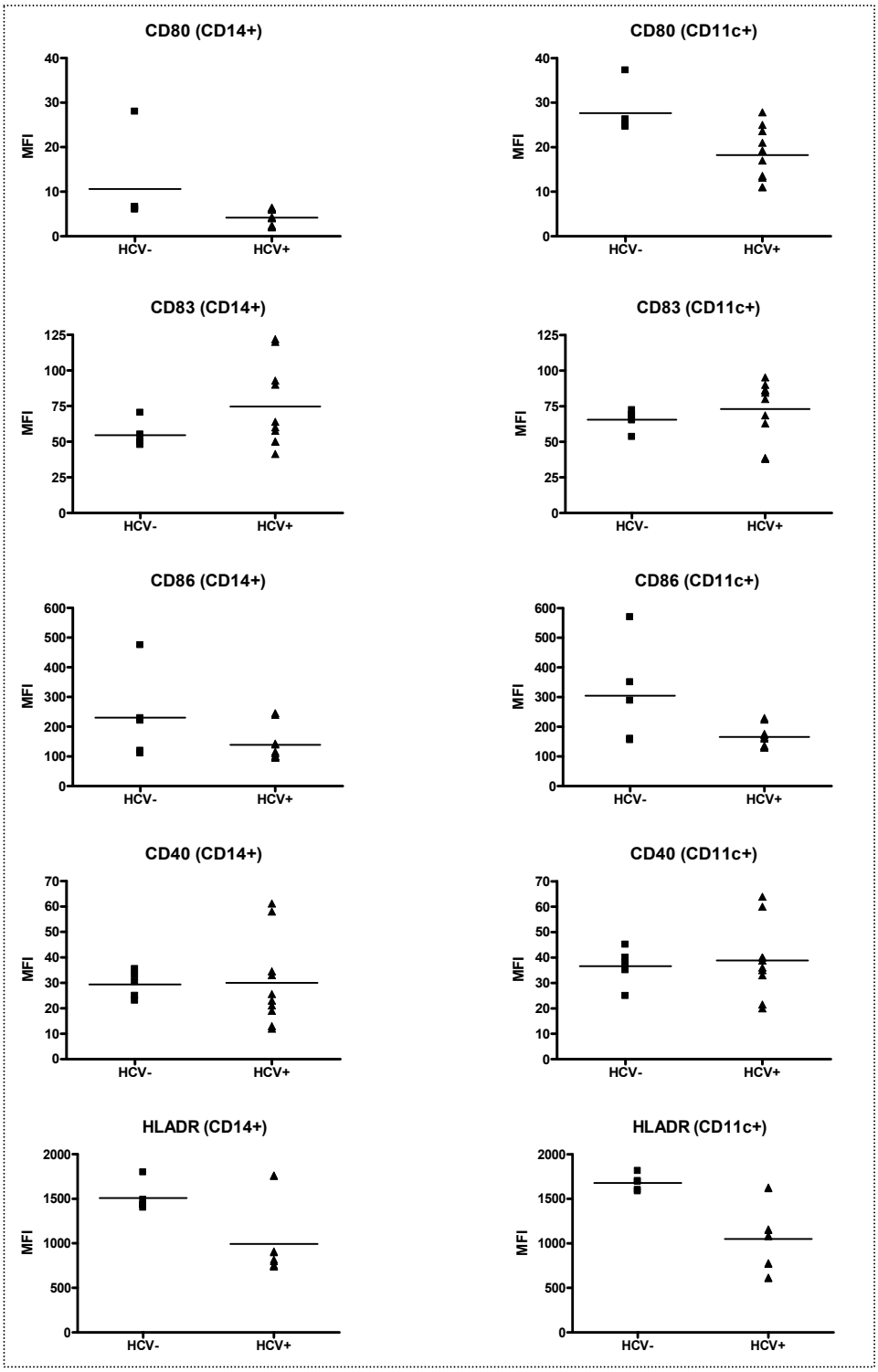

Fig. 9. Basal level expression of surface maturation/activation markers, indicated as Mean Fluorescence Index (MFI), on PBMCs from healthy (HCV-) and HCV positive (HCV+) subjects. CD14+ = monocytes; CD11c+ = mDCs.

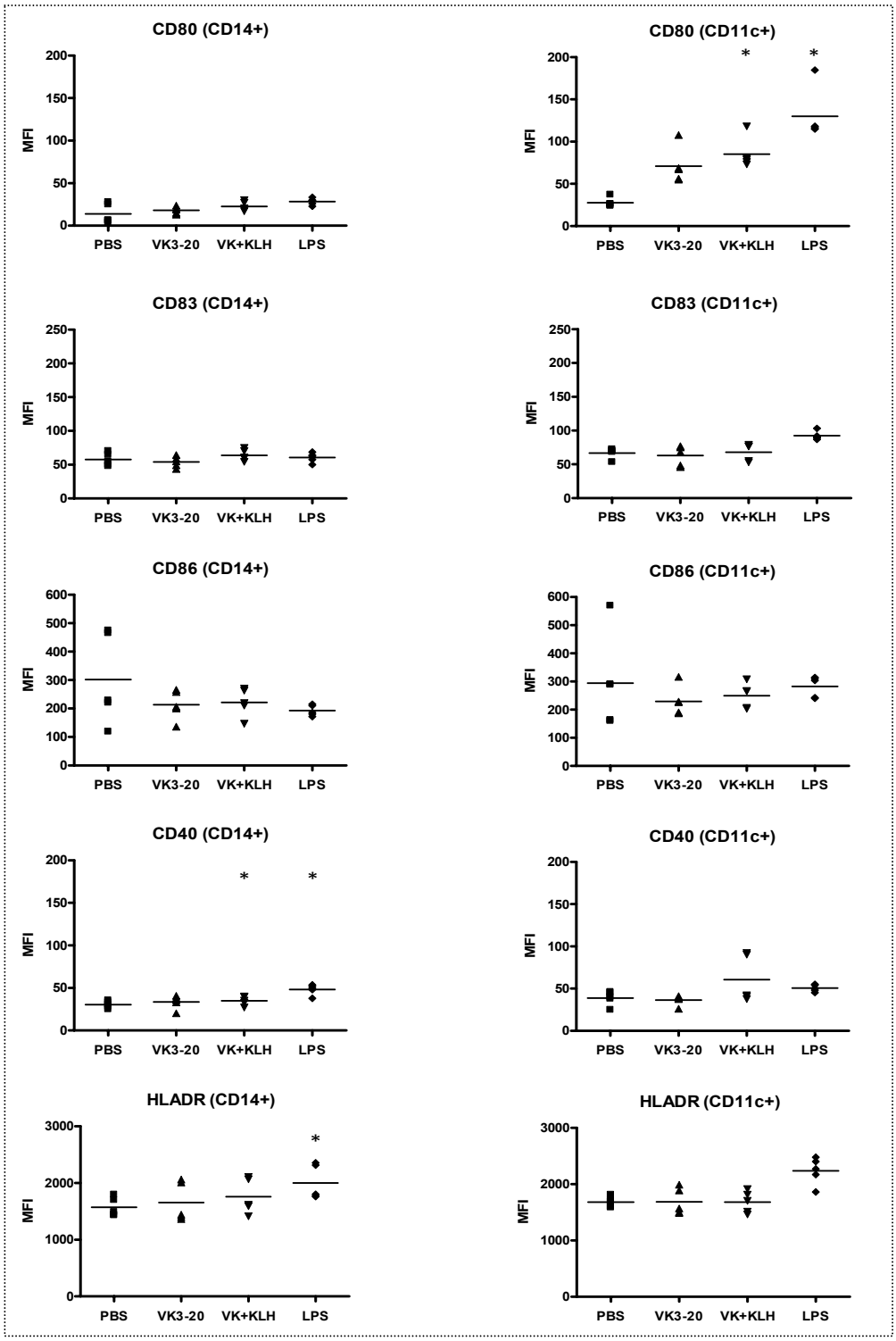

Fig. 10. Expression of surface maturation/activation markers, indicated as MFI, induced by VK3-20, VK3-20-KLH and LPS in APCs from control subjects. CD14+ = monocytes; CD11c+ = mDCs. * P < 0.05.

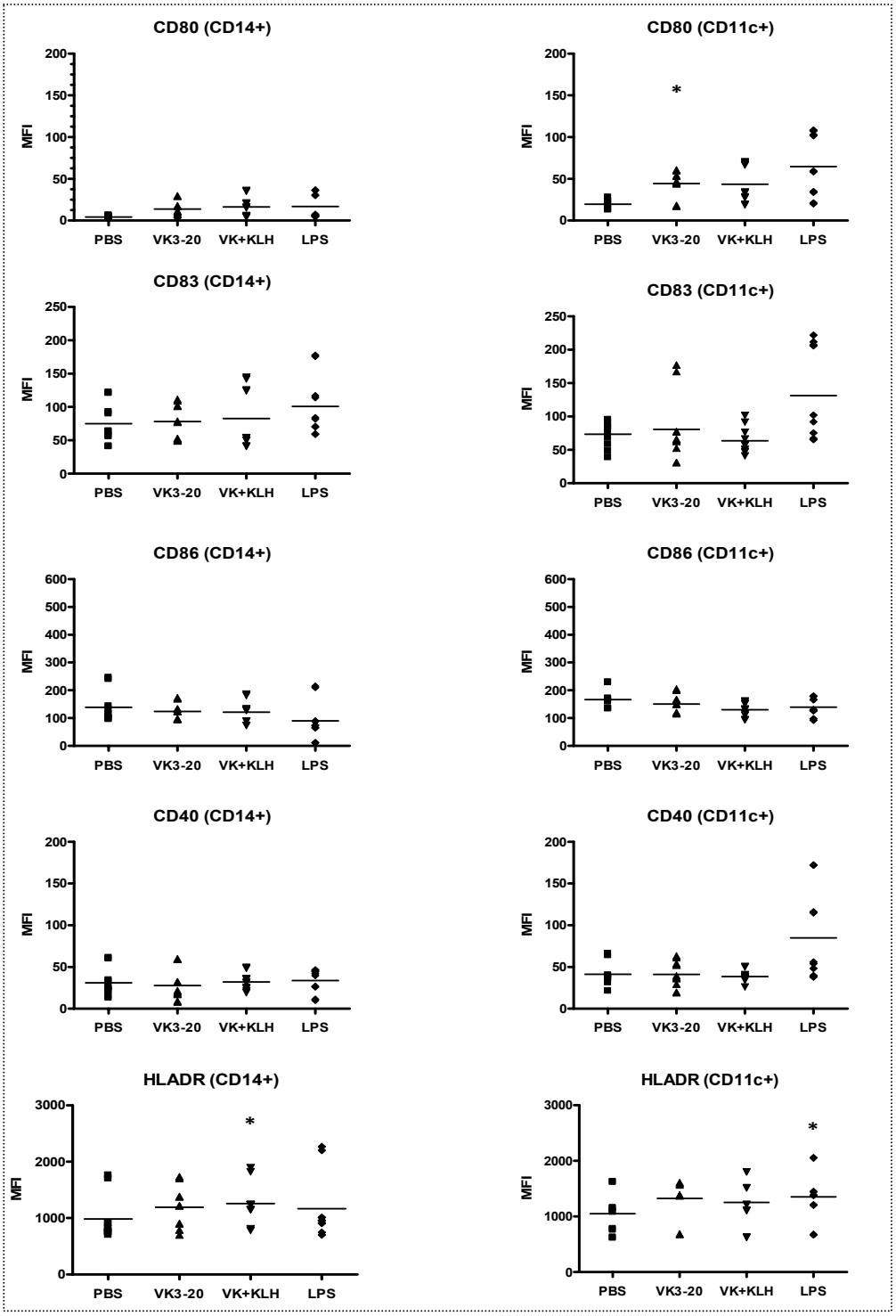

Fig. 11. Expression of surface maturation/activation markers, indicated as MFI, induced by VK3-20, VK3-20-KLH and LPS in APCs from HCV+ subjects. CD14+ = monocytes; CD11c+ = mDCs. * $P < 0.05$.

In addition, the levels of IL-2, IL-5, IL-6, IL-10, IFN-γ and TNF-α were assessed by ELISA in the supernatant of stimulated PBMCs. Both VK3-20 protein and VK3-20-KLH conjugate induced a significant production of TNF-α, IL-6 and IL-10 ($p < 0.05$) with no statistically significant differences; moreover, the level of induction in samples from healthy controls and HCV seropositive subjects was largely comparable (Fig. 12-13).

The overall results indicate that there is no significant difference in the immunological effects exerted by VK3-20-KLH compared to VK3-20.

Therefore, subsequent analysis of gene expression profile was performed on PBMCs stimulated with the recombinant VK3-20 protein.

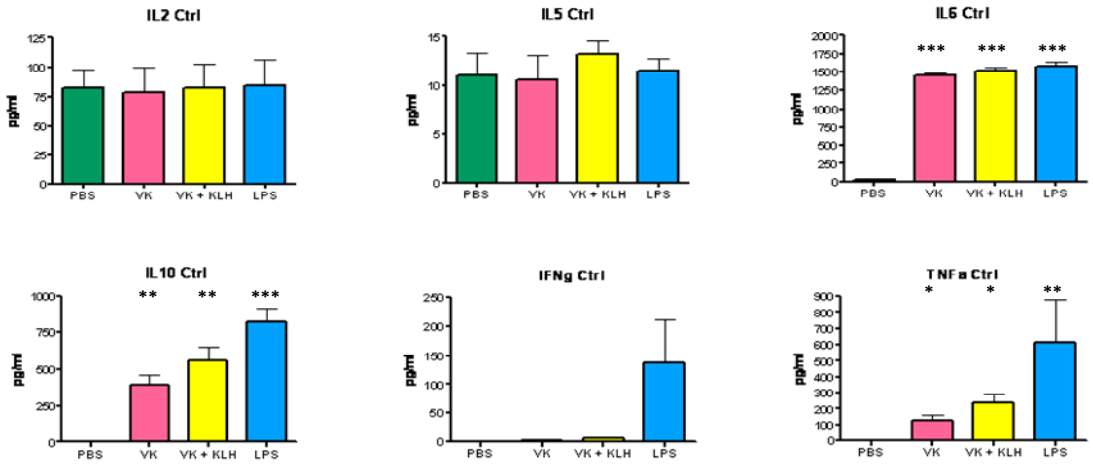

Fig. 12. Cytokines analysis in supernatants of PBMCs from control subjects induced by VK3-20, VK3-20-KLH and LPS. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

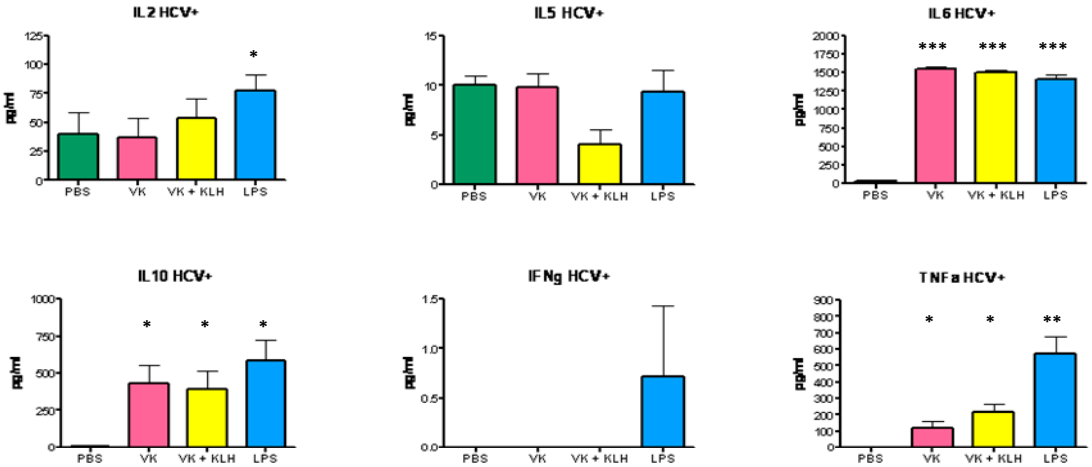

Fig. 13. Cytokines analysis in supernatants of PBMCs from HCV+ subjects induced by VK3-20, VK3-20-KLH and LPS. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.4. Gene expression profile induced by VK3-20 protein stimulation

The gene expression profile of samples from 4 healthy controls and 5 HCV seropositive subjects was analyzed. Total RNA, from samples of non-stimulated (PBS) and stimulated (VK3-20 or LPS) PBMCs after 24h or 6 days incubation, was purified and the quality of extracted RNA was verified with the LabChip GX/GXII Electrophoresis System. Discrete 28S and 18S rRNA bands, as well as a 28S/18S rRNA area ratio close to 2 were considered appropriate for subsequent analyses (Fig. 14A-14B).

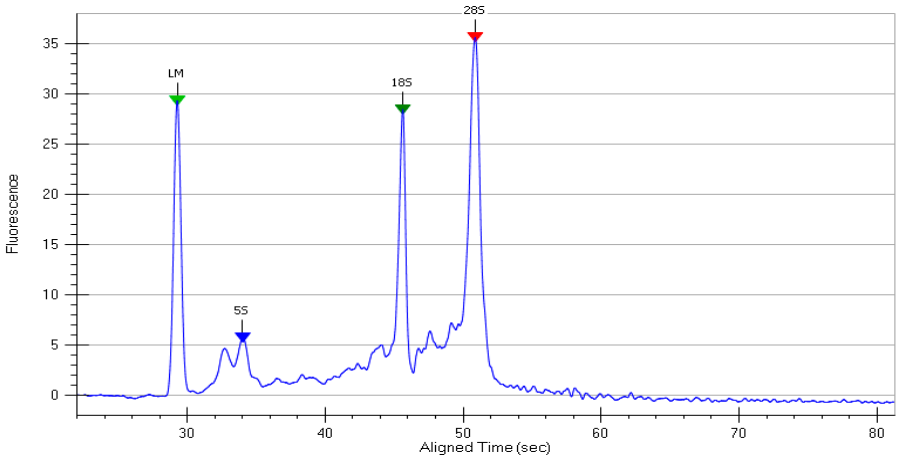

Fig. 14A. RNA quality control. Representative Electropherogram of total RNA extracted from a sample included in the analysis.

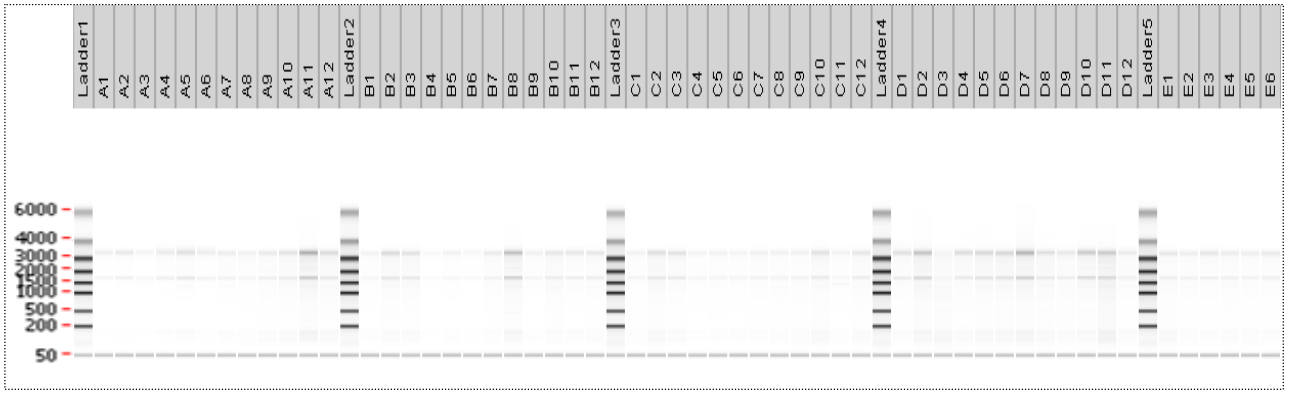

Fig. 14B. RNA quality control. Gel image evaluation of RNA integrity and 28S/18S rRNA ratio.

3.4.1. Primary unsupervised analysis

The cooperative action of the innate and adaptive immune system is the mechanism by which an effective protective immune response is mounted against a non-self antigen, including a pathogen, a vaccine antigen or cancer cells.

The comprehensive analysis of interactions, over time, between the components of the immune response, is definitely useful in predicting the responsiveness to an immunogenic antigen.

In this perspective, the molecular signature induced *ex vivo* by VK3-20 molecule in unfractionated PBMCs was evaluated at 24h and 6 days post-induction, in order to trace the dynamic pattern of immune system gene expression.

To this aim, samples from non-stimulated (PBS) and stimulated (VK3-20 or LPS) PBMCs after 24h or 6 days incubation were compared by unsupervised analysis.

According to filtering parameters described in Materials and Methods section, 6'562 genes differentially expressed were selected for the unsupervised analysis.

A clear cut separation in two main clusters matching the "time point" (24h or 6 days) was observed (Fig. 15). Moreover, within the 24h cluster a clear separation between PBS treatment and VK3-20 or LPS treatments was observed, with the VK3-20 and LPS treated samples from healthy controls mostly clustering apart from the HCV positive-related samples. On the contrary, neither a clear separation of samples based on treatment nor on HCV status was observed after 6 days (Fig. 15).

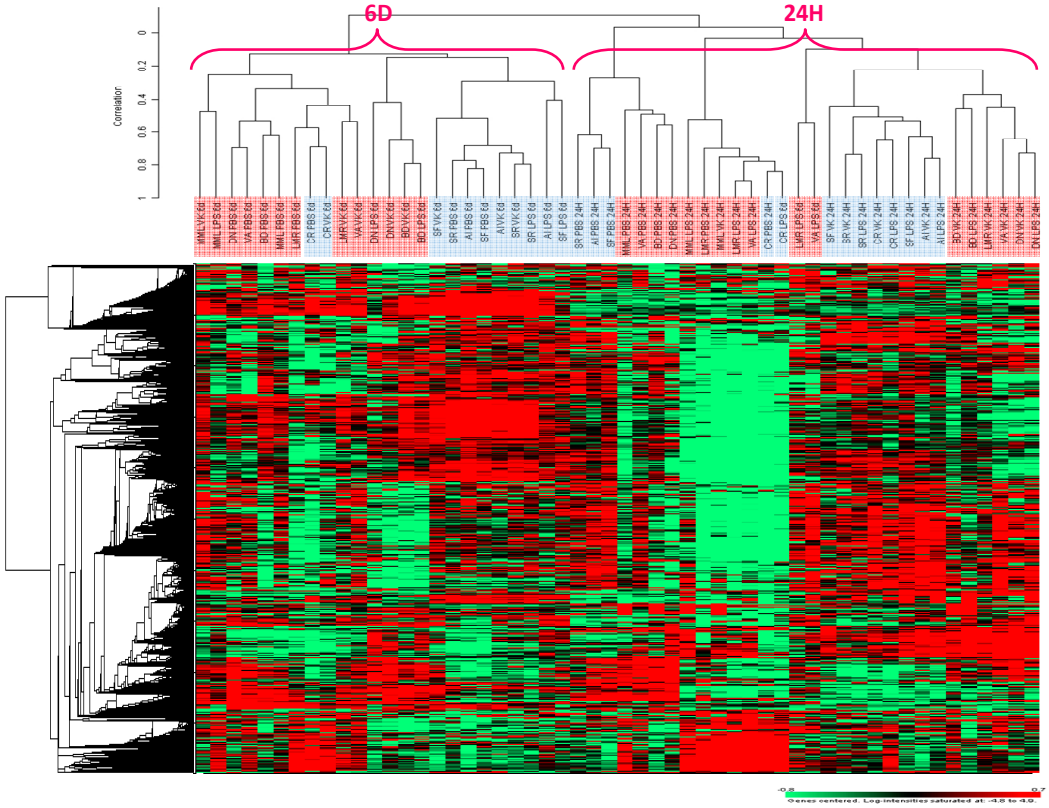

Fig. 15. Unsupervised hierarchical clustering. Unsupervised analysis including all samples from 4 healthy controls (light blue) and 5 HCV seropositive subjects (light red). Red indicates over-expression; green indicates under-expression; black indicates unchanged expression; gray indicates no detection of expression. Each row represents a single gene; each column represents a single sample. The dendrogram at the left of matrix indicates the degree of similarity among the genes examined by expression patterns. The dendrogram at the top of the matrix indicates the degree of similarity between samples.

3.4.2. Supervised analysis of *ex vivo* vaccinated PBMCs

The baseline gene expression profile of samples from HCV+ and HCV- subjects was evaluated by a pair-wise comparison analysis (Fig. 16).

The analysis identified 148 genes differentially expressed, of which 108 genes were shown to be up-regulated in samples from the HCV+ subjects and the remaining 40 genes were up-regulated in the samples from HCV- subjects. Interestingly, the gene signature of the HCV+ L.M.R. sample was intermediate between the HCV+ and HCV- samples, with an overall down-regulation of the identified genes.

Concerning the HCV+ subjects, most of the 108 up-regulated genes fell into several biological functions, including the defense and inflammatory responses (Table 3). The list of genes related to the inflammatory response is shown in Table 4.

Most of the remaining 40 genes were not annotated or associated to any significant cluster-function.

Such results indicate that HCV chronic infection induces a polarization of the gene expression towards an inflammatory response.

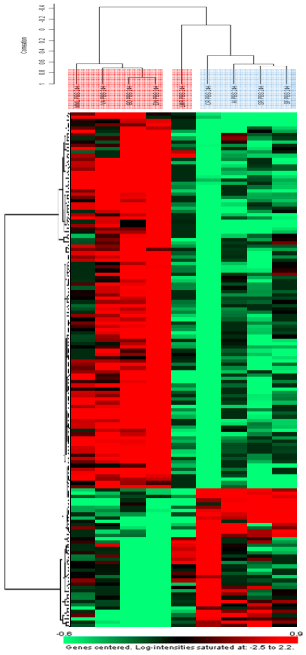

Fig. 16. Heat map of the gene signature identified by Class Comparison Analysis. Analysis comparing gene sets in non-stimulated PBMCs from HCV seropositive subjects (light red) and healthy controls (light blue) after 24h incubation. The expression pattern of the genes is shown, each row represents a single gene.

Table 3. DAVID analysis of baseline gene expression profile identified in HCV+ subjects. The p-value refers to the significance of the association of the gene ontology terms with the genes identified (count).

GENE ONTOLOGY TERM	COUNT	PERCENTAGE	P-VALUE
Response to wounding	17	15.88	9.36E-08
Glycosylation site: N-linked (GlcNAc...)	46	42.99	2.94E-07
Glycoprotein	47	43.92	3.22E-07
Vacuole	12	11.21	5.73E-07
Lytic vacuole	10	9.34	7.93E-06
Lysosome	10	9.34	7.93E-06
Disulfide bond	34	31.77	1.09E-05
Defense response	15	14.01	1.74E-05
Inflammatory response	11	10.28	2.58E-05

Table 4. Genomic signature of genes of inflammatory response up-regulated in HCV+ subjects. The p-value refers to the significance of the association of the specific gene ontology term with the gene list.

INFLAMMATORY RESPONSE (p-value: 2.6 E-5)

AFFYMETRIX_EXON_GENE_ID	GENE SYMBOL	GENE NAME
7920238	S100A12	S100 calcium binding protein A12
7920244	S100A8	S100 calcium binding protein A8
7905571	S100A9	S100 calcium binding protein A9
8086600	CCR1	chemokine (C-C motif) receptor 1
8072678	HMOX1	heme oxygenase (decycling) 1
7923907	IL10	interleukin 10
8058905	CXCR1	interleukin 8 receptor, alpha
7961142	OLR1	oxidized low density lipoprotein (lectin-like) receptor 1
8096301	SPP1	secreted phosphoprotein 1
8097903	TLR2	toll-like receptor 2
7897877	TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B

The gene expression profile of PBMCs *ex vivo* stimulated with VK3-20 protein was, subsequently, analyzed.

Normalized microarray expression data were evaluated to assess the effect of treatment by pair-wise comparisons between stimulated (VK3-20 treated) and non-stimulated (PBS) PBMCs from both HCV seropositive subjects and healthy controls. Integrated analyses were performed to identify the signature processes and pathways induced by VK3-20 treatment after 24 hours or 6 days and to compare VK3-20 treatment effects at 24 hours and 6 days.

The analysis comparing gene sets in stimulated PBMCs and non-stimulated PBMCs from HCV seropositive subjects and healthy controls after 24h incubation identified 503 genes differentially expressed. Among them, 300 genes were shown to be up-regulated and 203 genes were down-regulated in stimulated PBMCs (Figure 17). A separation in sub-clusters matching the HCV status was observed within the main VK3-20 cluster. No clear separation of samples based on HCV status was observed within the cluster of the PBS treatment (Figure 17).

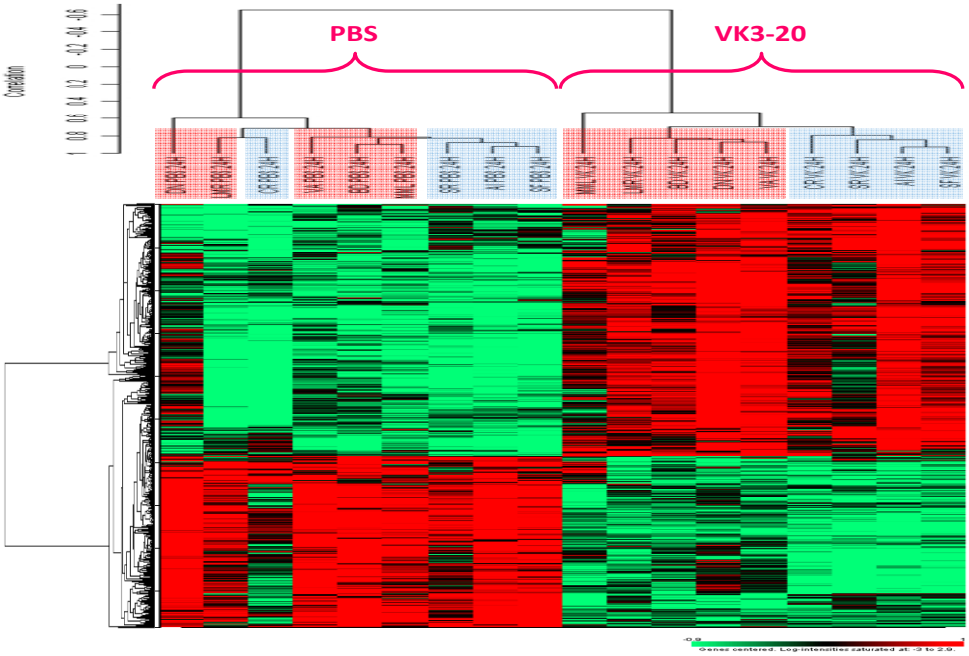

Fig. 17. Heat map of the gene signature identified by Class Comparison Analysis. Analysis comparing gene sets in stimulated and non-stimulated PBMCs from HCV seropositive subjects (light red) and healthy controls (light blue) after 24h incubation. The expression pattern of the genes is shown, each row represents a single gene.

Up-regulated genes were analyzed using DAVID Bioinformatics Database (http://david.abcc.ncifcrf.gov) for associations with particular Gene Ontology (GO) terms. Indeed, the top 20 genes up-regulated after treatment with the VK3-20 protein, showing the most significant association with the Immune Response GO term are listed in Table 5.

Table 5. Genomic signature of up-regulated genes identified in Figure 17. The p-value refers to the significance of the association of the specific gene ontology term with the gene list.

IMMUNE RESPONSE GO TERM (P-VALUE 1.1E-18)		
AFFYMETRIX_EXON_GENE_ID	**GENE SYMBOL**	**GENE NAME**
7953749	CLEC4D	C-type lectin domain family 4, member D
7960900	CLEC4E	C-type lectin domain family 4, member E
8154233	CD274	CD274 molecule
7909332	CD55	CD55 molecule, decay accelerating factor for complement (Cromer blood group)
8024792	EBI3	Epstein-Barr virus induced 3
7979269	GCH1	GTP cyclohydrolase 1
7906613	SLAMF7	SLAM family member 7
8066038	SLA2	Src-like-adaptor 2
8128939	TRAF3IP2	TRAF3 interacting protein 2
7921434	AIM2	absent in melanoma 2
7983910	AQP9	aquaporin 9
8006594	CCL18	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)
8160879	CCL19	chemokine (C-C motif) ligand 19
8048864	CCL20	chemokine (C-C motif) ligand 20
7996022	CCL22	chemokine (C-C motif) ligand 22
8014361	CCL23	chemokine (C-C motif) ligand 23
8014369	CCL3	chemokine (C-C motif) ligand 3
8019731	CCL3L3	chemokine (C-C motif) ligand 3-like 3; chemokine (C-C motif) ligand 3-like 1
8006602	CCL4	chemokine (C-C motif) ligand 4
8019651	CCL4L1	chemokine (C-C motif) ligand 4-like 1; chemokine (C-C motif) ligand 4-like 2

The analysis comparing gene sets in stimulated and non-stimulated PBMCs after 6 days incubation identified 149 genes differentially expressed. Among them, 123 were shown to be up-regulated and 26 were down-regulated in stimulated PBMCs from both HCV seropositive subjects and healthy controls (Figure 18). Even in this case a clear cut separation in sub-clusters matching the HCV status was observed within the main VK3-20 cluster (Figure 18)

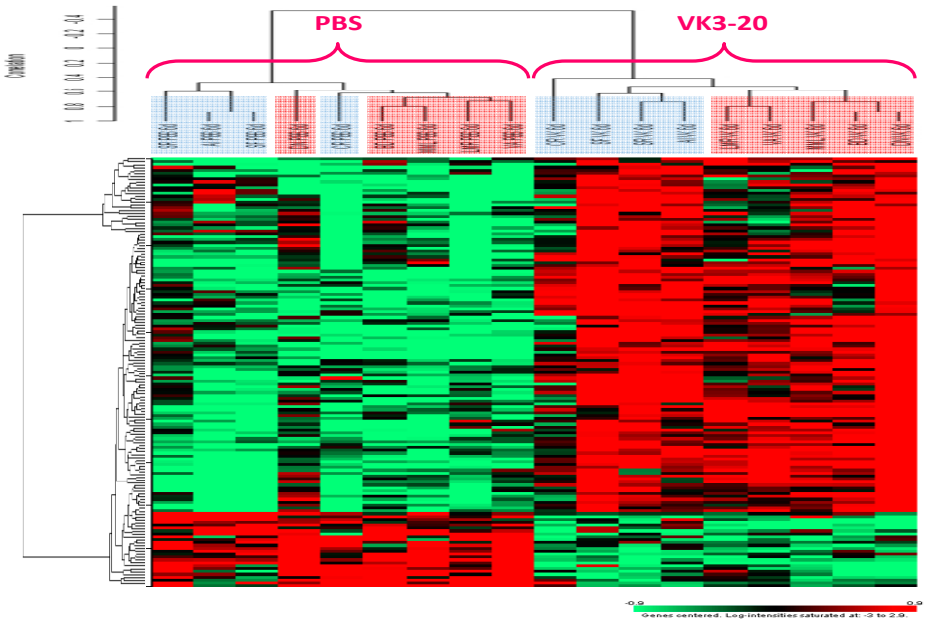

Fig. 18. Heat map of the gene signature identified by Class Comparison Analysis. Analysis comparing gene sets in stimulated and non-stimulated PBMCs from HCV seropositive subjects (light red) and healthy controls (light blue) after 6 days incubation. The expression pattern of the genes is shown, each row represents a single gene.

The genes up-regulated after treatment with VK3-20, showing the most significant association with the Response to Wounding GO term are listed in Table 6.

Table 6. Genomic signature of up-regulated genes identified in Figure 18. The p-value refers to the significance of the association of the specific gene ontology term with the gene list.

RESPONSE TO WOUNDING GO TERM (P-VALUE 2.8E-8)		
AFFYMETRIX_EXON_GENE_ID	**GENE SYMBOL**	**GENE NAME**
8063386	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta
7920244	S100A8	S100 calcium binding protein A8
8173287	VSIG4	V-set and immunoglobulin domain containing 4
8071899	ADORA2A	adenosine A2a receptor
8093294	CCR2	chemokine (C-C motif) receptor 2
8095886	CXCL13	chemokine (C-X-C motif) ligand 13
8030860	FPR2	formyl peptide receptor 2
7974851	HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
8007931	ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
8054722	IL1B	interleukin 1, beta
7968556	KL	klotho
7898057	PDPN	podoplanin
8062108	PROCR	protein C receptor, endothelial (EPCR)
7981068	SERPINA1	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1
8048283	SLC11A1	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1
7993624	SYT17	synaptotagmin XVII; synaptotagmin VII
7982597	THBS1	thrombospondin 1
8146500	LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
8106743	VCAN	versican

The analysis comparing gene sets in stimulated PBMCs after 24h and 6 days incubation identified 851 genes differentially expressed. Among them, 210 genes were shown to be up-regulated and 641 genes were down-regulated in stimulated PBMCs after 24h (Figure 19). The stimulation with VK3-20 induced a gene expression profile that was more similar within each group of analyzed subjects (i.e. HCV seropositive vs healthy controls) after 24h incubation compared to 6 days (Figure 19).

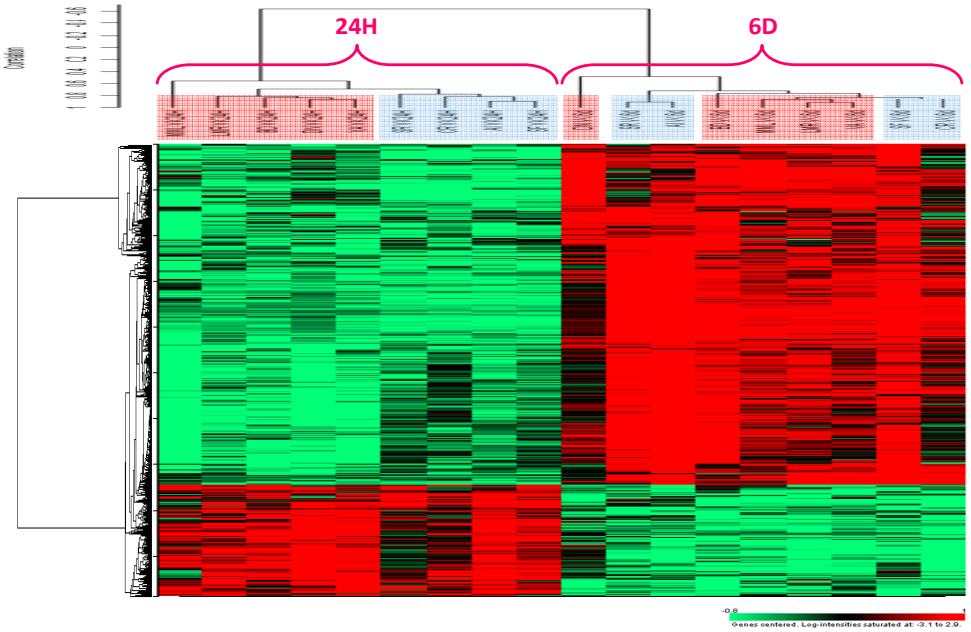

Fig. 19. Heat map of the gene signature identified by Class Comparison Analysis. Analysis comparing gene sets in stimulated PBMCs after 24h and 6 days incubation. The expression pattern of the genes is shown, each row represents a single gene.

The genes up-regulated after 24h of stimulation with the VK3-20 protein were significantly associated with Cytokine Activity (Table 7A).

On the other hand, most of the 641 genes up-regulated after 6 days incubation, clustered into several groups related to cell cycle activity, including: mitosis, nuclear division, nucleotide binding, protein-DNA complex assembly, chromatin organization. Indeed, the 20 top genes showing the most significant association with the Cell Cycle Gene Ontology term are listed in Table 7B.

Table 7A. Genomic signature of up-regulated genes identified, in Figure 19, after 24h stimulation. The p-value refers to the significance of the association of the specific gene ontology term with the gene list.

CYTOKINE ACTIVITY GO TERM (P-VALUE 7.5E-17)

AFFYMETRIX_EXON_GENE_ID	GENE SYMBOL	GENE NAME
8024792	EBI3	Epstein-Barr virus induced 3
8116818	BMP6	bone morphogenetic protein 6
8048864	CCL20	chemokine (C-C motif) ligand 20
8014369	CCL3	chemokine (C-C motif) ligand 3
8019731	CCL3L1	chemokine (C-C motif) ligand 3-like 3; chemokine (C-C motif) ligand 3-like 1
8019651	CCL4L1	chemokine (C-C motif) ligand 4-like 1; chemokine (C-C motif) ligand 4-like 2
8095697	CXCL13	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
8095886	CXCL13	chemokine (C-X-C motif) ligand 13
8100994	CXCL2	chemokine (C-X-C motif) ligand 2
8100984	CXCL3	chemokine (C-X-C motif) ligand 3
8100977	CXCL5	chemokine (C-X-C motif) ligand 5
8006999	CSF3	colony stimulating factor 3 (granulocyte)
8044541	IL1F9	interleukin 1 family, member 9
8044574	IL1RN	interleukin 1 receptor antagonist
8054712	IL1A	interleukin 1, alpha
8054722	IL1B	interleukin 1, beta
7923907	IL10	interleukin 10
7909271	IL24	interleukin 24
8142120	NAMPT	nicotinamide phosphoribosyltransferase
8075316	OSM	oncostatin M
8100971	PPBP	pro-platelet basic protein (chemokine (C-X-C motif) ligand 7)
8119898	VEGFA	vascular endothelial growth factor A

Table 7B. Genomic signature of up-regulated genes identified, in Figure 19, after 6 days stimulation. The p-value refers to the significance of the association of the specific gene ontology term with the gene list.

CELL CYCLE GO TERM (P-VALUE 1.6E-49)

AFFYMETRIX_EXON_GENE_ID	GENE SYMBOL	GENE NAME
8112327	CKS1B	CDC28 protein kinase regulatory subunit 1B
8156290	CKS2	CDC28 protein kinase regulatory subunit 2
8071212	CDC45	CDC45 cell division cycle 45-like (S. cerevisiae)
7945014	CHEK1	CHK1 checkpoint homolog (S. pombe)
7936408	DCLRE1A	DNA cross-link repair 1A (PSO2 homolog, S. cerevisiae)
7947110	E2F8	E2F transcription factor 8
8130374	FBXO5	F-box protein 5
8077731	FANCD2	Fanconi anemia, complementation group D2
7985829	FANCI	Fanconi anemia, complementation group I
8059838	HJURP	Holliday junction recognition protein
8102560	MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)
8019857	NDC80	NDC80 homolog, kinetochore complex component (S. cerevisiae)
7924096	NEK2	NIMA (never in mitosis gene a)-related kinase 2
7906930	NUF2	NUF2, NDC80 kinetochore complex component, homolog (S. cerevisiae)
7987636	OIP5	Opa interacting protein 5
7963157	RACGAP1	Rac GTPase activating protein 1 pseudogene; Rac GTPase activating protein 1
8090448	RUVBL1	RuvB-like 1 (E. coli)
8020254	SEH1L	SEH1-like (S. cerevisiae)
8056572	SPC25	SPC25, NDC80 kinetochore complex component, homolog (S. cerevisiae)
7989915	TIPIN	TIMELESS interacting protein

In addition, the gene expression profile of PBMCs, from healthy subjects, *ex vivo* stimulated with LPS, as a positive activator of genes involved in the pathway of immune response, was analyzed and compared to the gene expression profile of PBMCs *ex vivo* stimulated with VK3-20.

To this aim, a pair-wise comparison between non-stimulated (PBS) and LPS-stimulated PBMCs from the HCV- subjects was performed. The analysis showed 580 genes differentially expressed, among them 384 were up-regulated after LPS treatment and 196 were down-regulated (Fig. 20A).

In parallel, a pair-wise comparison between non-stimulated (PBS) and VK3-20-stimulated PBMCs from the HCV- subjects was performed. The analysis showed 329 genes differentially expressed, among them 218 were up-regulated after VK3-20 treatment and the remaining 111 were down-regulated (Fig. 20B).

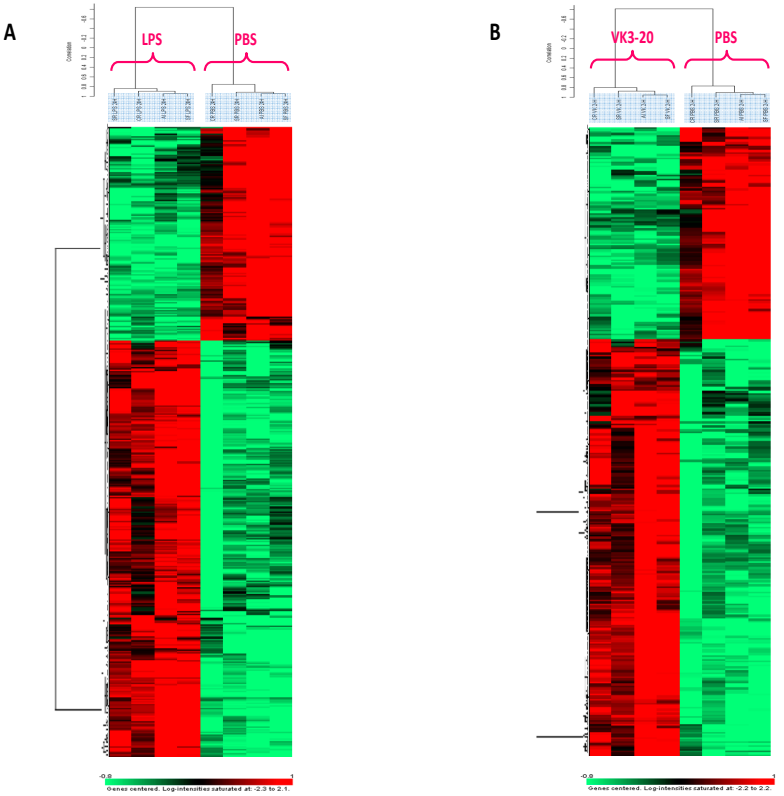

Fig. 20 Heat maps of the gene signature identified by Class Comparison Analyses. Analyses comparing gene sets in LPS stimulated PBMCs from HCV- subjects **(A)** and VK3-20 stimulated PBMCs from HCV- subjects **(B)** after 24h incubation. The expression pattern of the genes is shown, each row represents a single gene.

It is relevant that 180 genes were up-regulated after treatment with both LPS and VK3-20, indicating a strong overlapping effect induced by VK3-20 and LPS in the regulation of genes (i.e. chemokines and cytokines) involved in the process of defense response pathway (most significant association) (Table 8).

Moreover, among genes significantly up-regulated only after LPS treatment, many were involved in the pathway of immune response, including CD83 (see figure 7), which plays a central role in antigen presentation, and CD274, which is specifically up-regulated on macrophages and DCs in response to LPS, inducing IL-10 production, which, in turn, inhibits $CD4^+$ T cell function [75-79]. Table 9 summarizes the results obtained.

Interestingly, among genes down-regulated, only, after LPS treatment, we found CD86 (see figure 7), which is involved in costimulatory signal, essential for T cell proliferation and IL-2 production, by binding CD28 for stimulatory signal or CTLA-4 for inhibitory signal [80].

Table 8. Genomic signature induced by LPS and VK3-20 stimulation in healthy controls (overlapping genes). The p-value refers to the significance of the association of the specific gene ontology term with the gene list.

DEFENSE RESPONSE GO TERM P-VALUE 1.7E-18		
AFFYMETRIX_EXON_GENE_ID	**GENE SYMBOL**	**GENE NAME**
8031213	LILRA1	leukocyte immunoglobulin-like receptor, subfamily A, member 1
8039226	LILRA3	leukocyte immunoglobulin-like receptor, subfamily A, member 3
7906613	SLAMF7	SLAM family member 7
8115210	TNFAIP3	TNFAIP3 interacting protein 1
8071899	ADORA2A	adenosine A2a receptor
8030860	FPR2	formyl peptide receptor 2
8146092	IDO1	indoleamine 2,3-dioxygenase 1
8139207	INHBA	inhibin, beta A
8044574	IL1RN	interleukin 1 receptor antagonist
8043995	IL1R1	interleukin 1 receptor, type I
8054712	IL1A	interleukin 1, alpha
8054722	IL1B	interleukin 1, beta
7923907	IL10	interleukin 10
7931914	IL2RA	interleukin 2 receptor, alpha
8131803	IL6	interleukin 6 (interferon, beta 2)
8095680	IL8	interleukin 8
8077786	IRAK2	interleukin-1 receptor-associated kinase 2
7934161	PRF1	perforin 1 (pore forming protein)
8092134	PLD1	phospholipase D1, phosphatidylcholine-specific
8040080	RSAD2	radical S-adenosyl methionine domain containing 2
7897877	TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B
8045688	TNFAIP6	tumor necrosis factor, alpha-induced protein 6

Table 9. Genomic signature induced by LPS stimulation in healthy controls. The p-value refers to the significance of the association of the specific gene ontology term with the gene list.

DEFENSE RESPONSE GO TERM P-VALUE 1.5E-5

AFFYMETRIX_EXON_GENE_ID	GENE SYMBOL	GENE NAME
8116983	CD83	CD83 molecule
8160559	DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58
8015511	DHX58	DEXH (Asp-Glu-X-His) box polypeptide 58
7979269	GCH1	GTP cyclohydrolase 1
8178977	TAPBP	TAP binding protein (tapasin)
8072735	APOL1	apolipoprotein L, 1
8014361	CCL23	chemokine (C-C motif) ligand 23
7917875	F3	coagulation factor III (thromboplastin, tissue factor)
7909350	CR2	complement component (3d/Epstein Barr virus) receptor 2
8179351	CFB	complement factor B
8056285	IFIH1	interferon induced with helicase C domain 1
8045539	KYNU	kynureninase (L-kynurenine hydrolase)
8083594	PTX3	pentraxin-related gene, rapidly induced by IL-1 beta
8098611	TLR3	toll-like receptor 3
8180061	TAP1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)

3.4.3. Ingenuity Pathways Analysis

The Ingenuity Pathways Analysis (IPA) was performed to understand the biological role of genes differentially expressed in the supervised comparisons.

Gene expression changes induced by *ex vivo* "vaccination" after 24h or 6 days incubation were obtained by subtracting the $\log_2$ expression value induced by PBS (non-stimulation/baseline) from the one induced by VK3-20.

The human pathway lists were determined by "Ingenuity System Database", setting the significance threshold of t-test at 0.05.

Samples from VK3-20-stimulated PBMCs after 24h showed a strong up-regulation of genes involved in several pathways, including: Communication between Innate and Adaptive Immune Cells, Acute Phase Response Signaling, LXR/RXR (liver X receptor/retinoid X receptor) Activation, IL-6 Signaling, Dendritic Cell Maturation, Role of Cytokines in Mediating Communication between Immune Cells, TREM1 (triggering receptor expressed on myeloid cells) Signaling (Fig. 21).

Indeed, VK3-20 was able to induce the activation of genes of the Acute Phase Response, which is a rapid inflammatory reaction triggered by infection, tissue injury, neoplastic growth and immunological disorders. This is characterized by the increase of inflammatory factors (such as pro-inflammatory cytokines IL-1, IL-6, TNF-α) and the change in the concentration of several plasma proteins (the acute phase proteins), including: fibrinogen, haptoglobin, alpha-1-acid glycoprotein, alpha-1-antitrypsin, serum amyloid A, C-reactive protein [81]. Positive acute phase response proteins are detectable as early as 4-5 hours after a single inflammatory stimulus and their levels remain elevated for at least 24 hours, decreasing after 48 hours. Positive acute phase response proteins play a crucial role in opsonization of micro-organisms, complement activation and modulation of the immune response [82].

On the other hand, at 6 days post-stimulation, genes involved in the Role of Tissue Factor in Cancer, ILK (integrin linked kinase) Signaling, IL-17 Signaling and G-Protein Coupled Receptor Signaling ranked at the top and were up-regulated in PBMCs (Fig. 22).

Among such "late" pathways, the IL-17 Signaling is relevant to the present study. IL-17, also referred to as IL-17A, is a member of a family of cytokines primarily secreted by T cells [83], whose function is essential to a subset of $CD4^+$ T cells called T helper 17 (Th17) cells. IL-17 promotes the expansion and recruitment of innate immune cells, stimulating the production of anti-microbial peptides [84].

IL-17 binds to and signals through a transmembrane, heterotrimeric receptor complex, activating NF-kB and MAPK pathways via adapter molecule TRAF6. Other signaling pathways include Ras/ERK and the JAK/PI3K/AKT, that lead to the induction of pro-inflammatory cytokines, chemokines, matrix metalloproteinases and anti-microbial peptides [85]. Indeed, IL-17 is a unique cytokine, playing a central role in integrating adaptive and innate immune responses.

Other significant canonical pathways positively modulated after VK3-20 stimulation are listed in Figures 21 and 22.

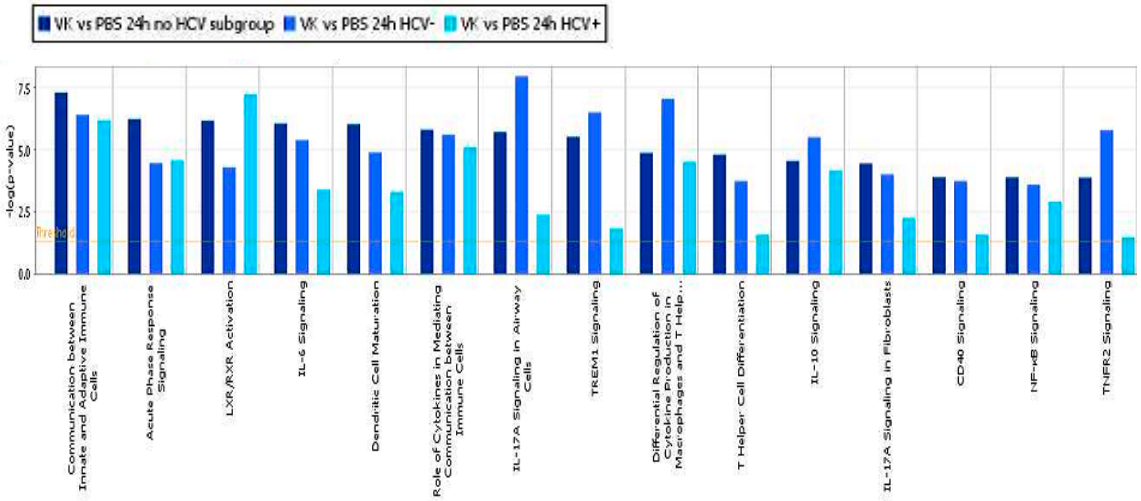

Fig. 21. Ingenuity Pathways Analysis. Analysis of canonical pathways up-regulated in stimulated PBMCs compared to non-stimulated PBMCs after 24h incubation.

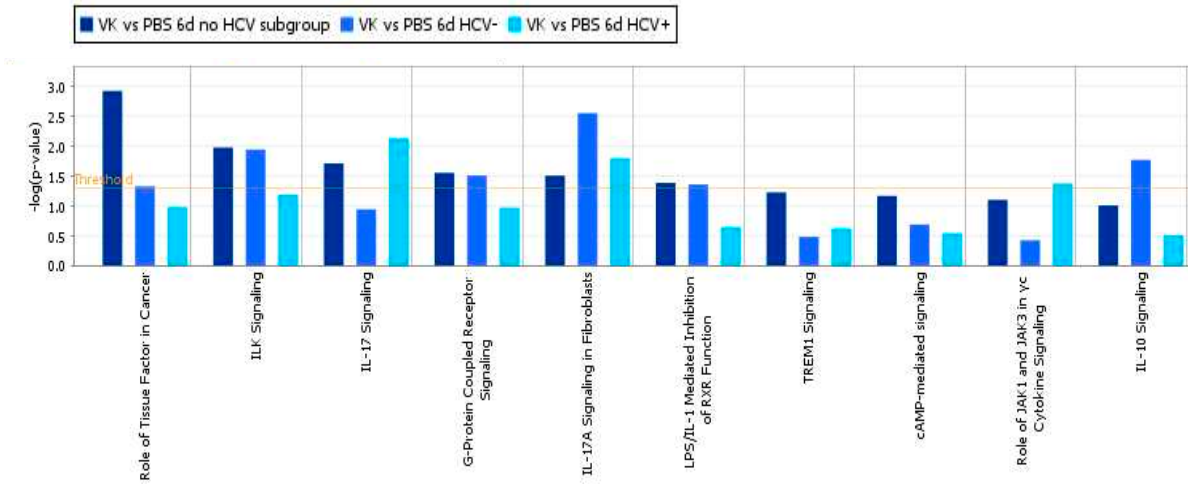

Fig. 22. Ingenuity Pathways Analysis. Analysis of canonical pathways up-regulated in stimulated PBMCs compared to non-stimulated PBMCs after 6 days incubation.

Moreover, a comparison analysis of top canonical pathways activated by VK3-20 after 24h and 6 days incubation both in HCV seropositive subjects and in healthy controls was performed and results are shown in Figure 23.

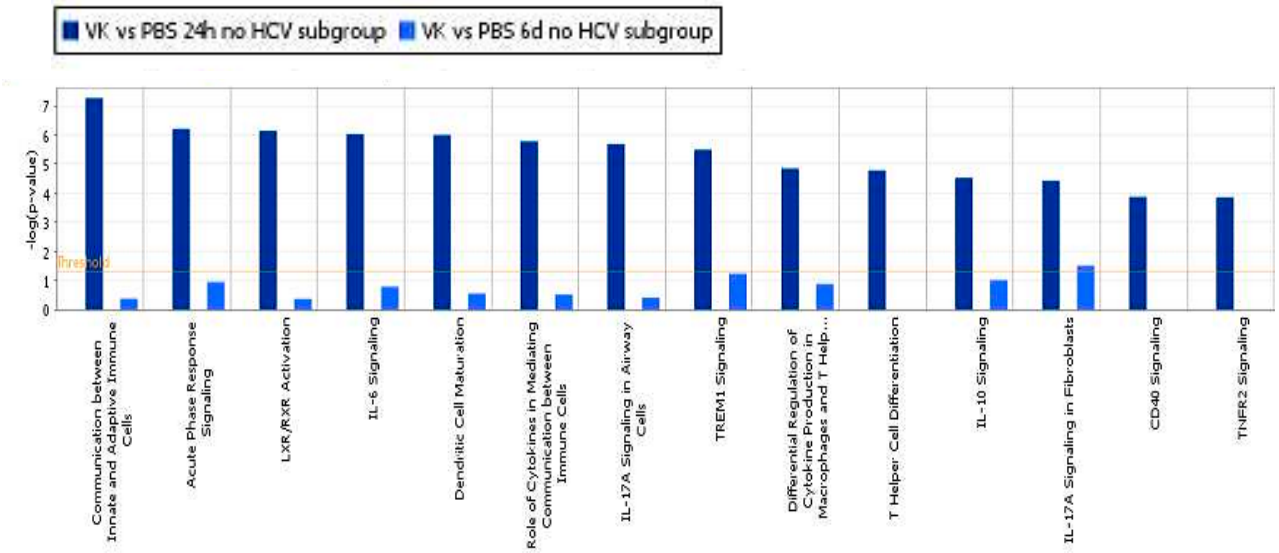

Fig. 23. Ingenuity Pathways Analysis. Comparison analysis of canonical pathways up-regulated in stimulated PBMCs after 24h and 6 days incubation.

Among differentially expressed genes, modulated by VK3-20 after 24h and 6 days incubation, Ingenuity Pathways Analysis identified a specific network related to the Inflammatory Response (Fig. 24-27). The analysis showed that VK3-20 induced a gene signature in which NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) protein complex plays a crucial role. Indeed, NF-κB complex is known to be involved in cellular responses to several stimuli, including stress, cytokines, ultraviolet irradiation and bacterial or viral antigens [86]. NF-κB protein complex is, also, known to play a critical role in regulating the immune response to infections [87]. Incorrect regulation of NF-κB has been linked to cancer, inflammatory and autoimmune diseases as well as viral infections [88].

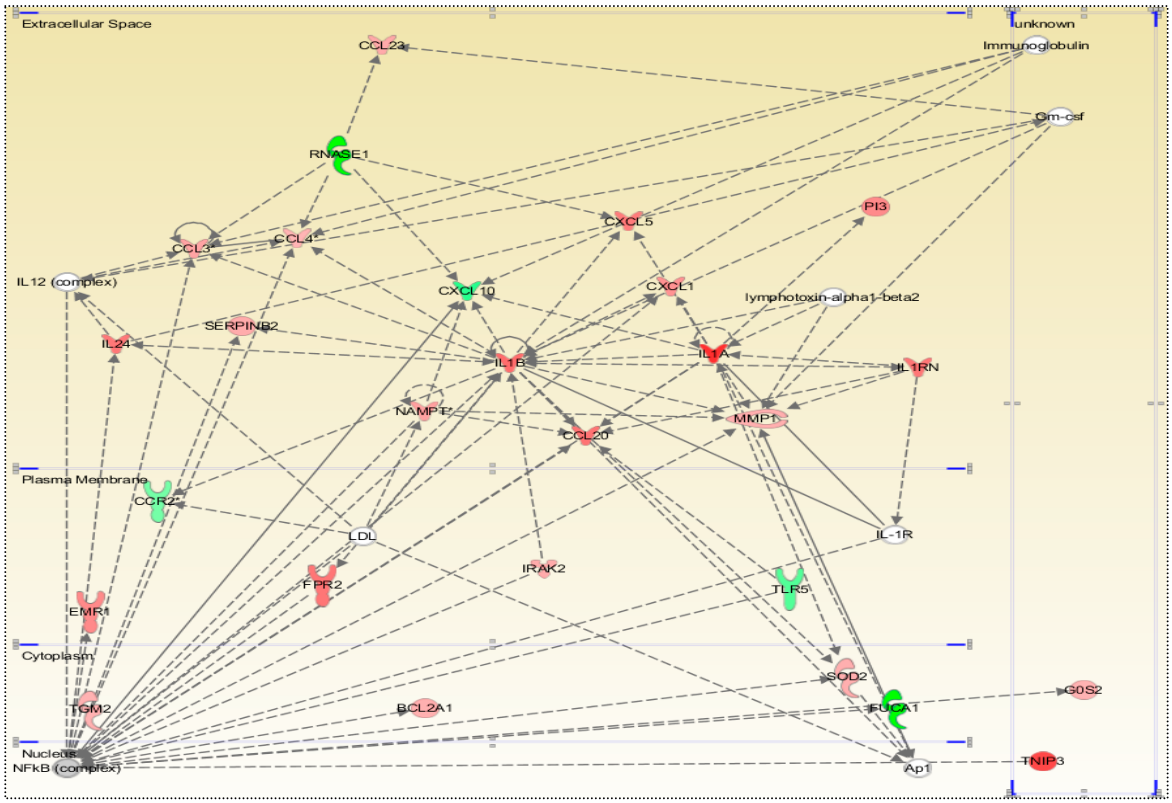

Fig. 24. Inflammatory Response Network in stimulated PBMCs after 24h incubation.

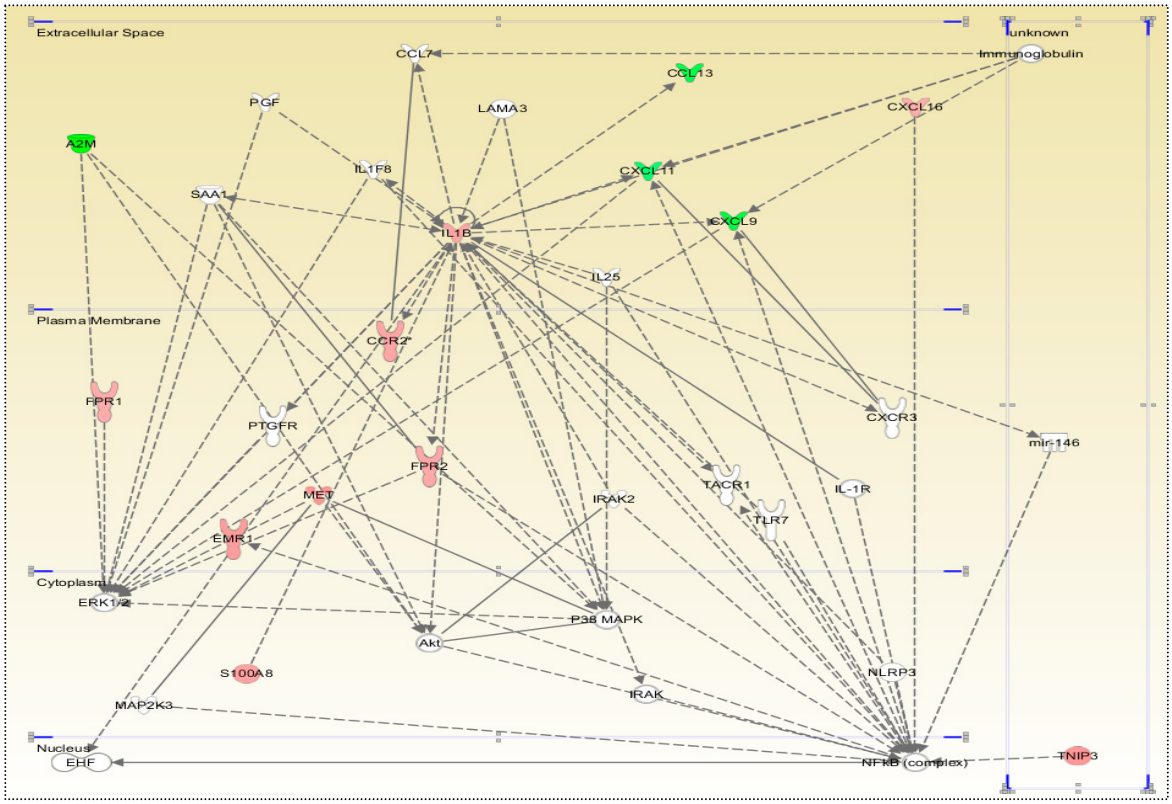

Fig. 25. Inflammatory Response Network in stimulated PBMCs after 6 days incubation.

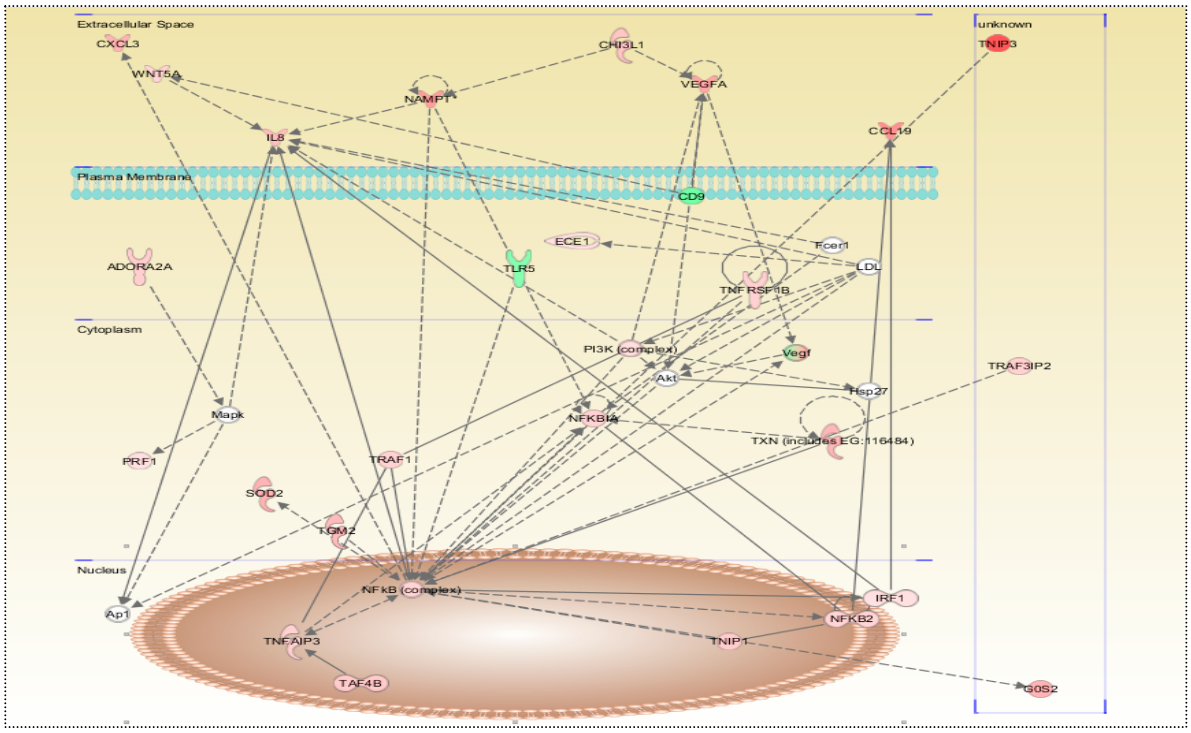

Fig. 26. Inflammatory Response Network in stimulated PBMCs from HCV- subjects after 24h incubation.

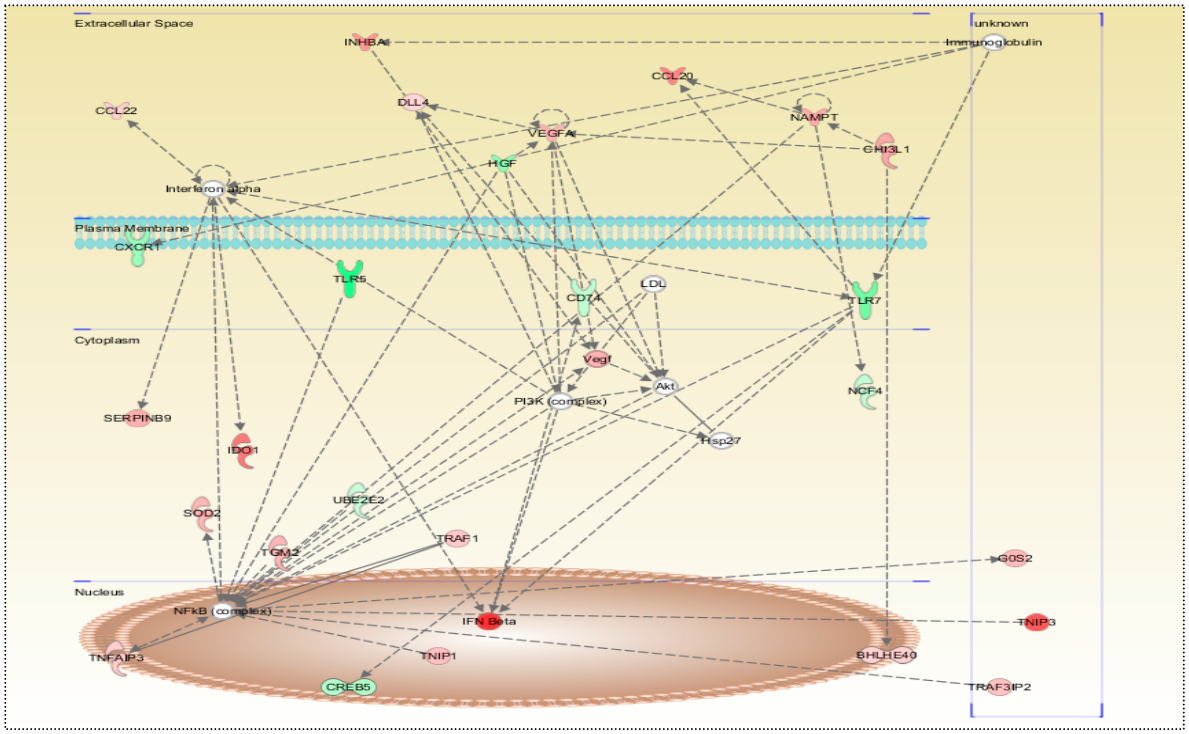

Fig. 27. Inflammatory Response Network in stimulated PBMCs from HCV+ subjects after 24h incubation.

Furthermore, the analysis of top biological functions of significantly up-regulated genes identified in response to VK3-20 stimulation after 24h and 6 days incubation was performed and results are shown in Figures 28-30.

In particular, genes whose function is involved in inflammatory response, cellular growth and proliferation, cancer and immunological disease ranked at the top and were up-regulated in VK3-20 stimulated PBMCs after 24h (Fig. 28).

On the other hand, genes involved in immune cell trafficking, inflammatory response, hematological system development and function were up-regulated in VK3-20 stimulated PBMCs after 6 days (Fig. 29).

Moreover, a comparison analysis of top functions activated by VK3-20 after 24h and 6 days incubation both in HCV seropositive subjects and in healthy controls was performed and results are shown in Figure 30.

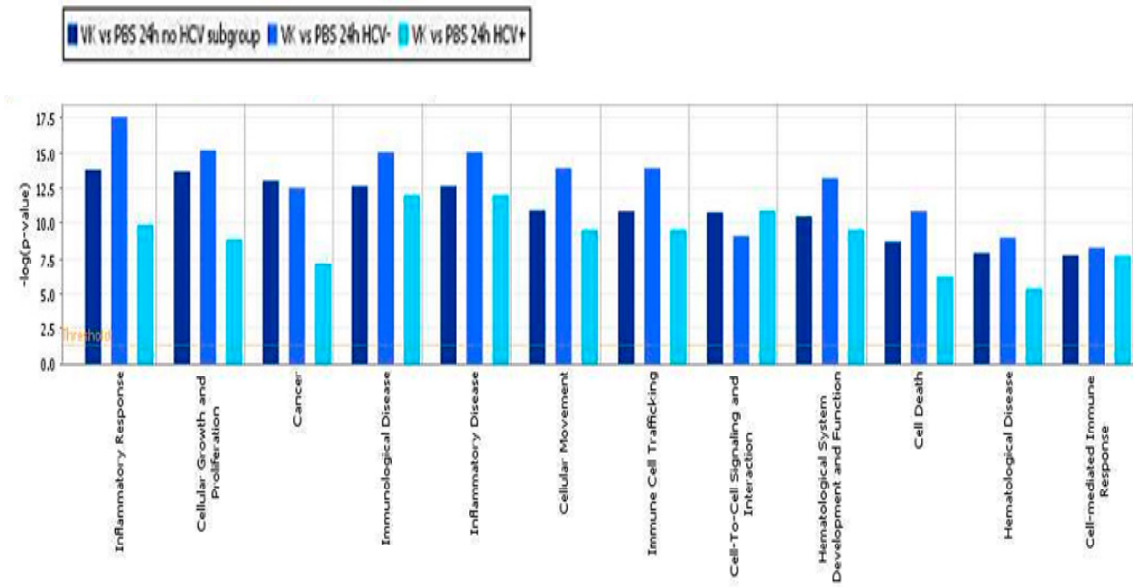

Fig. 28. Ingenuity Pathways Analysis. Analysis of top functions in stimulated PBMCs compared to non-stimulated PBMCs after 24h incubation.

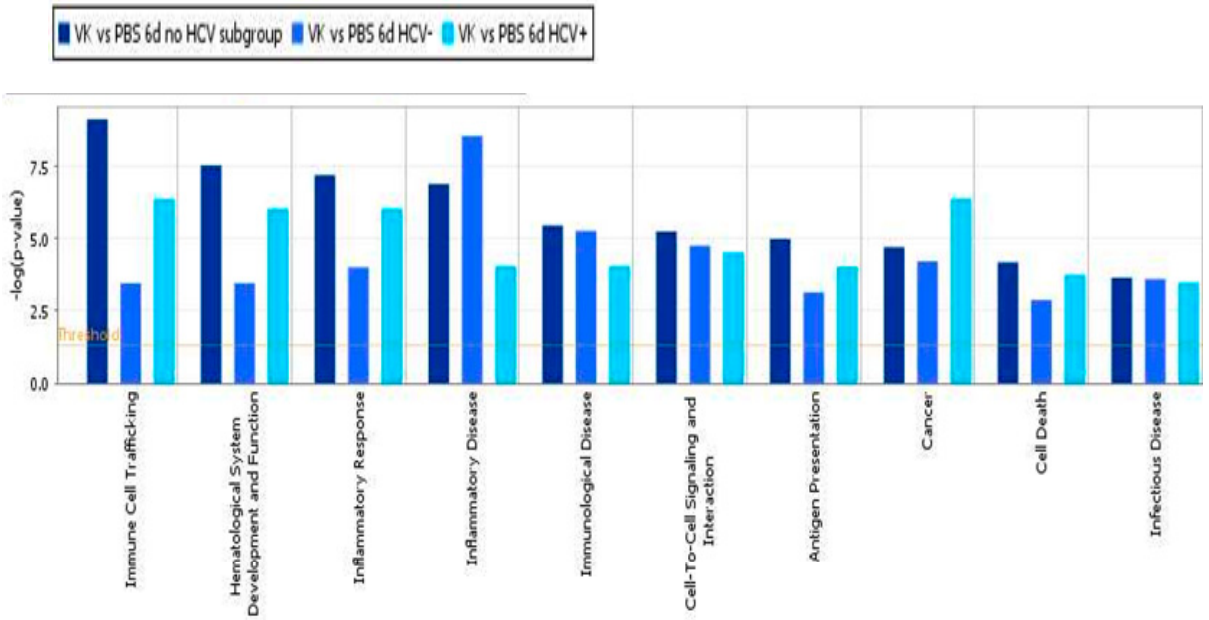

Fig. 29. Ingenuity Pathways Analysis. Analysis of top functions in stimulated PBMCs compared to non-stimulated PBMCs after 6 days incubation.

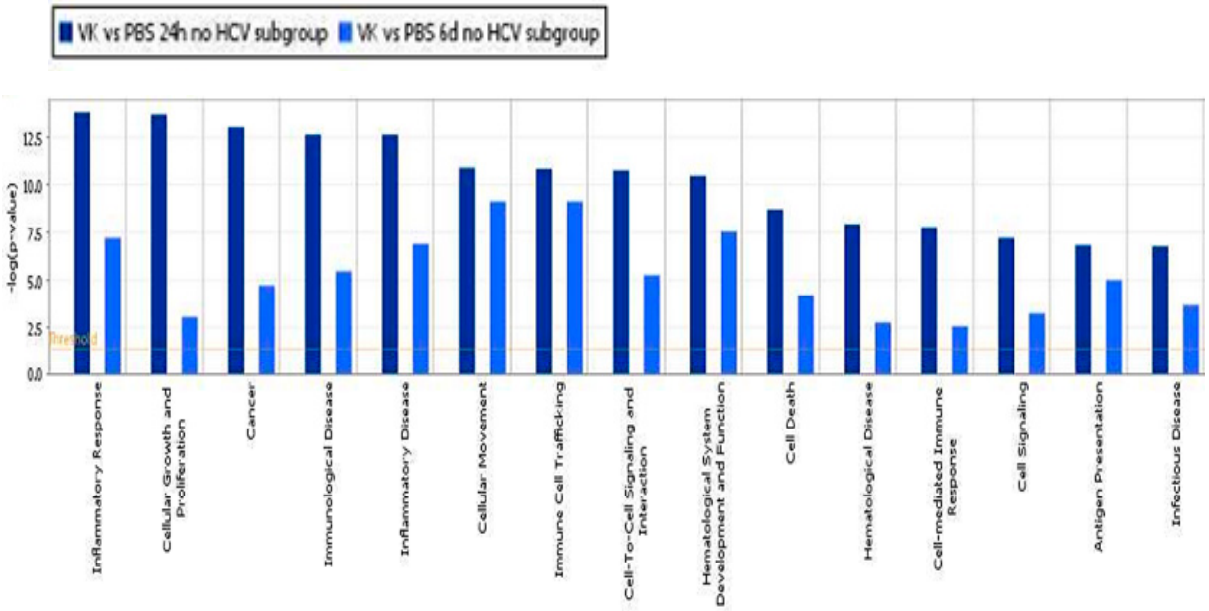

Fig. 30. Ingenuity Pathways Analysis. Comparison analysis of top functions in stimulated PBMCs after 24h and 6 days incubation. Genes involved in inflammatory response, cellular growth and proliferation, cancer and immunological disease ranked at the top.

3.4.4. Integrated analysis of the immune signature induced by recombinant VK3-20 protein

The large number of genes modulated by VK3-20 and to some extent involved in the pathways of immune response has prompted us to investigate in more detail their specific role.

In this framework, the immune signature of PBMCs *ex vivo* stimulated with the recombinant VK3-20 protein was analyzed. To this aim, normalized microarray expression data were evaluated to determine the effect of VK3-20 on the immune response, by pair-wise comparisons between VK3-20-stimulated and non-stimulated PBMCs, using a specific CGAP (Cancer Genome Anatomy Project) immunology gene list for supervised analysis.

The resulting immune networks were visualized using Cytoscape (http://www.cytoscape.org), an open source software for complex network analysis and visualization; gene function was assigned based on DAVID Bioinformatics Database.

The analysis comparing gene sets in stimulated and non-stimulated PBMCs from healthy controls after 24h incubation identified 58 CGAP genes differentially expressed. Among them, 36 genes were shown to be up-regulated and 22 genes were down-regulated in stimulated PBMCs (Fig. 31A).

The same analysis performed on samples from HCV seropositve subjects identified 54 CGAP genes differentially expressed. Among them, 23 genes were shown to be up-regulated and 31 genes were down-regulated in stimulated PBMCs (Fig. 31B).

The analysis comparing gene sets in VK3-20-stimulated and non-stimulated PBMCs from healthy controls after 6 days incubation identified 24 CGAP genes differentially expressed. Among them, 17 were shown to be up-regulated and 7 were down-regulated in stimulated PBMCs (Fig. 32A).

The same analysis on samples from HCV seropositve subjects identified 27 genes differentially expressed. Among them, 11 genes were shown to be up-regulated and 16 genes were down-regulated in stimulated PBMCs (Fig. 32B). All genes differentially expressed are listed in the heat maps in Figures 31-32.

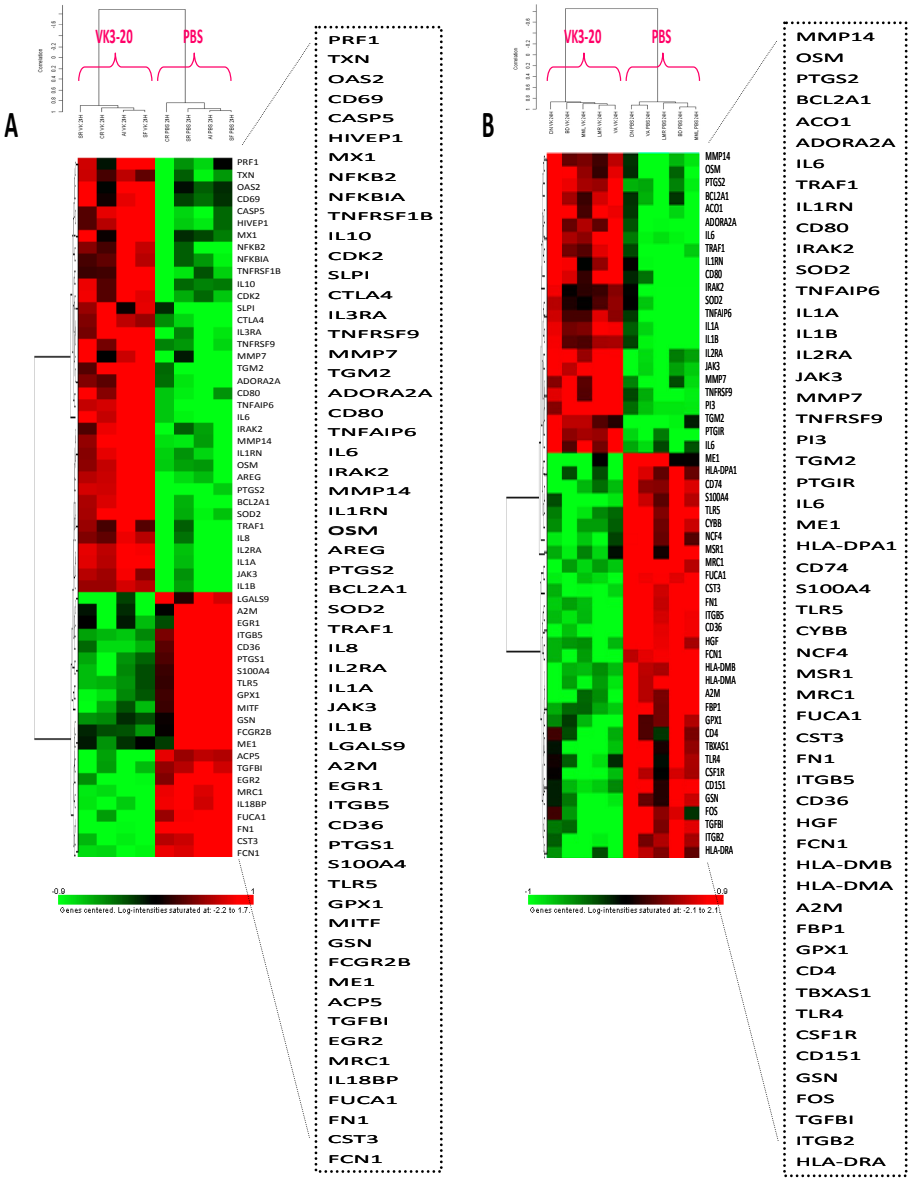

Fig. 31. Heat map of the immune gene signature identified by Class Comparison Analysis. Analysis comparing immune genes in stimulated vs non-stimulated PBMCs from healthy controls (A) and HCV seropositive subjects (B) after 24h incubation. All genes differentially expressed are listed.

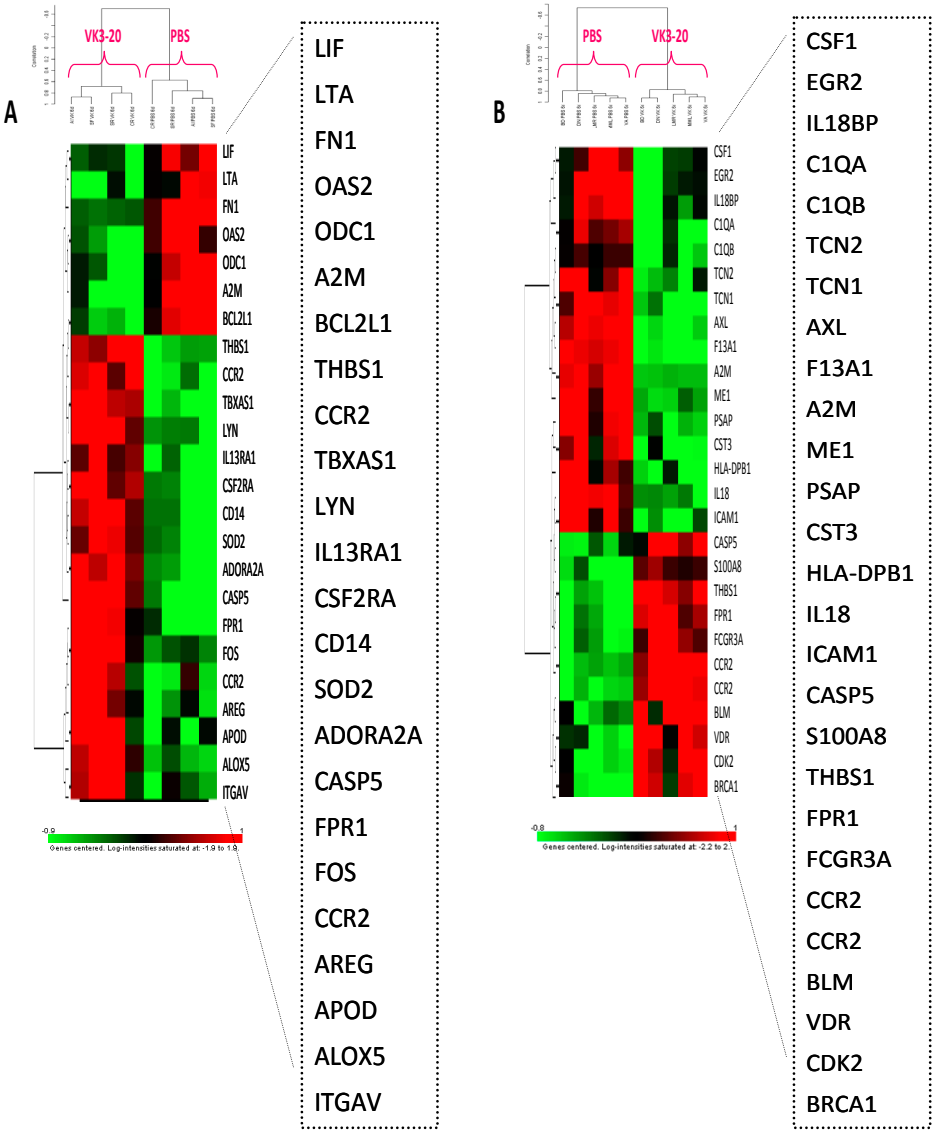

Fig. 32. Heat map of the immune gene signature identified by Class Comparison Analysis. Analysis comparing immune genes in stimulated vs non-stimulated PBMCs from healthy controls (A) and HCV seropositive subjects (B) after 6 days incubation. All genes differentially expressed are listed.

The integrated analysis of modulated genes was performed using Cytoscape and results are shown in Figures 33-36.

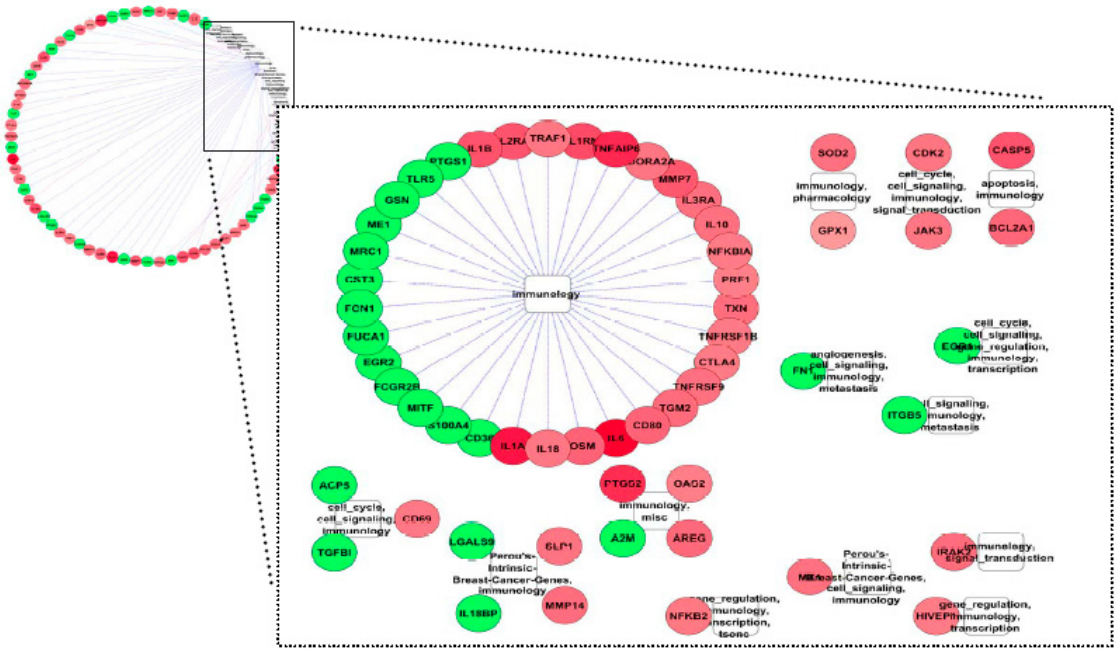

Fig. 33. Cytoscape analysis. Integrated analysis of significantly modulated immune genes from HCV- stimulated PBMCs, incubated for 24h. Red indicates over-expression; green indicates under-expression.

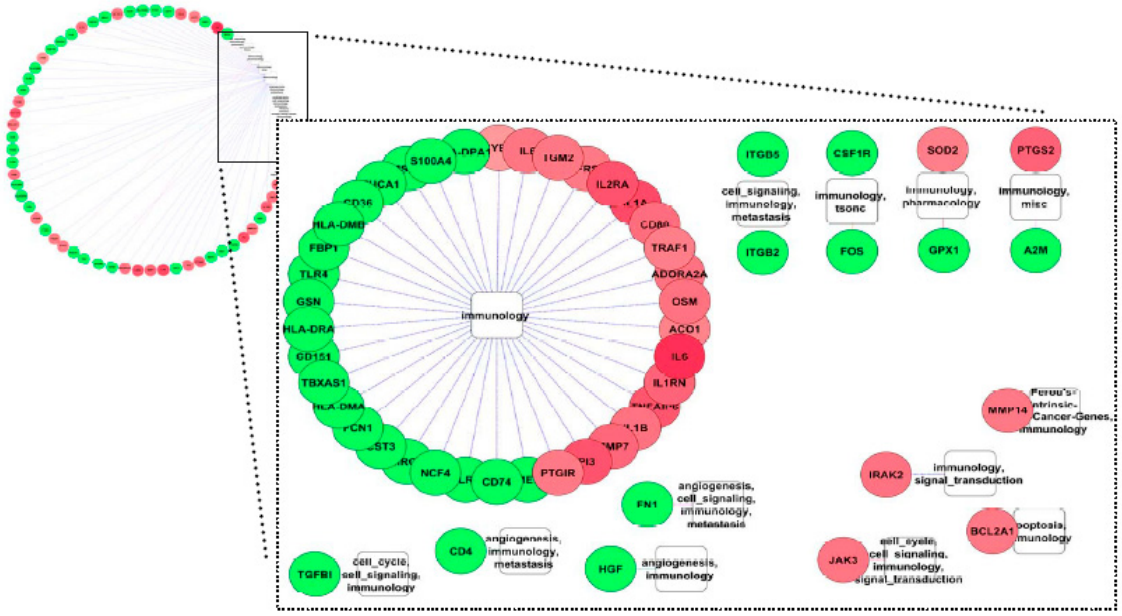

Fig. 34. Cytoscape analysis. Integrated analysis of significantly modulated immune genes from HCV+ stimulated PBMCs, incubated for 24h. Red indicates over-expression; green indicates under-expression.

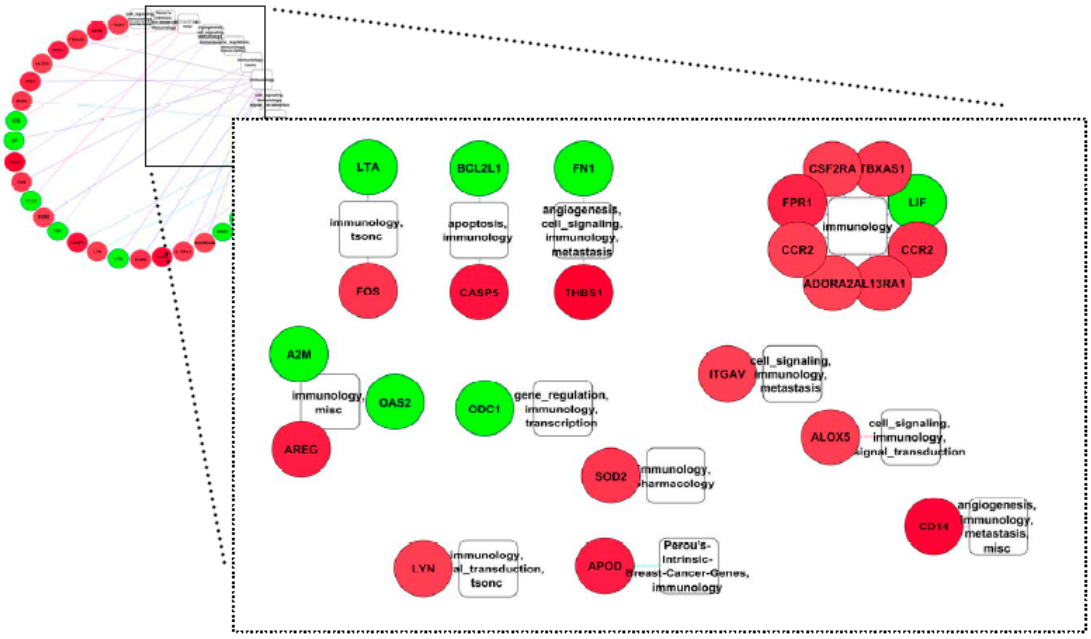

Fig. 35. Cytoscape analysis. Integrated analysis of significantly modulated immune genes from HCV- stimulated PBMCs, incubated for 6 days. Red indicates over-expression; green indicates under-expression.

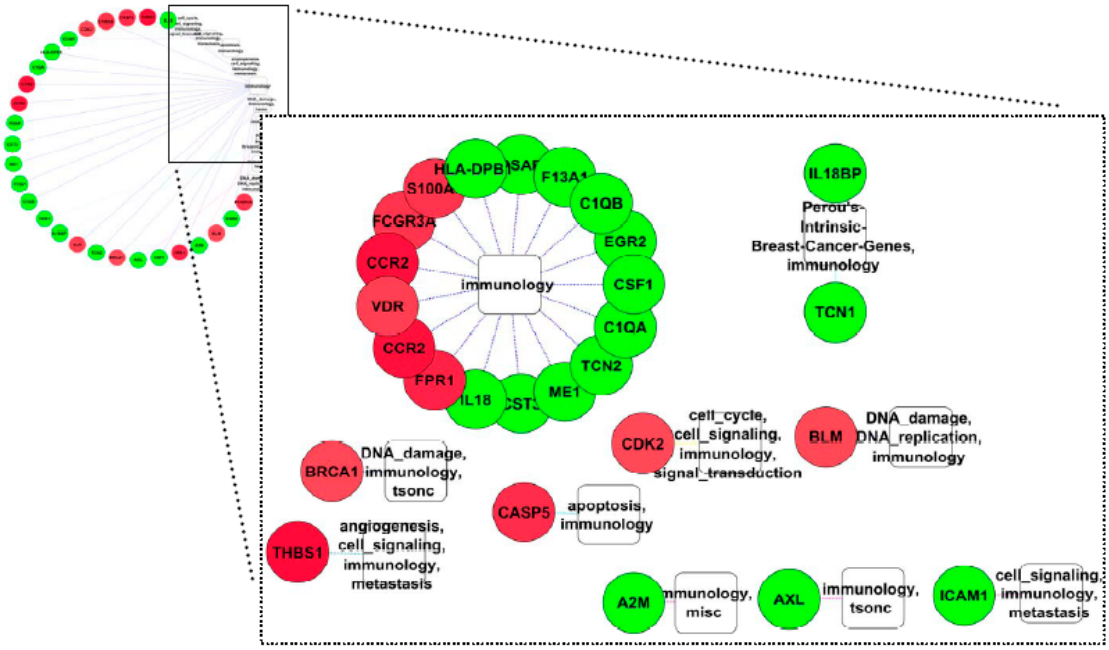

Fig. 36. Cytoscape analysis. Integrated analysis of significantly modulated immune genes from HCV+ stimulated PBMCs, incubated for 6 days. Red indicates over-expression; green indicates under-expression

In particular, the immunology networks from stimulated PBMCs incubated for 24h (Fig. 33-34) was characterized by a significant up-regulation of genes of the cytokine and cytokine receptor families, as well as of downstream signaling cascades, including IL-1, IL-6, OSM (oncostatin M), IL-10, TNFRSF1B (tumor necrosis factor receptor superfamily member 1B) [89], TNFAIP6 (tumor necrosis factor alpha-induced protein 6) [90], IRAK2 (interleukin-1 receptor-associated kinase-like 2) [91], TRAF1 (TNF receptor-associated factor 1) [92] and JAK3 [93].

Indeed, both IL1A and IL1B were found up-regulated after 24h stimulation. IL-1 cytokine is known to be produced by activated macrophages, it is an important mediator of the inflammatory response and is involved in several cellular activities, including cell proliferation, differentiation and apoptosis [94].

In particular, IL-1 shares with TNF-α similar receptor mechanisms and downstream pathways [95]. Indeed, the potent pro-inflammatory activity of these cytokines is triggered by the activation of the same set of transcription factors (i.e. MAPK8/JNK and NF-kB) [95]. The individual signal transduction pathways initiated by these two cytokines are integrated in a common signaling cascade involving the so called TNF receptor associated factors (TRAFs).

Our results, strongly suggest a VK3-20-mediated activation of IL-1 and TNF-α signaling pathways, given the up-regulation of several genes, including IRAK2, which becomes associated with the IL-1 receptor (IL1R) upon stimulation, participating in IL1-mediated up-regulation of NF-kB through TRAF6 [91].

Moreover, we found that TRAF1 was, also, up-regulated [92]. The protein is a member of the TNF receptor associated factor protein family. TRAF1 is able to form a heterodimeric complex with TRAF2, which is required for TNF-alpha-mediated activation of MAPK8/JNK and NF-kB.

Furthermore, several genes specifically induced by IL-1 and TNF-α were found to be up-regulated by VK3-20 stimulation, including TNFAIP6, which plays an important role in protease network associated with inflammation [90] and BCL2-related protein A1 (BCL2A1), which is a direct transcription target of NF-kB [96]. Finally, NFKB2 subunit was found to be up-regulated, as well.

Besides the up-regulation of several mediators of innate immunity, such as chemokines (i.e. IL-8) and pro-inflammatory cytokines including IL-1, TNF-α and IL-6, our results strongly suggest the activation of an early adaptive immune response, based on cytokine modulation of T cell activity.

In this framework, IL-2 receptor alpha (IL2RA) was found to be up-regulated. It is well known that IL-2/IL-2R interaction is able to stimulate T cell growth, differentiation and survival via activation of the expression of specific genes, including cyclin E, which binds G1-phase cyclin-dependent kinase

2 (CDK2), which we found to be up-regulated, and BCL2 (BCL2A1) which is required for T cell survival [97].

The binding of the receptor for IL-2 activates the Ras/MAPK, as well as JAK/Stat and PI3-kinase/Akt signaling pathways [98].

In particular, JAK3, which is mostly expressed in T cells and NK cells, where it is involved in cytokine signaling transduction via type I cytokine receptor family (i.e. IL-2R, IL-4R, IL-7R, IL-9R, IL-15R) was found to be up-regulated, as well [98].

Moreover, the evidence of the up-regulation of several surface activation markers of the immune cells, such as IL3RA (CD123), CD80, CD69 and tumor necrosis factor receptor superfamily member 9 (TNFRSF9) [99,100], confirms our preliminary results, stressing the ability of VK3-20 to induce the expression of activation markers and co-stimulatory molecules on circulating APCs after 24h incubation [66].

Indeed, it is well known that CD80, which is found on activated B cells and monocytes, is able to provide a costimulatory signal necessary for T cell activation and survival [101].

Moreover, the activation of T cells, both in vivo and in vitro, is known to induce the expression of CD69, which appears to be the earliest inducible cell surface glycoprotein acquired during lymphoid activation [102]. CD69 is involved in T cell proliferation, and works as a signal-transmitting receptor in lymphocytes [103].

Finally, TNFRSF9 (CD137) is known to be expressed by activated T cells, DCs, follicular dendritic cells, natural killer cells, granulocytes and cells of blood vessel walls at sites of inflammation. CD137 enhances T cell proliferation, IL-2 secretion, survival and cytolytic activity. CD137 has been shown to interact with TRAF2, a member of the TNF receptor associated factor protein family, required for TNF-alpha-mediated activation of MAPK8/JNK and NF-kB signaling [104].

All 33 genes modulated at 24h post-stimulation in both healthy controls and HCV+ subjects were analyzed using the DAVID Bioinformatics Database (http://david.abcc.ncifcrf.gov) for associations with particular Gene Ontology terms. Indeed, genes mainly related to the response to wounding, inflammatory response, programmed cell death, defense response, regulation of cell proliferation and cytokine-mediated signaling pathway were identified (Table 10). In addition, the specific genes involved in inflammatory response, programmed cell death and cytokine-mediated signaling pathway are listed in Tables 11-13.

Table 10. DAVID analysis of up-regulated and down-regulated genes identified in Fig. 33-34. The p-value refers to the significance of the association between the gene ontology term and the genes identified (count).

GENE ONTOLOGY TERM	COUNT	PERCENTAGE	P-VALUE
Response to wounding	15	45.4	4.17E-12
Inflammatory response	11	33.3	2.25E-09
Programmed cell death	12	36.3	7.91E-08
Defense response	12	36.3	8.45E-08
Regulation of cell proliferation	13	39.3	1.06E-07
Cytokine-mediated signaling pathway	6	18.1	5.80E-07
Acute-phase response	5	15.1	2.22E-06
IL-1 receptor binding	4	12.1	5.50E-06
Positive regulation of cell communication	8	24.2	9.41E-06
Immunoregulation	3	9	4.01E-05
Acute inflammatory response	5	15.1	7.97E-05
Positive regulation of T cell proliferation	4	12.1	1.04E-04
Regulation of T cell activation	5	15.1	1.58E-04
Positive regulation of mononuclear cell proliferation	4	12.1	3.06E-04
Cytokine activity	5	15.1	8.73E-04

Table 11. Genomic signature of modulated genes identified in Fig. 33-34.

INFLAMMATORY RESPONSE

AFFYMETRIX_EXON_GENE_ID	GENE NAME	GENE SYMBOL	MODULATION
8071899	adenosine A2a receptor	ADORA2A	UP
8045688	tumor necrosis factor, alpha-induced protein 6	TNFAIP6	UP
8044574	interleukin 1 receptor antagonist	IL1RN	UP
8054712	interleukin 1, alpha	IL1A	UP
8054722	interleukin 1, beta	IL1B	UP
7931914	interleukin 2 receptor, alpha	IL2RA	UP
8131803	interleukin 6 (interferon, beta 2)	IL6	UP
8077786	interleukin-1 receptor-associated kinase 2	IRAK2	UP
7924499	toll-like receptor 5	TLR5	DOWN
7960947	alpha-2-macroglobulin	A2M	DOWN
8058765	fibronectin 1	FN1	DOWN

Table 12. Genomic signature of modulated genes identified in Fig. 33-34.

CYTOKINE-MEDIATED SIGNALING PATHWAY

AFFYMETRIX_EXON_GENE_ID	GENE NAME	GENE SYMBOL	MODULATION
8035351	Janus kinase 3	JAK3	UP
8054712	interleukin 1, alpha	IL1A	UP
8054722	interleukin 1, beta	IL1B	UP
8131803	interleukin 6 (interferon, beta 2)	IL6	UP
8077786	interleukin-1 receptor-associated kinase 2	IRAK2	UP
8066214	transglutaminase 2	TGM2	UP

Table 13. Genomic signature of modulated genes identified in Fig. 33-34.

PROGRAMMED CELL DEATH

AFFYMETRIX_EXON_GENE_ID	GENE NAME	GENE SYMBOL	MODULATION
7990818	BCL2-related protein A1	BCL2A1	UP
8163825	TNF receptor-associated factor 1	TRAF1	UP
8071899	adenosine A2a receptor	ADORA2A	UP
8054712	interleukin 1, alpha	IL1A	UP
8054722	interleukin 1, beta	IL1B	UP
7931914	interleukin 2 receptor, alpha	IL2RA	UP
8131803	interleukin 6 (interferon, beta 2)	IL6	UP
8075316	oncostatin M	OSM	UP
8130556	superoxide dismutase 2, mitochondrial	SOD2	UP
8066214	transglutaminase 2	TGM2	UP
8157582	gelsolin (amyloidosis, Finnish type)	GSN	DOWN
8180346	glutathione peroxidase 1	GPX1	DOWN

A lack of expression for the overall mentioned cytokines and downstream signaling factors, was observed in PBMCs incubated for 6 days (Fig. 35-36), suggesting a loss of VK3-20 effect over time, which needs further investigation.

In particular, PBMCs from the HCV+ subjects showed a down-regulation of genes of the pathway "adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains" (Table 14).

Such observation strongly suggests the need for use of specific adjuvant strategy in order to retain and/or potentiate the effect of the antigen molecule over time.

Table 14. Genomic signature of down-regulated genes identified in Fig. 36. The p-value refers to the significance of the association of the specific gene ontology term with the gene list.

ADAPTIVE IMMUNE RESPONSE GO TERM (p-value 9.3E-7)		
AFFYMETRIX_EXON_GENE_ID	**GENE SYMBOL**	**GENE NAME**
7898793	C1QA	complement component 1, q subcomponent, A chain
7898805	C1QB	complement component 1, q subcomponent, B chain
8025601	ICAM1	intercellular adhesion molecule 1
7951686	IL18	interleukin 18 (interferon-gamma-inducing factor)
7942300	IL18BP	interleukin 18 binding protein

The above mentioned results have been further confirmed by parallel cytokine evaluation in supernatant of stimulated PBMCs after 24h or 6 days incubation (Fig. 37-38), confirming our previous data [66].

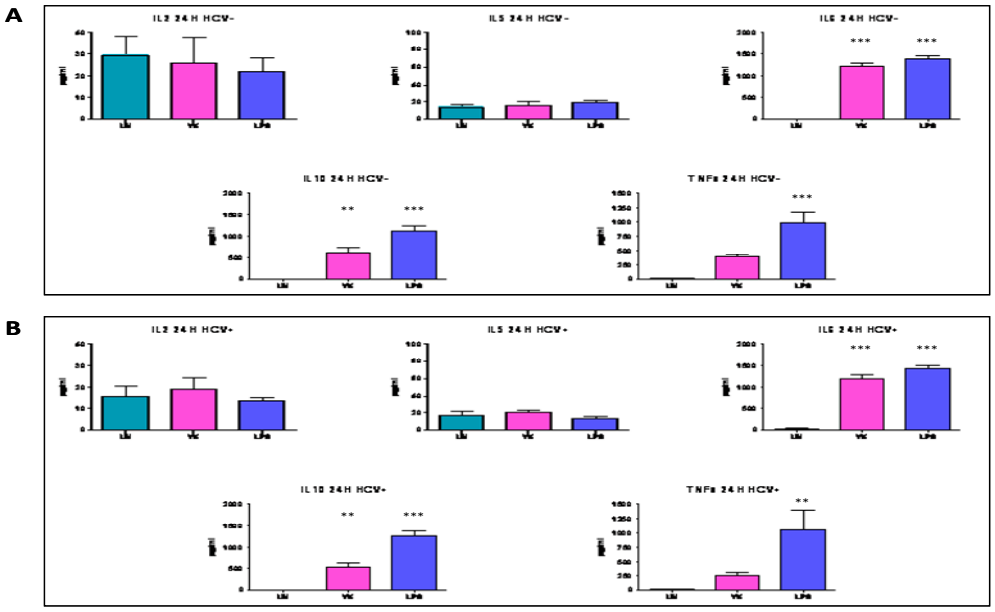

Fig. 37. Analysis of cytokines production in supernatants of PBMCs from healthy controls (A) and HCV+ subjects (B) induced by VK3-20 and LPS after 24h incubation. ** P < 0.01, *** P < 0.001.

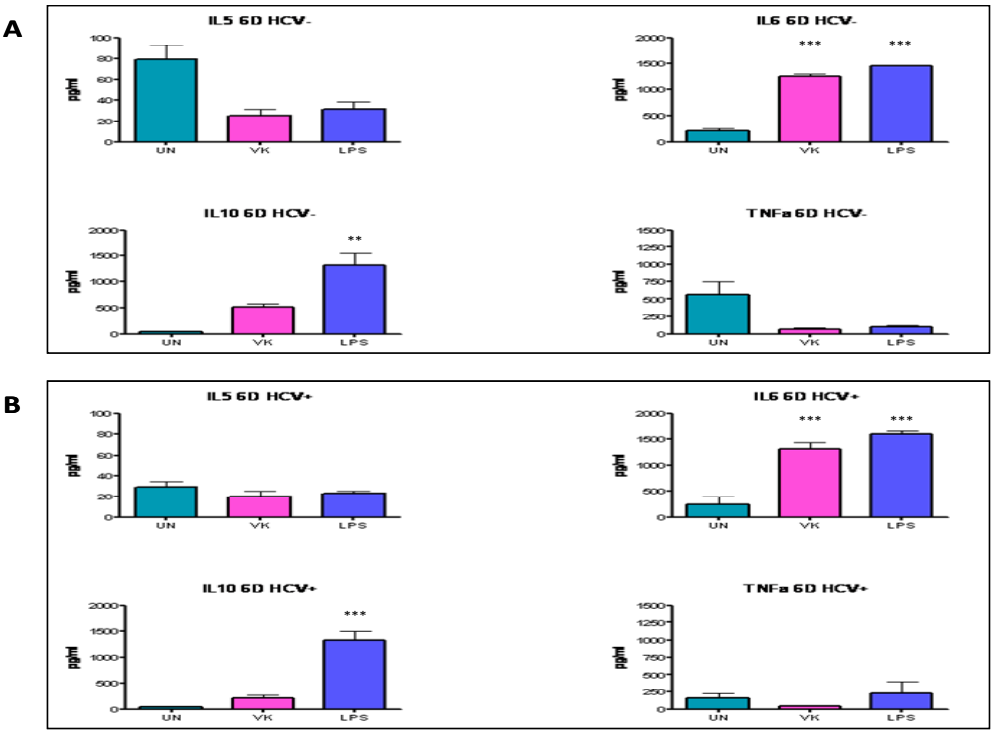

Fig. 38. Analysis of cytokines production in supernatants of PBMCs from healthy controls (A) and HCV+ subjects (B) induced by VK3-20 and LPS after 6 days incubation. ** P < 0.01, *** P < 0.001.

4. DISCUSSION

In the quest of specific molecular mechanisms involved in the activation of the immune response to HCV-related B cell NHL, here we report the results of a multivariate and multiparametric analysis of the innate and early adaptive immune response induced in PBMCs from HCV seropositive subjects and healthy controls, following *ex vivo* "vaccination" with the most represented VK3-20 light-chain protein idiotype, expressed by clonal B cells of HCV-related type II MC as well as NHL [43].

The immune signature induced by VK3-20 and/or VK3-20-KLH was evaluated, based on flow cytometry analysis as well as cytokine profile analysis of stimulated PBMCs.

Preliminary results showed that the basal and VK3-20-induced expression of activation markers and co-stimulatory molecules in the evaluated circulating antigen presenting cells (APCs) was largely comparable between HCV seropositive subjects and healthy controls [66].

Overall, the activation markers showed a trend of increased expression in all circulating cells, although the most evident and consistent pattern was observed in the $CD123^+$ pDCs and/or $CD11c^+$ mDCs [66].

No significant difference was observed in results obtained from monocyte derived dendritic cells (MDDCs) and circulating APCs, confirming our previous results and results from other groups [51,55,57,105]. The overall expression pattern suggested maturation/activation induced by VK3-20, although for some specific markers and in some patients the trend did not reach statistical significance.

Results obtained in parallel with lipopolysaccharide (LPS), used as a positive activation factor, confirmed the responsiveness of circulating APCs from both groups analyzed in the preliminary analysis [66].

Nonetheless, some HCV+ individuals showed a complete lack of maturation induced by VK3-20 in circulating APCs, strongly suggesting the need for individual evaluations to identify possible impairments in response to the specific immunogen.

These preliminary results confirm and extend data from others showing a normal expression of surface molecules involved in antigen-specific T cell activation on immature and mature DCs from HIV-1-infected and HCV/HIV-co-infected individuals [106-108]. Furthermore, monocyte derived DCs from either HCV-infected or HCV/HIV-co-infected subjects have been previously shown to

stimulate a mixed leukocyte reaction in purified, allogeneic $CD4^+$ T cells comparable to the one observed with DCs derived from healthy donors [109-111].

Moreover, the analysis of the cytokine profile induced by VK3-20 showed a significant production of IL-4, IL-6, IL-10 and TNF-α in PBMCs from HCV seropositive subjects and control subjects, with the highest levels observed in the samples treated with the highest concentration of VK3-20 (15 μg/ml) ($p < 0.05$). No increase in the production of IL-2 and IFN-γ was observed in the control as well as HCV+ group [66].

Indeed, the production of IFN-γ is known to be inhibited by IL-10 [112], with a sequential detrimental effect on the IL-12-mediated induction of IFN-γ production by NK and T cells [113]. Therefore, the high levels of IL-10 induced by VK3-20 could explain the lack of increased production of IFN-γ in both groups. The very similar response observed in HCV seropositive subjects, regardless of the diagnosis of type II MC or NHL, would suggest the absence of an *in vivo* priming for the VK3-20.

In this regard, the evaluation of VK3-20 expression on the clonal B cell populations of these subjects would be of great interest.

In order to verify the possible induction of a more potent immune response, a conjugated form of VK3-20 protein (i.e. VK3-20-KLH) was, also, tested.

Our results indicate that the expression of activation markers and costimulatory molecules on monocytes and $CD11c^+$ mDCs was largely comparable between healthy controls and HCV seropositive subjects, with no statistically significant differences between VK3-20 and VK3-20-KLH treatment.

In addition, the levels of IL-2, IL-5, IL-6, IL-10, IFN-γ and TNF-α were assessed in supernatants of stimulated PBMCs. Both VK3-20 protein and VK3-20-KLH conjugate induced a significant production of TNF-α, IL-6 and IL-10 ($p < 0.05$) with minor differences, suggesting comparable effects exerted by VK3-20-KLH and VK3-20 treatment.

The overall preliminary results represent a proof-of-concept and confirm the possibility of screening donor susceptibility to an antigen treatment using circulating APCs, without the need of purification and *ex vivo* selection of DCs, simplifying the identification of "responsive" vaccinees.

The analysis of the immunological effects induced by VK3-20 in PBMCs stimulated *ex vivo* has been further performed using a systems biology [51] approach to obtain a global picture of the induced immune response.

To this aim, global gene expression profile of samples from healthy controls and HCV seropositive subjects "*ex vivo* vaccinated" with VK3-20 was analyzed.

The primary unsupervised analysis showed a distinct gene signature pattern induced by VK3-20 treatment after 24h or 6 days incubation. Moreover, VK3-20 treatment induced the segregation of the HCV-related samples from control samples, indicating the identification of specific gene signature patterns peculiar to the HCV-status.

A supervised analysis was performed by pair-wise comparisons between stimulated and non-stimulated PBMCs to identify the signature processes and pathways with respect to the VK3-20 treatment.

In particular, the analysis identified 503 genes differentially expressed between VK3-20 stimulated and non-stimulated PBMCs after 24h incubation, regardless the HCV seropositivity status. Among them, 300 genes were shown to be up-regulated in stimulated PBMCs, which were further analyzed using the DAVID Bioinformatics Database for annotation and 50 of them showed a significant association with the Immune Response Gene Ontology term.

The analysis comparing gene sets in stimulated and non-stimulated PBMCs after 6 days incubation identified 149 genes differentially expressed. The up-regulated genes showed a significant association with the Response to Wounding GO term.

Finally, the analysis comparing gene sets in stimulated PBMCs after 24h and 6 days identified 851 genes differentially expressed. The genes up-regulated after 24h incubation showed the most significant association with the Cytokine Activity GO term, whereas the genes up-regulated after 6 days incubation showed the most significant association with the Cell Cycle GO term.

Up-regulated genes identified in each individual class comparison analysis were further evaluated and classified according to the "Ingenuity System Database".

Indeed, samples from stimulated PBMCs after 24h showed a positive modulation of genes involved in Communication between Innate and Adaptive Immune Cells, Acute Phase Response Signaling, LXR/RXR Activation, IL-6 Signaling, Dendritic Cell Maturation, Role of Cytokines in Mediating Communication between Immune Cells, TREM1 Signaling. Whereas, genes involved in the pathways of ILK Signaling, IL-17 Signaling, G-Protein Coupled Receptor Signaling were positively modulated in PBMCs from samples stimulated for 6 days.

Among differentially expressed genes modulated by VK3-20 after 24h and 6 days incubation, Ingenuity Pathways Analysis identified specific networks related to the Inflammatory Response in which NF-κB protein complex plays a crucial role.

The immune signature of *ex vivo* stimulated PBMCs was further evaluated using a specific immunology gene set for supervised analysis and the resulting immune networks were displayed using Cytoscape.

In particular, the immunology network of stimulated PBMCs incubated for 24h was characterized by a significant up-regulation of genes of cytokine and cytokine receptor families, as well as of the downstream signaling cascades, including: IL-1, IL-6, OSM, IL-10, TNFRSF1B, TNFAIP6, TRAF1 and JAK3, confirming ELISA results.

Nevertheless, the up-regulation of such cytokines was not detectable in the expression data from stimulated PBMCs after 6 days incubation. Therefore, the cytokine profile observed in ELISA tests performed on 6 days supernatants was, definitely, correlated to an earlier production (i.e. at 24h) with a consequent residual effect.

Moreover, gene expression data showed the up-regulation of surface activation markers of the immune cells, such as CD123, CD80, CD69 and TNFRSF9, confirming our preliminary results on immune signature induced by VK3-20 stimulation after 24h incubation [66].

In summary, we demonstrated that systems biology approaches not only allow the observation of a global picture of vaccine-induced innate immune responses but can be, also, used to predict subsequent adaptive immune response.

In particular, our results strongly suggest the induction of the innate and early adaptive immune response in PBMCs *ex vivo* stimulated with the most represented VK3-20 light-chain protein idiotype expressed by B cell clones sustaining the HCV-related type II MC as well as NHL, suggesting its potential use as a preventive, as well as a therapeutic vaccination strategy.

5. MATERIALS AND METHODS

5.1. Enrolled subjects

Peripheral blood was obtained by venipuncture from HCV seronegative volunteers and HCV seropositive subjects. All human specimens were obtained at the Infectious Disease Unit of "G. Rummo" Hospital (Benevento) under informed consent, as approved by the Institutional Review Board and processed at the National Cancer Institute in Naples.

5.2. PBMCs isolation and MDDCs preparation

Fresh human PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation and plated in six-well plates at a concentration of approximately $1x10^7$ cells/well in a maximum volume of 3 ml/well for induction [56,114]. Isolated PBMCs were then cultured for 24h (short-term culture) or for 6 days (medium-term culture) in RPMI 1640 medium.

Alternatively, MDDCs were generated as described previously [114], with minor modifications. Briefly, isolated PBMCs were enriched for $CD14^+$ monocytes by negative selection with a cocktail of monoclonal antibodies (MAbs) from StemCell Technologies (Vancouver, British Columbia, Canada), according to the instructions of the manufacturer. Typically, greater than 80% of the cells were $CD14^+$ after enrichment, as verified by flow cytometry. The isolated monocytes were allowed to adhere to plastic by plating in six-well plates at $1x10^6$ cells per ml in RPMI 1640 medium for 2h. Adherent monocytes were washed with RPMI 1640 medium and were then cultured for 6 days in DC culture medium supplemented with 50 ng of recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF; R&D Systems, Minneapolis, Minn.) per ml and 1,000 U of recombinant interleukin-4 (rIL-4; R&D Systems, Minneapolis, Minn.) per ml.

5.3. Cell culture medium

PBMCs culture medium consisted of RPMI 1640 (Life Technologies, Carlsbad, CA) supplemented with 2 mM L-glutamine (Sigma), 10% fetal calf serum (Life Technologies) and 2% penicillin/streptomycin (5,000 I.U./5mg per ml, MP Biomedicals). Recombinant interleukin-2 (rIL-2; R&D Systems, Minneapolis, Minn.) was added at a concentration of 75 U/ml for medium-term culture (6 days).

MDDCs culture medium consisted of RPMI 1640 (Life Technologies, Carlsbad, CA) supplemented with 2 mM L-glutamine (Sigma), 1% non-essential amino acids (Life Technologies), 1% sodium pyruvate (Life Technologies), 50 μM 2-mercaptoethanol (Sigma) and 50 μg of gentamicin (Life Technologies) per ml.

5.4. Cell treatment

PBMCs or MDDCs were pulsed with serial dilutions of the recombinant VK3-20 protein (15, 5 and 1.5 μg/ml) provided by Areta International (Gerenzano, Italy). In parallel, cells were pulsed with 4 μg/ml of lipopolysaccharide (LPS), as positive control. PBS was used as negative control. After 24h incubation, PBMCs and MDDCs were harvested, washed with 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.2) without Calcium and Magnesium and analyzed by flow cytometry.

Alternatively, PBMCs were incubated for 24 hours (short-term culture) with the recombinant VK3-20 protein (15 μg/ml) or the recombinant VK3-20-KLH (15 μg/ml) protein, provided by Areta International (Gerenzano, Italy). In parallel, cells were pulsed with 8 μg/ml of LPS, as positive control. Furthermore, PBMCs were incubated for 6 days (medium-term culture) with the same concentration of recombinant VK3-20 protein or LPS, added at day 0 and 3. PBS was used as negative control.

At the end of the incubation, PBMCs were harvested, washed with 1X PBS (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4, pH7.2) without Calcium and Magnesium and analyzed by flow cytometry or stored frozen at -80°C in RNA later (Ambion, Austin, TX).

All cell supernatants were collected for quantification of cytokine production by enzyme-linked immunosorbent assay (ELISA).

5.5. Flow cytometry

MDDCs and short-term culture PBMCs were incubated for 30 min at 4°C with human monoclonal antibodies specific for CD40, CD80, CD83, CD86, HLA-DR, CD11c, CD123 and CD14 (BD Pharmingen, San Diego, CA), washed and then analyzed with a FACScalibur flow cytometer (BD Pharmingen). Data analysis was carried out with WinMDI2.8 Software. A Paired t test was performed, all p-values were two-tailed and considered significant if less than 0.05.

5.6. Cytokine analysis

At the time of cell harvesting, supernatants were collected and analyzed. Cytokine production was assessed using the Instant ELISA system (Bender Medsystems*)* for quantitative detection of human cytokines, according to the manufacturer's instructions. Data acquisition was performed using the Sirio-S ELISA reader. A Paired t test was performed, all p-values were two-tailed and considered significant if less than 0.05.

5.7. RNA purification and microarray hybridization

Total RNA from PBMCs was purified using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. The purity of total RNA preparation was verified by 260:280 nm ratio (range, 1.8-2.0), at NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). In addition, phenol contamination was checked and a 2.0-2.2 ratio at 260:230 nm was considered acceptable. Integrity of extracted RNA was evaluated using the LabChip GX/GXII Electrophoresis System (Caliper LifeSciences) and a RNA Quality Score (RQS) >8 was considered acceptable for RNA quality.

Single-stranded cDNA was obtained with the Ambion WT Expression Kit (Applied Biosystems) according to manufacturer's instructions from a starting material of 200 ng total RNA per sample. The sense strand cDNA was fragmented and labeled using the Affymetrix GeneChip WT Terminal Labeling Kit (Applied Biosystems) according to manufacturer's instructions.

After hybridization on Human Gene 1.0 ST Arrays (Affymetrix) for 16h at 45°C at 60 rpm in a Hybridization Oven 640 (Affymetrix), slides were washed and stained on a Fluidics Station 450 (Affymetrix). Scanning was performed on a GeneChip Scanner 3000 7G (Affymetrix) and Affymetrix GCOS software was used to perform image analysis and generate raw intensity data (CEL files). Initial data quality was assessed by background level and pairwise correlation among samples. A $\log_2$ base transformation was applied to the data before the arrays were normalized. Microarray intensity data of probe sets were normalized by RMA, which includes global background adjustment and quantile normalization.

5.8. Statistical Analysis

5.8.1. Unsupervised Analysis

For the unsupervised analysis a low-stringency filtering was applied, selecting the genes differentially expressed in 80% of all experiments with a >3 fold change ratio in at least one experiment. Hierarchical cluster analysis was conducted on the selected genes according to Eisen et al. [115]; differentially expressed genes were visualized by Treeview and displayed according to the central method [116].

5.8.2. Supervised Analysis

Supervised class comparison was performed using BRB ArrayTool developed at NCI, Biometric Research Branch, Division of Cancer Treatment and Diagnosis. Three subsets of genes were explored. The first subset included genes up-regulated in stimulated (VK3-20 treated) PBMCs compared to non-stimulated (PBS treated) PBMCs after 24h incubation, the second subset included genes up-regulated in stimulated PBMCs compared to non-stimulated PBMCs after 6 days incubation, the third subset included genes up-regulated in stimulated PBMCs after 24h incubation compared to stimulated PBMCs after 6 days incubation, from both HCV seropositive subjects and healthy controls.

Class comparison analyses were tested for an univariate significance threshold set at a p-value < 0.001. Gene clusters identified by the univariate t-test were challenged with two alternative additional tests, an univariate permutation test (PT) and a global multivariate PT.

Class comparison and hierarchical clustering were employed to determine the pattern of response and results are illustrated as a heat map of significance values. All analyses were performed using R and Cytoscape (http://www.cytoscape.org); gene function was assigned based on Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://www.david.abcc.ncifcrf.gov) and Gene Ontology (http://www.geneontology.org).

5.9. Ingenuity Pathways Analysis

Ingenuity Pathways Analysis (IPA, www.ingenuity.com) was employed to elucidate the relationships and connections between differentially expressed genes.

The IPA system transforms large data sets into a group of relevant networks containing direct and indirect relationships between genes, based on interactions contained in the Ingenuity Pathways Knowledge Base. An IPA ''network'' is a graphical representation of the molecular relationships between genes, represented as nodes, and biological interactions, represented as connecting lines between nodes. Gene networks are generated algorithmically based on connectivity in terms of expression, activation, transcription and/or inhibition.

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Immune signatures in human PBMCs of idiotypic vaccine for HCV-related lymphoproliferative disorders

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Abstract

Hepatitis C virus (HCV) is one of the major risk factors for chronic hepatitis, which may progress to cirrhosis and hepatocellular carcinoma, as well as for type II mixed cryoglobulinemia (MC), which may further evolve into an overt B-cell non-Hodgkin's lymphoma (NHL).

It has been previously shown that B-cell receptor (BCR) repertoire, expressed by clonal B-cells involved in type II MC as well as in HCV-associated NHL, is constrained to a limited number of variable heavy (VH)- and light (VL)-chain genes. Among these, the VK3-20 light chain idiotype has been selected as a possible target for passive as well as active immunization strategy.

In the present study, we describe the results of a multiparametric analysis of the innate and early adaptive immune response after *ex vivo* stimulation of human immune cells with the VK3-20 protein. This objective has been pursued by implementing high-throughput technologies such as multiparameter flow cytometry and multiplex analysis of cytokines and chemokines.

Introduction

Hepatitis C virus (HCV) is a Hepacivirus of the Flaviviridae family, mainly involved in hepatic disorders, including chronic hepatitis which may progress to cirrhosis in about 10-20% of cases and further to hepatocellular carcinoma in 1-5% of cirrhotic patients [1].

Subsequently, the virus has been implicated as one of the major risk factors for type II mixed cryoglobulinemia (MC), an autoimmune disease that may evolve into an overt B-cell non-Hodgkin's lymphoma (NHL) in about 10% of MC patients [2-5]. Several studies have contributed to establish the causative role of HCV infection in the etiopathogenesis of MC, showing the presence of the viral RNA and/or anti-HCV antibodies in a range of 70 to 100% of MC [6-8]. Furthermore, the clinical evolution of MC is closely linked to the natural history of the underlying HCV chronic infection [9,10].

The most accredited pathogenetic mechanism of MC during HCV chronic infection is the persistent immune stimulation sustained by viral proteins which, in turn, may result in production of cross-reactive autoantibodies, including cryoglobulins [11,12]. Chronic stimulation of the B-cell by HCV epitopes may produce the expansion of B-cell subpopulations with dominant genetic characteristics. In particular, the interaction between HCV E2 protein and CD81 molecule, an almost ubiquitous tetraspannin present on B-cell surface, has been shown and it may lead to a strong and sustained polyclonal stimulation of B-cell compartment [13]. Furthermore, the t (14,18) translocation observed in 85% of the patients affected by HCV-related type II MC might lead to abnormally elevated expression of Bcl-2 protein with consequent inhibition of apoptosis and increased B-cell survival [14]. This multistep process may ultimately lead to B-cell NHL as late complication of the MC syndrome [9,15].

The clonality of expanded B cells can be defined by the analysis of the antigen-binding region (so called

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idiotype, Id) of the immunoglobulin produced and expressed by the B-cell clone. According to the variety of Ids identified, the lymphoproliferative disorder may be sustained by mono-, oligo- or polyclonal B cells. It has been previously demonstrated that the B-cell receptor (BCR) repertoire expressed by clonal B-cells involved in HCV-associated type II MC as well as in NHL is not random, with V1-69, V3-7, V4- 59 variable heavy (VH)- and still more variable κ (VK)3-20 and VK3-15 light (VL)-chain genes being the most represented [16-18]. These data suggest a model of antigen-driven origin for these lymphoproliferative disorders with the recognition of a limited number of HCV antigens [18,19].

The constrained heterogeneity of Ids shared by such patients strongly suggests the possibility of targeting one or few idiotypes to hit and eliminate the B cell clone sustaining the HCV-associated NHL. One strategy is to generate idiotype-specific MAbs to be employed in a selective passive immunization [20]. An alternative strategy is to use an idiotype vaccine [21] in order to elicit an active humoral/cellular immune response as preventive and/or therapeutic approach against the expansion of the B cell clone sustaining the HCV-associated NHL.

We have previously shown that a multivariate and multiparametric analysis can predict the innate and early adaptive immune response induced by a vaccine molecule in human monocyte-derived dendritic cells (MDDCs) as well as whole peripheral blood mononuclear cells (PBMCs) using an ex-vivo experimental setting. This systems biology approach involves high-throughput technologies such as global gene expression profiling, multiplex analysis of cytokines and chemokines, and multiparameter flow cytometry, combined with computational modeling [22-26].

In the present study, we performed a multiparametric analysis of the innate and early adaptive immune response after *ex vivo* stimulation with the VK3-20 light chain protein, the idiotype most frequently identified on B cell clones sustaining the HCV-associated type II MC and NHL. This objective has been pursued using freshly isolated circulating human PBMCs.

Materials and methods

Enrolled subjects

Peripheral blood was obtained by venipuncture from 5 healthy volunteers and 10 HCV positive patients. All human specimens were obtained and processed at the National Cancer Institute in Naples under informed consent, as approved by the Institutional Review Board.

Cell culture medium

PBMCs culture medium consisted of RPMI 1640 medium (Life Technologies, Carlsbad, CA) supplemented with 2 mM L-glutamine (Sigma), 10% fetal calf serum (Life Technologies) and 2% penicillin/streptomycin (5,000 I.U./5 mg per ml, MP Biomedicals).

MDDCs culture medium consisted of RPMI 1640 medium (Life Technologies, Carlsbad, CA) supplemented with 2 mM L-glutamine (Sigma), 1% non-essential amino acids (Life Technologies), 1% sodium pyruvate (Life Technologies), 50 μM 2-mercaptoethanol (Sigma) and 50 μg of gentamicin (Life Technologies) per ml.

PBMC isolation and MDDC preparations

Fresh human PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation and plated in six-well plates at a concentration of approximately 1×10^7 cells/well in a maximum volume of 3 ml/well for induction. Alternatively, MDDCs were generated as described previously [24,27], with minor modifications. Briefly, isolated PBMCs were enriched for CD14+ monocytes by negative selection with a cocktail of monoclonal antibodies (MAbs) from StemCell Technologies (Vancouver, British Columbia, Canada), according to the instructions of the manufacturer. Typically, greater than 80% of the cells were CD14+ after enrichment, as verified by flow cytometry. The isolated monocytes were allowed to adhere to plastic by plating in six-well plates at 1×10^6 cells per ml in RPMI 1640 medium for 2 hrs. Adherent monocytes were washed with RPMI 1640 medium and were then cultured for 6 days in DC culture medium supplemented with 50 ng of recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF; R&D Systems, Minneapolis, Minn.) per ml and 1,000 U of recombinant interleukin-4 (rIL-4; R&D Systems, Minneapolis, Minn.) per ml.

Cell treatment

PBMCs or MDDCs were pulsed with serial dilutions of the recombinant VK3-20 protein (15, 5 and 1.5 μg/ml) provided by Areta International (Gerenzano, Italy) (Patent PCT/IB2008/001936). In parallel, cells were pulsed with 4 μg/ml of lipopolysaccharide (LPS), as positive control. PBS was used as negative control. After 16-h incubation, PBMCs and MDDCs were harvested and washed with 1× PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2) without Calcium and Magnesium.

Flow cytometry

PBMCs and MDDCs were incubated for 30 min at 4°C with human monoclonal antibodies specific for CD40, CD80, CD83, CD86, HLA-DR, CD123, CD11c and CD14 (BD Pharmingen, San Diego, CA), washed and then analysed with a FACScalibur flow cytometer (BD Pharmingen). Data analysis was carried out with WinMDI2.8 Software.

Multiplex cytokine analysis

At the time the cells were harvested, the supernatants were also collected and stored frozen until analyzed. Cytokine production was assessed using the BD™ Cytometric Bead Array (CBA) tool (Becton Dickinson and Company), according to the instructions of the manufacturer. Data acquisition was performed using a FACScalibur flow cytometer (BD Pharmingen), the analysis was performed with the BD CBA Analysis Software.

Statistical analyses

Intergroup comparisons were performed with the Mann-Whitney U test (for univariate nonparametric group analysis). All p-values were two-tailed and considered significant if less than 0.05.

Results

Clinical parameters of subjects included in the analysis

Fifteen subjects were enrolled in the study. Ten subjects were HCV positive patients, of whom 2 were males and 8 were females (P1 - P10). Four of them were diagnosed with NHLs and only one of them showed a type II MC (Table 1). Five healthy subjects were enrolled as controls (C1 - C5), matched for age and life style.

VK3-20 protein induces comparable maturation phenotype in MDDCs and PBMCs of control subjects

Freshly derived PBMCs and immature MDDCs were obtained from healthy HCV-negative subjects and were incubated with 1.5 μg/ml, 5 μg/ml or 15 μg/ml of the VK3-20 protein. After a 16-hr stimulation, the expression of surface maturation/activation markers, such as CD40, CD80, CD83, CD86 and HLA-DR was examined. The results showed the up-regulation of all markers in PBMCs in CD14+ monocyte population as well as CD123+ plasmacytoid DC (pDC) or CD11c+ myeloid DC (mDC) (Fig. 1). Furthermore, MDDCs showed patterns of activation comparable to circulating mDCs and pDCs (Fig. 2).

Table 1 Clinical parameters of enrolled subjects

	SEX	HCV	MC	NHL
C1	M	Neg	Neg	Neg
C2	M	Neg	Neg	Neg
C3	F	Neg	Neg	Neg
C4	F	Neg	Neg	Neg
C5	F	Neg	Neg	Neg
P1	F	Pos	Neg	Neg
P2	M	Pos	Neg	Neg
P3	F	Pos	Neg	Neg
P4	M	Pos	Neg	Neg
P5	F	Pos	Neg	Neg
P6	F	Pos	n.d.	Follicular
P7	F	Pos	n.d.	Marginal
P8	F	Pos	n.d.	Diffuse large B cell
P9	F	Pos	Pos	Diffuse large B cell
P10	F	Pos	n.d.	Neg

n.d. = not done.

Quantification of cells expressing activation markers in the subsets of circulating monocytes, pDC and mDC cells showed a trend of partial dose-response at increasing concentrations of the VK3-20 protein, indicating a specific activation/maturation activity on the circulating antigen presenting cells (APCs) (Fig. 3). The expression of CD40 and CD80 markers showed similar pattern of induction (data not shown).

The similar levels of activation/maturation observed in MDDCs and in PBMCs, regardless the marker of cell population used for gating, confirmed the feasibility of such analysis using "unselected" PBMCs, as previously reported [22,25].

The VK3-20 protein induces maturation phenotype in PBMC

Given the comparable results observed in MDDC and in PBMC, subsequent analyses on samples from the enrolled subjects were performed only on circulating monocytes, pDC and mDC and the VK3-20-induced expression of the markers was evaluated in terms of mean fluorescence index (MFI).

The basal expression of the markers was largely comparable between control and HCV+ subjects in the considered cell populations (Fig. 4A to 4C). The only exception is represented by basal CD83 expression, which shows a trend of higher expression in the CD11c + mDC population of HCV+ subjects (Fig. 4A).

The stimulation with VK3-20 protein induces a trend of increased expression of the activation/maturation markers in all circulating cells, from control and HCV seropositive subjects, although the most evident and consistent pattern is observed in the CD123+ pDC and/or CD11c+ mDC cells (Fig. 5 and 6A to 6C).

In particular, the lowest dose of VK3-20 used in the experimental system (1.5 μg) appears to be already sufficient to induce an increased expression of the activation markers in cells from both groups of subjects.

In control subjects, VK3-20 induced the most evident effect on the expression of CD86 in the circulating monocytes, pDCs and mDCs(Fig. 5B). On the contrary, the effect was significantly evident for all evaluated markers in the circulating cell populations from HCV + subjects (Fig. 6A to 6C). This observation suggests that overall the HCV seropositivity status does not significantly affect the responsiveness to an immunogenic stimulus (i.e., VK3-20) of circulating APC populations.

Cytokine production in VK3-20-loaded PBMCs

In order to evaluate the impact of the VK3-20 protein stimulation on the production of cytokines involved in

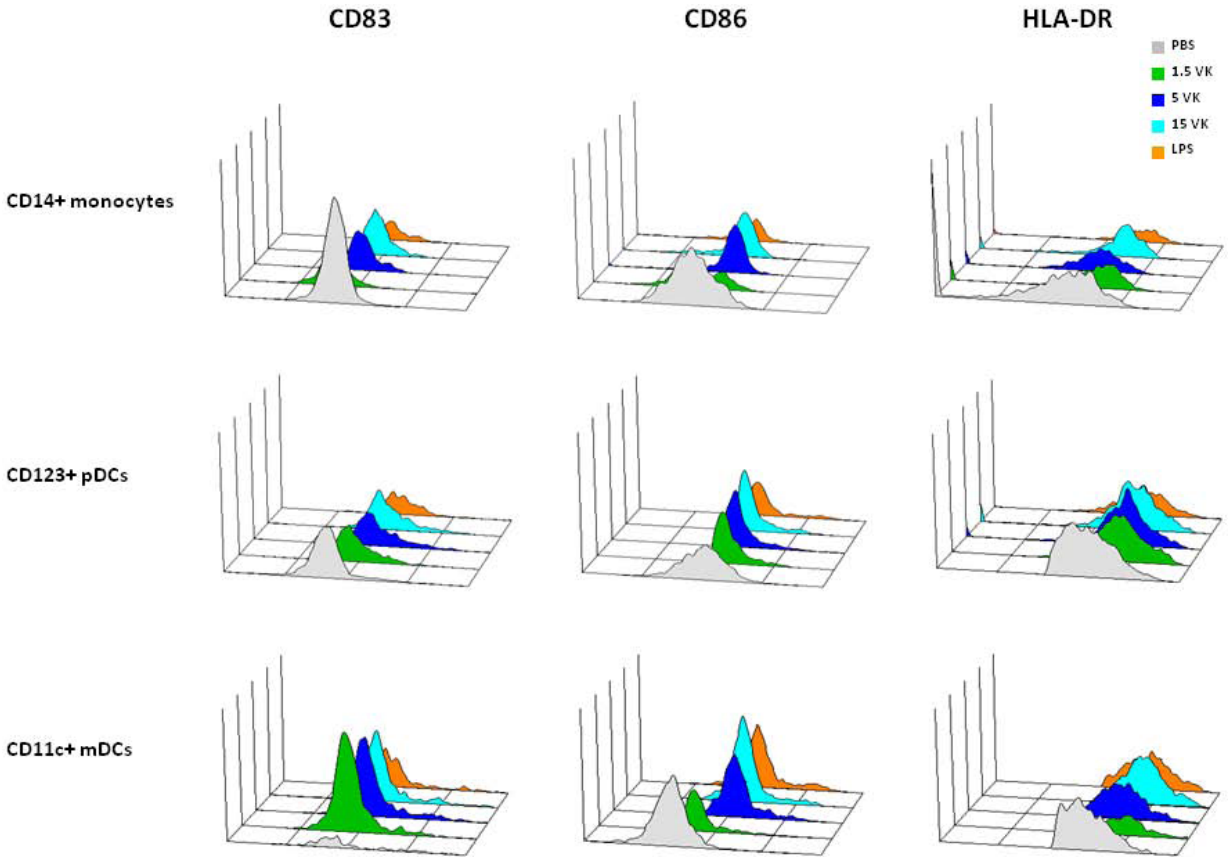

Figure 1 PBMCs were incubated with increasing doses of VK3-20 protein for 16 hrs. The expression of CD83, CD86 and HLADR was analysed by FACScalibur flow cytometer in CD14+ monocytes, CD123+ pDCs and CD11c+ mDCs. Data analysis was carried out with WinMDI2.8 Software. One representative experiment is shown.

T-helper-cell activation, the levels of IL-2, gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), IL-6, IL-4 and IL-10 were assessed in the supernatant of PBMCs stimulated with the VK3-20 protein.

The average basal level of all evaluated cytokines showed no significant difference between HCV positive patients and control subjects (Fig. 7). Cell treatment with the VK3-20 protein did not induce any increase in the production of Th1 cytokines (IL-2 and IFN-γ). On the contrary, the VK3-20 protein induced a significantly higher production of the Th2 cytokines (IL-4, IL-6, IL-10, and TNF-α) in PBMCs from HCV seropositive and control subjects, with the highest levels observed in the samples treated with the highest concentration of VK3-20 (15 μg) ($p < 0.05$) (Fig. 8 and 9). The levels of Th2 cytokines induced in the HCV+ samples were significantly higher than those observed in control samples ($p < 0.01$).

Discussion

The multivariate and multiparametric analysis described in the present study shows that the basal and VK3-20-induced expression of activation markers and co-stimulatory molecules in the evaluated circulating antigen presenting cells (APCs), CD14+ monocyte as well as CD123+ plasmacytoid DC (pDC) or CD11c+ myeloid DC (mDC) populations, is largely comparable between HCV-seropositive and control subjects. Overall, the markers show a trend of increased expression in all circulating cells, although the most evident and consistent pattern is observed in the CD123+ pDC and/or CD11c+ mDC cells. No significant difference was observed between results obtained in human monocyte-derived dendritic cells (MDDCs) and circulating APCs, confirming previous results from us and other groups [22,25,28,29].

The overall expression pattern suggests maturation/activation induced by VK3-20, although for some specific markers and in some patients the trend does not reach statistical significance. This observation suggests that the HCV seropositivity status does not significantly impair the immune activation status and the responsiveness of circulating APC populations to the VK3-20 immunogenic stimulus. Results obtained in parallel with

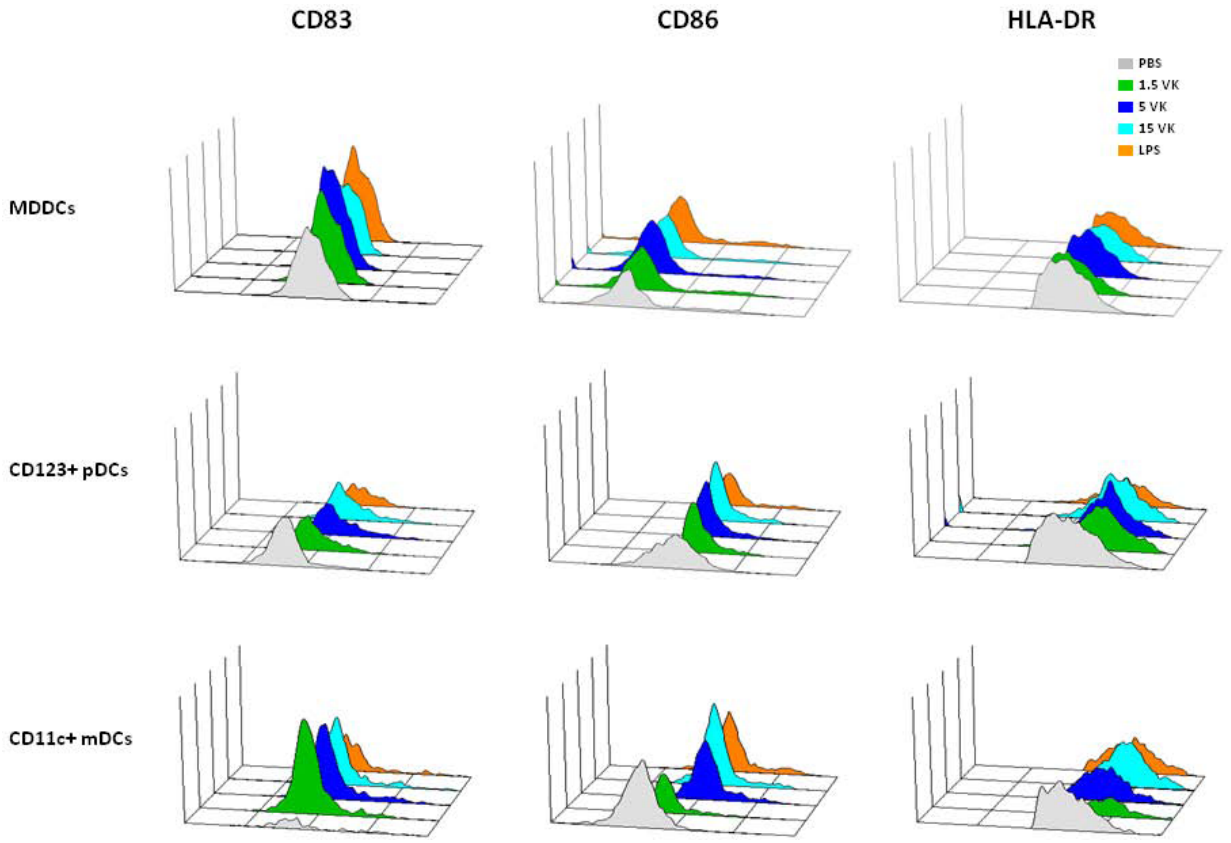

Figure 2 Comparative analysis of the expression of surface maturation/activation markers (CD83, CD86, HLADR) performed on stimulated MDDCs, CD123+ pDCs and CD11c+ mDCs.

lipopolysaccharide (LPS) used as a positive activation factor, confirm the responsiveness of circulating APCs from both groups analyzed in the present study. Nonetheless, some HCV+ individuals show a complete lack of maturation induced by VK3-20 in circulating APCs, strongly suggesting the need for individual evaluations to identify possible impairments in response to this immunogen.

The present results confirm and extend data from others showing a normal expression of surface molecules involved in antigen-specific T-cell activation on immature and mature DCs from HIV-1-infected and hepatitis C virus (HCV)-HIV-coinfected individuals [30-32]. Furthermore, monocyte-derived DCs from either HCV-infected or HCV-HIV-coinfected subjects have been previously shown to stimulate a mixed leukocyte reaction in purified, allogeneic CD4+ T cells comparable to that with DCs derived from healthy donors [33-35].

The average basal level of the Th2 (TNF-α, IL-6, IL-4, and IL-10) cytokines is significantly higher ($p < 0.02$) in HCV-seropositive compared to control subjects. On the contrary, Th1 cytokine levels are equivalent in the two groups. These results suggest a Th2 polarization induced by an established HCV infection, as previously extensively reported [36-39].

VK3-20 induced a significantly higher production of the analysed Th2 cytokines in PBMCs from HCV-seropositive and control subjects, with the highest levels observed in the samples treated with the highest concentration of VK3-20 (15 μg/ml) ($p < 0.05$). Furthermore, the levels of Th2 cytokines induced in the HCV+ samples were significantly higher than those identified in the control samples ($p < 0.01$), suggesting the persistence of a prevalent Th2 status. No increase in the production of Th1 cytokines (IL-2 and IFN-γ) was observed ($p < 0.4$) in the control as well as HCV+ group. In particular, the production of IFN-γ is known to be inhibited by IL-10 [40], with a sequential detrimental effect on the IL-12-mediated induction of IFN-γ production by NK and T cells [41-43]. Therefore, the high levels of IL-10 and TNF-α induced by VK3-20 could explain the lack of increased production of IFN-γ in both groups. The observed discrepancy between the VK3-20 concentration necessary for the maximal induction of activation markers (1.5 μg/ml) and the one necessary for the

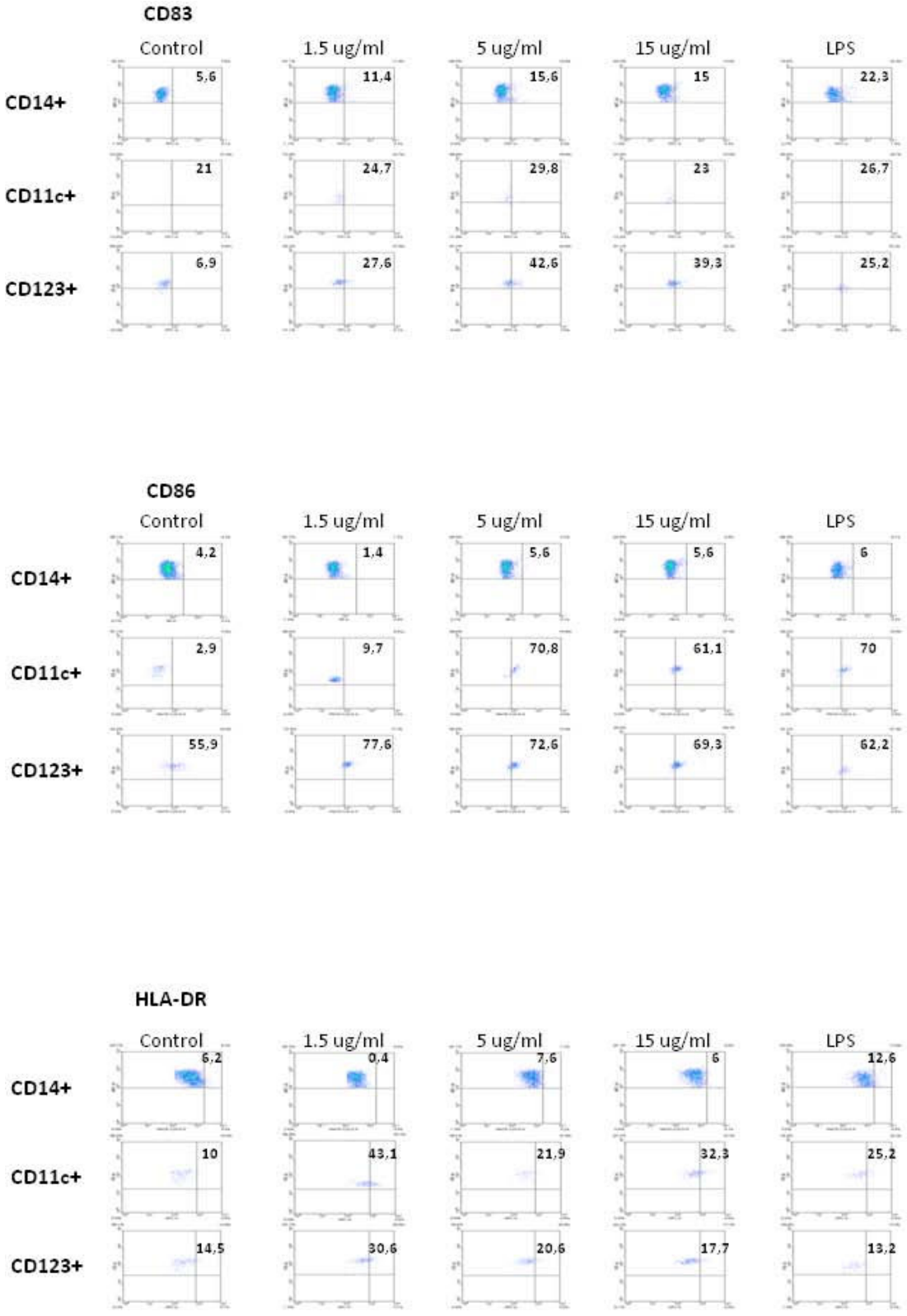

Figure 3 6-color flow cytometric analysis was performed on VK3-20 protein-stimulated monocytes, mDC/pDC cell populations and immunophenotype analysis of surface maturation/activation marker expression is shown. Values in each quadrant represent the percentage of positive cells.

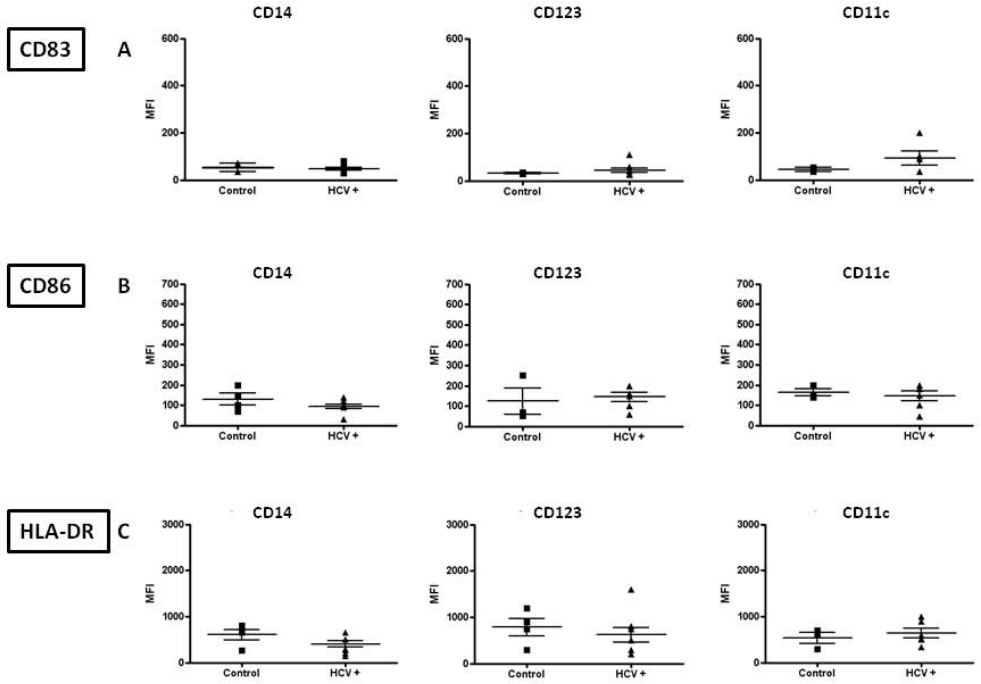

Figure 4 Basal level expression of surface maturation/activation markers, indicated as Mean Fluorescence Index (MFI), on PBMC-derived monocytes and DC from control and HCV positive (HCV+) subjects. CD14 = CD14+ monocytes; CD123 = CD123+ pDCs; CD11c = CD11c+ mDCs.

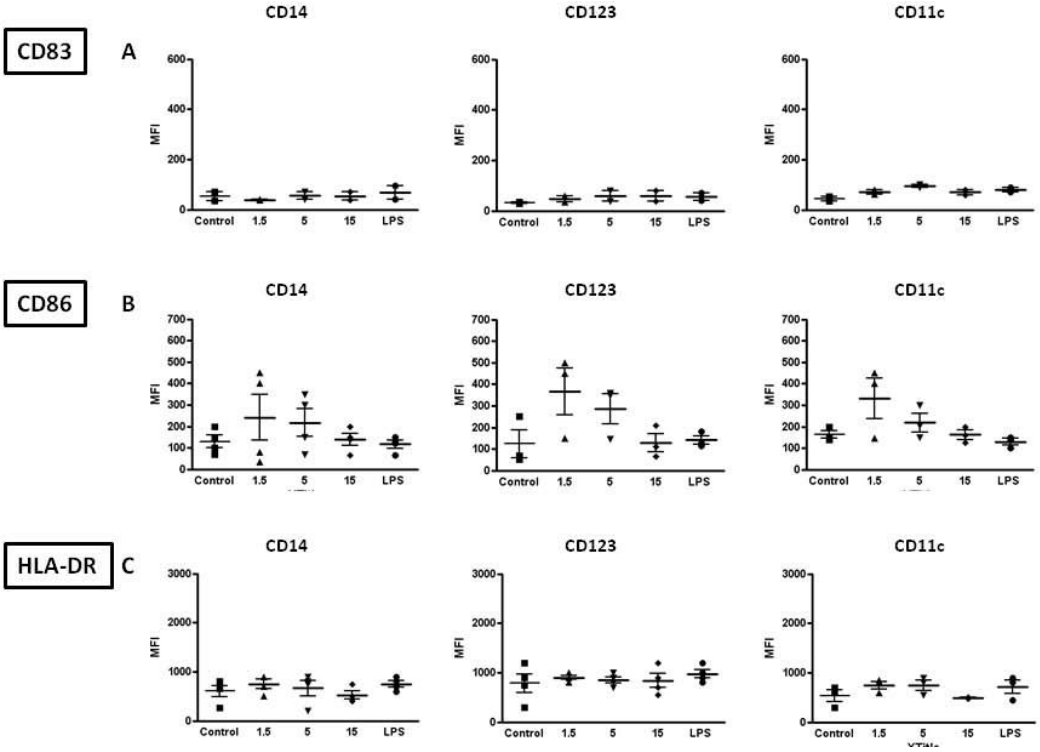

Figure 5 Expression of surface maturation/activation markers, indicated as Mean Fluorescence Index (MFI), induced by the indicated concentrations of VK3-20 and LPS in PBMC-derived monocytes and DC from control subjects. CD14 = CD14+ monocytes; CD123 = CD123+ pDC; CD11c = CD11c+ mDC.

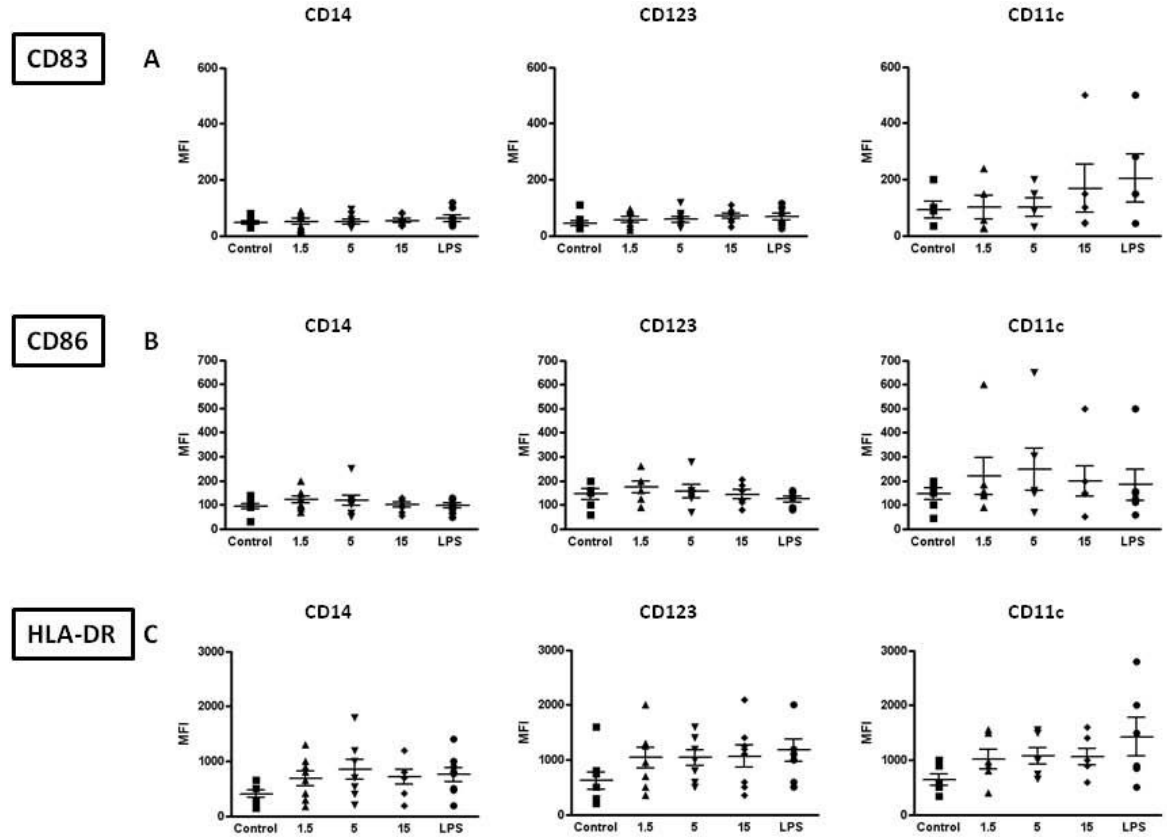

Figure 6 Expression of surface maturation/activation markers, indicated as Mean Fluorescence Index (MFI), induced by the indicated concentrations of VK3-20 and LPS in PBMC-derived monocytes and DC from HCV seropositive subjects. CD14 = CD14+ monocytes; CD123 = CD123+ pDC; CD11c = CD11c+ mDC.

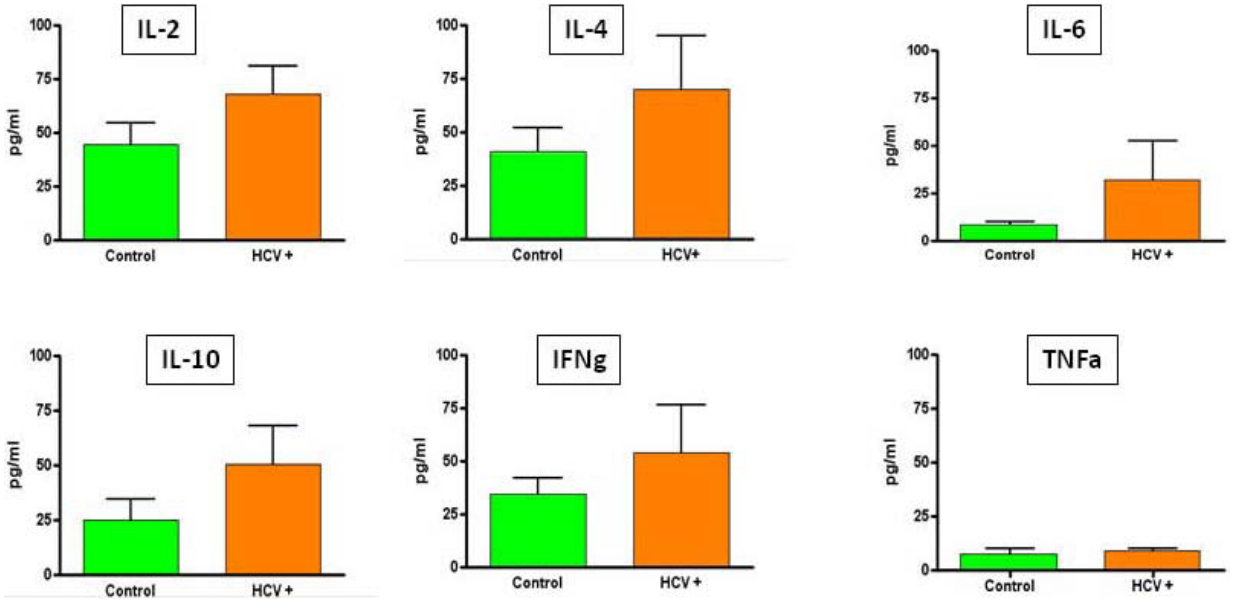

Figure 7 Analysis of basal level production of Th1 and Th2 cytokines in supernatants of PBMCs from control and HCV positive (HCV+) subjects.

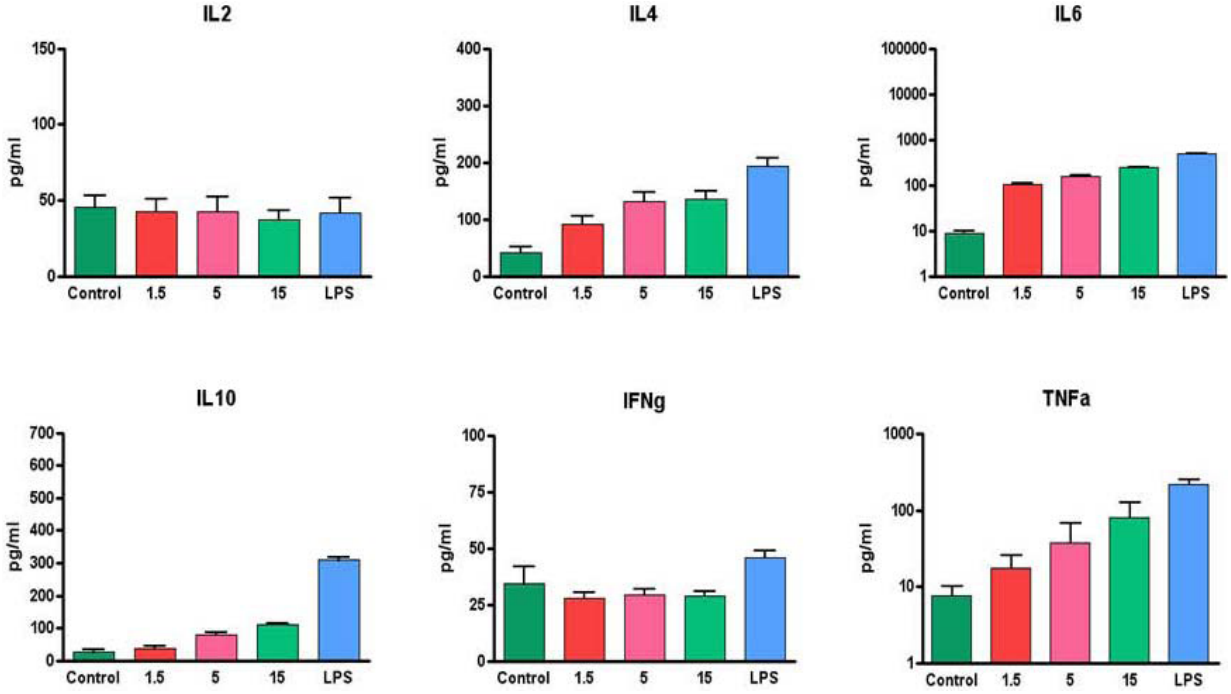

Figure 8 Analysis of Th1 and Th2 cytokines in supernatants of PBMCs from control subjects induced by the indicated concentrations of VK3-20 and LPS.

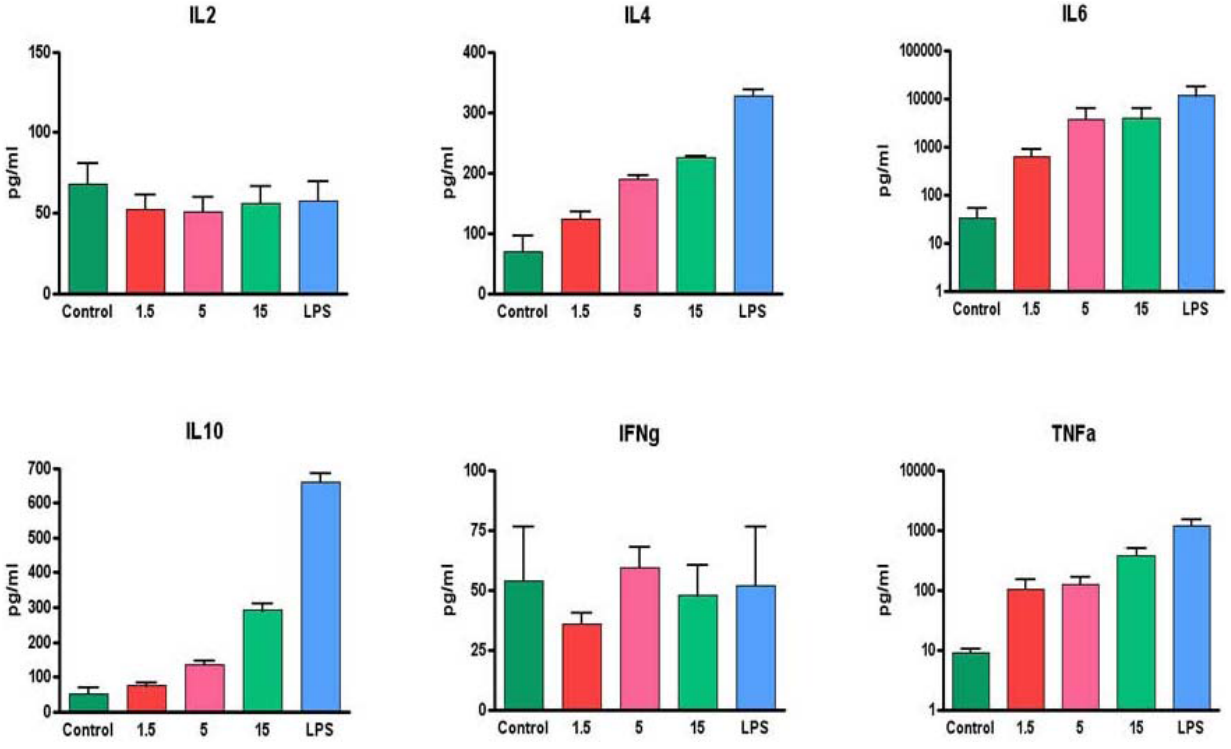

Figure 9 Analysis of Th1 and Th2 cytokines in supernatants of PBMCs from HCV seropositive subjects induced by the indicated concentrations of VK3-20 and LPS.

maximal induction of cytokine expression (15 μg/ml) may suggest a different pathway of activation involved in the two independent biological effects, which need further investigation.

The similar response observed in HCV-seropositive subjects, regardless of the diagnosis of type II MC or NHL, would suggest the absence of an *in vivo* priming for the VK3-20. In this regard, the expression of VK3-20 in the clonal B-cell populations of these subjects is currently under evaluation.

The impairment of basal and antigen-induced production of Th1-polarizing cytokines for HCV-seropositive individuals is in concordance with our previous observations on PBMCs from HIV infected subjects exposed *ex vivo* to a VLP-based HIV vaccine model [25,44].

The overall results here described represent a proof-of-concept and confirm the possibility of screening donor susceptibility to an antigen treatment using circulating APCs, CD14+ monocytes as well as CD123+ plasmacytoid DC (pDC) or CD11c+ myeloid DC (mDC) populations, without the need of purification and *ex vivo* selection of DCs, simplifying the identification of "responsive" vaccinees and providing mechanisms of eventual failures in individuals enrolled in clinical trials. When necessary, additional and more detailed studies on fractionated cell types would allow identification and a better characterization of the individual cells involved in mediating the *in vivo* response. In conclusion, our results indicate that circulating APCs from HCV-seropositive patients can be functional in active autologous immunotherapy strategies. In particular, the results strongly suggest the induction of the innate and early adaptive immune response by the protein corresponding to the VK3-20 light chain of the idiotype most frequently identified on B cell clones sustaining the HCV-associated type II MC and NHL. Therefore, its use as preventive as well as therapeutic vaccination strategy appears to be feasible and potentially effective. However, specific Th1-driving adjuvant strategies might be necessary to obtain the sought efficient therapeutic effect.

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Authors' contributions

LB designed the study and wrote the paper; AP conducted the cellular inductions and cytokines evaluations; MLT conducted the statistical analyses; MN conducted the cytofluorimetric analyses; GC supervised the cytofluorimetric analyses; GB, AdR and OP provided the clinical samples; LR and VS provided the VK3-20 protein; DM, VdR participated to the design of experiment and evaluation of data; RD and FMB supervised the whole project.

All authors read and approved the final manuscript.

Competing interests

MLN is the CEO of Areta International S.r.l., who provided the VK3-20 protein for the study. The authors declare that they have no competing interests.

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MINIREVIEW

Translating Tumor Antigens into Cancer Vaccines[▽]

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Vaccines represent a strategic successful tool used to prevent or contain diseases with high morbidity and/or mortality. However, while vaccines have proven to be effective in combating pathogenic microorganisms, based on the immune recognition of these foreign antigens, vaccines aimed at inducing effective antitumor activity are still unsatisfactory. Nevertheless, the effectiveness of the two licensed cancer-preventive vaccines targeting tumor-associated viral agents (anti-HBV [hepatitis B virus], to prevent HBV-associated hepatocellular carcinoma, and anti-HPV [human papillomavirus], to prevent HPV-associated cervical carcinoma), along with the recent FDA approval of sipuleucel-T (for the therapeutic treatment of prostate cancer), represents a significant advancement in the field of cancer vaccines and a boost for new studies in the field. Specific active immunotherapies based on anticancer vaccines represent, indeed, a field in continuous evolution and expansion. Significant improvements may result from the selection of the appropriate tumor-specific target antigen (to overcome the peripheral immune tolerance) and/or the development of immunization strategies effective at inducing a protective immune response. This review aims to describe the vast spectrum of tumor antigens and strategies to develop cancer vaccines.

CANCER IMMUNOTHERAPY

Cancer immunotherapy may be classified into passive as well as active strategies, with the latter being specific or nonspecific (117). Passive or "adoptive" immunotherapy is based on administration of antitumor antibodies or transfer of tumor-reactive lymphocytes. Active immunotherapy is aimed either at eliciting a specific *de novo* host immune response against selected tumor antigens (Ags) by employing cancer vaccines or at amplifying the existing antitumor immune response by administering nonspecific proinflammatory molecules or adjuvants. In this context, considering the disappointing results up to now, the quest for specific and selective tumor antigens for developing tumor-specific cancer vaccines, optimal delivery systems (i.e., dendritic cell [DC]-based vaccines), adjuvants, and strategies to overcome immune tolerance and regulatory T (Treg) cell responses is the main goal for several research groups and leading health care companies.

QUEST FOR THE APPROPRIATE TUMOR ANTIGEN

The role of the immune system in tumor containment and/or "rejection" has been studied for decades, showing the possibility of inducing an immune response able to reject an experimentally transplanted tumor. However, the "immunosurveillance of tumors" theory independently postulated by Burnet (19–21) and Thomas (173) has not held the original promise, and much skepticism has been raised by different authors. More recently, the original concept of immunosurveillance has been further elaborated by Schreiber et al. (53, 54) into the "cancer immunoediting" hypothesis, which postulates three main phases: elimination, equilibrium, and escape. In particular, in the elimination phase, cells of the innate and adaptive immune responses may eradicate the developing tumor and protect the host from tumor formation. If the elimination process is not successful, the tumor cells may enter the equilibrium phase and be immunologically shaped by immune "editors" to produce new populations of tumor variants. These variants may eventually evade the immune system and become clinically detectable in the escape phase (53, 54).

The cells playing a key role in this process have been identified in both the innate (e.g., natural killer cells, natural killer T cells, macrophages, and dendritic cells) and the adaptive (e.g., $CD4^+$ Th1 and $CD8^+$ T cells) immune systems, whose final goal is to kill the antigen-bearing tumor cells. More recently, a relevant role for an additional subset of $CD4^+$ T helper cells (named Th17) in the immune response to cancer has been proposed and described by several authors (reviewed in reference 203).

However, different approaches have failed to induce an effective antitumor immune response, suggesting the notion of "nonimmunogenicity" of tumors (69). However, more recently it has been shown that the low tumor immunogenicity is not due to the lack of target "tumor" antigens but to their inability to induce an effective immune response. Among several possible biological reasons, this would be consequent to the growth of tumors in the absence of an inflammation process necessary to establish the tissue microenvironment essential to

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recruit and induce activation and maturation of the antigen-presenting cells (APCs), which represent the key point of initiating an effective adaptive humoral and cellular immune response.

In this perspective, the search for human tumor antigens as potential targets for cancer immunotherapy has led to the discovery of several molecules expressed mainly or selectively on cancer cells.

Antigens used in cancer vaccines, indeed, should preferably be molecules differently expressed on normal and tumor cells; however, most antigens are derived from mutated or modified self-proteins, which may induce immune tolerance (61). This aspect represents a challenge for the appropriate design of vaccines that have to overcome such tolerance in order to elicit specific antitumor immunity without "undesired" autoimmunity side effects (130).

MULTIPLE "UNDEFINED" ANTIGENS

It is well known that tumors show the accumulation of several genetic modifications in somatic cells (63, 186), which provide cancer cells with the selective growth advantage to initiate clonal expansion (66).

In this context, cancer genes have been originally studied based on their possible relation to cancer (59). More recently, high-throughput technologies have enabled the identification of mutated genes in cancers without any hypothesis-driven bias, whose number has resulted to be surprisingly high, with a functional heterogeneity broader than previously thought (140, 169). These studies have been performed in breast, colorectal, pancreatic, and lung cancers and glioblastoma and, overall, have identified almost 400 candidate cancer genes (CAN-genes) (48, 83, 134, 199). Interestingly, systems-level analyses show that, despite the low degree of overlap in terms of gene identity, cancer signatures converge on specific biological processes, as defined by significant molecular and functional associations between genes and/or proteins (68, 163, 170).

Considering the high number of potential tumor antigens for each individual type of cancer, the concept of immunizing with whole tumor cells to avoid the exclusion of potentially relevant antigens from the vaccine is still valid. A further advantage is that since whole tumor cells express an array of antigens, this vaccine approach circumvents the major histocompatibility complex (MHC) restriction and the need for specific patient-tailored epitope identification. The efficacy of autologous tumor cells as a cancer vaccine has been tested in several clinical trials targeting different tumor types, including colorectal cancer (67, 185) and melanoma (3, 12). Alternatively, to overcome the limitations of patient-tailored vaccines (e.g., standardization of large-scale production, variability in the quality and composition of the vaccines, and lack of reliable comparative analysis of clinical outcome), the use of allogeneic tumor cell lines as cancer vaccines has been tested for prostate cancer (114, 160, 162).

However, the effectiveness of such a vaccine strategy is dramatically hampered by the immune system's inherent tolerance to several tumor antigens, as they may be expressed by normal tissues or presented to T cells in a non-stimulatory context. As a consequence, the breaking of tolerance and the containment of immune suppression need a potent and specific immune stimulus combining antigens and immunological adjuvants (reviewed in references 32 and 192). Whole tumor cell vaccines can be made more immunogenic by modifying tumor cells to express costimulatory molecules and/or cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), which has proven to be more effective than others in inducing recruitment, maturation, and function of dendritic cells (DCs), the most potent type of antigen-presenting cell (APC) (50, 57, 81).

In particular, vaccination with genetically engineered, irradiated melanoma cells, modified to secrete GM-CSF, was shown to improve tumor antigen presentation through increased DC and macrophage recruitment, enabling the generation of effective melanoma-specific $CD4^+$ and $CD8^+$ T cells, CD1-restricted NKT cells, and antibodies (51, 57). Although the advantages of using GM-CSF as an adjuvant for cancer vaccines have been reported, recent observations have suggested the potential of GM-CSF to induce immune suppression, which may negatively impact the management of cancer patients (reviewed in reference 34). Whole tumor cell vaccines expressing high levels of costimulatory molecules are currently being pursued to treat aggressive cancers such as acute myeloid leukemia (AML) (26, 31).

Another approach based on multiple "undefined" antigens takes into account the heat shock proteins (HSPs), which are ubiquitous, intracellular molecular protein chaperons whose expression increases under conditions of elevated temperatures and metabolic stress (105) to enhance the antigen processing and presentation by MHC molecules (167). Consequently, HSPs are associated with a large repository of peptides consisting of self-peptides as well as the entire antigenic peptide repertoire of cancer cells. Therefore, the cross-presentation and cross-priming of tumor antigens mediated by HSPs may result in a valid strategy, especially when the amount of antigen is a limiting factor (reviewed in references 15 and 119). Heat shock protein-based vaccines have been shown to be effective in mice when the HSP was purified from tumor cells matching the implanted tumor (166, 171, 179). Vaccines based on proteins complexed with HSPs, purified from autologous tumors (HSP-protein complex), have been evaluated in clinical trials targeting different cancers. In particular, the immunogenicity and efficacy of HSP-protein complex 96 (HSPPC-96; vitespen) made of tumor peptides associated with the heat shock protein gp96 has been extensively assessed in preclinical and clinical trials for a wide range of cancers, including phase I and II trials in colorectal cancer (110), melanoma (7, 138), and renal cell carcinoma (82) and two phase III studies of melanoma and renal cell carcinoma (165, 172, 197, 198). Furthermore, a full-length human papillomavirus type 16 (HPV16) E7 antigen fused to HSP65 from *Mycobacterium bovis* BCG (HspE7) (33) has been evaluated in a phase II clinical trial, resulting in lesion regression in women with grade III cervical intraepithelial neoplasia (CIN III) (55, 183).

DEFINED ANTIGENS

Cancer vaccines based on defined specific tumor antigens should elicit a very specific effector and memory cell response

TABLE 1. List of most relevant TAAs recognized by T cells[a]

Shared Antigens		Type of tumor	Normal tissue distribution
Cancer-testis (CT) Ags	BAGE GAGE MAGE NY-ESO-1 SSX	melanoma, lymphoma, lung, bladder, colon and breast carcinomas	spermatocytes/spermatogonia of testis, placenta, ovary cells
Differentiation Ags	Gp100 Melan-A/Mart-1 Tyrosinase PSA CEA Mammaglobin-A	melanoma, prostate cancer, colon and breast carcinomas	melanocytes, epithelial tissues, prostate, colon
Overexpressed Ags	p53 HER-2/neu livin survivin	esophagus, liver, pancreas, colon, breast, ovary, bladder and prostate carcinomas	ubiquitous (low level)
Unique Antigens		**Type of tumor**	**Normal tissue distribution**
Unique Ags	β-catenin-m β-Actin/4/m Myosin/m HSP70-2/m HLA-A2-R17OJ	melanoma, non-small cell lung cancer, renal cancer	N/A
Unique/Shared Antigens		**Type of tumor**	**Normal tissue distribution**
Tumor-associated Carbohydrate Ags	GM2 GD2 GD3 MUC-1 sTn globo-H	melanoma, neuroblastoma, colorectal, lung, breast, ovarian and prostate cancer	epithelial tissues (e.g., renal, intestinal, colorectal)

[a] References for antigens listed here are reported in the text. N/A, not applicable.

with a limited chance of inducing autoimmunity. Such an approach may have opposite biological effects. One effect is the possible undesired selection and expansion of tumor variants which lack the target tumor antigen and are biologically resistant to the vaccine-induced immune response. Such tumor variants, however, may in turn induce a beneficial effect, broadening the immune response against newly expressed antigens not present in the original vaccine in a process defined "epitope spreading" (23, 144).

Since the identification of MAGE-1, the first gene reported to encode a human tumor antigen recognized by T cells (182), a large number of tumor antigens have been described (Table 1). Initial classification was based on expression profiles, with tumor-specific antigens (TSAs) being expressed only by cancer cells and tumor-associated antigens (TAAs) representing the mutated counterparts of proteins expressed by normal tissues. The currently accepted classification, however, includes only TAAs, which are divided into shared and unique TAAs and further classified into class I and class II HLA-restricted TAAs, according to the HLA allele restriction (reviewed in reference 125).

Among the shared TAAs, the following three main groups can be identified: (i) cancer-testis (CT) antigens, (ii) differentiation antigens, and (iii) widely occurring, overexpressed antigens.

Among shared tumor-specific antigens, cancer-testis (CT) antigens are expressed in histologically different human tumors and, among normal tissues, in spermatocytes/spermatogonia of the testis and, occasionally, in placenta. CT antigens result from the reactivation of genes which are normally silent in adult tissues (46) but are transcriptionally activated in different tumor histotypes (45). Many CT antigens have been identified and used in clinical trials, although little is known about their specific functions, especially with regard to malignant transformation. This group of TAAs includes MAGE-A1 (30, 177), NY-ESO-1 (78), and SSX-2 (1).

Differentiation antigens are shared between tumors and the normal tissue of origin and found mostly in melanomas and normal melanocytes (Gp100, Melan-A/Mart-1, and Tyrosinase) (4, 89–91, 131, 190), although they are also found in epithelial tissues and tumors such as prostate tumors (prostate-specific antigen [PSA]) (37, 38) and breast carcinomas (mammaglobin-A) (79). Moreover, expression of several oncofetal antigens appears to be increased in many adult cancer tissues, including carcinoembryonic antigen (CEA), which is highly expressed in colon cancer (178). TAAs from this group, despite

representing self-antigens, have been and still are commonly used in current cancer vaccination trials, often together with CT antigens.

Widely occurring, overexpressed TAAs have been detected in different types of tumors as well as in many normal tissues, and their overexpression in tumor cells can reach the threshold for T cell recognition, breaking the immunological tolerance and triggering an anticancer response. Among the most interesting TAAs of this group are the antiapoptotic proteins (livin and survivin) (154, 155), hTERT (116, 187, 188), and tumor suppressor proteins (e.g., p53) (2, 180).

Unique TAAs, on the other hand, are products of random somatic point mutations induced by physical or chemical carcinogens and therefore expressed uniquely by individual tumors and not by any normal tissue, representing the only true tumor-specific antigens (Ags) (reviewed in reference 133). Such Ags characterize each single neoplasm and were shown to be diverse between tumors induced in the same animal or even in different tissue fragments from the same tumor nodule (61, 141, 200). A relevant feature of unique Ags is their potential resistance to immunoselection if the mutated protein is crucial to the oncogenic process and thus indispensable for maintaining the neoplastic state. As a consequence, unique Ags should elicit an immune response clinically more effective than that of shared Ags. However, identification of unique tumor antigens for solid human tumors requires sequencing of the whole genome of each individual tumor in order to identify mutated genes and select peptides whose motifs are predicted to be presented by the patient's HLA alleles. Moreover, each tumor bears highly heterogeneous sets of defects in dozens of different genes (25, 83, 85, 142, 199) which need to be further verified for their substantial contribution to the tumor development and progression and, consequently, for their relevance as vaccine targets (58).

On the contrary, unlike for the solid tumors, the strategy of identifying unique TAAs is relatively easy and feasible for tumors of hematological origin, such as B cell lymphomas, for which the target antigen is well known, being represented by the immunoglobulin idiotype (Ig Id) included in the B cell receptor (BCR). Therefore, sequencing analysis for the identification of cancer-related mutations can be selectively focused on the Ig Id, which can be used for developing a patient-specific vaccine (8, 168). More recently, however, it has been demonstrated that the BCR repertoire expressed by clonal B cells sustaining hepatitis C virus (HCV)-associated non-Hodgkin's lymphoma (NHL) is not random, with a restricted representation of immunoglobulin idiotypes in different patients (28, 43, 137). As a consequence, it is possible to overcome the limitations of tailor-made individual vaccines by designing shared idiotype vaccines to elicit immunity, targeting the B cell clone sustaining the HCV-associated NHL in a broad spectrum of patients (18, 44).

An additional class of tumor antigens is represented by tumor-associated carbohydrate antigens (TACAs), which are glycans uniquely or excessively expressed on the cancer cell surface (143) and correlate with various stages of cancer development (42, 65). However, TACAs do not elicit T cell responses and are usually poorly immunogenic given their structural similarity to normal antigens (80). Nevertheless, conjugation to a carrier protein increases their immunogenicity, enhancing the presentation of carbohydrate antigens to antigen-presenting cells as well as induction of helper T cell activation (92, 106). In particular, keyhole limpet hemocyanin (KLH) has been shown to be the most effective carrier for TACAs (86), and KLH conjugates of GM2 for melanoma (27) as well as those of sTn for breast cancer (73) have both entered phase III clinical trials. However, the disappointing outcomes of these vaccine clinical trials, in terms of time-to-disease progression and overall survival, have driven the generation of several forms of fully synthetic carbohydrate vaccines, which have been shown to be immunogenic regardless of the use of a protein carrier or external adjuvant (6, 13, 22, 76, 176, 194). Unfortunately, despite all efforts, TACA-based cancer vaccines have failed to induce sufficient T cell-mediated immune responses in cancer patients, and none has been approved for clinical use yet (64).

APPLICATION OF DEFINED ANTIGENS AS CANCER VACCINES

Most cancer vaccine clinical trials have been performed with peptide-based vaccines, employing either cancer-testis antigens or differentiation TAAs, and despite the induction of a high frequency of specific T cells, the clinical outcomes have been disappointingly limited (29, 132, 136, 148, 149) (Table 2). There are many possible reasons for these unsatisfactory results, including immune tolerance induced by shared TAAs (35, 112) and limited cytotoxic T lymphocyte (CTL) expansion due to activation of regulatory T lymphocytes (122). Furthermore, single peptides elicit a $CD8^+$ T cell response with a narrow epitope specificity, which may result in limited immunological efficacy and in the induction of immune escape mechanisms (146).

Several strategies have been adopted to overcome such limitations, including the introduction of inflammatory cytokines in the vaccination protocol, such as alpha interferon (IFN-α) (94, 135) and interleukin-2 (IL-2) (104, 147, 150, 151), with conflicting results. An alternative strategy is to generate peptide variants of TAAs (47, 72, 84), including mimotopes, heteroclitic peptides, altered-peptide ligands, and superagonists, introducing MHC-anchor residue modifications (127, 181), systematic residue substitutions (161), combinatorial peptide libraries (111, 139), and genetically encoded peptide libraries (39, 191). However, considering the overall disappointing results of clinical trials testing such peptide variants, additional strategies have been developed to broaden the repertoire of responding T cells by introducing amino acid substitutions in the peptide-MHC binding surface (16, 74, 102).

Significant improvement in the immunogenicity of single-peptide vaccines has been achieved using long peptides deriving either from the chemical linkage of multiple immunogenic epitopes (71, 159) or from naturally occurring linked CTL and Th epitopes, as shown for human papillomavirus (HPV) E6-E7 proteins (93, 193), the CT antigen NY-ESO-1 (202), and HER-2/neu (49, 95). The enhanced immunological potency of long peptides, which do not bind directly to MHC class I molecules as 8-mer to 10-mer CTL epitopes do, is most likely due to their efficient presentation to CTL precursors through processing by DCs (9, 14, 113, 156), which should dramatically reduce transient CTL responses or tolerance (174, 175). Furthermore,

TABLE 2. Peptide-based cancer vaccine clinical trials for most representative tumors[a]

Tumor	N.	Antigen (# of trials)	Phase
Acute Myelocytic Leukemia	10	WT1 (5), PR1 (5)	I/II
Breast	32	E75 (2); p53 (2); HER-2/neu (9)	I/II
Colorectal	18	ras (5); CEA (4)	I/II
Liver	6	AFP (2); CEA (1)	I/II
Lung	20	URLC10 (6); ras (4); HER-2 (2); VEGFR1 and 2 (3); mutant p53 (2)	I/II
Melanoma	115	MAGE (13); gp100 (54); MART-1 (36); Tyrosinase (32); NY-ESO-1 (4)	I/II
Ovarian	16	p53 (4); NY-ESO-1 (3); HER-2 (3)	I/II
Uterine	8	HPV16 E7 (4); Survivin (1); mutant p53 (1)	I/II
Pancreas	14	ras (4); VEGFR1 and 2 (3); MUC-1 (1); Survivin (1)	I/II

[a] Further information on current cancer vaccine clinical trials is available at www.clinicaltrials.gov. N. = number of total clinical trials registered for the corresponding tumors.

long peptides may persist longer in inflamed lymph nodes in close proximity to the vaccination site, resulting in the clonal expansion of IFN-γ-producing effector T cells and improved antitumor CTL response (14). Finally, the presence of multiple immunogenic epitopes in long peptides would ensure the interaction with different HLA class I and class II alleles, eliciting a broad T cell response against many epitopes, including the immunodominant ones, and reducing the emergence of tumor escape variants.

DENDRITIC CELLS AS AN ANTIGEN DELIVERY SYSTEM

An effective vaccine needs to efficiently hit the innate immune system and, downstream, the adaptive humoral and cellular immunity to elicit an adequate level of effector cells and establish the immunological memory. To this aim, vaccine strategies effective in activating both innate and adaptive immunity are actively pursued by several groups, and among the different possible strategies, dendritic cell (DC)-based vaccines represent one of the most promising strategies (reviewed in references 96 and 126).

Dendritic cells can be generated *in vitro* from $CD34^+$ progenitor cells derived from the patient's bone marrow or peripheral blood, and maturation can be obtained *ex vivo* with a cocktail of several cytokines.

The original protocol for DC generation has been designed, including GM-CSF and IL-4 (121, 157), but it has been shown that functionally distinct DC subsets can be generated by using different cytokines. Indeed, activated monocytes induced with IFN-α/β, thymic stroma lymphopoietin (TSLP), tumor necrosis factor (TNF), or IL-15 will differentiate into IFN DCs, TSLP DCs, TNF DCs, or IL-15 DCs, respectively, able to induce different types of immune responses (5). For example, melanoma-peptide-pulsed IL-15 DCs are much more efficient than IL-4 DCs in inducing antigen-specific CTL differentiation *in vitro* (52), whereas IFN-α DCs show improved activation of T helper cells (128). Similarly, different DC maturation pathways may significantly impact their capacity to elicit T cell immunity (99). Indeed, GM-CSF/IL-4 DCs activated with a cocktail of IFN-α, poly(I:C), IL-1β, TNF, and IFN-γ are much more effective in inducing specific CTLs than "gold standard" DCs matured with a cocktail of macrophage cytokines, including IL-1β/TNF/IL-6/prostaglandin E2 (PGE2) (109). Moreover, PGE2 can skew the differentiation of T helper cells to Th2 cells, blocking the production of IL-12 p70 (87, 88).

Matured DCs are then loaded with tumor antigens as peptides (24), tumor lysates (121), or apoptotic debris (129), which are processed and presented on the DC surface in the context of MHC class I and II molecules. The matured and antigen-loaded DCs are then transferred back to the patients for the generation of an antitumor immune response (reviewed in references 5 and 157) (Fig. 1).

Concerning the strategy of using tumor lysates or apoptotic debris as source of tumor antigens, the original and standard procedure is to use autologous DCs loaded with autologous tumor cells, both derived from the treated patient. However, the preparation of sufficient amounts of autologous tumor cells might be a significant limiting factor, and to overcome this hurdle, the use of allogeneic tumor cell lines as sources of tumor antigens has been proposed. Tumor cell lines, indeed, share many TAAs with the patients' autologous tumor cells and can be efficiently expanded *in vitro*. Cellular fusions generated by autologous DCs and allogeneic tumor cell lines have been shown to induce antigen-specific polyclonal CTLs, with cytotoxic activity against autologous tumor cells (10, 97, 98). A further possible alternative is to use allogeneic DCs from healthy donors as a fusion partner, given that unprimed T cells from an individual react against the foreign MHC antigens of another individual. It has been demonstrated, indeed, that fusions of both autologous and allogeneic DCs are effective in inducing antitumor immunity in humans (62), although this approach can be applied only in selected situations (reviewed in reference 96).

The effectiveness of DC-based vaccines has been demonstrated in specific tumor stages, and very recently, the first autologous cellular immunotherapy has received FDA approval for the treatment of asymptomatic or minimally symptomatic metastatic hormone-refractory prostate cancer (HRPC). Sipuleucel-T consists of autologous PBMCs loaded with recombinant human prostatic acid phosphatase (PAP) linked to granulocyte-macrophage colony-stimulat-

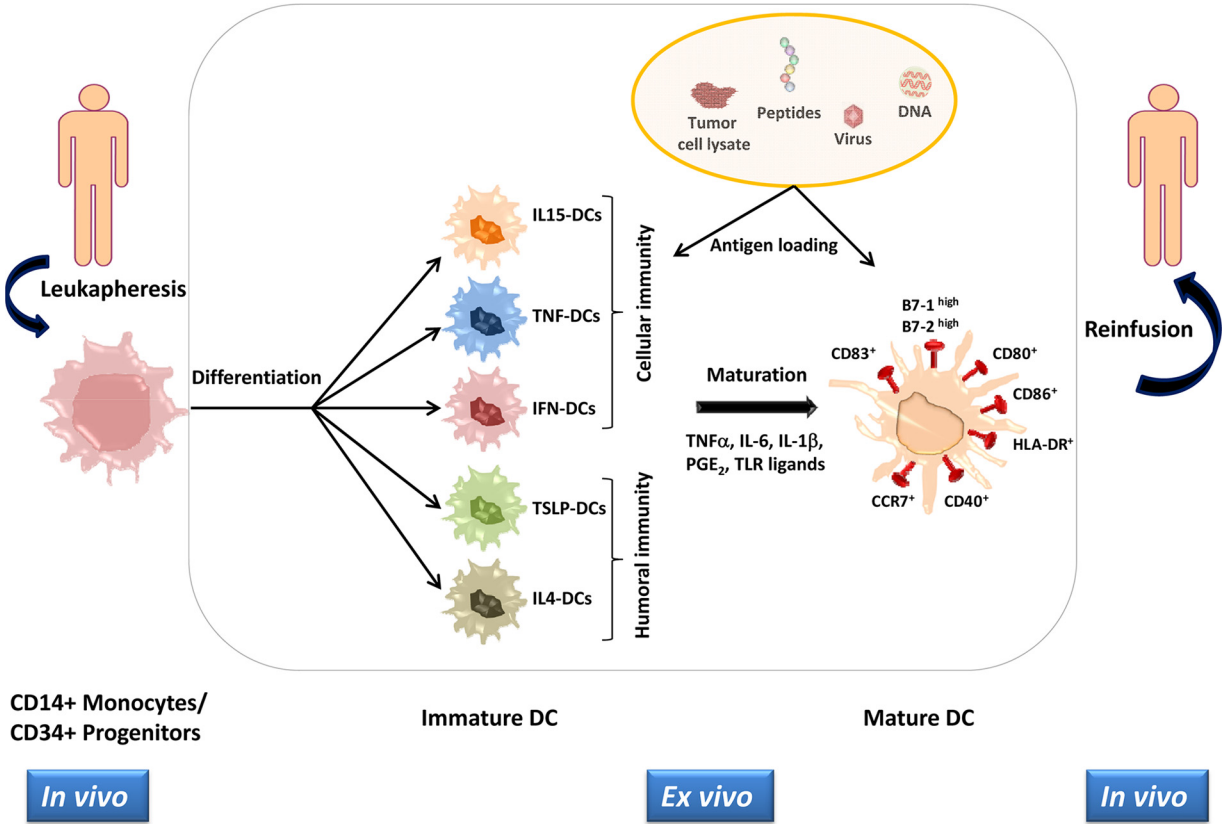

FIG. 1. Schematic representation of a DC-based vaccine preparation. $CD14^+$ monocytes or $CD34^+$ hematopoietic progenitors are derived from patients. Different DC subsets are generated *in vitro*, with distinct specialization in driving adaptive immunity to the Th1 or Th2 response. Mature DCs are loaded with one of the indicated sources of tumor antigens and reinfused in the patient. The most relevant cell markers characterizing the different activation stages of DCs are indicated.

ing factor (PAP–GM-CSF), which has proven to be effective in phase III clinical trials (70).

However, results from multiple clinical trials with DC-based cancer vaccines have been contradictory, and only fractions of the enrolled patients show potent antitumor immune responses (reviewed in references 96 and 103). Several reasons may account for this modest clinical outcome, including the reproducible efficiency of DC generation and the possible induction of adaptive $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T (Treg) cells in the presence of transforming growth factor β (TGF-β) or IL-10 derived from the tumor microenvironment (189).

HARNESSING IMMUNE TOLERANCE AND Treg CELLS

Naturally occurring regulatory T (Treg) cells account for 5% to 10% of peripheral $CD4^+$ T cells (60, 152), whose key role is to inhibit self-reactive effector T cells, inducing peripheral T cell tolerance (152). Moreover, Treg cells have been found to be increased in peripheral blood and tumors in a variety of human cancers (75, 107, 196), resulting in poorer prognosis and reduced survival (153, 158, 195, 201). The presence of an increased percentage of circulating Treg cells may, indeed, represent a major obstacle to the success of cancer vaccines, and partial depletion of Treg cells has been shown to enhance DC vaccine-induced immune responses in cancer patients (41, 123). In this perspective, cancer vaccines may be more effective when combined with therapeutic interventions aimed at eliminating and/or controlling naturally occurring $CD4^+$ $CD25^+$ regulatory T cells. Studies done in the 1980s showed that pretreatment with cytostatic drugs (i.e., cyclophosphamide) was significantly enhancing the efficacy of adoptive cancer immunotherapy in preclinical (124) as well as clinical (11) settings. Several clinical trials are currently ongoing to assess the efficacy of cyclophosphamide to control Treg cells and improve the immune response to cancer vaccines in humans (56). Alternative strategies to eliminate and/or control naturally occurring Treg cells are represented by the use of a recombinant IL-2 diphtheria toxin conjugate (Ontak), which has been shown to enhance tumor-specific T cell responses to vaccines (41, 118) as well as to improve immune responses in patients with metastatic melanoma (108).

Furthermore, depletion of $CD4^+$ $CD25^+$ Treg cells may also be achieved using an anti-CD25 monoclonal antibody (MAb), as shown for melanoma or breast cancer vaccine (77, 115, 145).

The role of Toll-like receptor (TLR) agonists as inhibitors of Treg cell function (36, 184) is still controversial. It has been reported, indeed, that different TLR agonists can be effective in limiting tumor progression when used as adjuvants to coadministered a cancer vaccine (120, 164) or as stand-alone immunotherapeutics, eliciting an immune response to tumor self-antigens (17, 100, 101). Nevertheless, such effects are not

univocal, given that TLR agonists may induce differentiation, proliferation, or activation of Treg cells. Human $CD4^+$ $CD25^+$ Treg cells stimulated with the TLR5 agonist flagellin, indeed, show enhanced expression of Foxp3 and increased suppressive function (40).

CONCLUSIONS AND FUTURE DIRECTIONS

The cancer vaccine field is constantly growing and generating a considerable amount of information in terms of antigen target identification and delivery as well as immune modulation, which will represent the knowledge platform to accomplish the ultimate aim of developing an effective cancer vaccine. However, large-scale clinical trials of current strategies and protocols have not yet proved to be as efficacious as needed for complete tumor regression.

Several reasons may account for these disappointing results, including (i) tumor evasion from immune recognition, (ii) inefficient induction of high-affinity adaptive immunity, and (iii) tumor-induced immunosuppression. Each of these aspects needs to be addressed and possibly solved in order to increase the chances of success.

In this discovery process, the systems biology approach can have a great impact not only on the comprehension of multiple pathways involved in tumor development and progression but also on the dissection of molecular mechanisms involved in the efficient induction of effective innate and adaptive immunity. Such an approach, ultimately, will have a significant impact on cancer vaccine development for the identification of both novel potential target antigens and molecular prediction markers of immunogenicity.

This would represent the real switch from the "empirical" to the "knowledge-based" age of cancer vaccinology, enabling the development of strategies with enhanced therapeutic efficacy to significantly improve the quality of life of cancer patients.

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Dendritic cells in the pathogenesis and treatment of human diseases: a Janus Bifrons?

Dendritic cells (DCs) represent the bridging cell compartment between a variety of nonself antigens (i.e., microbial, cancer and vaccine antigens) and adaptive immunity, orchestrating the quality and potency of downstream immune responses. Because of the central role of DCs in the generation and regulation of immunity, the modulation of DC function in order to shape immune responses is gaining momentum. In this respect, recent advances in understanding DC biology, as well as the required molecular signals for induction of T-cell immunity, have spurred many experimental strategies to use DCs for therapeutic immunological approaches for infections and cancer. However, when DCs lose control over such 'protective' responses – by alterations in their number, phenotype and/or function – undesired effects leading to allergy and autoimmune clinical manifestations may occur. Novel therapeutic approaches have been designed and currently evaluated in order to address DCs and silence these immunopathological processes. In this article we present recent concepts of DC biology and some medical implications in view of therapeutic opportunities.

KEYWORDS: autoimmunity ■ cancer ■ cell therapy and immunotherapy ■ dendritic cells ■ HIV

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The main function of immune system is to protect our body from invading foreign pathogens or newly generated expanding cells (i.e., cancer cells) by neutralizing cell-free microorganisms or killing targeted cells. To efficiently fulfill its function, the immune system must distinguish self from nonself antigens in order to minimize collateral immunopathological consequences, which may possibly lead to a wide repertoire of autoimmune diseases.

In this respect, dendritic cells (DCs) perform an important function in the generation and regulation of immunity [1]. They circulate throughout the body and constantly sense the surrounding environment for invading pathogens, malignant cells or other inflammatory signals. This sentinel function of DCs, as well as the expression of several immune-modulating molecules, makes DCs unique in their capacity to stimulate naive T cells and shape the T-cell immune response into different effector T-cell subsets. This function is mediated by pathogen recognition receptors (PRRs) that detect foreign antigens (i.e., pathogen-associated molecular patterns [PAMPs]); activating specific signaling pathways to drive biological and immunological responses [2,3]. Among the PRRs, Toll-like receptors (TLRs) are widely expressed on innate immune cells (including DCs, macrophages, mast cells and neutrophils), endothelial cells and fibroblasts, and their expression is regulated in response to several factors, including foreign antigens, vaccines and cytokines [4–7].

In addition to TLRs, other important families of PRRs are plasma-membrane and cytoplasmic receptors, including the C-type lectins; which recognize a range of microbial stimuli from pathogens such as HIV, hepatitis C virus, *Helicobacter pylori*, and *Mycobacterium tuberculosis* [8,9], and NOD receptors; which recognize components of intracellular bacteria [10]. In addition, DCs express a wide variety of receptors for inflammatory mediators that are released upon damage. As an example, some malignant cells express so-called damage-associated molecular patterns such as heat-shock proteins [11], uric acid [12] and high mobility group box 1 [13].

Once DCs have captured a foreign nonself antigen, they undergo a complex maturation process, remodeling from 'antigen-capturing' into 'antigen-presenting' cells. Indeed, they display foreign antigens complexed with MHCs on their surface, to enable recognition of the antigen by T cells through their T-cell receptor. This interaction, which is also referred to as signal 1, is an essential step to drive the adaptive immune response. However, in the absence of a second 'costimulation' signal, signal 1 is insufficient to activate naive T cells and will result in T-cell anergy or deletion [14–16]. Costimulatory molecules for signal 2, such as CD80 and CD86, can

either amplify or regulate the interaction between DCs and T cells. Finally, DCs can polarize naive T cells into Th1, Th2, Th17 or regulatory T (Treg) cell through the necessary third signal; provided by the secretion of cytokines such as IL-12, IL-23 or IL-10, as well as the expression of surface molecules, such as OX40-L or ICOS-1 (FIGURE 1) [17–20]. From the insights above, it is evident that alterations in the number, phenotype and/or function of DCs have been implicated in various human inflammatory and autoimmune immunopathological diseases [21,22].

Considering the key role of DCs in the activation and polarization of T cells in the response to external (i.e., microbial) and internal (i.e., cancer cells) nonself antigens, their manipulation for therapeutic approaches is evaluated in many diseases. During the past decade, progress has been made with regard to cellular therapies that have been designed to elicit or amplify immune responses. DC-based activation immunotherapies, so-called 'positive' vaccinations, have been widely used for cancer [23,24] and infectious diseases (mainly HIV) [25]. On the other hand, DC-based 'negative' immunotherapies aiming to reduce, suppress or redirect an existing immune response in autoimmune disorders and allergies is still in its infancy, but is gaining interest [26].

In this regard, we envisage that the administration of DCs will be integrated as a substantial component of future, individualized treatment modalities, for a broad scope of medical fields. In this article, we will focus on the role of DCs in the (immune) pathogenesis and treatment of human disease, including cancer, infectious diseases, autoimmune disorders and allergies.

DC-subsets

Dendritic cells were originally described by Steinman and Cohn [27]. Variations among the tissue distribution of DCs, and the differences in their phenotype and function, indicate the existence of heterogeneous populations of DCs. Indeed, several subsets of DCs have been characterized and their fundamental role in either immunity or tolerance has been described [28].

In general, DCs are classified into two subsets: plasmacytoid (pDCs) and myeloid or conventional DC (mDC or cDC), which show several distinct phenotypic and biological features [29]. While pDCs originate from a lymphoid progenitor cell in lymphoid organs, a myeloid progenitor cell differentiates towards different DC populations in the bone marrow [30]. Subsequently, DC subsets circulate throughout the body: Langerhans cells (LCs) migrate towards the

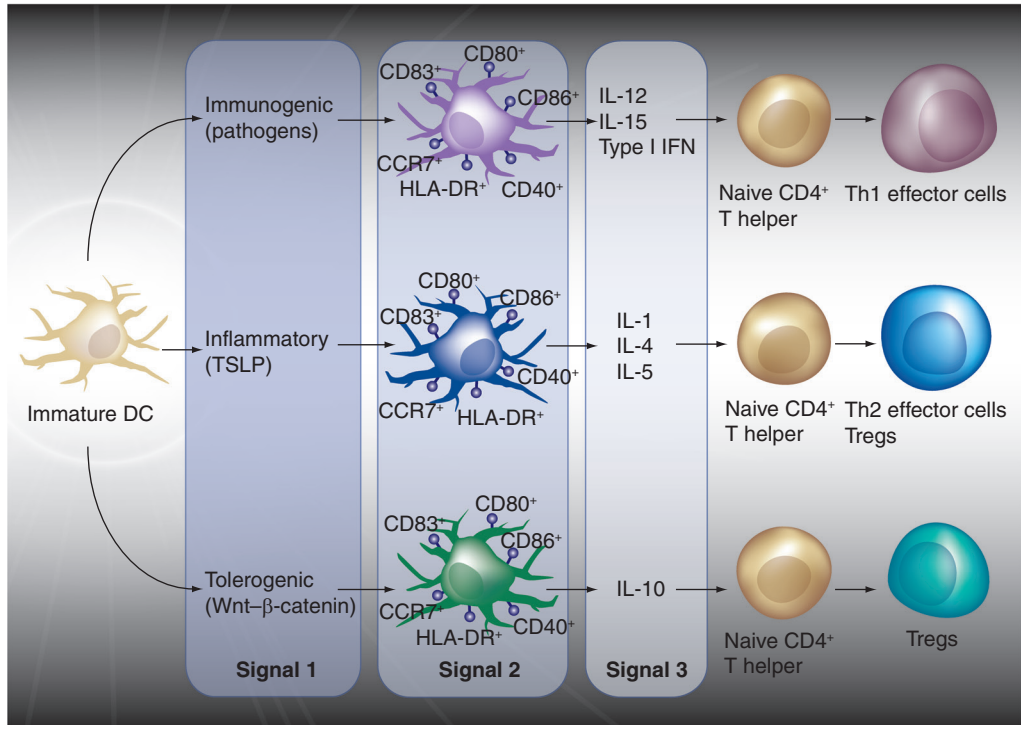

Figure 1. Dendritic cell maturation upon different stimuli and subsequent signals required for T helper cell differentiation. The distinct effector T cells derived from naive T helper cells are represented using a color-code strategy matching the DC subpopulation which provide the corresponding signals.
DC: Dendritic cell; Treg: Regulatory T cell; TSLP: Thymic stromal lymphopoietin.

skin epidermis, while interstitial DCs migrate towards the skin dermis and various other tissues (airways, liver and intestines). Circulating and migrating DCs are found in the blood and in the afferent lymphatics, respectively.

In human blood, differences in DC subsets can be identified based on differential expression of TLRs [31], cytokine receptors and cytokines [32], as well as a difference in migratory potential [33]; indicating a different function in induction and regulation of the immune response (reviewed in [34]). pDCs express CD303 (BDCA-2), CD304 (BDCA-4) and CD123 (IL-3Rα) and release high amounts of type I IFN in response to viral stimuli [35]. In particular, BDCA-2$^+$ pDCs are considered the front line in antiviral immunity owing to their capacity to rapidly produce high amounts of type I IFN in response to viruses [36]. Moreover, such pDC subsets demonstrate an efficient cross-presentation of antigens, inducing both CD4$^+$ and CD8$^+$ antiviral T-cell responses [37], representing the optimal subset to be exploited for preventive and therapeutic antiviral vaccines. However, a second distinct subset of pDCs has been recently described that inhibits replication of cancer cells and kills them in a contact-dependent fashion [38].

On the other hand, cDCs are characterized by their expression of CD1c (BDCA-1), CD11c and HLA-DR, and can produce IL-12p70, IL-6, TNF-α and IL-10 in response to bacterial stimuli and CD40 ligation [39]. Recently, several groups have characterized cDCs expressing CD141 (BDCA-3) as the human counterpart of CD8α DCs in mice [40–43]. By contrast to human blood CD1c$^+$ DC, the CD141$^+$ DC subset is characterized by the expression of high levels of TLR3, but not TLR4, 5 or 7 [40], and displays the unique expression of the C-type lectin CLEC9A [41] and the chemokine receptor XCR1 [42,43]. In addition, CD141$^+$ DCs produce high levels of IL-12p70, CXCL10 and IFN-β, and are of profound importance in the induction of T-cell responses by cross-presentation [40–43]. Although CD141$^+$ DCs represent only 3–4% of all DCs in human blood, the data mentioned above suggest that CD141$^+$ DCs will be the major human DC subset involved in the induction of cytotoxic T lymphocyte (CTL) responses against tumors and many pathogens.

In human skin, instead, two different mDC subsets have been characterized: epidermal LCs and dermal interstitial DCs (dermal DCs), with the latter subset further subdivided into CD1a$^+$ DCs and CD14$^+$ DCs [44]. In particular, human CD14$^+$ dermal DCs induce naive CD4$^+$ T cells to differentiate into cells with properties of follicular helper T cells [45], which are the class of effector T helper cells that regulate the step-wise development of antigen-specific B-cell immunity *in vivo* [46].

On the other hand, LCs induce both CD4$^+$ and CD8$^+$ T cells and are more efficient in cross-presenting peptides from protein antigens to CD8$^+$ T cells. Moreover, CD8$^+$ T cells primed by LCs acquire potent cytotoxicity, and are able to efficiently kill target cells, including tumor cell lines that express peptide-HLA complexes only at low amounts [45]. Therefore, the overall data suggest that dermal DCs would be more appropriate for vaccines aimed at inducing humoral responses, and LCs more appropriate for vaccines aimed at inducing cellular responses.

DCs in human disease

Cancer

There is a clear link between cancer and inflammation, confirmed by the presence of inflammatory cells, chemokines and cytokines in the early-stage tumor microenvironment [47]. DCs are also frequently present in the intra- and peritumoral area [48], recruited by several chemokines and cytokines [49,50], and contributing to either tumor growth or regression.

Studies examining DC subset distribution in tumors have demonstrated that epithelial, stromal, marginal and peritumoral areas can harbor different DC types [51–55]. In particular, some studies have shown that high numbers of overall tumor-infiltrating DC are associated with improved outcome for patients with primary tumors [56–60], whereas other studies have shown that this is true only in the presence of specific DC subtypes, for example DCs positive for both CD1a and HLA-DR [55,61,62]. In addition to the number and subtype of infiltrating DCs, also the location of infiltration has also been shown to have a prognostic value, depending on the tumor type [52,54,55]. In patients with colorectal cancer, indeed, the presence of HLA-DR-positive DCs in the tumor stroma or CD1a-expressing DCs in the advancing edge has been correlated with an adverse survival outcome [54]. On the contrary, in patients with tongue carcinoma; peritumoral CD1a-positive DCs have been associated with improved patient survival and a reduced recurrence [63].

At present, the crucial role of DCs in the generation of protective antitumor immune responses has been established, and is related to their capacity to link innate and adaptive immunity by presenting tumor-associated antigens

to T cells and inducing tumor-specific type 1 T-cell responses [48]. Furthermore, the efficacy of inducing such type 1 T-cell responses by DCs also seems to be strongly dependent also on bi-directional cross-talk between DCs and NK cells [64].

However, the efficacy of antitumor immune responses mediated by DCs may be suboptimal given that, although proinflammatory cytokines can mature DCs, the most powerful danger signals for DC activation are PAMPs, which are absent if tumoral transformation is not virus-induced [65,66]. Moreover, tumor cells and the tumor environment can hamper DC function through so-called immune escape mechanisms [67]. Indeed, tumour-derived inhibitory factors might be involved in downregulating or altering DC function, which includes the inhibition of DC differentiation, maturation, antigen processing and migration out of the tumor into the lymph nodes [68–70]. In this regard, cancer patients have fewer circulating DCs [68,71–74] and intratumoral DCs are mostly characterized by an immature phenotype [51,68,69]. Immature DCs (iDCs) and immature myeloid cells are, indeed, recruited by tumor-derived factors (e.g., VEGF, IL-10, prostaglandin E2 (PGE2), COX-2 and gangliosides) and can directly suppress specific T-cell responses [68,71,72,75–78]. Moreover, the development of myeloid-derived suppressor cells and tumor-associated macrophages can contribute to DC inhibition, and further inhibit the immune containment of tumor growth [79–82].

The sum of such immune suppressive factors hampering the biological functions of DCs not only facilitates the tumor escaping from immune control, but also reduces dramatically the success of cancer immunotherapy. In order for DC-based vaccines to be successful, highly immunostimulatory DCs are required to activate T cells and NK cells and simultaneous counteraction of immunosuppressive factors might be necessary to achieve optimal clinical benefit.

Viral infectious diseases

Viruses are one of the major targets of the immune system, and DCs are key determinants of the pathogenetic outcome of viral infections. Several PAMPs, such as the high repetition of capsomers and/or peplomers on virion surfaces and genomic viral RNA or DNA, are recognized as markers of viral invasion by PRRs on DCs [2,7]. In particular, DCs play a key role not only in directly sensing the presence of pathogens, but also in orchestrating the interactions between the other innate immune cell types, and facilitating the elicitation of antiviral defenses such as secretion of type I IFNs and defensins [26,28]. Moreover, the interaction between PRRs and components of microbes or viruses triggers a downstream signaling cascade whose end point is the activation of transcription factors (e.g., IRF3, IRF7, AP-1 and NF-κB) inducing the activation of inflammatory cytokine genes, such as *TNF*-β, *IL-6*, *IL-1*β and *IL-12*, as well as the upregulation of costimulatory molecules such as CD80, CD86 and CD40 on DCs [83]. Viral antigens presented by DCs in the context of MHC class I will induce $CD8^+$ CTLs, while those presented in the context of the MHC class II will induce $CD4^+$ T helper cells. In the latter case, according to the pattern of cytokines secreted by DCs, antigen-specific activated $CD4^+$ T helper cells can be directed into Th1, Th2 or Treg cell polarization, ultimately driving the adaptive immune system toward either a cellular T cell (sustained by $CD8^+$ CTLs), a humoral antibody or a tolerance response, respectively [84–89]. pDCs prevalently participate in the antiviral defense, inducing clonal expansion of T cells in lymph nodes [90], release of type I IFN [91] and antigen-specific $CD8^+$ cytotoxic activity [37].

Several viruses have developed defensive strategies in order to counteract the DC-mediated antiviral activity and to promote their own persistence, by favoring the production of Th1-suppressive cytokines and altering cytokine secretion. Indeed, inducing a shift toward a Th2 response is known to cause detrimental effects by promoting inflammation, allergy and ultimately viral persistence [92,93]. Alternatively, viral infections may affect distinct stages of DC generation, from hematopoietic progenitor differentiation to maturation [93–97], or interfere with antigen presentation process [97], thereby promoting viral spread by inhibiting the induction of efficient CTL responses that are crucial for viral control [98].

Furthermore, the numbers of and balance between the two subtypes of DCs seems to have a significant impact on the clinical outcome of viral infections. Severity of disease, indeed, has been found to be inversely correlated with the number of circulating pDCs and a blunted IFN-α release response during the early Phases of infection [99–101]. A reduced number of pDCs, besides individual genetic characteristics, may be virally determined, as shown in chronic hepatitis C virus [102] or HIV [103] infections. Finally,

viruses may replicate inside of DCs, perturbing or even subverting their physiology and giving rise to a more severe disease dependent on immunopathology [104–108].

Autoimmunity

Clinical autoimmunity arises as a result of an altered balance between the autoreactive cells and the compensating regulatory mechanisms. While T and B cells have been identified as the pathogenic effector cells in disease, the contribution of DCs in the initiation and perpetuation of inflammatory responses in autoimmune diseases is more and more being appreciated in the recent years [109–111]. Indeed, because of their committed antigen-presenting capacity and central role in the enhancement and regulation of cell-mediated immune reactions, DCs are thought to play a pivotal role in the immunopathogenesis of several autoimmune disorders [21,112].

The main mechanisms whereby DCs induce and maintain peripheral tolerance include the generation of Treg cells from naive T cells and the expansion of pre-existing Treg cells, the release of IL-10 and other immune-regulatory cytokines, and the promotion of T cell anergy or depletion. The phenotype and functional features of the different Treg cell populations have been extensively reviewed elsewhere [113–115]. Ohnmacht *et al.* confirmed a role for DCs in preventing autoimmunity under steady-state conditions, by constitutive ablation of all DCs subsets in mice, which resulted in breaking tolerance to self-antigens and development of spontaneous fatal autoimmunity [116]. In addition, depletion of pDCs during the acute or relapse Phase of experimental autoimmune encephalomyelitis, a mouse model for multiple sclerosis (MS), exacerbates the disease, underlining the regulatory role of DCs in limiting inflammation during autoimmunity [117].

Several studies have confirmed this involvement by demonstrating accumulation of DCs at the site of inflammation, such as the synovial tissue and fluid in rheumatoid arthritis [118–120], the CNS in MS [121–124], and the salivary glands and conjunctiva of ocular surface in Sjögren's syndrome [125]. Although, in some autoimmune diseases, reduced circulating levels of DC subsets have been observed in the peripheral blood of patients, this can likely be consequent to the recruitment or migration of DCs from the blood to the inflammatory site [118]. In that study, indeed, Jongbloed *et al.* demonstrated decreased expression of CD62L on DC in the synovial fluid of rheumatoid arthritis patients as compared with circulating DCs in the peripheral blood, indicative of migration. In addition, the chemokines CCL3, CCL4, CCL5 and CCL19 are highly expressed in brain tissue of MS patients [126,127], and may play a role in chemoattracting DCs (expressing the CCR5 and CCR7 receptors) found in the peripheral blood, CSF and MS lesions of such patients [128,129]. The link between CCR5 expression and the clinical severity of MS is further supported by the observation that in MS patients expressing a truncated allele of the *CCR5* gene and encoding a nonfunctional CCR5 receptor, the onset of MS is delayed [130] and the risk of recurrent clinical disease activity is decreased [131].

Furthermore, an altered phenotype and function of DCs in the peripheral blood and inflamed tissues of patients has been reported in several studies. In patients affected by systemic lupus erythematosus (SLE), systemic sclerosis or Sjögren's syndrome, pDCs are known to be hyperactivated and release high levels of IFN-α, in response to immune complexes containing DNA- or RNA-bound to specific autoantibodies [132–135]. The IFN-α promotes the differentiation of monocytes into mDCs which in turn promote the expansion of autoreactive T cells [136]. By contrast, pDCs characterized by impaired maturation, decreased or delayed expression of activation markers and reduced secretion of type I IFN have been observed in MS patients [137,138]. Moreover, pDCs isolated from the CNS can actively suppress the ability of mDCs to promote Th1 or Th17 differentiation [117]. One possible explanation for the discrepant results obtained in these studies is the existence of numerous subsets of DCs with opposite roles in promoting tolerogenic versus inflammatory responses. Indeed, two different subsets of pDCs have been recently reported in MS patients [139]: pDC1 ($CD123^{high}$ and $CD58^{low}$) which promote the induction of IL-10 secreting T regulatory type 1 (Tr1) cells and are abundantly present in steady state conditions, and pDC2 ($CD123^{low}$ and $CD58^{high}$) that promote differentiation of Th17 cells and are present in higher numbers in inflammatory conditions. In addition, monocyte-derived DCs and freshly isolated mDCs from patients with SLE [140], RA [141] and MS [142–144] display upregulated levels of activation markers (i.e., CD86, CD80 and HLA-DR), whereas monocytes and mDCs from SLE patients and progressive MS patients fail to upregulate PD-L1, a negative costimulatory molecule [143,145]. Furthermore, this activated phenotype of monocyte-derived

DCs and mDCs is accompanied by an enhanced secretion of immune-modulatory cytokines, including IL-12p70, IL-18 and IL-23 [146–149].

The remarkable regulatory role of DCs, in terms of their ability to induce antigen-specific tolerance, makes them a very attractive target for development of cellular therapies in autoimmune diseases. However, the pathological role of DCs, in terms of DC dysfunction reported in aforementioned investigations, imply that DC function needs to be restored in order to exploit the use of monocyte-derived DCs as an immunotherapeutic approach with regard to autoimmunity. In this framework, novel strategies that regulate DC development and differentiation, in view of harnessing their tolerogenic capacity, are being developed. Indeed, a wide spectrum of biologicals and pharmaceuticals have the ability to differentiate tolerogenic DCs, both *in vitro* and *in vivo*. Based on this knowledge, vaccination with *ex vivo* modulated tolerogenic DCs as treatment modality for autoimmune disorders is gaining momentum.

Role of DCs in allergic inflammation

Similarly to autoimmune disease, DCs are also suggested to contribute to the pathogenesis of allergic disorders. A significant rapid accumulation of myeloid and pDCs to the inflammatory site after allergen challenge has been observed [22,150–152], which directly correlates with a rapid decrease of circulating DCs in the peripheral blood [153,154]. Observed in allergic children [155] and adults [156], especially in asthmatic subjects. In addition, DCs from allergic patients have a more pronounced mature phenotype, such as higher expression of surface markers of DC maturity (CD83, CD86, HLA-DR and CD1c), which correlates with the heightened immune responsiveness [152,157,158]. Moreover, circulating DCs in children with allergic asthma produce a Th2-polarizing cytokine pattern upon TLR stimulation [159]. Allergens directly trigger PRRs on DCs which, upon allergen encounter, undergo maturation and migrate to the local lymph nodes in order to activate effector T and B cells [160–163]. In particular, it has been recently shown that TLR4 is the specific receptor for both airborne and contact allergens, such as the main house dust mite allergen, Derp2 [164], and nickel [165], activating the downstream signal transduction pathway and subsequent pro-inflammatory cytokine production. The key role of inflammatory DCs in the allergic immune response is further underlined by the fact that their depletion at the time of allergen challenge abrogates all allergic manifestations in the airways and intestine [166–168], which are restored by subsequent readministration of DCs [169].

DC-based therapeutic strategies

Immune-stimulatory DCs

The understanding of the pivotal role played by DCs in initiating the immune response and directing the adaptive immunity to foreign antigens allows a knowledge-based design of DC-based vaccine strategies (reviewed in [170]). This knowledge will help to targeting specific subsets, according to the desired outcome.

In order to develop DC-based immunotherapies, DCs can be generated *in vitro* either from $CD34^+$ progenitor cells, derived from patients' bone marrow or peripheral blood $CD14^+$ monocytes; differentiation can be achieved using a cocktail of cytokines, leading to the generation of distinct DC subsets. Indeed, monocytes induced with GM-CSF and IL-4, IFN-α/β, TSLP, TNF or IL-15, differentiate into GM-CSF/IL-4 DCs, IFN-α DCs, TSLP-DCs, TNF-DCs or IL-15 DCs, endowed with distinct functional characteristics [171]. Of note, melanoma-peptide-pulsed IL-15 DCs were shown to be much more efficient than IL-4 DCs in inducing antigen-specific CTL differentiation *in vitro* [172], whereas IFN-α DCs showed improved activation of helper T cells [173]. Similarly, different DC maturation cocktails may significantly impact upon the capacity of DCs to elicit T cell immune responses [174]. Indeed, GM-CSF/IL-4 DCs activated with a cocktail of IFN-α, poly I:C, IL-1β, TNF-α and IFN-γ, were shown to be much more effective in inducing specific CTLs than DCs matured with a cocktail of cytokines, including IL-1β, TNF-α, IL-6 and PGE2 (Figure 1) [175]. Subsequently, DCs loaded with antigens either as peptides, mRNA, tumor lysates or apoptotic debris, may lead to the generation of $CD4^+$ and $CD8^+$ T cell immune response [23,176].

DC-based cancer vaccines

The recent US FDA approval for sipuleucel-T, autologous PBMCs loaded with recombinant human prostatic acid phosphatase linked to GM-CSF, for therapeutic treatment of prostate cancer [177,178], has represented a significant advancement in the field of cancer vaccinology and a boost for new studies in the field of DC-targeted immunotherapy. The approval was based on results of randomized double-blind placebo-controlled multicenter trials, demonstrating that patients who received sipuleucel-T

had a median overall survival of 25.8 months, compared with 21.7 months for patients who received the control treatment (p = 0.032; HR: 0.775; 95% CI: 0.61–0.98). However, no difference in time-to-progression between the two groups was observed.

Undoubtedly, the increased overall survival of 4 months in vaccinated patients, and the cost of approximately $US100,000 for the treatment, does not represent the optimal cost-effective scenario for a treatment. However, unlike several previous attempts in the field, this is the

Table 1. Phase I/II clinical trials for cancer using autologous monocyte-derived dendritic cells.

Cancer type	Antigen	Patients (n)	Route of administration	Clinical response and stable disease	Immunological response	Ref.
Immunogenic DC						
Melanoma	Tumor lysate	10	id.	3/10	ND	[248]
		9	id.	1/9	ND	[249]
		40	id.	3/40	Yes	[250]
		50	id.	Yes	Yes	[251]
		38	id./sc.	9/33	ND	[252]
	Apoptotic tumor cells	16	id.	8/16	Yes	[253]
		10	id./iv.	3/10	Yes	[254]
	Gp100 peptides	12	iv.	2/12	Yes	[255]
		27	id./iv.	3/27	Yes	[256]
	Melan-A/MART-1 and/or NA17-A peptides	14	in./intralymph	0/14	Yes	[257]
	MART-1 peptides	16	id.	4/16	Yes	[258]
Breast cancer	Tumor lysate	12	sc.	2/12	Yes	[259]
	MUC-1 peptides	10	sc.	NA	Yes	[260]
Lung cancer	p53	54	sc.	NA	Yes	[261]
	Tumor lysate	15	id.	2/15	Yes	[262]
	Allogeneic cell line	14	id.	0/14	Yes	[263]
	Necrotic tumor cells	8	intranodal	2/8	Yes	[264]
Renal carcinoma	Tumor lysates	24	sc.	10/20	Yes	[265]
		9	sc.	6/9	Yes	[266]
		18	in.	9/18	Yes	[267]
		14	id.	1/12	ND	[268]
	MUC-1 peptides	20	sc.	6/20	Yes	[269]
Colorectal cancer	Tumor lysate	26	id.	4/17	Yes	[270]
	CEA DNA/mRNA	24	iv.	6/24	Yes	[271]
		14	sc./id.	1/12	Yes	[272]
	CEA peptides	10	in.	2/10	Yes	[273]
Hepatocellular carcinoma	Tumor lysate	10	in.	3/7	Yes	[274]
		31	iv.	4/31	ND	[275]
		35	iv.	10/35	ND	[276]
Glioma/glioblastoma	Tumor lysate	10	id.	2/10	Yes	[277]
		24	id./intratumoral	4/24	Yes	[278]
	Tumor-derived peptides	12	id.	1/12	Yes	[279]
		22	in.	9/22	Yes	[280]
Leukemia	None	6	in.	0/6	Yes	[281]
		10	sc.	4/10	Yes	[282]
	Tumor lysate	12	id.	8/12	Yes	[283]
	WT 1mRNA	10	sc.	2/10	Yes	[284]

More than 200 clinical trials have been performed using DC to modulate human disease. This table provides an overview after a systematic literature search from clinical trials that investigated clinical response and enrolled at least six patients.
DC: Dendritic cell; id.: Intradermal; in.: Intranasal; iv.: Intravenous; NA: Not applicable; ND: Not determined; sc.: Subcutaneous.

first therapeutic cancer vaccine to demonstrate effectiveness in Phase III clinical trials, by prolonging the life of patients who have advanced to the late stage disease, metastatic, asymptomatic, hormone-refractory prostate cancer. It definitely shows the feasibility and effectiveness of therapeutic cancer vaccines and represents a starting point from which to move forward.

Indeed, the task of therapeutic vaccines in cancer requires overcoming the critical challenges represented by the quest for specific and selective tumor antigens and optimal delivery systems [179–186].

With respect to identify an optimal antigen delivery system, many different approaches are currently being pursued and those based on DCs, either by *ex vivo* loading or by *in vivo* targeting, are among these [170,187]. In particular, considering the distinct ability of the different subsets of DCs in inducing the two arms of the adaptive immunity, the exploitation of specific subsets of DCs to elicit the desired adaptive response is foreseen. As described previously, indeed, LCs are very efficient in cross-presenting peptides to $CD8^+$ T cells, which acquire potent cytotoxicity and are able to efficiently kill target cells, including tumor cell lines that express peptide-HLA complex, only at low amounts [45]. Such biological effects appears to be related to prevalent production and secretion of IL-15 cytokine [188].

In this framework, differentiation of monocytes into DCs with cocktails including GM-CSF and IL-15 will generate cells with the phenotype and characteristics of LCs, which are far more efficient *in vitro* in priming melanoma antigen-specific $CD8^+$ T cells than DCs derived with GM-CSF and IL-4 [172,189]. Alternatively, IL-4 DCs activated with a cocktail of IFN-α, poly I:C, IL-1b, TNF and IFN-γ are able to induce significantly more melanoma-specific CTLs *in vitro* than DCs matured with the 'standard' cocktail of IL-1b, TNF, IL-6 and PGE2) [175].

However, results from multiple clinical trials with DC-based cancer vaccines (Tables 1) have been contradictory and only fractions of the enrolled patients show potent antitumor immune responses (reviewed in [190–192]). Several reasons may account for this modest clinical outcome, including the reproducible efficiency of DC generation, as well as the possible induction of adaptive $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells in the presence of TGF-β or IL-10 derived from tumor microenvironment [81,193]. All such microenvironment-immunosuppressive factors need to be considered and counterbalanced to hopefully improve the clinical outcome of cancer vaccines.

Different strategies are currently being pursued in order to improve the efficacy and outcome of DC-based cancer vaccines. Along this path, indeed, chemotherapy drugs at low doses have been reported to increase DC function [194,195] and to enhance cross-priming for tumor antigens of apoptotic cancer cells [196]. In particular, low-dose cyclophosphamide selectively increases migratory or pDCs, resulting in enhanced antigen presentation, augmented cytokine secretion and some inhibition of Treg cells [197]. Furthermore, it induces an increased expression of DC maturation markers [198] and regulates the production of type 1 IFNs; promoting the evolution of the $CD44^{high}$ memory T-cell response [199]. Finally, it can condition the tumor microenvironment by modulating the expression of tumor antigens and accessory molecules of T-cell activation or inhibition [200].

An additional strategy is the exploitation of biological effects of TLR agonists, whose antitumor affect was described many years before their actual discovery [201] and are listed (i.e., agonists to TLR3, 4, 7/8 and 9) among the immunotherapeutic agents with the highest potential to treat cancer [202]. Such molecules, indeed, induce a series of effects on DCs which will ultimately lead to initiating T cell immunity (i.e., antigen uptake, processing and presentation, DC maturation and T-cell activation [203]) for a potential anticancer therapy [204–206].

Although two TLR agonists, Bacillus Calmette-Guerin and imiquimod have been approved by the FDA for clinical use as monotherapy for cancer [207–210], while others are in human clinical trials, of which several represent promising vaccine adjuvants as they are potent DC activators, augment T-cell responses and downregulate suppressive effects of Treg cells (reviewed in [211]).

DC-based vaccines for HIV

Because of the ability of DCs to prime potent adaptive immunity, the concept of DC-based therapeutic vaccination is also an attractive option for HIV [24,212]. In particular, several ongoing clinical trials are based on DCs *ex vivo* loaded with autologous HIV-1 antigens in the form of apoptotic cells, inactivated virus and viral RNA or DNA [25,213–215]. Both therapy-naive and highly active antiretroviral therapy (HAART)-treated individuals have been enrolled in clinical trials, in order to evaluate the impact of HAART on the immunotherapeutic strategies. Combined antiretroviral therapies (HAART), indeed, result in partial immune recovery [216], which should improve the immune response to

vaccines. Other authors believe it is better to vaccinate patients naive for therapy to maximize the endogenous antigen load and to minimize potentially immunosuppressive effects of antiviral therapy [217].

The first human DC-based clinical trial was designed based on protein-pulsed autologous IL-4 DCs and tested in HIV-infected patients naive for HAART [218]. The administration of the formulation was well tolerated, enhancing HIV-specific T-cell reactivity, however, no effect on viral load was observed.

A second human trial used *ex vivo* generated monocyte-derived IL-4 DCs loaded with aldrithiol-2 (AT-2)-inactivated virus, showing the efficacy of the immunotherapy using inactivated virus and cytokine-matured DCs in patients with chronic HIV infection naive to HAART [219]. An effective HIV-1-specific T-cell response with sustained viral suppression of over 90% was observed in almost 50% of vaccinated subjects (8/18), demonstrating that strong virus-specific $CD4^+$ T helper cells are necessary to induce and sustain virus-specific $CD8^+$ effector functions. Interestingly, the therapeutic vaccine remained effective in close to half of the vaccinated patients a year after the vaccination, which demonstrated a direct correlation between the percentage of HIV-1-Gag-specific $CD8^+$ T cells expressing perforin, and the decline in plasma viral load [220].

Similarly, a trial testing the efficacy of autologous DCs loaded with heat-inactivated virus was performed in patients under HAART. Partial control of viremia was observed for 24 weeks after treatment interruption; however, the increased virus-specific $CD4^+$ and $CD8^+$ T-cell response was weak and transient [221]. Considering the discordant results in the two aforementioned clinical trials, the role of HAART or the effect of AT-2 inactivated virus versus heat-inactivated virus is still to be clarified. More recently, a clinical trial based on autologous DCs (IL-4 DC) pulsed with HIV peptides and influenza A matrix protein peptide was performed on HAART treated patients; the vaccine was safe and well tolerated, even though it did not show any significant effect on the viral load [222].

In addition, a clinical trial carried out to test the efficacy of viral mRNA-electroporated IL-4 DCs on HAART individuals demonstrated a proliferative response of $CD8^+$ T cells to HIV antigens in seven out of nine treated patients [223]. The effect on viral load was not evaluated in this trial because the vaccinees did not undergo treatment interruption. Furthermore, a Phase I/II clinical trial based on monocyte-derived DCs loaded with a mixture of HIV-1 Gag, Pol and Nef antigen lipopeptides, covalently linked to a palmitoyl-lysylamide moiety, is nearing completion [301], for therapeutic vaccination in chronically HIV-1-infected patients on HAART (**Table 2**) [224].

Tolerogenic DCs

While immunogenic DCs have been used in clinical trials to mainly treat cancer patients, the undisputed role of DCs in the pathogenesis of autoimmune diseases opens new avenues for harnessing tolerogenic DCs for clinical application. The use of DCs has become, indeed, a popular research track to repair and modulate autoimmunity, as well as to induce specific tolerance in organ transplants or allergy. DC-based immunotherapeutic strategies for autoimmune and allergic diseases can be developed either by targeting antigen to steady-state DCs *in vivo*, by modulating DC function *in vivo* or by culturing the cells *in vitro*, pulsing with antigen and injecting them back into patients.

Table 2. Phase I/II clinical trials for viral diseases using dendritic cell-based immunotherapy.

Patient population	Antigen	Patients (n)	Route of administration	Viral response	Immunological response	Ref.
Viral infectious diseases						
Hepatitis B	HBsAg	19	sc.	Yes	ND	[285]
	HBcAg 18–27 peptide and HBV PreS244–53 peptide	380	iv.	Yes	Yes	[286]
Hepatitis C	HCV-derived peptide	6	id.	No	Yes	[287]
Human cytomeglovirus	HCMV-derived peptide	24	sc.	Yes	Yes	[288]
HIV-1	Heat-inactivated virus	24	sc.	Yes	Yes	[289]
	HIV-1 derived DNA/mRNA	29	sc.	No	Yes	[290]
		10	id.	No	Yes	[223]
	HIV-1-derived peptides	18	iv./sc.	Yes	Yes	[222]

HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCMV: Human cytomeglovirus; id.: Intradermal; iv.: Intravenous; ND: Not determined; sc.: Subcutaneous.

Antigens coupled to antibodies specific for DC markers, such as 33D1 or DEC-205, have already been used in a mouse model to deliver antigens to DCs *in vivo*, resulting in antigen-specific tolerance which could not be broken by injection of the same peptide in the Freund's adjuvant [225,226].

Alternatively, DC functions *in vivo* can be interfered either by suppressing immunogenic functions or by promoting tolerogenic activity. For instance, TNF-α blockers have been extensively used and validated as an efficacious treatment for RA, Crohn's disease and psoriasis [227,228]. Although it remains to be established whether neutralization of TNF-α has a direct effect on DC function or cytokines produced by DCs, this approach clearly represents one of the greatest successes of immunology. In addition, the therapeutic efficacy of an anti-IL-12p40 monoclonal antibody has been demonstrated in patients with active Crohn's disease [229] and psoriasis [230,231], but not in MS patients [232].

Similarly, several new molecules have been identified that are able to alter DC function in allergic inflammation, resulting in immune tolerance and thus possibly leading to a long-lasting effect on the natural course of allergic disease. As an example, a specific small molecule compound (VAF347) that interferes *in vitro* with the function of monocyte-derived DC was also shown to be effective *in vivo* in allergic airway inflammation in a mouse model of asthma [233].

Administration of iDC has already been shown to induce antigen-specific T-cell tolerance. Indeed, when iDCs pulsed with influenza matrix protein and keyhole limpet hemocyanin, a general stimulator of $CD4^+$ T cells, were injected, a decline in influenza-specific $CD8^+$ IFN-γ secreting T cells was observed, while peptide-specific IL-10-secreting T cells appeared [234,235].

In this framework, an important issue to consider when designing DC-based immunotherapeutic strategies is that tolerogenic DC, after *in vivo* trafficking to the inflammatory microenvironment, will receive maturation signals and will incite unwanted T-cell responses. Therefore, such side effect needs to be blocked. Several reports demonstrate that exposure to anti-inflammatory cytokines and immune-suppressive agents can condition DCs to a tolerogenic state [28]. DCs generated in the presence of anti-inflammatory factors, such as vitamin A, vitamin D3, PGE2, indoleamine 2,3-dioxygenase, IL-10, TGF-β, retinoids, hepatocyte growth factor and vasoactive intestinal peptides display a stable tolerogenic phenotype that might be resistant to *in vivo* maturation stimuli [236–242]. Recently, we have shown that the *in vitro* exposure to IL-10 of monocyte-derived DCs from MS patients results in IL-10-, but not IL-12-secreting DCs with low expression levels of CD80/86 and an effective capacity to suppress myelin-specific T-cell responses *in vitro* [Cools *et al.*, Manuscript Submitted]. Importantly, further *in vitro* treatment of DC with maturation stimuli did not induce phenotypic changes or modifications in the cytokine secretion profile.

Aforementioned results suggesting that DCs can induce antigen-specific T-cell tolerance *in vitro* as well as *in vivo*, have prompted a number of groups to translate these findings into clinical application. A Phase I clinical trial using vitamin D3-treated tolerogenic DCs will be started in RA patients at Newcastle University (Table 3) [243,244,302]. Furthermore, the genetic manipulation of DCs by overexpressing immune regulatory molecules or inhibiting or silencing immune stimulatory molecules, promotes tolerogenic function. In line with this, a first safety study using tolerogenic DCs treated with antisense oligonucleotides targeting the primary transcripts of the CD40, CD80 and CD86 costimulatory molecules, has recently started at the University of Pittsburg [303].

Conclusion

Aforementioned investigations focusing on a better understanding of the role of DCs in the immunopathogenesis of these diseases will guide future research into the development of new and alternative therapeutic interventions. Indeed, dissecting the mechanisms of DC-activity in inflammatory conditions is invaluable in the

Table 3. Phase I/II clinical trials for autoimmune diseases using tolerogenic dendritic cells.

Patient population	Antigen	Patients (n)	Route of administration	Clinical response	Immunological response	Ref.
Tolerogenic DC: autoimmune diseases						
Diabetes	Not specified	15	id./sc.	ongoing	NA	[303]
Rheumatoid arthritis	Autologous synovial fluid	12	Into the knee joint	ongoing	NA	[302]

DC: Dendritic cell; id.: Intradermal; NA: Not applicable; sc.: Subcutaneous.

rational design of drugs and therapeutics that can reprogram the balance between immunity and tolerance, in the control of autoimmune diseases, cancer and chronic infections.

The key role of DCs is not only in connecting the innate immunity and the adaptive immune response, but also in driving the type of effector immune response. Such a role is predominantly efficacious in protecting us from external (e.g., microbial pathogens) or internal (e.g., cancer cells) dangers. However, an excessive or misleading response may result in a pathogenetic effect, leading to autoimmunity or allergy. Considering such properties, DCs are being targeted as a therapeutic approach either to exploit their immunopotentiating activity (DC-based vaccine) or to inhibit their immunopathogenetic effects (antiallergic therapies). In particular, the exploitation of the distinct immunological features of the different DC subtypes should enable us to significantly improve the efficacy of vaccine strategies. However, the clinical effects of such strategies are still limited for the complexity of the immunoregulatory environment, which exerts a significant immune inhibition effect. Also, the first cancer immunotherapy approved for clinical use, sipuleucel-T, shows limited efficacy. Nevertheless, it represents an extraordinary chance to acquire valuable information which will help to improving the immunological effectiveness and clinical outcomes of DC-based immunotherapeutic strategies.

Future perspective

Dendritic cells have become an attractive cell type for therapeutic manipulation of the immune system, in order to enhance insufficient immune responses in infectious diseases and cancer or to attenuate excessive immune responses in allergy and autoimmunity. Novel strategies to improve DC-induced tumor immune responses are under investigation. The recent identification that human CD141$^+$ DCs to cross-present antigens has clear implications for the design of new therapies to treat cancers and infectious diseases with improved efficacy. However, although Poulin *et al.* [41] made a first attempt to delineate *in vitro* culturing conditions for the generation of CD141$^+$ DCs from human progenitor cells, further optimization of such protocols is necessary to allow for their use in adoptive transfer immunotherapy approaches. In addition, targeting antigen to the newly identified unique targets on CD141$^+$ DCs (i.e., CLEC9A [41] and XCR1 [43]) will provide novel approaches to harness human DC subsets.

Furthermore, depending on the type of antigen, human DC subsets may cross-present for different functional outcomes, such as priming or tolerance [245]. This will be an important area for further investigation.

In addition, stronger activation and maturation stimuli for DCs will boost research in this field, and it is likely that TLR ligands will play a major role in the development of new vaccine adjuvants in the 21st century.

The prospected enhanced therapeutic value in patients of these *ex vivo* selective generated DC-vaccines needs to be proven. However, such observations may have a great impact to assess the specific immune responses elicited *in vivo* targeting selective DC subsets by using antigens linked to specific anti-DC receptor antibodies. Preclinical studies, indeed, demonstrate that the specific targeting of antigens to selective DC subset *in vivo* results in considerable potentiation of antigen-specific T cell immunity [226,246,247], suggesting that such strategy could be successfully translated into humans.

In conclusion, we anticipate that novel DC-based vaccination strategies will most likely be focussed on a multitargeted approach, based on a better understanding of DC biology in order to attain the goal of effective immunotherapy in cancer. In addition, vaccination with tolerogenic DCs, in combination with regulatory T cells, might gain clinical interest in the treatment of autoimmune disorders and allergy.

Executive summary

- Although the exact genesis and development of the different types and subsets of dendritic cells (DCs) has not yet been fully delineated, differences in the expression of various molecules predicts that they have different functions. This is supported by recent groundbreaking work on the subdivision of human DC into subsets and the investigation of their respective functions.
- Alteration in the number, phenotype and/or function of dendritic cells has been observed in inflammation, such as in cancer, infectious diseases and autoimmune immunopathological diseases in humans. These observations underline the central role of DCs in maintaining the delicate balance between immunity and tolerance.
- By elucidating the means for *ex vivo* generation and modulation of distinct DC subsets, DC-based therapeutic strategies have gained momentum both in order to ameliorate and attenuate immune responses. However, many questions remain to be answered and future research is warranted.
- Combination of DC-based therapeutic vaccines and adjuvanting strategies to counteract the immunosuppressive tumor microenvironment should significantly improve the clinical outcome.

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- ▪ of interest
- ▪▪ of considerable interest

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