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"The crosstalk between Dendritic and Natural Killer cells together with the function of Tumor Associated Macrophages (TAM) represent novel cancer-related targets for Human Immunodeficiency Type-1 Protease Inhibitors"

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“The crosstalk between Dendritic and Natural Killer cells together with the function of Tumor Associated Macrophages (TAM) represent novel cancer-related targets for Human Immunodeficiency Type-1 Protease Inhibitors”

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LIST OF PUBLICATIONS

This dissertation is based upon the following publications:

1. **Ciaglia E**, Giardino Torchia ML, Masci AM, Vitiello L, Mavilio D, La Sala A and Racioppi L. **Dendritic cells/Natural Killer cross-talk: a novel target for Human Immunodeficiency Type-1 Protease Inhibitors.** (Submitted for publication).

2. Illario M, Giardino-Torchia ML, Sankar U, Ribar TJ, Galgani M, Vitiello L, Masci AM, Bertani FR, **Ciaglia E,** Astone D, Maulucci G, Cavallo A, Vitale M, Cimini V, Pastore L, Means AR, Rossi G, Racioppi L. **Calmodulin-dependent kinase IV links Toll-like receptor 4 signaling with survival pathway of activated dendritic cells.** Blood 2008 Jan 15;111(2):723-31.

ABSTRACT

Inflammatory conditions and infections in selected organs increase the risk of cancer. In the tumor microenvironment, smoldering inflammation contributes to proliferation and survival of malignant cells, angiogenesis, metastasis and subversion of adaptive immunity.

The human immunodeficiency virus (HIV) infection is characterized by increased risk of several solid tumors due to its inherent nature of weakening the immune system. Recent observations point to a lower incidence of some cancers in patients treated with protease inhibitor (PI) cocktail such as HAART (Highly Active Anti-Retroviral Therapy).

Human Immunodeficiency Type-1 Virus protease inhibitors (HIV-1-PIs) originally designed to block selectively the aspartic protease of HIV-1, also shown the ability to modulate a variety of biological functions, including the immune response, by mechanisms largely independent from their anti-viral activity. Herein, we investigate the effects of PIs on differentiation programs of monocytes toward: (a) dendritic cells (DC); (b) Tumor Associated Macrophages-like cells (TAM-like).

Differentiation of human circulating monocytes in the presence of PIs led to generation of DC with atypical phenotype, including low level of Cd1a, and DC-SIGN, a receptor that enables DC to bind HIV-1 virions in tissues, and carry them to lymphonodes. Moreover, DC generated in the presence of ritonavir also fail to terminally differentiate, and secrete lower amounts of IL-12 and IL-15, in response to bacterial endotoxin (LPS). This phenomenon parallels their inability to prime NK cells, and become resistant to NK-mediated cytotoxicity.

The exposure of monocytes to certains PIs determines generation of Tumour Associated Macrophages-Like cells with an atypical phenotype, including higher level of the co-stimulatory molecules CD86, and lower

expression of ILT3, a receptor playing an imunosuppressive role. Accordingly, in response to LPS, TAM-like cells generated in the presence of PIs, secrete lower amount of MM9 and VEGF, a phenomenon accompanying their ability to release more GM-CSF.

Altogether, these findings demonstrate the ability of PIs to modulate the differentiation programs of human monocytes. The remakable property of certain PIs to modulate phenotypes and functionalities of DC and TAM, might open novel perspectives for immune-intervention aimed to manipulate the cancer inflammatory milieu.

1. BACKGROUND

1.1 Mechanisms that link inflammation and cancer

It was in 1863 that Rudolf Virchow noted leucocytes in neoplastic tissues and made a connection between inflammation and cancer. On these bases, he suggested that the "lymphoreticular infiltrate" reflected the origin of cancer at sites of chronic inflammation. Over the past ten years our understanding of the inflammatory microenvironment of malignant tissues has supported Virchow's hypothesis, and the links between tumour and inflammation have implications for prevention and treatment. Furthermore, usage of non-steroidal anti inflammatory agents is associated with protection against various tumors, a finding that to a large extent mirrors that of inflammation as a risk factor for certain cancers.

Table 1 lists some cancers where the inflammatory process is a cofactor in carcinogenesis.

Panel 1: **Some associations between inflammation and cancer risk**

Malignancy	Inflammatory stimulus/condition
Bladder	Schistosomiasis
Cervical	Papillomavirus
Ovarian	Pelvic inflammatory disease/talc/tissue remodelling
Gastric	*H pylori* induced gastritis
MALT lymphoma	*H pylori*
Oesophageal	Barrett's metaplasia
Colorectal	Inflammatory bowel disease
Hepatocellular	Hepatitis virus (B and C)
Bronchial	Silica, asbestos, cigarette smoke
Mesothelioma	Asbestos
Kaposi's sarcoma	Human herpesvirus type 8

Table 1. From Balkwill F and Mantovani A, Lancet 2001; 357: 539–45

About 15% of the global cancer burden is attributable to infectious agents, and inflammation is a major component of these chronic infections. Furthermore, increased risk of malignancy is associated with the chronic

inflammation caused by chemical and physical agents, and autoimmune and inflammatory reactions of uncertain aetiology (Balkwill and Mantovani 2001). For example, there are strong associations between alcohol abuse, which leads to inflammation of the liver and pancreas, and cancers of these organs. Cigarette smoking, asbestos and silica exposure are each associated with inflammation of the lung and lung carcinoma; inflammatory bowel disease (IBD) is associated with colon cancer; infections with Helicobacter pylori is associated with gastric carcinoma; chronical viral hepatitis is associated with liver cancer; infections with Schistosoma spp. is associated with bladder and colon carcinoma; infection with some strains of HPV is associated with cervical cancer; and infection with EBV is associated with Burkitt lymphoma and nasopharyngeal carcinoma. (Coussens et Werb 2002, Shacter and Weitzman, 2002, Hussain et al. 2003, Fox and Wang 2007, Dobrovolskaia and Kozlov, 2005).

Key features of cancer-related inflammation (CRI) include the infiltration of white blood cells such as DCs, T cells, NK cells and prominently tumor-associated macrophages (TAMs); the presence of polypeptide messengers of inflammation [cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1, IL-6, IL-12, chemokines such as CCL2 and CXCL8] and the occurrence of tissue remodeling and angiogenesis. (Colotta et al. 2009).

Recent efforts have shed new light on molecular and cellular circuits linking inflammation and cancer (Mantovani et al. 2008). Two pathways have been schematically identified; in the intrinsic pathway, genetic events causing neoplasia initiate the expression of inflammation-related programs that guide the construction of an inflammatory microenvironment. Of note, it is reported that the rearrangements of the *RET* receptor tyrosine kinase gene generating *RET-PTC* oncogenes, specific to papillary thyroid carcinoma (PTC), when exogenously expressed in primary normal human thyrocytes, induce the expression of a large set of genes involved in inflammation and tumor invasion, including those encoding chemokines (CCL2, CCL20, CXCL8, and

CXCL12), chemokine receptors (CXCR4), cytokines (IL1B, CSF-1, GM-CSF, and G-CSF), matrix-degrading enzymes (metalloproteases and urokinase- type plasminogen activator and its receptor), and adhesion molecules (L-selectin). Selected relevant genes (CCL20, CCL2, CXCL8, CXCR4, L-selectin, GM-CSF, IL1B, MMP9, UPA, and SPP1-OPN) were found up-regulated also in clinical samples of PTC, particularly those characterized by *RET-PTC* activation, local extrathyroid spread, and lymph node metastases, when compared with normal thyroid tissue or follicular thyroid carcinoma, demonstrating that the *RET-PTC1* oncogene activates a proinflammatory program and provide a direct link between a transforming human oncogene, inflammation, and malignant behaviour (Borrello et al. 2005). It is reported that other oncogenes representative of different molecular classes and mode of action (tyrosine kinases, ras–raf, nuclear oncogenes and tumor suppressors such as von Hippel-Lindau tumour suppressor (VHL), and phosphatase and tensin homologue (PTEN) share the capacity to orchestrate proinflammatory circuits (e.g. angiogenetic switch; recruitment of myelo-monocytic cells) (Mantovani et al. 2008) predisposing to cancer.

In the extrinsic pathway, are the inflammatory conditions cited before to facilitate cancer development by a variety of mechanisms (Balkwill and Mantovani 2001). I will now look in more detail at the mechanisms by which cytokines and chemokines might act to promote cancer also summerized in next figure 1.

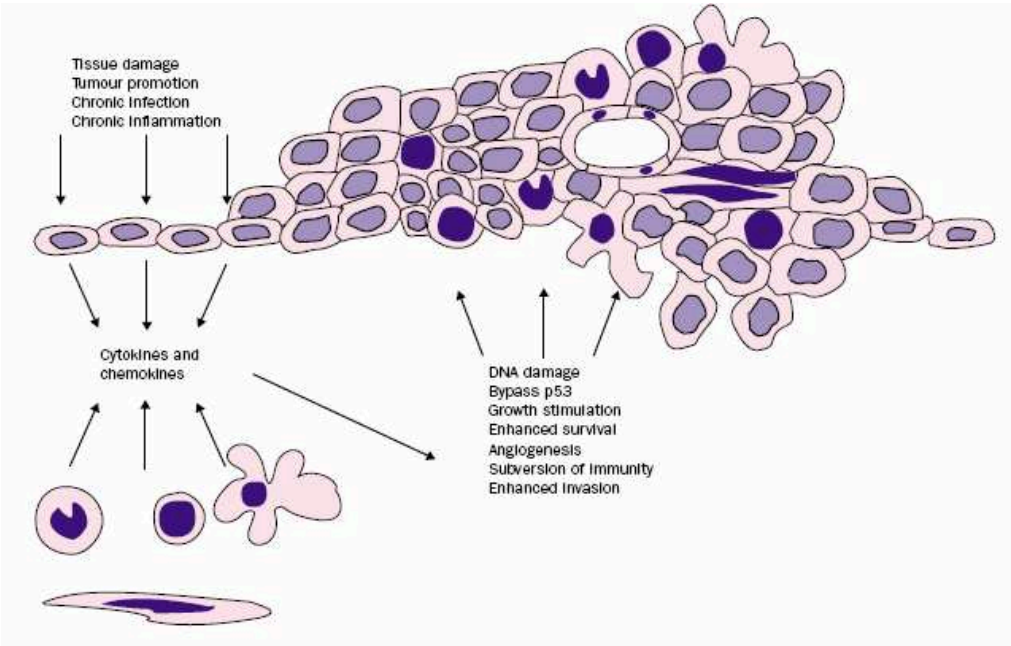

Figure 1: Chronic inflammation, tissue damage, and chronic infection may stimulate cytokines and chemokines that contribute to development of malignant disease (Balkwill F and Mantovani A, 2001).

Mediators of inflammation as growth and survival factors

Cytokines and chemokines have the potential to stimulate tumour-cell proliferation and survival and some of them may also act as autocrine growth and survival factors for malignant cells. IL-6 is a growth factor for haematological malignancies (Tricot 2000); IL-1 has growth stimulating activity for gastric carcinoma that may be related to genetic predisposition (B-Omar 2000) and for myeloid leukaemias; and growth of melanomas is promoted by IL-8 and related chemokines (Hanghnegahdar et al. 2000) .

Angiogenesis

The inflammatory cell infiltrate, particularly TAM, may contribute to tumour angiogenesis, and there are many reports of associations between macrophage infiltration, vascularity, and prognosis (Leek et al. 1999). Moreover TNF, IL-1, and IL-6 can stimulate production of angiogenic factors such as VEGF. Inflammatory macrophages also produce TGF-β1 that is itself angiogenic and induces production of VEGF. Chemokines also have a role.

Some CXC chemokines (eg, IL-8) are proangiogenic whereas others such as IP-10 (CXCL10) have antiangiogenic activity (Keane and Strieter 1999). In addition, CC chemokines may inhibit or stimulate angiogenesis indirectly, via their influence on TAM.

Invasion and metastasis

Cytokines and chemokines affect various stages in the process of metastasis. TNF-α and CC chemokines can induce production of proteases important for invasion in both tumour cells and macrophages. In one skin tumour model, paracrine matrix metalloproteinase-9 production by inflammatory cells was implicated in epithelial hyperproliferation, angiogenesis and increased malignant potential, and skin tumour development was reduced in mice genetically "knocked out" for this protein. TNF-α and IL-1 increase the expression of adhesion molecules on endothelial cells (Ekbom et al. 1990, Negus et al. 1997). IL- 18 derived from the endothelium may be the ultimate mediator of one tumour cytokine-induced adhesion molecule (Tricot 2000) 10. Certain cells have receptors for adhesion molecules and use these molecular tools, typical of migrating leucocytes, to seed at distant anatomical sites (Martin Padura et al. 1991) .

Furthermore, chemokine agonists induce migration or proliferation of some tumour cells (Hanghnegahdar et al. 2000). Receptors that are essential for lymphocyte and dendritic cell homing to lymph nodes (Allavena et al. 2000), could play a role in lymphatic dissemination of certain carcinomas. Thus, tumour cells use the same molecular tools (adhesion molecules, cytokines, chemokines, chemokine receptors) and pathways as leucocytes to spread to distant anatomical sites during inflammation.

Subversion of immunity

The prevalence of Th2 cells is common to tumours, suggesting that this polarization may be a general strategy to subvert immune responses against tumours. Chronic exposure to high cytokine concentrations (IL-4, IL-13, IL-

10,) in the tumour microenvironment may set in motion a vicious cycle leading to skewing towards a type II inflammatory response (Sica et al. 2000).

Some viruses encode chemokines and their inhibitors and receptors. Of particular interest is human herpesvirus type 8, which is involved in the pathogenesis of Kaposi's sarcoma. The virus genome codes for three chemokines which are selective attractants of polarized Th2 cells. The virus-encoded chemokines might subvert immunity by activating type 2 responses and diverting effective Th1 defence mechanisms (Sozzani et al. 1998, Endres et al. 1999).

Genome Instability

Recent data suggest that an additional mechanism involved in cancer-related- inflammation (CRI) is the induction of genetic instability by inflammatory mediators either directly inducing DNA damage (via reactive oxygen) or affecting DNA repair systems and altering cell cycle checkpoint. For example HIF-1α, which is induced in cancer cells by inflammatory cytokines (TNF and IL-1β), PGE2 (Jung et al. 2003) and reactive oxygen and nitrogen species (Sandau et al. 2000) downregulate mismatch repair (MMR) proteins MSH2 and MSH6 by displacing c-Myc from MSH2/MSH6 promoters (Koshiji et al. 2005).

Hydrogen peroxide produced by inflammatory cells inactivates MMR members by damaging the enzymes at the protein level; NO-induced upregulation of DNA methyltransferase results in extensive methylation of the cytosine bases, which is associated with promoter silencing and loss of gene expression of the MMR member hMLH1 (Fleisher et al. 2000). In fact by immunohistochemistry, decreased levels of hMLH1 proteins are seen in gastric epithelial cells in H.pylori-positive patients (Mirzaee et al. 2008) and in colitis-associated cancers, hMLH1 hypermethylation is observed in a substantial proportion of patients (Fleisher et al. 2000). The nucleotide excision repair pathway, which serves to repair a variety of DNA lesions caused by UV radiation, mutagenic chemicals and chemotherapeutic agents, appears to be

affected by IL-6 that in multiple myeloma cells induces hypermethylation, and thus defective function, of the key nucleotide excision repair component hHR23B (Hodge et al. 2005). HIF-1α induces the microRNA-373 that downregulates the expression of the nucleotide excision repair component RAD23B (Crosby et al. 2009). Chromosomal instability (CI) results in abnormal segregation of chromosomes and aneuploidy. In most cancers with CI, proteins of the mitotic checkpoints are disregulated (Rajagopalan et al. 2003). As a consequence, cancer cells fail to halt the cell cycle until DNA repair can be executed. Inactivation of p53 may play a role in CI (Tomasini et al. 2008). The p53 pathway protects cells from transformation by inducing apoptosis upon DNA damage and CI. p53 deficiency and a defective mitotic checkpoint in T lymphocytes increase CI through aberrant exit from mitotic arrest (Baek et al. 2003). Loss of p53 and p73 are associated with increased aneuploidy in mouse embryonic fibroblasts (Talos et al. 2007). The proinflammatory cytokine migration inhibitory factor suppresses p53 activity as a transcriptional activator (Hudson et al. 1999). NO and its derivatives inhibit the function of p53 (Calmels et al. 1997, Cobbs et al.2003) and are associated with p53 mutations (Ambs et al. 1999, Marshall et al. 2000, Jaiswa et al. 2001, Wink et al. 1994). NO (Hmadcha et al. 1999) and the inflammatory cytokine IL-6 (Hodge et al. 2005) increase the activity of DNA methyltransferase, resulting in CpG island methylation. Malignant cells employ matrix metalloproteinases (MMPs) to penetrate the extracellular matrix and basement membrane and to invade distant tissues. Recent data suggest that MMPs produced by tumours and by the inflamatory cells may also function as oncogenes by promoting CI. MT1-MMP, which is present also in the pericentrosomal compartment, compromises normal cytokinesis inducing aneuploidy. A potential target of MT1-MMP is pericentrin, an integral centrosomal/midbody protein required for centrosome performance and chromosome segregation (Golubkov et al. 2007). MMP-3, which is upregulated in many breast cancers (Stemlicht et al. 2001), also mediates CI in

cultured cells and in transgenic mice (Stemlicht et al. 1999, Lochter et al. 1997). Expression of MMP-3 in cells stimulates the production of Rac1b (Matos et al. 2003), an hyperactive alternative splicing form of Rac1, which stimulates ROS production which can cause oxidative DNA damage and CI.

This cancer genetic instability through accelerated somatic evolution leads to a genomically heterogenous population of expanding cells naturally selected for their ability to proliferate, invade distant tissues and evading host defenses (Colotta et al. 2009).

The intrinsic and extrinsic pathways converge on the inhibitor of NF-kB kinase/ NF-kB (IKK/ NF-kB) signaling pathway, which is activated by many proinflammatory cytokines. NF-kB is a transcription factor that regulates the expression of many genes whose products can suppress tumor cell death; stimulate tumor cell cycle progression; enhance epithelial-to-mesenchymal transition, which has an important role in tumor invasiveness; and provide newly emerging tumors with an inflammatory microenvironment that supports their progression, invasion of surrounding tissues, angiogenesis, and metastasis (Dobrovolskaia and Kozlov 2005, Karin 2006).

In conclusion, CRI is a key component of tumors and may represent the seventh hallmark of cancer, providing further impetus for studies targeted to the inflammatory microenvironment of tumors (Colotta et al. 2009) (Figure 1.2).

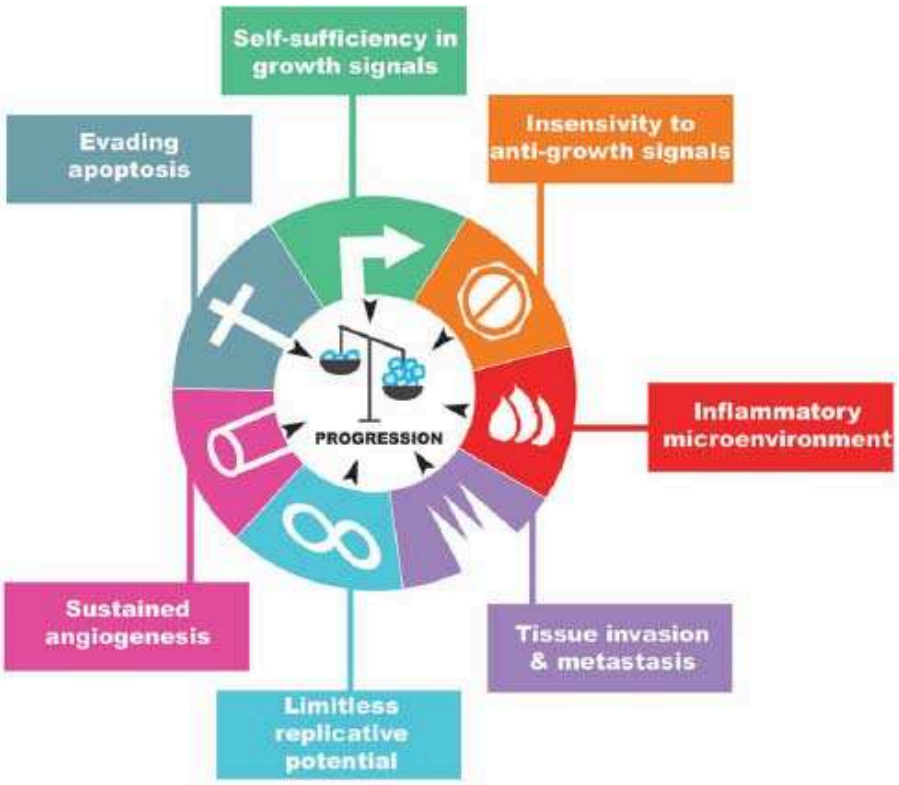

Figure 1.2 Inflammation as the seventh hallmark of cancer. An integration to the six hallmarks of cancer. (Colotta et al. 2009)

1.2 An overview of inflammation

To fully understand the role of inflammation in the evolution of cancer, it is important to understand what inflammation is and how it contributes to physiological and pathological processes such as wound healing and infection.

Inflammation is the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. Inflammation is a protective attempt by the organism to remove the injurious stimuli as well as initiate the healing process for the tissue. Inflammation is not a synonym for infection. Even in cases where inflammation is caused by infection, the two are not synonymous: infection is caused by an exogenous pathogen, while inflammation is the response of the organism to the pathogen.

In the absence of inflammation, wounds and infections would never heal and progressive destruction of the tissue would compromise the survival of the organism (Ferrero-Miliani et al. 2007, Coussens and Werb 2002).

Inflammation can be classified as either *acute* or *chronic*. *Acute inflammation* is the initial response of the body to harmful stimuli and is achieved by the activation and directed migration of leukocyte (neutrophils, monocytes and eosinophils) from the blood into the injured tissues. For neutrophils, a four-step mechanism is believed to coordinate recruitment of these inflammatory cells to sites of tissue injury and to the provisional extracellular matrix (ECM) that forms a scaffolding upon which fibroblast and endothelial cells proliferate and migrate, thus providing a nidus for reconstitution of the normal microenvironment (Chettibi et al. 1999). These steps involve: activation of members of the selectin family of adhesion molecules (L- P-, and E-selectin) that facilitate rolling along the vascular endothelium; triggering of signals that activate and upregulate leukocyte integrins mediated by cytokines and leukocyte-activating molecules; immobilization of neutrophils on the surface of the vascular endothelium by means of tight adhesion through α4β1 and α4β7 integrins binding to endothelial vascular cell-adhesion molecule-1 (VCAM-1) and MadCAM-1, respectively; and transmigration through the endothelium to sites of injury, presumably facilitated by extracellular proteases, such as matrix metalloproteinases (MMPs).

A number of chemokines, which possess a relatively high degree of specificity for chemoattraction of specific leukocyte populations (Rossi and Zlotnik 2000, Homey et al. 2002), recruits downstream effector cells and dictates the natural evolution of the inflammatory response.

Neutrophils (and sometimes eosinophils) are the first recruited effectors of the acute inflammatory response. Monocytes, which differentiate into macrophages in tissues, are next to migrate to the site of tissue injury, guided by chemotactic factors. Once activated, macrophages are the main source of

growth factors and cytokines, which profoundly affect endothelial, epithelial and mesenchymal cells in the local microenvironment. The process of acute inflammation is initiated also by cells already present in all tissues, mainly resident macrophages, dendritic cells, histiocytes, Kuppfer cells and mastocytes. At the onset of an infection, burn, or other injuries, these cells undergo activation and release inflammatory mediators responsible for the clinical signs of inflammation.

Prolonged inflammation, known as *chronic inflammation*, leads to a progressive shift in the type of cells which are present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process. (Cotran et al. 1998).

In tumor development, the major driving force is chronic inflammation secondary to persistent infection with a parasite, bacterium, or virus (Karin and Greten 2005).

Infectious organisms (parasite, bacterium or virus) trigger inflammation through activation of receptors that recognize pathogen-associated molecular patterns (PAMPs), such as cell wall components and nucleic acids (Medzhitov 2001). At least four families of mammalian innate immune receptors that recognize PAMPs have been identified; these are known as pattern recognition receptors (PRRs) and include TLRs, nucleotide-binding oligomerization domain–like (NOD-like) receptors (NLRs), C-type lectin receptors (CLRs), and triggering receptors expressed on myeloid cells (TREMs) (Akira et al.2006, Fritz et al. 2006, Robinson et al. 2006, Klesney-Tait et al. 2006). The interaction between PAMPs and PRRs results in the activation of innate immune cells and initiation of host responses whose major purpose is to eliminate and kill invading organisms (Karin and Greten 2005). However, inadequate pathogen eradication, prolonged inflammatory signaling, and defects in antinflammatory mechanisms can all lead to chronic inflammation and benefit tumor development (Han and Hulvetich 2005).

Inflammatory cells in tumour microenvironment.

The inflammatory microenvironment of tumours is characterized by the presence of host leucocytes both in the supporting stroma and in tumour areas (Negus et al. 1997). Tumour infiltrating leucocytes may contribute to cancer growth and spread, and to the immunosuppression associated with malignant disease.

Macrophages and *dendritic cells* infiltrate tumours (Scarpino et al. 2000, Mantovani et al. 2002). In the tumour microenvironment many signals polarize these mononuclear phagocytes which can express different functional programmes. Fully polarized type I and type II (or alternatively activated) macrophages are the extremes of a continuum of functional states. Tumor-derived and T cell-derived cytokines stimulate *tumor associated macrophages* (TAM) to acquire a polarized type II phenotype. These functionally polarized cells, and similarly oriented or dysfunctional and immature dendritic cells present in tumors, play a key role in the subversion of adaptive immunity and in inflammatory circuits which promote tumor growth and progression (Solinas et al. 2009, Fricke and Gabrilovich 2006).

1.3 Tumor –Associated-Macrophages (TAM)

The tumor mass is undoubtedly a multifaceted show, where different cell types, including neoplastic cells, fibroblasts, endothelial, and immune-competent cells, interact with one another continuously. Macrophages represent up to 50% of the tumor mass, and they certainly operate as fundamental actors. (Solinas et al. 2009).

Polarization of macrophage function.

Macrophages constitute an extremely heterogeneous population, which could be divided schematically into two main classes: M1 and M2 (Figure 1.3). Blood monocytes differentiating in the presence of LPS/IFN-γ mature into M1-polarized cells (classically activated macrophages). They produce high levels of IL-12, IL-1, IL-23, TNF-α, and CXCL10 and are characterized by cytotoxic

activity against microorganisms and neoplastic cells, expression of high levels of ROI, and capability as APCs with high expression of the co-stimulatory molecule CD86. On the other hand, when monocytes differentiate in the presence of IL-4, IL-13, IL-10, or corticosteroids, they mature into M2 macrophages (alternatively activated macrophage) which secrete IL-10, CCL17, CCL22, CCL18, IL-1ra, and IL-1R decoy. M2 cells are active workers of the host, promoting scavenging of debris, angiogenesis, remodeling, and repair of wounded/damaged tissues. Within the tumor mass, they exert the same functions favoring tumor promotion. In addition, M2 macrophages control the inflammatory response by down-regulating M1-mediated functions and adaptive immunity (Solinas et al. 2009). They are in fact the major source of the soluble and membrane-bound imunoglobulin-like transcript 3 (ILT3) which may be responsible for the immuno-escape mechanisms of tumors. Both membrane-bound ILT3 (mILT3) and soluble ILT3(sILT3) inhibited T cell proliferation in mixed lymphocyte culture (MLC), anergizing CD4+ Th cells, suppressing the differentiation of IFN-gamma producing CD8+ cytotoxic T cells, and inducing the differentiation of alloantigen-specific CD8+ T suppressors in primary 7-day MLC (Kim-Schulze et al. 2006). Furthermore, it is reported that in a humanized severe combined mmunodeficiency (SCID) mouse model, soluble and membrane ILT3 induce CD8+ T suppressor cells and prevent rejection of allogeneic tumor transplants. Furthermore, patients with carcinoma of the pancreas produce the soluble ILT3 protein, which induces the differentiation of CD8+ T suppressor cells and impairs T cell responses in mixed lymphocyte culture. These responses are restored by anti-ILT3 mAb or by depletion of sILT3 from the serum suggesting that ILT3 depletion or blockade is crucial to the success of immunobiotherapy (Cortesini 2007).

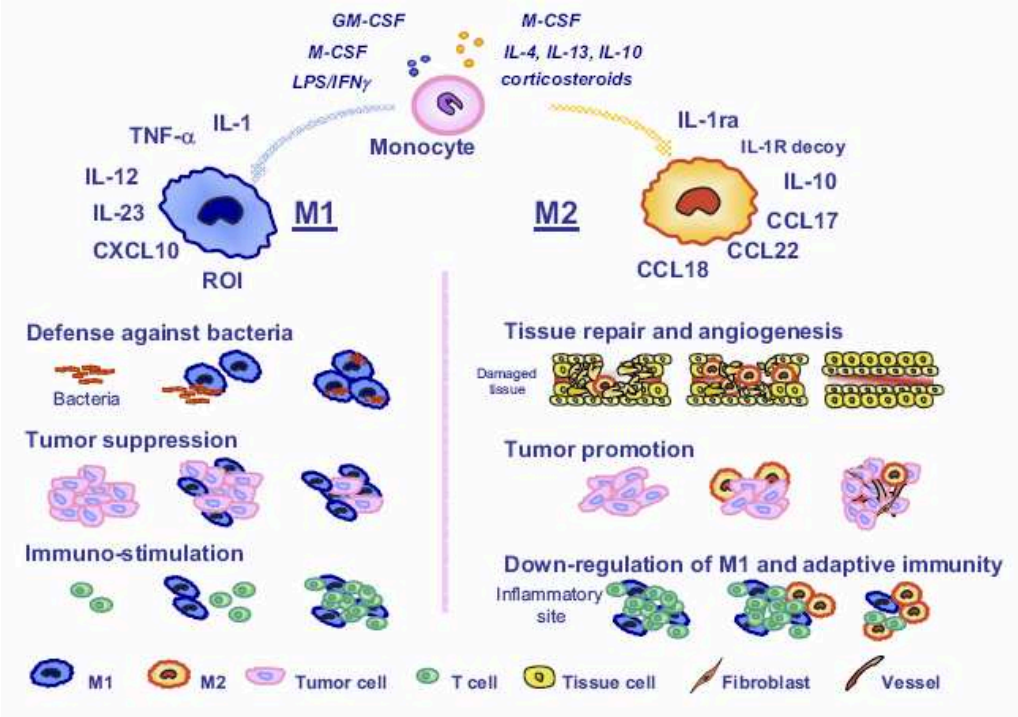

Figure 1.3. Polarization of macrophage function. (Solinas G et al. 2009)

TAM originate from blood monocytes recruited at the tumor site (Mantovani et al. 1992) by molecules produced by neoplastic and by stromal cells. The chemokine CCL2, earlier described in 1983 as a tumor-derived chemotactic factor, is the main player in this process and experimental and human studies correlate its levels with TAM abundance in many tumors, such as ovarian, breast and pancreatic cancer (Balkwill 2004). TAM themselves produce CCL2, suggesting the action of an amplification loop, and anti-CCL2 antibodies combined with other drugs have been considered as an anti-tumor strategy. Other chemokines involved in monocyte recruitment are CCL5, CCL7, CXCL8, and CXCL12, as well as cytokines such as VEGF, PDGF and the growth factor M-CSF. Moreover, monocytes could be attracted by fibronectin, fibrinogen and other factors produced during the cleavage of ECM proteins induced by macrophage and/ or tumor cell-derived proteases (Allavena et al. 2008).

When monocytes reach the tumor mass, they are surrounded by several microenvironmental signals such as IL-3 and M-CSF, able to induce their differentiation toward mature macrophages (now called TAM) and to shape the "new" cells as needed by the tumor (CSFs, IL-4, IL-10, and TGF-β). Tumor-molded macrophages resemble M2-polarized cells and play a pivotal role in tumor growth and progression. As shown in figure 1.4 TAM actively work for the tumor: they produce several molecules that sustain malignant cell survival (M-CSF, VEGF, PDGF, FGF, TGF Beta, MMPs) and they abundantly secrete matrix proteins and several proteases such as serine proteases, MMPs, and cathepsins, which act on cell– cell junctions, modify the ECM composition, and promote basal membrane disruption. Moreover, TAM actively contribute to build up the tumor matrix architecture by producing several matrix proteins, including secreted protein acid rich in cysteine, which modulate collagen density, leukocyte and blood vessel infiltration. TAM preferentially localize in tumor hypoxic regions, and hypoxia activates in these cells a specific proangiogenic program. Low oxygen conditions promote HIF-1 and HIF-2 expression with subsequent overexpression of proangiogenic molecules. Of note, TAM express VEGF almost exclusively in a vascular and perinecrotic areas of human breast carcinomas. Among chemokines, hypoxia tightly regulates the expression of CXCL12, HIF-1-dependent potent chemoattractant for endothelial cells, and its receptor CXCR4. In addition to CXCL12, TAM release other chemokines involved in angiogenic processes such as CCL2, CXCL8, CXCL1, CXCL13, and CCL5. For instance, levels of CXCL5 and CXCL8 were associated with increased neovascularization and correlated inversely with survival. Finally, TAM affect adaptive immune responses significantly by recruiting and stimulating Tregs (IL10, CCL17, TGF, CCL22) and recruiting Th2 lymphocytes (CCL17, CCL22), which in turn inhibit Th1 cells, and by inducing anergy of naıve T cells (CCL18) (Solinas et al. 2009).

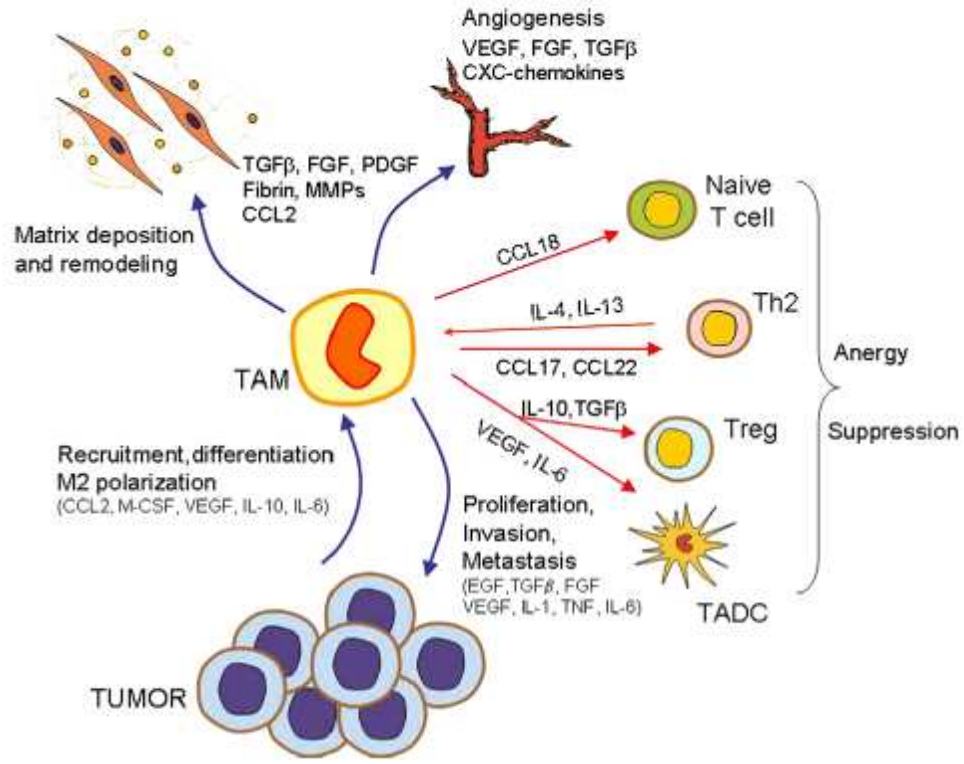

Figure 1.4 Protumoral functions of tumor-associated macrophages (TAM) and interplay with tumor cells. (Sica A et al. 2008)

1.4 Dendritic Cells.

Dendritic cells (DCs) are the most potent antigen-presenting cells of the immune system. They serve as the sentinels that capture antigens in the periphery, process them into peptides and present these to lymphocytes in lymph nodes. DCs play a key role in regulating immunity. Several DC-subsets exist, including myeloid-DCs (MDCs), plasmacytoid-DCs (PDCs) and Langerhans cells (LC). DCs not only instruct T- and B-lymphocytes, but also activate Natural Killer cells and produce interferons, thus linking the innate and adaptive immune system. (Bancherau et al. 2000). Inflammatory-mediators and especially the Toll like receptor (TLR) family of proteins play a pivotal role in inducing the immune activation program in DCs. TLRs recognize pathogen-associated-molecular-patterns (PAMPS) like LPS or flagellin and signal to alert immune cells in general, and DC in particular (Akira et al. 2006). DC activation, also referred to as DC maturation, thus results in immunity.

Mature DC produce pro-inflammatory cytokines. In contrast, resting DC or DC receiving immune-inhibitory signals, such as IL-10 and/or corticosteroids, induce immune tolerance via T cell deletion and induction of suppressive T cells, now termed regulatory T cells. Several mouse models have demonstrated that the immunological outcome depends upon the DC activation state; mature immune-activating DC protect mice from a tumor or pathogen, whereas tolerogenic DC induce tolerance against transplanted tissues. Hence, DC act at the interface of immunity and peripheral tolerance (Steinman et al. 2003).

Among the inflammatory cytokines produced by DC, it is reported that IL-12 has a dual opposite effect: antitumor property relying on its ability to promote Th1 adaptive immunity and CTL responses by stimulating the production of IFN-γ from T and natural killer (NK) cells, and reducing IL-4 mediated suppression of IFN-γ which results in enhancement of the cytotoxic activity of NK cells and CD8+ cytotoxic T lymphocytes (Trinchieri 2003), but also tumour- promoting role. In fact, it is reported that IL-12 plays a major role in sustaining the chronic phase of several inflammatory conditions that often degenarate in carcinoma such as colitis (Leach and Rennick 1998).

IL-15 is an other cytokine produced by activated DC, that has been shown to play a pivotal role in orchestrating immune-mediated tissue destruction in inflammatory disease (Mention et al. 2003, Kovesdy and Kalantar-Zadeh 2008). IL-15 is indispensable for the generation, maintenance, and homeostasis of local T and NK cell. IL-15 also induces proliferation of CD8+ T and NK lymphocytes in addition to enhancing their effector functions, including those associated with cytolysis and cytokine secretion. IL-15 also promotes perpetuation of chronic inflammation by mediating activation of monocyte and neutrophils and by preventing activation-induced cell death of activated CD8+ T cells (Waldmann 2006, Huntington et al. 2007). In addition to these positive modulatory effects on the activation pathways leading to persistent inflammation, IL-15 can block the negative regulatory pathways critical in maintaining immune homeostasis by inhibiting the anti-

inflammatory Smad-dependent signaling of TGF-β thereby, further aggravating ongoing inflammation (Benahmed et al. 2007).

The presence of DCs in human carcinomas has been largely documented, (Yang and Carbone 2004) and has been proposed to correlate with a more favorable prognosis (Tsujitani et al. 1990, Ishigami et al. 1998, Iwamoto et al. 2003). Ideally, DC should be recruited to the tumour site to initiate the immune response, and promote tumour rejection. In breast cancer, immature TADC are interspersed in the tumour mass, whereas mature dendritic cells are confined to the peritumoral area (Treiilleux et al. 2004). In papillary thyroid carcinoma TADC are also immature, but they tend to localise at the invasive edge of the tumour (Scarpino et al. 2000).

However, although DC can engulf tumor cells debrites, process and cross present tumor-associated antigens to cytotoxic T lymphocytes (CTLs) (Chan and Housseau 2008), the tumours microenvironment conteract this phenomenon by releasing a number of immunosuppressive factors, including IL6, VEGF, IL8 and IL10, that contributes to DC malfunction (Fricke and Gabrilovich 2006). Thus, tumour-associated-denditic cells (TADC) usually have an immature phenotype, with defective ability to stimulate T cells, a phenotype suggesting a controversial role for TADC in the immune response toward cancer cells.

1.5 Dendritic Cell-Natural Killer crosstalk

Natural killer (NK) cells are a population of large granular lymphocytes with a CD56+/CD3-phenotype. They are distinguishable from B and T lymphocytes by lack of antigen receptors. NK cells kill a variety of tumor cells, virus-infected cells and allogeneic cells in a non-major histocompatibility complex restricted manner, and provide the first line of immune defense (Trinchieri 1989, Moretta et al. 2002).

Although NK cells lack the antigen-specific receptors, they distinguish between normal cells and abnormal cells by their cell surface receptors. After binding to potential target cell, NK cell activating and inhibitory receptors interact with ligands and transmit signals, and then all the signals are integrated to determine whether NK cell stays and responds (Bottino et al. 2005, Long 1999, Moretta et al. 2001). The effector function of NK cells is regulated by a balance between the inhibitory signals delivered by the MHC class I-specific inhibitory receptors and the activating signals transmitted by activating receptors (Lanier 2005). NK cell effector function is mainly mediated through: 1) releasing cytoplasmic cytotoxic granules (granzyme and perforin) by exocytosis; 2) secreting proinflammatory cytokines (IFN-γ, TNF-α, etc.); and 3) the engagement of death receptors on target cells by their cognate ligands (e.g., FasL and TRAIL) on NK cells (Janeway and Medzhitov 2002). In course of DC-NK interplay, myeloid DCs by secreting NK-cells activating cytokines (IL-12, IL-15, type I IFNs), promote the secretion of pro-inflammatory cytokines and cytotoxicity of NK cells (Walzer et al. 2005).

Reciprocally, NK cells, traditionally considered to be major innate effector cells, have also recently been shown to play immunoregulatory 'helper' functions, being able to activate DCs and to enhance their ability to produce pro-inflammatory cytokines. (Walzer et al. 2005, Moretta et al. 2006, Degli-Esposti and Smyth 2005). In addition, once activated, NK cells acquire the capability of killing immature myeloid DCs (Moretta et al. 2002, Zitvogel 2002). This effect is due to the fact that immature DCs typically underexpress HLA-class I molecules that would protect from NK-mediated lysis. On the other hand, DCs that, after Ag uptake, undergo maturation, upregulate MHC-class I expression becoming essentially resistant to NK cells (Ferlazzo et al. 2003). It has been suggested that the NK-mediated killing of DCs may serve to keep in check the quality and the quantity of DCs undergoing maturation (‘editing’ process). According to this view, DCs that fail to express sufficient amounts of MHC molecules would be removed. Thanks to this mechanism NK

cells may prevent the survival of faulty DCs that after expression of CCR7 and migration to lymph nodes, would induce inappropriate, low-affinity, T-cell priming resulting either in Th2 responses or in a state of tolerization (Langenkamp 2000).

Indeed, only DCs undergoing this NK-mediated quality control would become fully mature and capable of inducing priming of protective and cytotoxic Th1 responses.

1.6 Emerging aspects of NK cell biology

In contrast to their protective role in various inflammatory conditions, NK cells can also act as mediators of innate immunopathology.In patients with chronic hepatitis B virus infection, a subset of NK cells contributes to liver inflammation by inducing hepatocyte death through a TRAIL-dependent mechanism (Dunn et al. 2007). In hepatitis B virus transgenic mice, NK cells also promote liver injury through NKG2D (Chen et al. 2007). Moreover, NK cells act detrimentally in experimental sepsis induced by *Streptococcus pneumoniae* or *Escherichia coli* by exacerbating inflammatory responses (Kerr et al. 2005, Badgwell et al. 2002). Consistent with these data, a potential contribution of NK cells has also been postulated in human inflammatory diseases such as arthritis (de Matos et al. 2007) and sarcoidosis (Katchar et al. 2005) .

Transgenic mice that express human NK cells-activating IL-15 specifically in enterocytes (T3b-hIL-15 Tg mice) develop villous atrophy and severe duodeno-dejunal inflammation with massive accumulation of NK-like CD8-lymphocytes in the affected mucosa that leads to a major propensity for the development of enteropathy associated CD8 T cell lymphoma. Finally, in humans NK cells have been shown to home to inflamed skin in various conditions, such as vernal keratoconjunctivitis (Lambiase et al. 2007), atopic dermatitis (Buentke 2002) and psoriasis (Ottaviani et al. 2006).

Thus another interesting mechanism of action of drugs that target cancer-related inflammation might be to prevent deleterious NK cell-driven inflammatory response.

1.7 HIV protease inhibitors: antiretroviral agents

Immunodeficiency is a hallmark of human immunodeficiency virus type 1 (HIV-1) disease and is characterized by a progressive decrease in CD4 T cells. The advent of new antiretroviral drugs, most notably HIV protease inhibitors, has generated new hope in the fight against AIDS. Development of HIV-protease inhibitors in the early 1990s followed the characterization of the crystal structure of HIV protease in 1989 (Navia MA et al. 1989). Inhibitors of HIV protease are peptidomimetics that generally contain a synthetic analogue of the peptide bond between phenylalanine and proline at positions 167 and 168 of the gag-pol polyprotein, which is the target of the HIV aspartyl protease (Flexner C 1998). This action prevents production of infectious viral particles. The first inhibitor of HIV protease developed that received FDA approval was saquinavir, followed by ritonavir, indinavir, nelfinavir, and amprenavir. Drugs approved more recently include lopinavir (in combination with ritonavir), atazanavir, fosamprenavir (a prodrug of amprenavir), tipranavir, and darunavir.

Used in combination with nucleoside inhibitors of HIV reverse transcriptase, protease inhibitors have led to impressive clinical outcomes. Such combined therapeutic regimens, known as highly active antiretroviral therapies (HAART), work by suppressing HIV replication and can lead to a large reduction in HIV plasma viraemia, restoration of normal numbers of CD4-positive T lymphocytes, immunological recovery, and reduction of morbidity and mortality related to HIV and opportunistic infections. The increase in CD4-positive T-cell counts and the immune restoration that occurs with HAART is most likely to depend on the following mechanisms: increased peripheral CD4-positive T-cell survival and proliferation, central renewal of lymphocytes, improvement of T-cell responses, and restoration of the T-cell

repertoire. Therefore, protease-inhibitor-based HAART owes its success to the ability to block HIV replication and promote subsequent immunological recovery (Sgadari et al. 2003).

1.8 Antitumour effects of antiretroviral therapy

Infection by human immunodeficiency virus (HIV) is associated with an increased risk of certain tumours, particularly Kaposi's sarcoma, non-Hodgkin's lymphomas and cervical cancer. However, the incidence of these cancers and the general tumour burden in HIV-infected patients has decreased significantly since the widespread use of highly active antiretroviral therapy (HAART). This effect cannot be solely explained by the ability of these drugs to suppress HIV replication and thereby reconstitute the immune system; in fact tumour development is not always correlated with a patient's viral load or level of immune reconstitution. Recent studies have shown that inhibitors of the HIV aspartyl protease, which are widely used in HAART, have direct anti-angiogenic and antitumour effects that are unrelated to their antiviral activity (Monini et al. 2004**).**

These direct antitumour effects of HAART could be related to specific actions of the protease inhibitors included in this therapeutic cocktail, such as ritonavir, saquinavir, indinavir and nelfinavir. Figure 1.5 summarizes the various steps in tumour progression and metastasis affected by HIV-protease inhibitors**.** These steps usually lead to progression of *in situ* carcinoma (**a**) to invasive cancer (**b**) and to metastasis formation and dissemination (**c**–**f**). Tumour outgrowth (**a**,**b**) is dependent on tumour neoangiogenesis and its net rate is determined by the balance between tumour cell proliferation versus apoptosis, invasive behaviour and the ability of tumour cells to evade the immune response. At concentrations similar or above therapeutic peak levels, HIV-PIs promote apoptosis and inhibit proliferation of tumour cells with little or no effects on survival and proliferation of normal cells (Gaedicke et al. 2002), whereas at therapeutic steady-state concentrations they inhibit tumour

angiogenesis and tumour-cell invasion. Furthermore, HIV-PIs have anti-inflammatory effects (**b**). As metastatic cell clones emerge, tumour cells loosen their contact with surrounding cells and the extracellular matrix (ECM). This leads to invasion of blood or lymphatic vessels and to extravasation of tumour cells at distant sites (**c**–**e**). These steps require the degradation of basement membranes and, at the same time, inhibition of apoptosis following loss of cell anchorage (anoikis), processes that are also inhibited by HIV-PIs. Finally, colonization of ectopic tissue by tumour cells (**f**) is required for establishment of metastases, and this process is similarly affected by HIV-PIs (Sgadari et al. 2002). During tissue invasion and establishment of metastases (**b**,**f**), activated endothelial cells, stromal cells and immune cells cooperate in basement membrane and ECM degradation, modify the ECM composition, release ECM-bound growth and angiogenic factors, and produce cytokines and chemokines that stimulate tumour-cell growth and migration, and recruit all these cell types at the invasive front. These processes are all affected by HIV-PIs through their ability to inhibit cytokine and chemokine production, cell activation, and basal membrane and ECM degradation and remodelling. The ability of these drugs to prevent tumour growth and progression might be mediated by their ability to inhibit proteasome function, resulting in the inhibition of NF-kB activity, and the activity of matrix metalloproteinases (Pajonk et al. 2002).

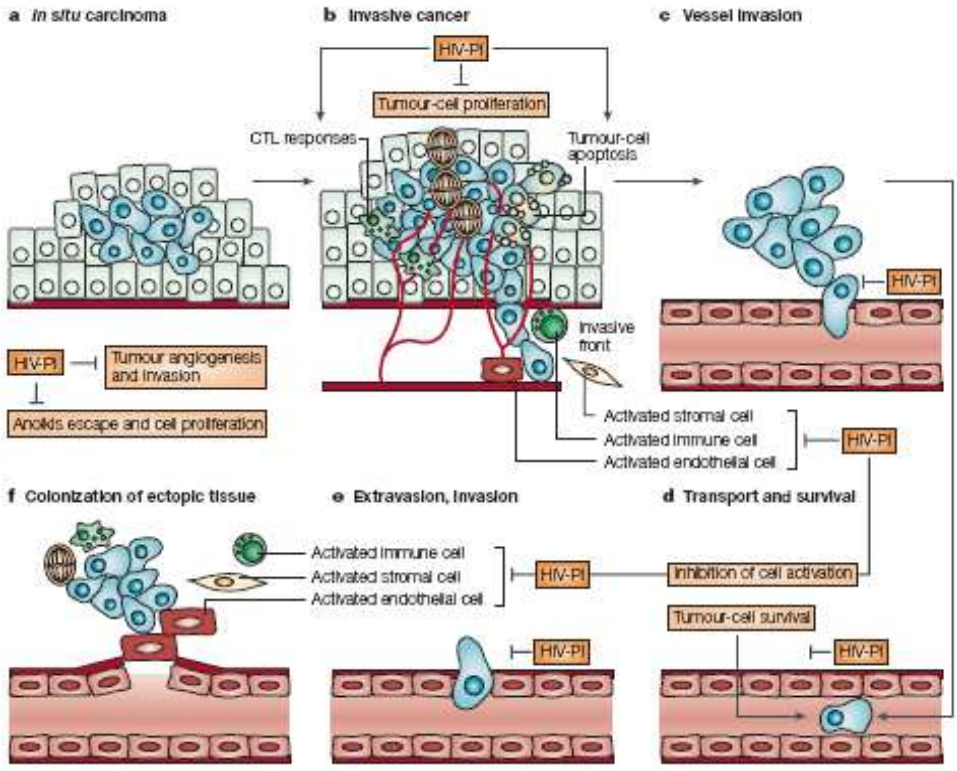

Figure 1.5 Steps in tumour progression and metastasis affected by HIV-protease inhibitors. (Monini P et al. 2004)

HIV-PIs exert also indirect effects on tumour affecting tumour-associated inflammation and tumour immunity in HIV-free model.

For example, ritonavir and saquinavir inhibit the production and/or release of inflammatory cytokines and chemokines including TNF-alpha, IL-6, and IL-8, by both peripheral-blood mononuclear cells and endothelial cells (Pati et al. 2002) This effect of HIV-PIs on inflammatory cytokines has been confirmed in treated patients, as PI-HAART has also been shown to inhibit TNF-alpha, IL-2 and IFN-γ production by peripheral-blood mononuclear cells from uninfected individuals who were treated with HIV-PIs for prophylactic intervention without acquiring HIV infection (Tovo 2000). Similarly, ritonavir inhibits the expression by endothelial cells of adhesion molecules, including VCAM1, ICAM1, and selectin E, which are known to mediate leukocyte recruitment at sites of inflammation (Pati et al. 2002). Pro-inflammatory

cytokines, chemokines and adhesion molecules are crucial in the development of Kaposi's sarcoma, as they mediate local inflammatory and immune responses to Kaposi's-sarcoma cells and to other KSHV-infected cells. Furthermore, they regulate survival, growth, invasion and eradication of most tumours. In fact, they lead to local stroma activation, basement-membrane and/or extracellular-matrix perturbation angiogenesis, and regulate local tumour immunity. In this context, HIV-PIs directly modulate antigen processing, T-cell survival (by the inhibition of T-cell apoptosis) and proliferative responses (Lu and Andrieu 2000, Sloand et al. 1999), and they might even affect T-cell priming, as they can inhibit dendritic-cell maturation and function (Gruber et al. 2001).

The most prominent mechanism underlying these last antitumour effects of HIV-Pis is likely to be MMP inhibition, that is not only responsible for the blockage of cell invasion and angiogenesis but it is also involved in several crucial immune and immunomodulatory functions, and in cancer-mediated immune suppression. (Sgadari et al. 2002, Lopez et al. 2000). Moreover, MMPs, including MMP2, act as potent modulators of local inflammation by activating or degrading inflammatory cytokines and chemokines present on the cell membrane, such as TNF-alpha, monocyte chemoattractant protein 3 nd IL-8 (Gearing et al. 1994, Ito et al. 2000, Schonbeck et al. 1998). Importantly, MMPs activate transforming growth factor-beta which, in turn, inhibits T cell responses against tumours (Gorelik and Flavell 2001, Yu and Stamenkovic 2000). MMPs can also cleave IL-2 receptor (Sheu et al. 2001), which is required for T-cell proliferation following antigen stimulation. These effects of MMPs are important determinants of tumour immune evasion, but might also explain the strong stimulatory effect of low HIV-PIs concentrations on T-cell proliferation and survival. Furthermore, as MMPs are required for leukocyte transmigration and tissue infiltration by inflammatory cells, the capability of ritonavir to inhibit CTL-dependent inflammatory responses could be mediated not only following the modulation of CTL-epitope processing by the

proteasome, but also through the inhibition of MMP activation or function in transmigrating lymphocytes (Kelleher et al. 2001).

2. AIM OF THE STUDY.

On last decade, an increasing number of reports have shown that PIs, originally designed to block the HIV-1 protease, can also exert remarkable immunomodulatory effects on multiple cell types by mechanisms not related to their anti-viral activity. Since these drugs have been widely used in HIV-1 therapy, prove their capability to intercept the inflammatory response, and identify their cell targets, might generate valuable information for their "offlabel" use in disorders where a modulation of inflammatory response is required.

A plethora of evidences identify the inflammatory response has a key component of mechanisms responsible for cell transformation, tumour growth and metastatic process, in human cancer. Hematopoietic cells, mostly from myeloid lineage, play a pivotal role in these processes by driving the immune response toward a beneficial anti-tumour pathway, or providing support to cancer cells. In this context, urges to propose novel immunomodulatory strategies aimed to modify cancer milieu, and drive the anti-tumors response toward most favorable routes.

Monocytes are the circulating progenitors of different cell types that can infiltrate cancer lesions, and has a powerful ability to shape the inflammatory response. For this reason, monocytes and their activation programs are attractive targets for immune therapies aimed to subvert the immune response in cancer-bearing individuals. The present study has been aimed to verify the capability of PIs to interfere with differentiation and activation programs of human peripheral monocytes.

Specifically, I verified the ability of a panel of PIs, widely used in HIV-1+ patients, to interfere with differentiation of monocytes toward:

(a) Dendritic cell lineage

(b) Tumor associated macrophage-like lineage.

3. MATERIALS AND METHODS

3.1 Media and Reagents.

The regular medium used throughout the study was RPMI 1640 (Invitrogen) supplemented with 2 mM L-glutamine, 50 ng/ml streptomycin, 50 units/ml penicillin, and 10% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, UT). Granulocytes monocytes-colony stimulating factor (GM-CSF) was purchased from Schering-Plough (Kenilworth, NJ) and used at a concentration of 50 ng/ml. Interleukin-4 (IL-4) was obtained from ImmunoTools and used at 1000U/ml.

Saquinavir, Ritonavir, Nelfinavir, Indinavir, Amprenavir were dissolved in dimethyl sulfoxide (Me_2SO) and used at 20uM. As controls, cells were either left untreated or were treated with a comparable concentration of Me_2SO but without HIV-1 protease inhibitor. Saquinavir, Ritonavir, Nelfinavir, Indinavir sulfate, Amprenavir were obtained through the NIH AIDS Research and Reference Reagent Program, Division af AIDS, NIAID, NIH.

3.2 Isolation and culture of NK cells, DCs and Tumour-Associated-Macrophage (TAM).

Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated by density gradient on Ficoll Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). Blood samples were obtained in accordance with the ethical committee requirements. NK cells were negatively selected by depleting by using an antibodies cocktail against lineages specific markers and magnetic beads (StemCell Technologies Inc.). According to cytometry, typical purified NK cells were 97% pure. Purified NK cells contained ≤3% contamination with other PBMC subsets as determined by the expression of CD3, TCR-α/β, TCR-γ/δ, CD19, or CD14. Polyclonal NK cells and NK cell subsets were activated in vitro with recombinant IL-2 (rIL-2; Roche) at 200 UI/m for 6 days.

To generate iDCs, monocytes were purified by positive selection with anti-CD14 conjugated magnetic microbeads (Miltenyi Biotec, Bologna, Italy). $CD14^+$ cells were than cultured at a concentration of 0.5–1 x 10^6 cells/ml in regular medium supplemented with GM-CSF (50 ng/ml) and IL-4 (1000U/ml) for 4–5 days to obtain cells with typical phenotype of iDCs. After 6 d of stimulation in culture, CD14dim-neg and CD1apos iDCs were induced to undergo maturation by incubation with LPS at 1 μg/ml (Sigma-Aldrich) for 24 h.

In order to generate Macrophage and TAM, myeloid cells were maintained in complete medium (CM) consisting of culture medium supplemented with 20 ng/ml GM-CSF. Macrophages (Mφ) were differentiated from $CD14^+$ monocytes cultured for 5 d in CM at 106 cells/ml. TA-Mφ were differentiated from $CD14^+$ monocytes cultured for 5 d in CM at 106 cells/ml with tumour ascites (diluted 1:10).

All cell culture was conducted at 37°C in humidified 5 % CO_2 atmosphere.

3.3 Flow Cytometry.

Cell phenotypes of DCs were analyzed by flow cytometry by using the following monoclonal antibodies conjugated: anti-HLA-I and anti-CD14 from Sigma; anti-CD1a, anti-CD86, anti-CD80, anti-CD83, anti-CD40, anti-HLA-DR, anti-HLA-ABC, anti-CD11c, anti-CD36, anti-CD54 from BD Biosciences, anti DC-SIGN from NIH research and reference reagent program.

To analyze T-cell programming, DCs generated in presence or absence of HIV-PIs (1×10^4 cells/well) were cocultered with allogeneic naïve CD45RA+ CD4+ Tcells (1×10^5 cells/well) in the presence of LPS for 10 days. Thereafter, T cells were stimulated with 10 ng/ml phorbol myristate acetate (PMA, Sigma-Aldrich) and 1 μg/ml ionomycin (Sigma-Aldrich) for 4 h and evaluated for IFN-γ and IL-4. For intracellular cytokine detection, Brefeldin A (5 μg/ml; Sigma) was added to the culture medium. Cells were then fixed and

permeabilized by using a cytokine staining kit following the manufacturer's instructions (Caltag Laboratories, Burlingame, CA). Antibodies against, IFN-γ and IL-4 were purchased from BD Biosciences. FACSCalibur cytometer and Cellquest software were used for these analyses (BD Biosciences).

Human NK cells analysis was performed with: anti–TCR-α/β (IgG1), anti–TCR-γ/δ (IgG1), anti-CD19 (IgG1), FITC-anti-CD14, PE-anti-CD107a purchased from Becton Dickinson, USA, FITC anti -CD3/PE-Cy5 –anti-CD56 purchased from Beckman-Coulter-Immunotech, Marseille, France.

Data were collected using a FACSCAlibur flow cytometer (Becton Dickinson, USA) and analyzed using FlowJo v6.3.3 (Treestar, Palo Alto, CA, USA).

Cell phenotypes of Macrophages (Mφ) and TA-Mφ were analyzed using FITC-labeled anti- ILT3 (R&D Systems), PE-labeled anti-CD86 (BD Pharmingen, San Diego, CA) and Per-CP-labeled anti-CD14 mAbs (Becton Dickinson, USA). Isotype control mAbs were from BD Pharmingen and Beckman Coulter. Results are expressed as mean fluorescence intensities (MFI) after subtraction of the value obtained with the control mAb.

FACSCalibur cytometer and Cellquest software were used for these analyses (BD Biosciences).

3.4 Proliferation Assay.

Freshly purified NK cells were cryopreserved until required as responders. Experiments were performed in triplicate in 96-well round plates with complete medium. NK cells were cocultured at a constant concentration of 2 x 10^5 NK cells/well with autologous mDCs (stimulators) in serial dilutions (10–1.50 x 10^3 cells/well). [^{3}H]Thymidine (0.037 Mbq per well; PerkinElmer Life Sciences) was added 18 h before harvest cell cultures, and incorporation of [3H]thymidine into the cells was quantified using a b-counter.

3.5 Analysis of NK-cell cytotoxicity by chromium release.

After 6 d of activation with rIL-2, NK cells were tested for cytolytic activity in a 4-h ^{51}Cr release assay. A total of 1×10^6 target cells (K562 or autologous DC) were labeled with 1mCi of $Na^{51}CrO_4$ for 1 h at 37°C.

Cells were then washed twice with complete medium and incubated with effector cells at an E:T ratio of 20:1. After incubation for 4 h at 37°C, a sample of supernatant was counted on a Microbeta Trilux Scintillation counter (PerkinElmer). Percentage of cytotoxicity was calculated using the formula (experimental-spons)/(maximum-spons) ×100%, where spons = release from targets incubated with medium alone and maximum = release from targets induced by 10% SDS (Sigma-Aldrich).

Saturating concentrations (10 μg/ml) of specific mAbs blocking NK cell receptors were added for the masking experiments performed with autologous DCs.

3.6 NK-DC cocolture.

NK-DCs were cocoltured at 1:1 ratio (2X 10^5 /well) in presence of LPS (10 μg/ml) in 48-well cell culture plates. After 16-h incubation, cell culture supernatants were collected and stored at -20° until analyzed for cytokine production and NK cells were collected and analyzed for CD107a degranulation assay.

3.7 CD107a degranulation assay.

r-IL2–activated purified NK cells were cocultered alone (no target control) or with K562 target cells at a 1:1 E:T ratio (2×10^5 effector cells: 2×10^5 target cells in a volume of 200 μl) in the presence of 20μl of PE-CD107a mAb for 3 h at 37°C in total. After the first 1 h 5 μl of the secretion inhibitor 2mM monensin (Sigma Aldrich, Munich, Germany) in 100% ethanol was added. At the end of coincubation, cells were washed in PBS and stained with mAbs (CD56, CD3) for flow cytometric analysis. NK cells were gated by

$CD56^{+}/CD3^{-}$ staining, and CD107a expression was determined based on background level of staining exhibited by no target control cells.

3.8 Cytokine secretion and mediators quantification.

The levels of IL-12p70 (IL-12) , TNF-α and IL-15 secreted by mDCs and that of MMP9, VEGF and GM-CSF secreted by 48h LPS- activated TA-Mφ were quantified by ELISA in the cell-free supernatants. (R&D Systems and Biosource International).

To detect the production of IFN-γ, freshly purified NK cells were cryopreserved until required and cocultured with autologous LPS matured DCs in 96-well round-bottom plates with complete medium. The mDC/NK cell ratio was 1:10. The supernatant of the cultures was collected after 24 h and assayed by ELISA (BD Biosciences).

To simultaneosly profile the relative levels of multiple cytokines and angiogenesis-related proteins in a single sample of TA-Mφ culture supernates were used the R&D Systems Human Cytokine Array Panel A and the R&D Systems Human Angiogenesis Array .Briefly 500 μL of conditioned media was used for each array shown. Cell density was 1 x 106 cells/mL.

Array signals from scanned X-ray film images were analyzed using image analysis software. Array images are from one minute exposures to X-ray film.

4. RESULTS AND DISCUSSION

4.1 HIV-1 protease inhibitor treatment affects the immune phenotype and LPS-induced terminal differentiation of DC.

To investigate the ability of HIV-1 PIs to interfere with the differentiation program of human DCs, I generated monocyte-derived DCs in the presence or absence of 20uM of Saquinavir (SQV), Ritonavir (RTV), Indinavir (IDV), Amprenavir (APV) or Nelfinavir (NFV). After 7 days, cell surface expression of typical differentiation markers (CD14, CD1a, CD11c, CD83), adhesion molecules (CD54, CD11a, CD11c), co-stimulatory molecules (CD80, CD86) and scavenger receptors (CD209, CD36) was tested by flow cytometry.

DC generated in regular medium with or without PIs (DC-PI and DC, respectively) showed comparable levels of CD54, CD11a, CD11c, MHC-I and -II, and CD80 (fig. 4.1A). Monocyte differentiation program toward DC lineage includes downregulation of CD14 and *de novo* synthesis of CD1a, events that were unaffected by the presence in the culture medium of the majority of tested PIs. Although the loss of CD14 was unaffected in DC generated in the presence of sqv and rtv, these cells showed an atypical phenotype including a barely detectable expression of CD1a, and a low expression of CD86 (fig. 4.1A). Of note, DC generated in the presence of each individual PIs showed a marked decrease in the level of CD209 (DC-SIGN), a molecule involved in the binding and spreading of the HIV-1 virions to T lymphocytes.

To investigate the capability of DC-PI to secrete cytokines and terminally differentiate, we exposed DC generated in the presence or absence of PIs to LPS, and after 24 hours we evaluated phenotype by flow cytometry and cytokines accumulated in supernatants. iDC generated in the presence of ind, ampr or nlf showed a comparable ability to terminally differentiate compared to DC generated in regular medium (fig. 4.1 B). On the contrary, iDC-sqv and iDC-rtv show a marked defect to up-regulate CD86 and *de novo* express CD83,

changes typically associated with terminal differentiation process, in response to LPS.

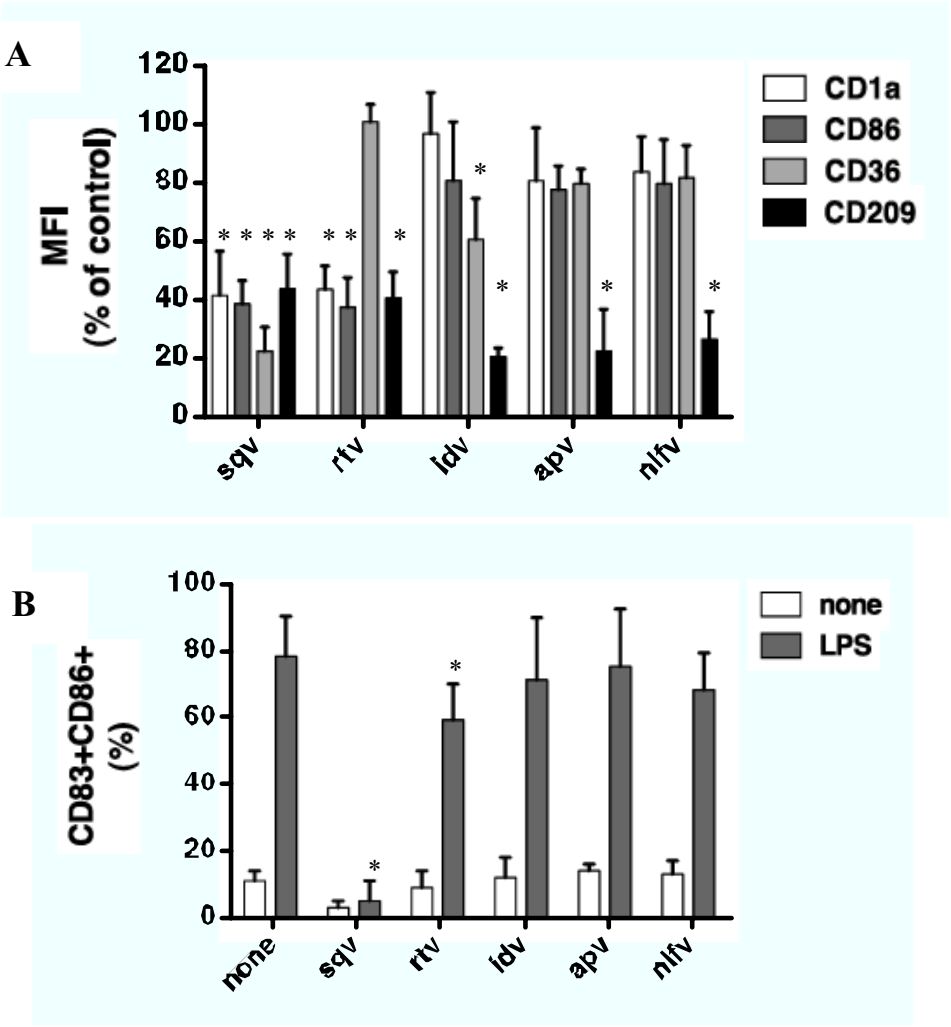

Figure 4.1 Influence of HIV-PIs on immunophenotype and maturation of DCs.
(A) iDCs generated in presence or absence of HIV-1 PIs were analyzed by flow cytometry. The histograms represent the percentage of cells positive for the indicated molecule. (B) iDCs generated in presence or absence of HIV-1 PIs were washed and then left in regular medium or exposed to LPS (1mg/ml) for 24 hours. Subsequently the expression levels of CD86 and CD83 at the surface of DCs were determined by flow cytometry. The histograms represent the percentage of cells positive for the indicated molecule.Asterisks indicate a statistically significant difference ($p < 0.05$).

4.2 HIV-1 protease inhibitor treatment affects the cytokine production of LPS-induced terminal differentiated DC.

To further investigate the effect of the HIV-PIs on terminal differentiation of DC, I examined by ELISA the amount of pro-inflammatory cytokines (TNF-alpha, IL-12, IL-15) produced in response to LPS. I focused my study on RTV rather than on SQV, considering that the latter is less used in clinical practice, because of the plethora of adverse effects and the low biodisponibility. In addition, I compared RTV effects to APV, the less effective drug in my experimental system. While LPS-dependent TNF-alpha and IL-12p40 induction were both unchanged, RTV treatment completely blocked the secretion of bioactive IL-12p70 and IL-15 (Figure 4.2). These findings further substantiate the results of previous studies demonstrating that, for example, ritonavir and saquinavir inhibit the production and/or release of inflammatory cytokines and chemokines including TNF-alpha, IL-6, and IL-8, by both peripheral-blood mononuclear cells and endothelial cells (Pati et al. 2002). This effect of HIV-PIs on inflammatory cytokines has been confirmed in treated patients, as PI-HAART has also been shown to inhibit TNF-alpha, IL-2 and IFN-γ. production by peripheral-blood mononuclear cells from uninfected individuals who were treated with HIV-PIs for prophylactic intervention without acquiring HIV infection (Tovo 2000). Because of the great importance of DC in the control of the inflammatory response, it was conceivable that HIV-PIs might exert their anti-inflammatory activity by impairing the immunostimulatory properties of this cell type, with a potential perturbation of the inflammatory circuits supporting tumor growth and progression.

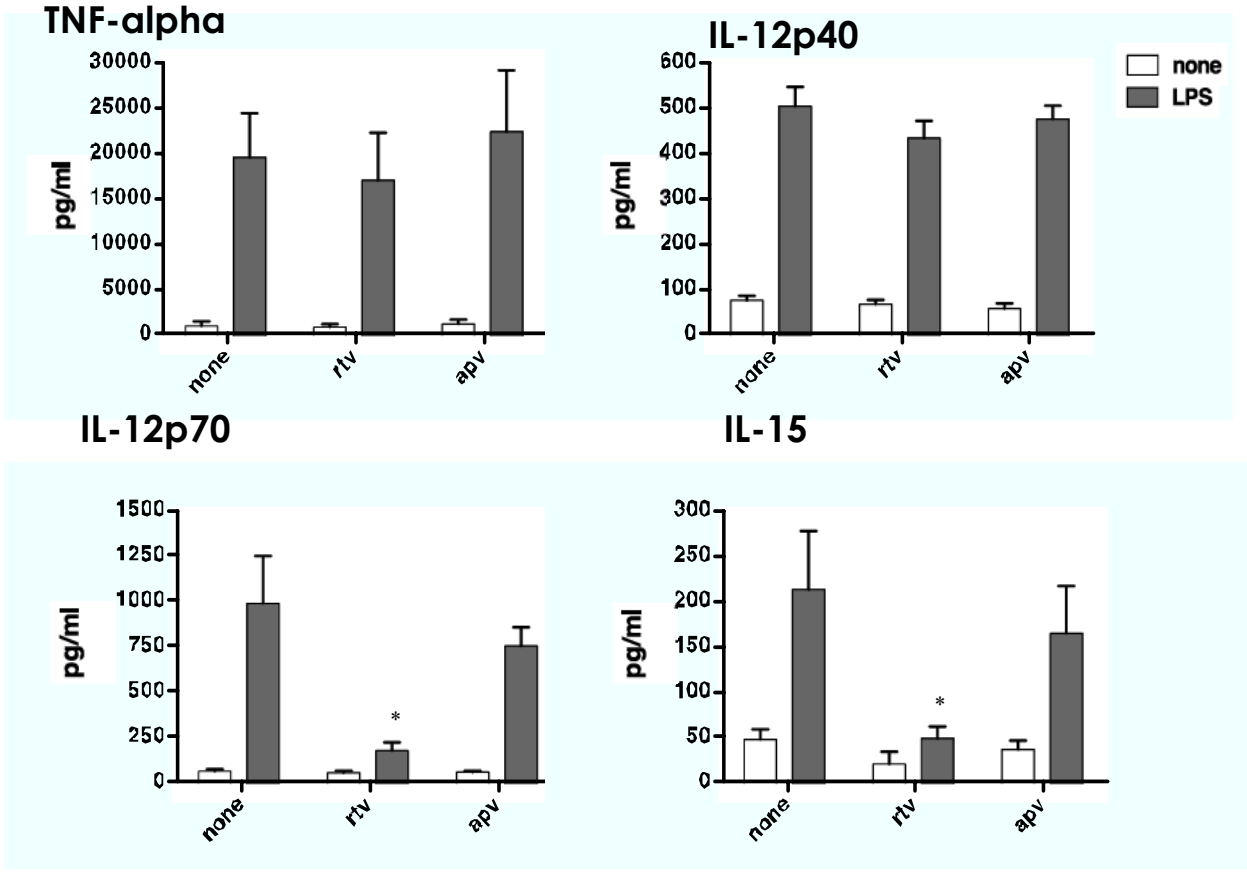

Figure 4.2 Impact of HIV-PIs on cytokine expression of DCs. iDCs generated in presence or absence of HIV-1 PIs were washed and then left in regular medium or exposed to LPS (1mg/ml) for 24 hours.Supernatants were collected and the levels of secreted cytokines were evaluated by ELISA. The results are presented as mean± s.e. of duplicate determinations.Asterisks indicate a statistically significant difference ($p<0.05$) between the indicated cytokines production of Ritonavir-treated DCs in comparison to untreated DCs.

4.3 Ritonavir impairs the polarization of CD4+ T cells toward a Th1 phenotype.

LPS-activated DCs efficiently direct the differentiation of naïve CD4+ T lymphocytes into IFN-γ-producing Th1 cells. To determine whether RTV inhibits this functional property, DCs that had been previously generated in presence or absence of the indicated drugs were cocultured with allogeneic naïve CD45RA+ CD4+ T cells in the presence of LPS. Thereafter, T cells were stimulated with PMA and ionomycin, and evaluated for IFN-γ and IL-4 production. As depicted in figure 4.3, Ritonavir profoundly impaired the capacity of LPS-activated DCs to induce differentiation into IFN-γ-producing

CD4+ T helper cells. This effect can be explained by the RTV-induced impairment of IL-12 secretion, since it has been shown that the differentiation of naïve CD4+ T cells into Th1 cells by LPS-stimulated DCs is critically dependent on this cytokine (Hsieh CS et al. 1993). These results reveal that the treatment with Ritonavir can substantially affect the potential of human DCs to induce programming of CD4+ T helper cells into Th1 cells which if on one hand may contribute to antitumor immunity, on the other by substainig the inflammatory network, can also have pro-tumour activity. Evidence indicates that NF-κB is important in determining this balance between the protumour and antitumour properties of different inflammatory cell type (Saccani A et al. 2006), thus NF-κB could be targeted to 're-educate' tumour-promoting immune cell towards an antitumour function.

The obtained results are in line with other studies indicating that Ritonavir can significantly inhibit CTL-dependent inflammatory response (Kelleher AD at al. 2001).

Overall the effects of this class of drugs on eukaryotic cells can be explained supposing their inhibitory effect on endogen proteases that share amino acid sequence homology and structure with that of the proteases of HIV. Several authors highlighted the role of proteases in the regulation of the differentiation and functional activity of DCs.

Recent studies reported indeed that ritonavir, by inhibiting the chymotrypsin-like activity of the proteasome impairs in a murine model the major histocompatibility complex class I restricted presentation of several viral antigens (Schmidtke G et al. 1999). Furthermore, it has been suggested that the proteasome inhibitor Bortezomib inhibited cytokine-induced maturation of human monocyte derived immature dendritic cells (DCs) (Subklewe et al. 2007). Even if the immunosuppresion by proteasome inhibition has been demonstrated in a murine model, this last work suggests that the effect might be mediated by the inhibition of DCs maturation (Subklewe et al. 2007).

Ritonavir also inhibits calpain activity in PC12 cell extracts. Recently this enzyme has been involved in the mechanisms that regulate the survival and the migration of neural and dendritic cells (Tremper –Wells B et al. 2002, Grynspan F et al.1997, Tremper-Wells B et al. 2005), suggesting that some of the effects of Ritonavir might result from the regulation of the calpains protease activity.

Finally, the effects of HIV-PIs are also determined by their action on the MMP that are involved in the differentiation process of human peripheral blood monocytes (Major TC et al. 2002), as well as in the DCs migration and in their functional response to the bacterial endotoxin (Ratzinger G et al. 2002, Lai WC et al. 2003).

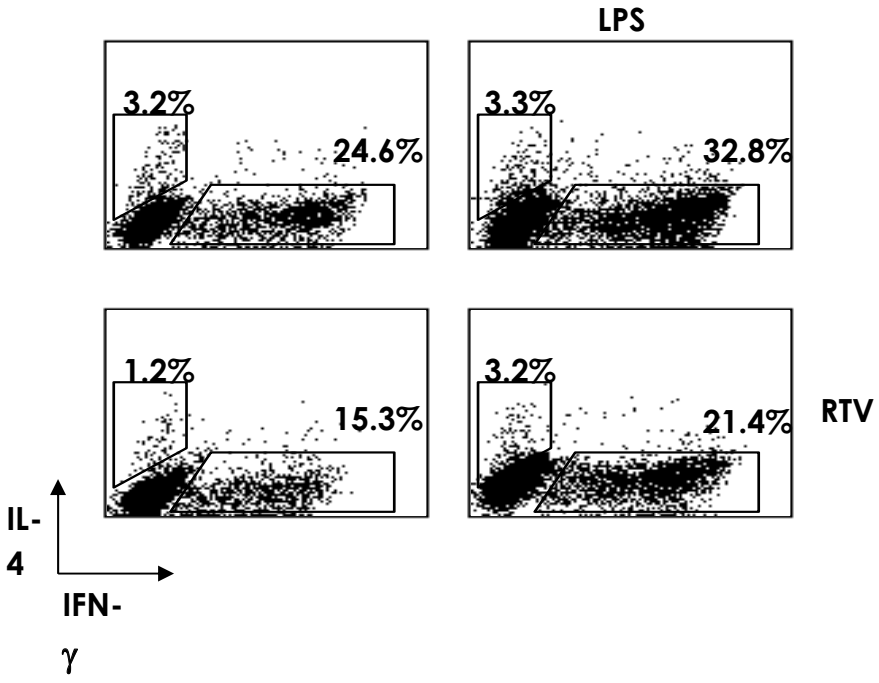

Figure 4.3 Effects of Ritonavir on the acquisition of T cells stimulatory activity of LPS primed DCs iDCs generated in presence or absence of Ritonavir were washed and then coincubated with allogeneic naïve CDRA+ CD4+ T helper cells in the presence of LPS for 10 days. The percentage of IFN-γ- orIL-4-producing CD4+ T helper cells was determined by flow cytometry. The results of one representative healthy donorout of four performed with similar results are depicted.

4.4 Inhibitory effects of Ritonavir on the capacity of mDC to activate autologous NK cells.

Given the central role of 12p70 and IL-15 cytokines in the DC-dependent NK-cells activation (Moretta A 2005, Ferlazzo G et al. 2002), I asked if RTV-treated DC preserved their ability to interplay with NK cells. I first examined the ability of LPS-treated mature DC (mDC) to activate these innate effector cells by inducing proliferation of autologous NK cells and IFN-gamma secretion. Therefore, iDCs generated in presence or absence of Ritonavir or Amprenavir were washed and then left in regular medium or exposed to LPS (10μg/ml). After 6 hours, mDCs (stimulator) were cocultered with autologous fresh NK cells (responders) at different ratios. Cells were harvested after 4 d of coincubation, and the proliferation of NK cells was measured by 3[H]thymidine incorporation. Long exposure to RTV decreased of about 50% the ability of mDC to sustain NK- cells proliferation (figure 4.4 A). In addition iDCs generated in presence or absence of HIV-PIs were washed and then left in regular medium or exposed to LPS (10μg/ml). After 18 h, freshly purified autologous NK cells were coincubated with iDC or mDC for 24h. Supernatants were collected and INF-γ concentration was determined by ELISA. As demonstrated in figure 4.4 B Ritonavir but not Amprenavir significantly reduced the ability of LPS-activated DCs to improve IFN-γ production by NK cells. For the first time I here showed the effects of HIV-protease inhibitors on a novel system: the crosstalk DC-NK. In particular, data obtained highlighted that Ritonavir treated DCs lose their capacity to efficiently stimulate NK cells, confirming and extending to a novel target the anti-inflammatory effects of HIV-PIs.

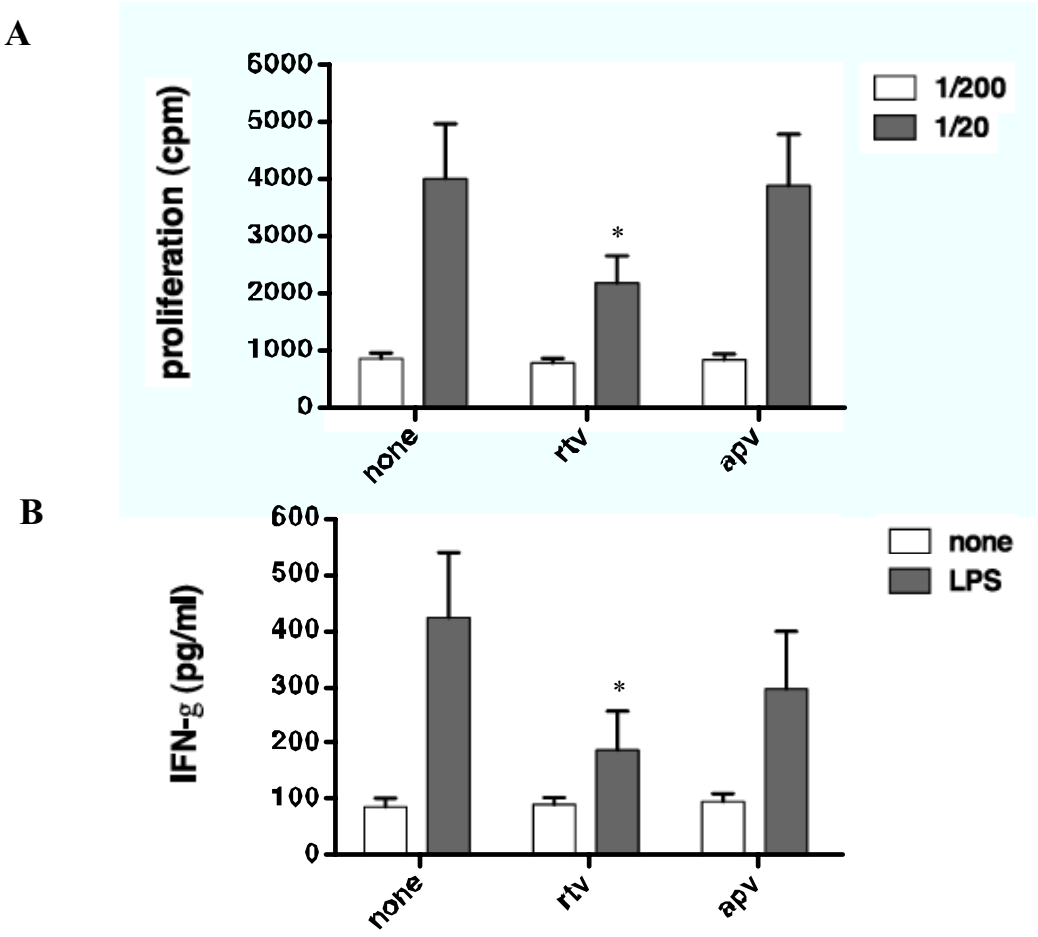

Figure 4.4 Influence of Ritonavir on the capacity of DCs to prime autologous NK cells. (A) iDCs generated in presence or absence of Ritonavir or Amprenavir were washed and then left in regular medium or exposed to LPS (10μg/ml). After 6 hours, mDCs (stimulator) were cocultered with autologous fresh NK cells (responders) at different ratios. Cells were harvested after 4 d of coincubation, and the proliferation of NK cells was measured by 3[H]thymidine incorporation.(B) iDCs generated in presence or absence of HIV-Pi were washed and then left in regular medium or exposed to LPS (10μg/ml). After 18 h, freshly purified autologous NK cells were coincubated with iDC or mDC for 24h. Supernatants were collected and INF-γ concentration was determined by ELISA. All data are presented as the mean ± SD of experiments conducted on 8 healthy donors. Asterisks indicate a statistically significant difference ($p< 0.05$).

4.5 Impact of Ritonavir on the ability of mDC to improve tumor-directed cytotoxicity of NK cells.

In line with precedents reports (Ferlazzo G et al. 2002), I reasoned that the lack of mDC cognate activation of NK-cells would affect their cytotoxic effect on a non-cognate susceptible cellular target. To test this hypothesis, I co-incubated mDC and NK-cells for 24 hours, and subsequently I analyzed the ability of DC-primed NK-cells to kill K562 target. CD107 cytometric analysis shows that K562 lysis is severely decreased by priming with RTV-treated mDC (Figure 4.5). These data suggest that the DCs-mediated enhancement of tumoricidal potential of NK cells was also markedly by Ritonavir.

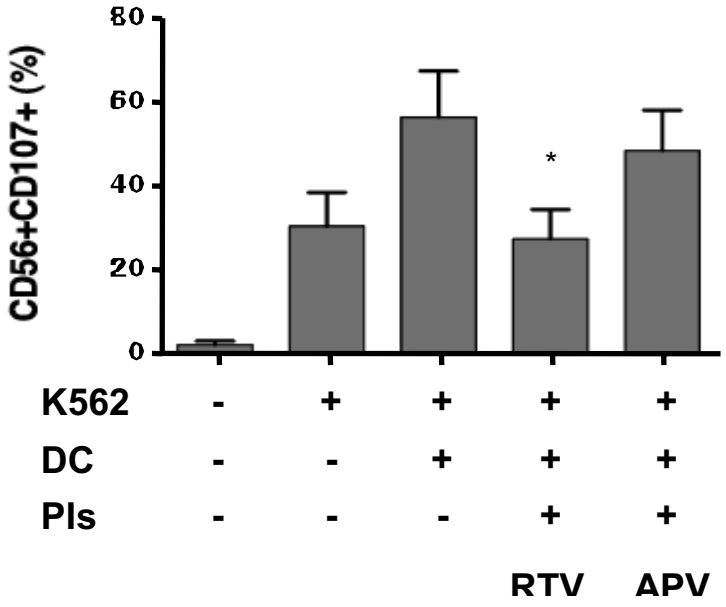

Figure 4.5 Ritonavir reduces the DCs-mediated enhancement of tumoricidal potential of NK cells. iDCs generated in presence or absence of Ritonavir or Amprenavir were washed and cultured with purified CD56+CD3- NK cells in the presence of LPS (10μg/ml). After 18 h, NK cells were separeted from adherent DCs and cocultured in round-bottom 96-well plates with K-562 target cells at an E/T ratio of 1:1 in presence of anti-CD107a mAb. After 4 h of culture, cells were stained with the two other indicated mAbs and analyzed by multiparametric flow cytometry. The histograms represent the percentage of $CD56^{+}CD3^{-}$ -gated NK cells staining positive for CD107 in the presence of targets following coincubation with treated or untreated mDC compared with control cultures incubated in the absence of mDC. All data are presented as the mean ± SD of experiments conducted on 8 healthy donors. Asterisks indicate a statistically significant difference ($p< 0.05$).

4.6 Impaired NK cell-mediated killing of Ritonavir treated mDC.

I next asked if NK-cells dependent killing of autologous iDCs were active in my system. This function depends on the engagement of NKp30 by still-undefined cellular ligands expressed by DCs. Indeed Cr 51+ release assay showes that non treated autologous iDC are efficiently killed by NK cells in an NKp30-dependent manner, as demonstrated by the ability of the specific mAb masking NKp30 (F252) but not of the specific mAb masking NKG2D (BAT221) to inhibit the NK killing activity (Figure 4.6 A). As expected untreated mDCs that, upregulate MHC-class I expression become essentially resistant to NK cells (Figure 4.6 B).

On the contrary, the treatment with Ritonavir increases the susceptibility to NK-cells mediated lysis of mDCs whereas iDC lysis was high irrespective to the treatment (Figure 4.6 A and B). This apparent paradox might be explained by the fact that RTV-treated DC failed to fully mature in response to LPS and even upon its stimulation RTV-treated DCs fail to express sufficient amounts of HLA-class I molecules that would protect from NK-mediated lysis (Ferlazzo G 2003).

All this set of results suggest that RTV can act on DC to disrupt their crosstalk with NK cells, leading to a deficient proliferation and activation. In addition, the major sensitivity to NK cells dependent lysis may lead to DC deficiency, thus amplifying the inhibitory effect on the adaptive immune response. These observations should be considered not only with respect to immunomodulatory aspects of HIV-1 treatment, but also to design new therapies for other inflammatory conditions that often degenerate in cancer.

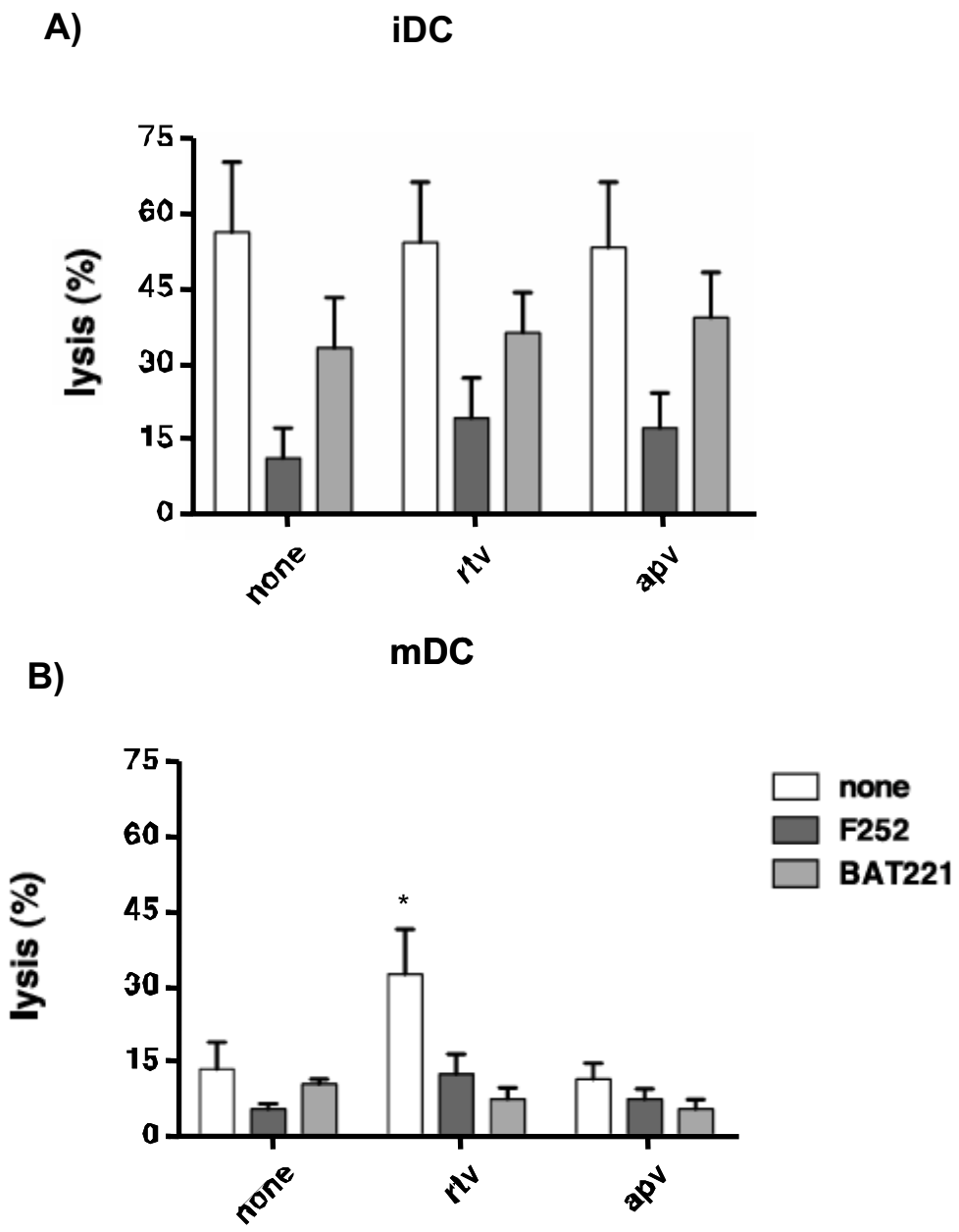

Figure 4.6 Impaired NK cell-mediated killing of Ritonavir treated mDC(A) Autologous iDC cytolysis exerted by rIL-2–activated NK cells purified from a healthy donor . (B) Autologous mDC cytolysis exerted by rIL-2–activated NK cells purified from a healthy donor NK cells were incubated either in the absence (baseline lysis) or presence of a specific mAb masking NKp30 (F252) or NKG2D (BAT221). The NK cell/DC ratio in all experiments was 10:1. All data are presented as the mean ± SD of experiments conducted on 8 healthy donors. Asterisks indicate a statistically significant difference ($p < 0.05$).

4.7 Ritonavir reduces the surface level of the inhibitory molecule ILT3 and upregulates that of the costimulatory molecule CD86 in established TAM.

Besides investigating the ability of DCs to sustain the pro-inflammatory circuits predisposing to cancer, I also evaluated whether RTV and APV may reverse the immunosuppressive properties of Tumor-associated macrophages (TAM), that are the major players of the cancer-related inflammation. To this purpose, I first tested RTV and APV for their ability to restore immunostimulatory molecule expression on established TAM. TAM were stimulated for 48 hr with LPS, and then CD86 expression was analyzed.

As a result, RTV- as well as APV-treated TAM recovered the ability to express higher levels of CD86 in response to LPS (Figure 4.7 A). Opposingly, analysis of surface molecules by flow cytometry also revealed that RTV-treated TAM expressed lower levels of the cell surface inhibitory molecule ILT3 than untreated TAM (Figure 4.7 B). Also the treatment with APV decreased ILT3 expression by TAM, but to a lower extent than RTV.

Up to now little is known about the regulation of ILT3 gene and protein expression. It is selectively expressed by myeloid antigen presenting cells such as monocytes, macrophages and dendritic cells, in which it displays a dual function of inhibitory receptor and antigen-capturing molecule. (Cella M et al. 1997). Recent data underline the critical role of ILT3 in the control of inflammation because the silencing of ILT3 expression in DC increase TLR responsiveness to their specific ligand as reflected in increased synthesis and secretion of proinflammatory cytokines (Chang CC et al. 2009). I can't hypothesize the mechanisms by which RTV might reduce ILT3 surface expression, however the reduction of its level by RTV really correlates with an antinflammatory condition (see later results). ILT3 signaling results in the inhibition of NF-kappaB pathway and of the transcription of costimulatory molecules (Suciu-Foca N and Cortesini R, 2007). On the other hand activation stimuli such as inflammatory conditions downregulate inhibitory ILT3 receptor

expression (Ju XS et al. 2004) These last specular aspects might explain the apparent discrepancy between the opposite effects exerted by RTV on CD86 molecule expression in DC and in TAM. In fact if on dendritic cells RTV and in general HIV-PIs reduce the surface levels of the costimulatory molecule CD86, in TAM RTV, by reducing the negative effect of ILT3 on its transcription, upregulates rather than downregulates, the level of CD86 costimulatory molecule. Overall these results show for the first time that APV but especially RTV switch monocyte differentiation into CD86high ILT3low M1 macrophages. I therefore tested whether these RTV-induced phenotypical modification were associated to the modulation of the functions of the TAM.

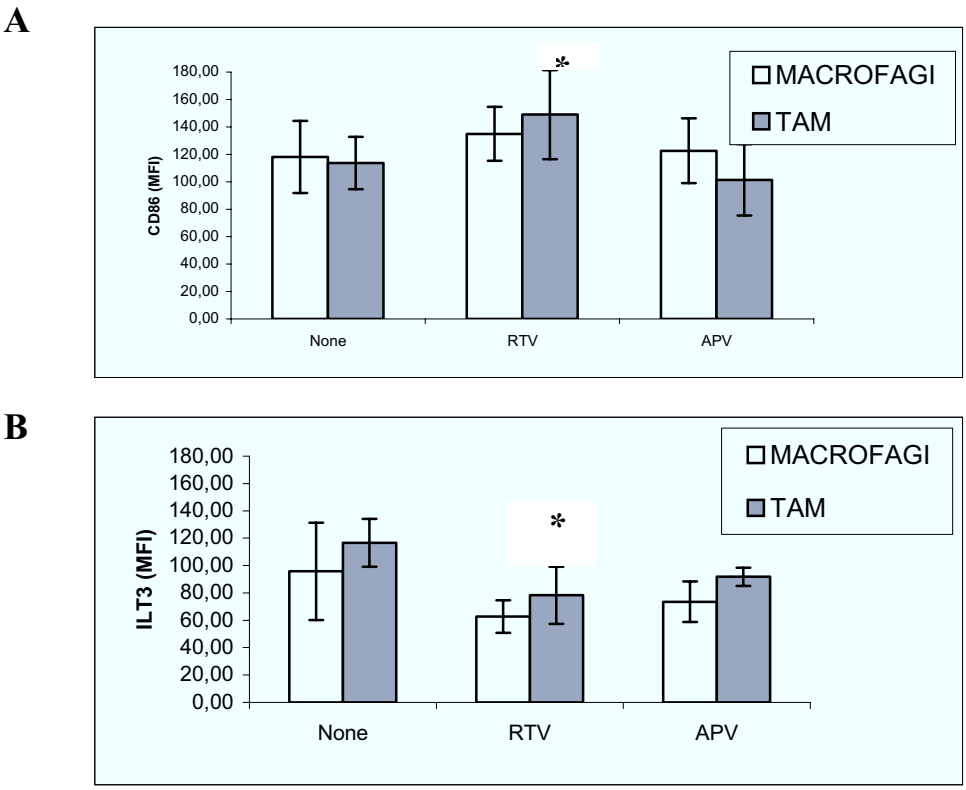

Figure 4.7 HIV PIs affect the immunophenotype of tumor-associated macrophages
Human blood monocytes cultured 5 days in the absence (macrophage) or presence of ascites (TAM), were stimulated or not with HIV-PIs before stimulation with 200 ng/ml LPS. After 48 hr, CD86 (A) and ILT3 (B) expression was analyzed by FACS. Results are expressed in MFI as mean of 6 separate experiments. * means $p < 0.05$.

4.8 RTV alters the secretion profile of different human cytokine and protumoral mediators by TAM.

TAM are characterized by the production of cytokine and mediators that promote tumor immune escape and progression. I thus tested the influence of RTV and APV on their expression by using two Proteome Profiler Array to detect multiple analytes in cell culture supernatants from untreated and treated TAM.

Array images are shown on the left and profiles created by quantifying the background-subtracted mean spot pixel densities are represented on the right. Array images are from one minute exposures to X-ray film.

The analysis of the Human Cytokine Array (Figure 4.8 A) shows that macrophages generated in the presence of RTV and APV secreted higher levels of GM-CSF, INF-γ and IL16 than TAM, while only RTV-treated TAM produced lower levels of the pro-inflammatory cytokine TNF-α and of the chemoattractant protein RANTES (CCL-5). In parallel, the analysis of the Human Angiogenesis Array shows that only macrophages generated in presence of RTV secreted reduced levels of MMP9, VEGF, Serpin E1 and of the chemoattractant MIP-1 alpha (CCL-3) (Figure 4.8 B) while the same analysis revealed that RTV-treated TAM secreted higher level of the GM-CSF, CXCL8 and Angiostatin proteins than TAM and APV-treated TAM.

In many tumours (for example, non-small-cell lung cancer and pancreatic carcinoma) it is the concert of different factors and the balance between protumor and antitumor cytokines and chemokines, that regulates tumour progression.

The data presented reflect the effective differential expression of many factors that contribute to carcinogenesis and the impact of the HIV-protease inhibitor Ritonavir on their modulation. TAM are key orchestrators of cancer-related inflammation, and as first, neoplastic cells together with TAM themselves actively lead monocyte recruitment, by producing chemoattrachtant mediators, from the blood into the tumor tissues to their own advantage,

Several “anti-macrophage” approaches are under evaluation currently. Interesting observations come from studies performed with chemokines and chemokine receptors as anti-cancer targets. For instance, in a breast cancer murine model, malignant cells recruit macrophages via the chemokine RANTES (CCL5), and treatment of murine breast cancers with Met-CCL5 (receptor antagonist) leads to a decreased number of infiltrating macrophages associated with a significantly reduced tumor size (Robinson et al. 2003). From the literature it is clear that protease inhibitors therapy reduced the high production of inflammatory cytokines (TNF- alpha, MIP-1alpha, MIP-1beta, RANTES, and INF-gamma) (De Luca A et al. 2000, Tovo PA 2000) in patients with more advanced HIV infection vivo. All this support the observations done in my experimental model, where ritonavir exerts an antitumor function by reducing the protein levels of inflammatory molecules linked to the recruitment of macrophages such as RANTES (CCL-5), MIP-1 alpha (CCL-3) and VEGF as well as that of TNF-alpha which exert a key role in the inflammatory circuits supporting tumor growth and progression. However, I also found an increase rather than a decrease in the level of the other pro-inflammatory cytokine, INF-gamma. This is just in apparent contrast with the antitumour potential of Ritonavir. It is enough to think that blood monocytes differentiating in the presence of INF-gamma mature into M1-polarized cells (classically activated macrophages) that offers defence against bacteria, tumor suppression and immuno-stimulation (Solinas G et al. 2009).

TAM contribute to tumor progression also by producing several factors which enhance neo-angiogenesis (VEGF), and the dissolution and remodeling of the interstitial matrix (metalloproteases, MMPs). I then investigated the effect of RTV and APV on the detected ELISA VEGF and MMP9. (see next results). On the other hand this process is counteracted by the production of factors that limit the tumour spread like endostatin. My data reveale that RTV reduces the protein levels of VEGF and MMP9, increasing that of the endostatin that is the most potent inhibitor of tumor anngiogenesis.

Finally, data showed that RTV can also affect the protein level of PAI-1/Serpin E1, whose expression is increased in primary tumor tissue of breast cancer patients, and correlate with tumor aggressiveness and poor clinical outcome (Annecke K et al. 2008).

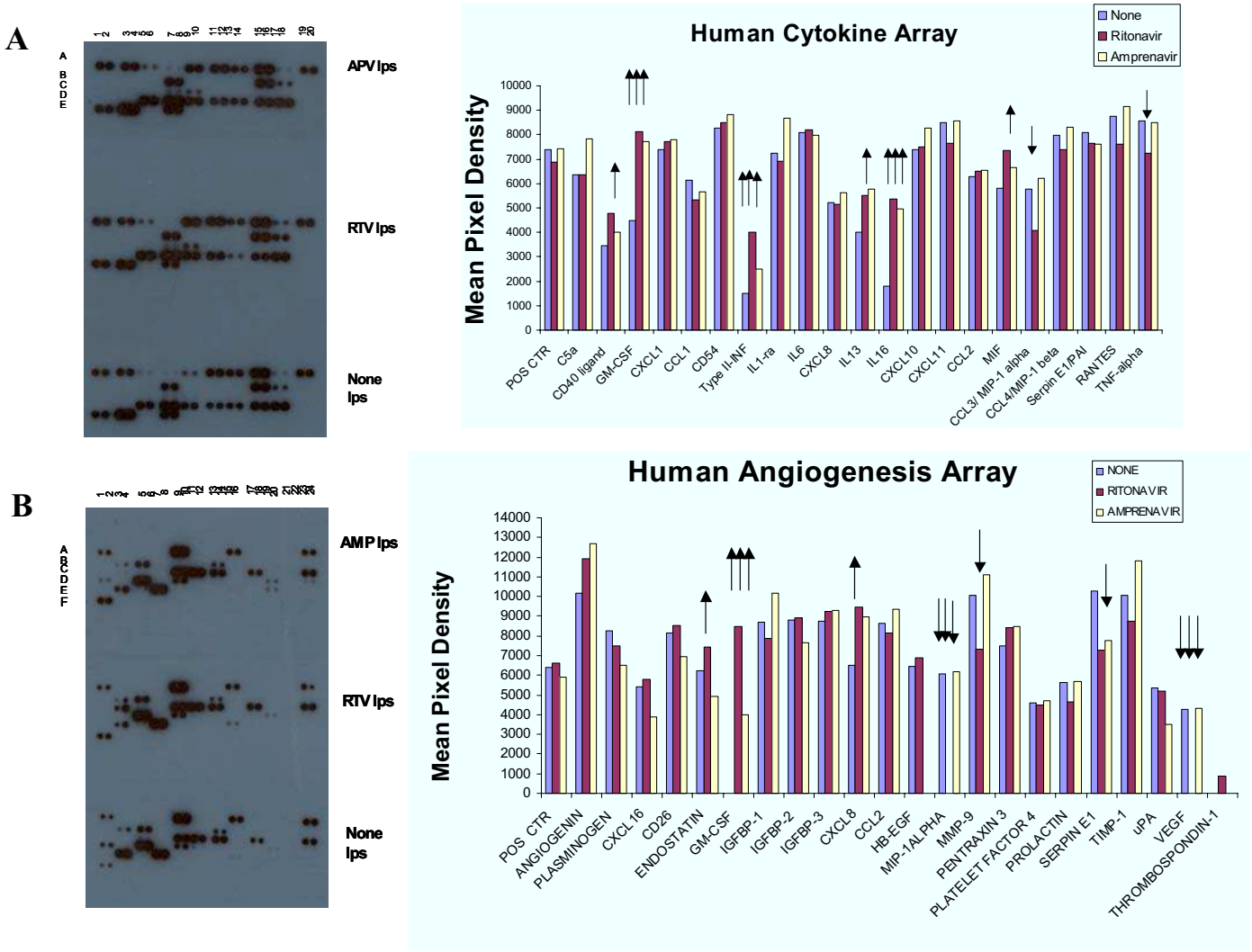

Figure 4.8. The human Cytokine and Angiogenesis arrays detects multiple analytes in TAM culture supernates generated in presence or absence of the indicated HIV-Pis. Array images are shown on the left and profiles created by quantifying the background-subtracted mean spot pixel densities are identified on the right. Array images are from one minute exposures to X-ray film.

4.9 RTV-treated TAM lose their protumoral properties.

To confirm the ability of RTV and APV to affect TAM-like cell functions, I next focused the attention on specific mediators seen modulated before and now assayed by ELISA in the cell-free supernatants. Thus, TAM generated in presence or absence of RTV and APV were stimulated for 48 hr with 200 ng/ml LPS and then MMP-9 (A), VEGF (B) and GM-CSF (C) were quantified in supernatants. Data showed that the macrophages generated in the presence of RTV secreted lower levels of MMP9 and VEGF than TAM and APV-treated TAM while RTV enhanced that of the immunostimulatory cytokine GM-CSF (Figure 4.9).

The data below confirmed the previous observations and contribute to better define the anti-angiogenic and anti-tumour activity of the Human Immunodeficiency Type-1 Protease Inhibitors, Ritonavir.

GM-CSF might be relevant to cancer patients in two broad areas: first, it drives hematopoietic precursor cells to mature granulocytes, macrophages, or dendritic cells (Wognum et al. 1994) and for this it is used clinically to accelerate bone marrow recovery and to increase the production of white blood cells to facilitate host defense and second, because of its pletora of effects on cancer cells. If on one hand it is reported that GM-CSF is aberrantly expressed in a number of different solid tumors (for example osteosarcoma (Rochet N et al, 1999), gliomas (Mueller MM et al., 1999) and pulmonary adenocarcinoma (Wislez M et al., 2001), where it correlates with an enhanced and metastatic potential and where it increases the number of granulocytes and macrophages in the tumor vicinity (Obermueller E et al., 2004) as well as that of the immunosuppressive immature myeloid cells (Serafini P, 2004); on the other hand recent data suggest that GM-CSF reduces VEGF activity by inducing secretion of the soluble form of VEGFR-1 by human monocytes/macrophages and thus by reducing biologically active VEGF available for angiogenesis (Eubank TD et al., 2004). Because Ritonavir increased the levels of GM-CSF produced by TAM, this result can give more details on the unpredicted anti-

tumor actions of this class of compounds, suggesting that Ritonavir in particular is effective in inhibiting tumour-associated angiogenesis and tumour cell invasion. This statement is corroborated also by the finding that in my experimental model RTV reduced the release by TAM of the angiogenic and monocyte chemoattrachtant factor VEGF and of the metalloprotease, MMP-9 which is involved in the degradation and remodelling of the matrix but also in the modulation of local inflammation (Sgadari C et al. 2002, Lopez et al. 2000); that could explain the ability of Ritonavir to reduce a set of inflammatory cytokine by TAM but also by dendritic cell. Data presented in the literature confirm that two other HIV-PIs, Indinavir and Saquinavir, effectively reduced the number of neo-formed vessels in a murine model of Kaposi Sarcoma (Sgadari C et al. 2002). HIV-Pis are also effective in inhibiting tumor-associated angiogenesis and tumor cell invasion in other xenograft human tumor models, including lung and breast adenocarcinoma and tumor of haematopoietic cell origin such as myelomonocytic or T-cell leukaemia and Burkitt Lymphoma (Monini P et al. 2003). In a separate set of experiments it is also reported that Saquinavir and Indinavir affect pathways involved in cell invasion and MMP activity, particularly MMP-2 proteolytic activation. (Sgadari C et al. 2002). The data shown extend the anti-angiogenetic activity of the HIV-PIs also to the Ritonavir, and indicated for the first time the specific cell type, TAM, a critical supplier of mediators to the carcinogenesis pathway, that is the target of the action of the drug.

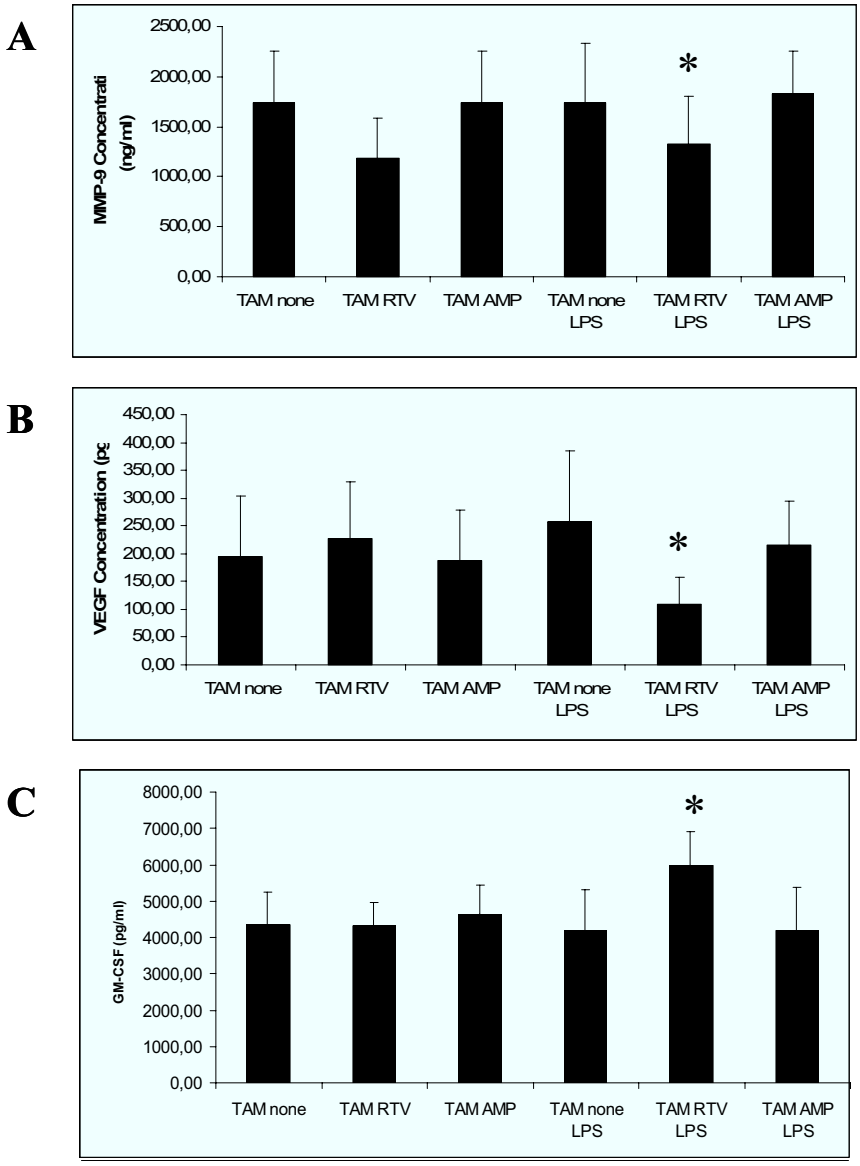

Figure 4.9 Ritonavir treated TAM loose their protumoral properties. TAM generated in presence or absence of the indicated HIV-PIs were stimulated 48 hr with 200 ng/ml LPS. MMP9 (a),VEGF (b) and GM-CSF (c) were quantified by ELISA in the cell-free supernatants.

5. CONCLUSIONS

A plethora of evidences identify the inflammatory response as a key component of mechanisms responsible for cell transformation, tumour growth and metastatic process, in human cancer. Thus, it urges to identify novel therapies aimed to revert the inflammatory status, and subvert immunosuppressive mechanisms operating in established cancer lesions.

In the present study, I have identified PIs used in the therapy of HIV-1 infection, as a promising class drugs with unexpected immunomodulatory activity. I demonstrated that PIs, and in particular ritonavir, interfere with differentiation programs of monocyte by modulating their ability to generate DC, and most notably, TAM.

The findings I have generated in the first part of the present study document the differential ability of PIs to affect phenotype and functionality of immature DC. Ritonavir has been identified as the PIs with the most remarkable immunomodulatory activity: DC generated in the presence of this drug fail to differentiate, secrete lower amount of pro-inflammatory cytokines, and lack the ability to polarize T-cells toward a Th1 phenotype. Ritonavir also impair DC-NK crosstalk, by reducing the ability of DCs to promote secretion of IFN-g, and cytotoxicity of NK cells.

The results obtained in the second part of my study, highlight for the first time the ability of ritonavir to interfere with phenotype and functions of monocyte derived TAM, by promoting the expression of co-stimulatory molecules, and decreasing the level of immunosuppressive ones. In parallel, TAM generated in the presence of ritonavir secrete lower level of mediators promoting tumor growth.

Altogether, these findings identify the differentiation programs of human monocytes as relevant targets of PIs activity. In this context, the remarkable property of certain PIs to modulate phenotypes and functionalities of DC, and

most notably TAM, might open novel perspectives for immune-intervention aimed to manipulate the cancer inflammatory milieu.

Finally the anti-angiogenic, anti-tumour and anti-inflammatory effects of PIs, their relatively low toxicity and the large body of data on their pharmacokinetics and tissue distribution, make them a suitable candidate for their rapid clinical 'repositioning' in the oncological field: in combination with the traditional antiblastic therapies, they might significantly contribute to the achievement of improved clinical goals without requiring imponent additional research efforts.

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I cannot end without thanking my family for the constant encouragement and love I have relied on throughout my life.

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Lastly, and most importantly I wish to thank my son, Stefano for the revolution he brought into my life. Now you don't understand why I had to be away some times, but some day you will. The success of finishing this thesis is my first present to my son.

I thank God for standing by me

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Calmodulin-dependent kinase IV links Toll-like receptor 4 signaling with survival pathway of activated dendritic cells

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Microbial products, including lipopolysaccharide (LPS), an agonist of Toll-like receptor 4 (TLR4), regulate the lifespan of dendritic cells (DCs) by largely undefined mechanisms. Here, we identify a role for calcium-calmodulin–dependent kinase IV (CaMKIV) in this survival program. The pharmacologic inhibition of CaMKs as well as ectopic expression of kinase-inactive CaMKIV decrease the viability of monocyte-derived DCs exposed to bacterial LPS. The defect in TLR4 signaling includes a failure to accumulate the phosphorylated form of the cAMP response element-binding protein (pCREB), Bcl-2, and Bcl-xL. CaMKIV null mice have a decreased number of DCs in lymphoid tissues and fail to accumulate mature DCs in spleen on in vivo exposure to LPS. Although isolated *Camk4*$^{-/-}$ DCs are able to acquire the phenotype typical of mature cells and release normal amounts of cytokines in response to LPS, they fail to accumulate pCREB, Bcl-2, and Bcl-xL and therefore do not survive. The transgenic expression of Bcl-2 in CaMKIV null mice results in full recovery of DC survival in response to LPS. These results reveal a novel link between TLR4 and a calcium-dependent signaling cascade comprising CaMKIV-CREB-Bcl-2 that is essential for DC survival. (Blood. 2008;111:723-731)

Introduction

Dendritic cells (DCs) are antigen presenting cells (APCs) that circulate in the blood and are also present in peripheral tissues and lymphoid organs. They are able to sustain and polarize the primary adaptive immune response and are involved in the mechanisms of tolerance toward self-antigens.[1-3] These cells recognize microbial products by using a variety of molecules expressed on their surface that enable them to detect infections in the periphery. Among these molecules, the Toll-like receptors (TLRs) bind pathogen-derived molecules to trigger the activation programs of DC, thus inducing the release of cytokines and driving DC migration to the T-cell zone.[4-6] Visualization of cellular interactions in intact lymphoid tissues reveals that DC–T cell conjugates must remain stable for up to 2 days for lymphocytes to become fully activated.[7] Therefore, the lifespan of DC is an essential factor in controlling the number of viable antigen-bearing DC in the T-cell zone, and in turn, to regulate the quality and magnitude of the adaptive immune response.

Agonists of TLR, including the Gram-negative bacterial lipopolysaccharide (LPS), control survival of DC by mechanisms only partially defined.[8] LPS signals DC via TLR4, an interaction that requires the lipopolysaccharide-binding protein and MD2, a TLR4-associated molecule.[4,6] Two distinct biochemical pathways are activated by this interaction. The "MyD88-dependent" cascade, involving Toll-interleukin-1 receptor domain adaptors MyD88 and Mal, regulates activation of the NF-kκB transcription factor and drives the synthesis of cytokines and the terminal differentiation program. The triggering of the "MyD88-independent" pathway requires TRIF and TRAM (a second set of Toll-interleukin-1 receptor domain adaptors) and stimulates phosphorylation and dimerization of IRF-3, a key event regulating the synthesis of interferon-γ. Several reports have suggested that TLR4 agonists activate antiapoptotic as well as proapoptotic pathways.[8-12] Recently, it has been proposed that LPS controls accumulation of both proapoptotic and antiapoptotic members of the Bcl-2 family of proteins and in so doing regulates the lifespan of DC.[8]

Calcium (Ca^{2+}) is a pervasive intracellular second messenger that initiates signaling cascades, leading to essential biologic processes such as secretion, cell proliferation, differentiation, and movement.[13] In DCs, many critical functions involve Ca^{2+} signaling. For example, apoptotic body engulfment and processing are accompanied by a rise in intracellular Ca^{2+} and are dependent on external Ca^{2+}.[14] In addition, chemotactic molecules produce Ca^{2+} increases in DC,[15-18] suggesting the involvement of a Ca^{2+}-dependent pathway in the regulation of DC migration. The role of a Ca^{2+}-dependent pathway in the mechanism regulating DC maturation is suggested by the

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opposite effects induced by Ca^{2+} ionophores or chelation of extracellular Ca^{2+} on this process.[19-21]

Many of the effects of Ca^{2+} are mediated via Ca^{2+}-induced activation of the ubiquitous Ca^{2+} receptor calmodulin (CaM).[22] In turn, Ca^{2+}/CaM stimulates a plethora of enzymes including those that comprise the family of multifunctional, serine-threonine kinases (CaMKs), 2 of which are CaMKII and CaMKIV.[23] These protein kinases have different tissue distributions, as CaMKII is ubiquitous[24] whereas CaMKIV is tissue-selective, and expressed primarily in brain, thymus, testis, ovary, bone marrow, and adrenal glands.[25] Whereas CaMKIV is expressed in immature thymocytes and mature T cells, it is absent in B cells. Previous studies have revealed roles for CaMKIV in regulating thymic selection as well as activation of naive and memory T cells. Moreover, CaMKIV plays a role in regulating the survival of hematopoietic progenitor cells.[26] Importantly, in addition to a rise in intracellular Ca^{2+}, activation of CaMKIV requires phosphorylation by an upstream CaMKK, leading to the suggestion that these 2 Ca^{2+}/CaM-dependent enzymes constitute a "CaM kinase cascade."

In this study, we demonstrate that CaMKIV is expressed in DC and plays a key role in the pathway linking the TLR4 with the control of DC lifespan by regulating the temporal expression of Bcl-2. These findings, which have been confirmed in human monocyte–derived DCs as well as in DCs derived from mice null for CaMKIV, reveal the importance of a CaMK cascade in mediating DC survival.

Methods

Mice and DCs

Mice were housed and maintained in the Levine Science Research Center Animal Facility located at Duke University under a 12-hour light, 12-hour dark cycle. Food and water were provided ad libitum, and all care was given in compliance within National Institutes of Health (NIH) and institutional guidelines on the use of laboratory and experimental animals under an approved Duke Institutional Animal Care and Use Committee protocol.

Camk4$^{-/-}$ mice were generated as previously described.[27] The *BCL-2* transgenic mice (a kind gift from Dr Tannishtha Reya, Duke University) have been previously described.[28]

BCL-2$^{tg/tg}$/*Camk4*$^{-/-}$ mice were generated by crossing *BCL-2*$^{tg/tg}$ with *Camk4*$^{+/-}$ mice to generate *BCL-2*$^{tg/tg}$/*Camk4*$^{+/-}$ hybrids. These hybrids were crossed to generate the *BCL-2*$^{tg/tg}$/*Camk4*$^{-/-}$ mice used in our experiments. All mice were screened by PCR to confirm the presence of the *BCL-2* transgene and the absence of the *Camk4* gene.

Mouse DCs were isolated from spleen, thymus, and lymph nodes of 4- to 8-week-old mice. $CD11c^+$ cells were positively selected using an anti-CD11 antibody (Miltenyi Biotech, Calderara di Reno, Italy). The purity of DC determined by flow cytometry was 80%-92%.

Human DCs were generated from $CD14^+$ monocytes isolated from peripheral blood of healthy donors (Miltenyi Biotech) cultured for 5 days in RPMI 1640 (Invitrogen, Carlsbad, CA), 10% fetal calf serum, 50 ng/mL granulocyte macrophage colony stimulating factor (GM-CSF; Schering-Plough, Kenilworth, NJ), and 250 ng/mL interleukin-4 (PeproTech, Rocky Hill, NJ). Phenotype was evaluated by cytometry. LPS was from Sigma (St Louis, MO).

Measurement of viability

The percentage of apoptotic cells was quantified using annexin V fluorescein isothiocyanate (FITC) kits (Bender MedSystem, Vienna, Austria) according to the manufacturer's instructions. Viable cells were evaluated by the exclusion of Trypan blue using a kit from Invitrogen.

Protein and RNA analyses

Immunoblots were performed as described.[29] Calpain inhibitors ALLM and ALLN were obtained from Calbiochem (San Diego, CA). Primary antibodies were: anti-CaMKII (Santa Cruz Biotech, Santa Cruz, CA), anti-CaMKIV (BD, San Jose, CA and Acris, Hidden Hausen, Germany), anti-actin (Sigma), anti-pCREB (phosphorylated form of the cAMP response element-binding protein), anti-pAkt, anti-Bcl-2 family proteins (Cell Signaling, Danvers, MA), anti-human Bcl-2 (BD). Binding was detected by horseradish peroxidase-conjugated secondary antibody and chemiluminescence (Amersham Pharmacia Biotech, Chalfont, United Kingdom). NIH Scion Image software version 1.61 (Bethesda, MD) was used to quantify bands.

RNA was isolated by using Trizol kits (Invitrogen), and first strand cDNA prepared by using SuperScript III (Invitrogen), according to the manufacturer's directions. PCR-based gene expression analysis was performed as reported elsewhere.[27] The sequences of all the primers used in this study are available on request.

Immunocytochemistry

$CD14^+$ monocytes were resuspended at 10^6 cells/mL in regular medium supplemented with IL-4 (1000 IU/mL, Immunotools, Friesoythe, Germany) and GM-CSF (50 ng/mL, Schering-Plough) and adhered to microscope slides coated with 0.05 mg/mL of poly-L-lysine in 24-well plates. DCs were fixed and permeabilized with the Cytofix/Cytoperm reagent (Becton Dickinson, Milan, Italy) according to the manufacturer's instruction and left in 3% bovine serum albumin solution in phosphate-buffered saline for 30 minutes at room temperature. DCs were then incubated with a rabbit polyclonal antibody to CaMKIV (0.5 μg/mL Acris Antibodies), stained with Alexa Fluor 594 goat anti-rabbit IgG (0.5 μg/mL Molecular Probes, Eugene, OR) and counterstained with Hoechst 33342 (Vector). Images were acquired with a DMIRE2 inverted confocal microscope (Leica Microsystems, Wetzlar, Germany) using a 40× lens at 40×/1.25 NA oil objective and processed using LCS software version 2.61 (Leica Microsystems). Internal photon multiplier tubes collected images in 8-bit, unsigned images at a 400-Hz scan speed. Hoechst 33342 fluorescence (Invitrogen) was excited with a mode-locked titanium-sapphire laser (Chameleon; Coherent, Santa Clara, CA; excitation wavelength: 740 nm, emission range: 410-470 nm). Two-photon intensity input was regulated with an amplitude modulator linked to the Leica Software System. Alexa Fluor 594 (Invitrogen) was excited by a helium-neon laser line (excitation wavelength: 543 nm, emission range: 600-700 nm). Line profiles of acquired images were performed with LCS 2.61 image analysis software (Leica Microsystems).

Flow cytometry

Antibodies used for human DC analysis: FITC-anti-CD14, phycoerythrin (PE)-anti-CD86, PE-CD1a, FITC-anti-CD83. Mouse DC staining were performed with: FITC-anti-I-A, PE-anti-CD8α, APC-anti-CD11c, FITC-anti-CD86, PE-anti-tumor necrosis factor (TNF), FITC-anti-IL-6. All of these antibodies were purchased from BD Pharmingen.

Lentiviral infection

The lentiviral constructs were generated and characterized by Kitsos et al.[26] Briefly, CaMKIV-WT and CaMKIV-K71M cDNA were cloned into Lenti-IRES-GFP vectors, and high titer control and recombinant viruses were prepared by pseudo-typing with VSV.G using a quadruple transfection protocol in 293T cells according to Follenzi et al.[30] Approximately 5×10^6 of immature monocyte-derived DCs were infected with the appropriate lentivirus at a multiplicity of infection of 5.2 days after infections GFP^+ cells were sorted by flow cytometry and cultured for an additional 18 hours in the presence of LPS (1 μg/mL), or left in regular medium.

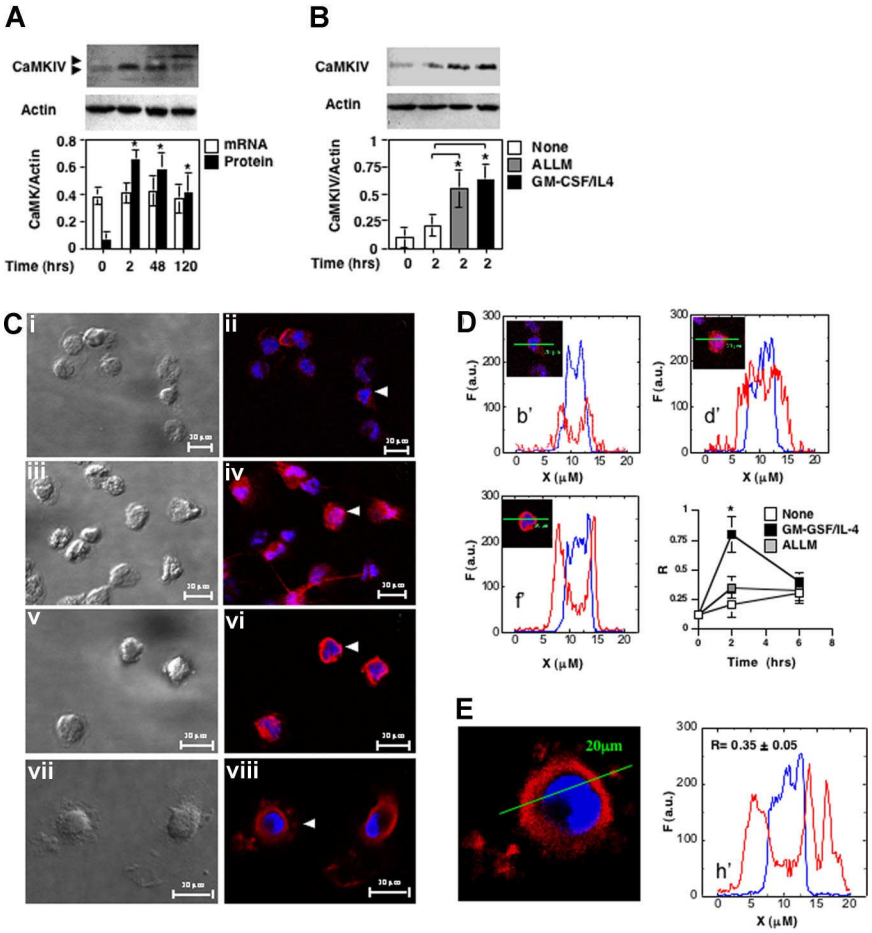

Figure 1. CaMKIV accumulates during differentiation of monocyte-derived dendritic cells. (A) $CD14^+$ mononuclear cells were cultured in the presence of GM-CSF and IL-4. Whole-cell lysates were prepared at the indicated times and analyzed by immunoblot with specific antibodies (CaMKIV and actin). Aliquots of cells were used to measure CaMKIV and actin mRNA levels by quantitative reverse transcription–polymerase chain reaction. Bottom panel shows mean (± SD) of optical density measurements expressed as the ratio between the CaMKs and actin bands (n = 4). $*P < .01$. (B) Calpain regulates CaMKIV accumulation in differentiating monocytes. $CD14^+$ mononuclear cells were cultured in the presence of ALLM, a selective calpain inhibitor (ALLM), or GM-CSF and IL-4 and analyzed for CaMKIV expression by immunoblot (top). The bottom panel shows mean (± SD) of the optical density measurements expressed as the ratio between the CaMKIV and actin bands (n = 4). $*P < .01$. (C-E) Intracellular distribution of CaMKIV in differentiating monocytes. (C) Transmission and confocal fluorescent immunocytochemistry images of CaMKIV expression in monocytes cultured for: 2 hours in regular medium (i,ii); 2 hours in the presence of GM-CSF/IL-4 or ALLM (iii, iv, v, and vi, respectively); and 120 hours in the presence of GM-CSF/IL-4 (vii,viii). (D) Line profiles of cells indicated by white arrows in the corresponding subpanels in panel C. The line segment is 20 μM; F indicates the fluorescence intensity in arbitrary units (a.u.). The bottom right graph shows the ratio (R) between the mean fluorescence intensity of Alexa Fluor 594 (CaMKIV) and Hoechst 33342 (nuclear staining) in the nuclear region along different line profiles. Means (± SD) represent 20 independent line profiles. $*P < .01$. (E) Expression and line profile analysis of CaMKIV in monocytes treated for 120 hours with GM-CSF/IL-4.

Results

CaMKIV accumulates during differentiation of human monocyte–derived DCs

$CD14^+$ cells were cultured in the presence of optimal amounts of GM-CSF and IL-4 and at different time points aliquots of cells were lysate to measure CaMKIV accumulation. Immunoblots showed a barely detectable amount of CaMKIV in freshly isolated monocytes (Figure 1A). However, within 2 hours after cytokine exposure CaMKIV was robustly up-regulated and remained so after 48 hours. After 120 hours of stimulus, cells have acquired the phenotype typical of immature DCs ($CD14^-$ $CD1a^+$ $CD86^+$ $CD83^-$; data not shown) and still expressed CaMKIV. No significant modulation in the amount of CaMKI occurred during the monocyte differentiation process (data not shown). Parallel analysis showed that CaMKIV mRNA remained stable during the differentiation process. Based on these findings, we reasoned CaMKIV expression likely to be largely regulated by a posttranscriptional mechanism.

Pharmacologic inhibition of calpain activity leads to the rapid accumulation of CaMKIV

Previous studies have suggested that accumulation of CaMKIV in neuronal cells is regulated by a Ca^{2+}-sensitive protease, calpain.[31] Thus, we evaluated CaMKIV expression in fresh isolated monocytes and in monocytes cultured for 2 hours in regular medium, with or without ALLM, a cell-permeable calpain inhibitor, or the cytokine cocktail composed of GM-CSF and IL-4. The immunoblot in Figure 1B reveals that the exposure to ALLM or GM-CSF/IL-4 resulted in a statistically significant and comparable accumulation of CaMKIV. This contention was confirmed using an additional calpain inhibitor ALLN (data not shown). Because the anti-CaMKIV antibody used recognizes the entire p55 molecule,[31] the barely detectable amount of p55 observed in untreated monocytes as well as the ability of calpain to increase its expression led us to hypothesize that a protease-dependent mechanism was likely to play a role in the control of CaMKIV accumulation in myeloid cells.

Confocal analysis of CaMKIV expression

To analyze the intracellular distribution of CaMKIV in differentiating monocytes, we used 2-photon confocal microscopy (Figure 1C-E). The image analysis confirmed a low level of CaMKIV in untreated monocytes and revealed that this kinase was primarily localized in close proximity to the nuclear membrane (Figure 1Ci,ii). Exposure to the cytokine cocktail or to ALLM induced a rapid increase in CaMKIV (Figure 1Ciii-vi). However, whereas inhibition of calpain activity did not stimulate nuclear accumulation of this kinase, a large amount of CaMKIV is detected in nuclei of monocytes exposed to GM-CSF/IL-4 for 2 hours. (Figure 1Ciii,iv). Finally, in monocytes treated with cytokines for 120 hours, conditions that generate a phenotype typical of DC, CaMKIV is detected predominantly in the perinuclear region as well as in spotted zones in proximity to plasma membranes (Figure 1Cvii,viii).

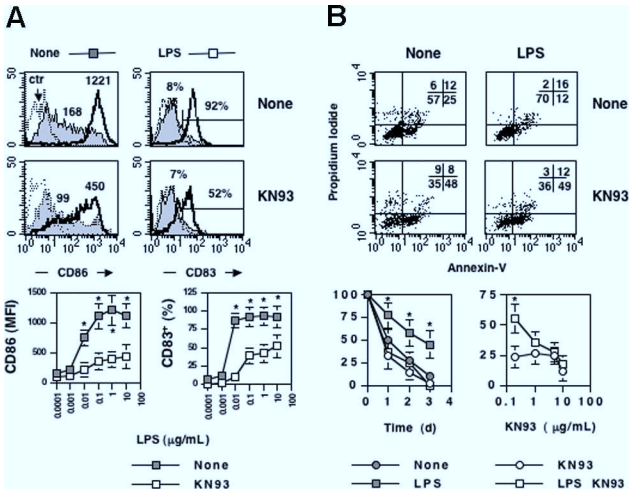

Figure 2. CaMKs regulate terminal differentiation and survival of monocyte-derived dendritic cells. Immature monocyte–derived DCs were cultured untreated or stimulated with LPS- (1 μg/μL) in the presence or absence of KN93 (10 μM), a selective inhibitor of the multifunctional CaMKs. After 24 hours, cells were recovered and double-stained with anti-CD86/anti-CD83 antibodies or with Annexin V/propidium iodide (A,B top). (A) Bottom: effects of KN93 on CD83 and CD86 expression as a function of the LPS dose. Mean (± SD) represents 6 independent experiments. (B) Bottom: effects of KN93 on survival of LPS-stimulated DC (LPS, 10 μg/mL) as a function of time or KN93 dose (left or right, respectively). Viability was calculated by trypan blue exclusion. Mean (± SD) represents 6 independent experiments.*$P < .01$. Values in panel A represent the mean fluorescente intensity of CD86 and the percentage of CD83 positive cells. Ctr refers to profiles of unstained cells. Values in panel B represent the percentage of cells in each quadrant.

A quantitative 20-μm line profile analysis of the acquired images confirmed a very low nuclear/perinuclear ratio of CaMKIV in unstimulated monocytes (Figure 1D, b′). This ratio is similar to that observed in freshly isolated cells and did not increase on culture in the absence of differentiating stimuli or in the presence of calpain inhibitors (Figure 1D, c′ and d′). Kinetic analysis showed that at a later time point (6 hours) the nuclear accumulation of CaMKIV in cytokine-treated cells decreases and CaMKIV returns to be localized mainly in the perinuclear region (Figure 1D bottom right). The quantitative analysis of DC images confirmed that, at this stage of differentiation, CaMKIV is located predominantly outside the nucleus (Figure 1E).

CaMKs regulate differentiation and survival of monocyte-derived DCs

To examine potential roles of the multifunctional CaMKs in the activation process of DCs, we tested the ability of KN93, a selective inhibitor of the multifunctional CaMKs (CaMKI, CaMKII, CaMKIV), to alter terminal differentiation and/or survival of DCs exposed to LPS. As shown in Figure 2A, KN-93 interfered with up-regulation of CD83 and CD86 induced by LPS (Figure 2A). To analyze the effect of KN93 on survival, we exposed DCs to increasing concentrations of the kinase inhibitor and double-stained cells at different time points with annexin-V and propidium iodide. Finally, we quantified the number of double-negative viable cells by flow cytometry or by using a trypan blue-exclusion assay (Figure 2B top and bottom, respectively). The exposure of DCs to LPS normally increases their lifespan: 50% of DCs treated with LPS were still viable after 2 days of culture compared with 25% of cells left in regular medium alone (Figure 2B). Probably because of its inhibitory effect on all 3 multifunctional CaMKs (I, II, and IV), high doses of KN93 (> 5 μM) also led to a decrease in the survival of unstimulated DCs (Figure 2B top and bottom left). However, at lower doses this drug exerted its effect preferentially on the LPS-stimulated DCs by preventing the prosurvival ability of the bacterial endotoxin with barely detectable effects on the viability of unstimulated DCs (Figure 2B bottom right). Of note KN92, a KN93 derivative that is 10-fold less potent that KN93 as a CaMK inhibitor, had no effect on differentiation and survival of DC at a concentration equivalent to the effective dose of KN93 (data not shown). These results suggest the importance of multifunctional CaM kinases in LPS-mediated DC survival.

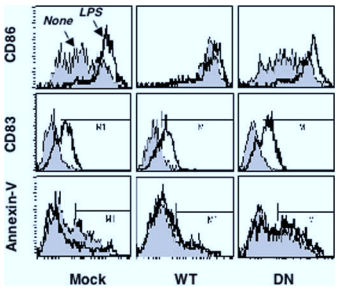

Figure 3. CaMKIV regulates survival of monocyte–derived DCs. Monocyte-derived DCs were infected with Lenti-IRES-GFP lentivirus expressing *Camk4*, *Camk4*-WT, or *Camk4*-K71M (Mock, WT, and DN, respectively). After 48 hours, cells were cultured for an additional 18 hours in the presence of LPS (1 μg/mL) or left untreated. Top: fluorescence-activated cell sorting (FACS) profiles of DC stained with CD86, CD83, and Annexin-V.

The ectopic expression of kinase-inactive CaMKIV decreases the viability of LPS-stimulated DCs

Our experiments using KN93 indicated that CaMKs play an important role in the activation programs triggered by TLR4 stimulation. However, because of the ability of KN93 to equivalently inhibit CaMKI, CaMKII, and CaMKIV, it is impossible to identify the relevant multifunctional CaMK family members. To directly investigate a role for CaMKIV in DC activation, we infected human immature monocyte–derived DCs with lentiviral vectors encoding wild-type or kinase-inactive *Camk4* (Lenti-IRES-GFP CaMKIV-WT and CaMKIV-K71M, respectively). Aliquots of DC were also infected with the control virus (Lenti-IRES-GFP). After 2 days, GFP^+ cells were sorted, washed, and cultured for additional 24 hours in the presence or absence of LPSs (1 μg/mL), before being analyzed by flow cytometry (Figure 3; Table 1). Although DCs infected with CaMKIV-K71M or control viruses (DN and Mock, respectively) left in regular medium expressed comparable amounts of CD86, infection of the cells with the CaMKIV-WT virus (WT) induced a significant up-regulation of this costimulatory molecule. However, neither CaMKIV-WT, nor

Table 1. Effects of CaMKIV on DC activation markers

	Mock	WT	DN
Regular medium			
CD86 (MFI)	350 ± 125	950 ± 320*	270 ± 160
CD83, %	10 ± 5	8± 2	7 ± 3
A-V, %	55 ± 14	28± 17*	63 ± 22
Viability, %	35 ± 12	67± 16*	32 ± 18
LPS			
CD86 (MFI)	1140 ± 250	1250 ± 305	1070 ± 360
CD83, %	52 ± 13	48± 20	51 ± 18
A-V, %	28 ± 10	23± 15	65 ± 19*
Viability, %	65 ± 11	62± 15	30 ± 16*

Means are plus or minus SD. MFI indicates mean fluorescence intensity; and A-V, annexin-V.

* indicates statistical significance (n = 3); $P < .01$.

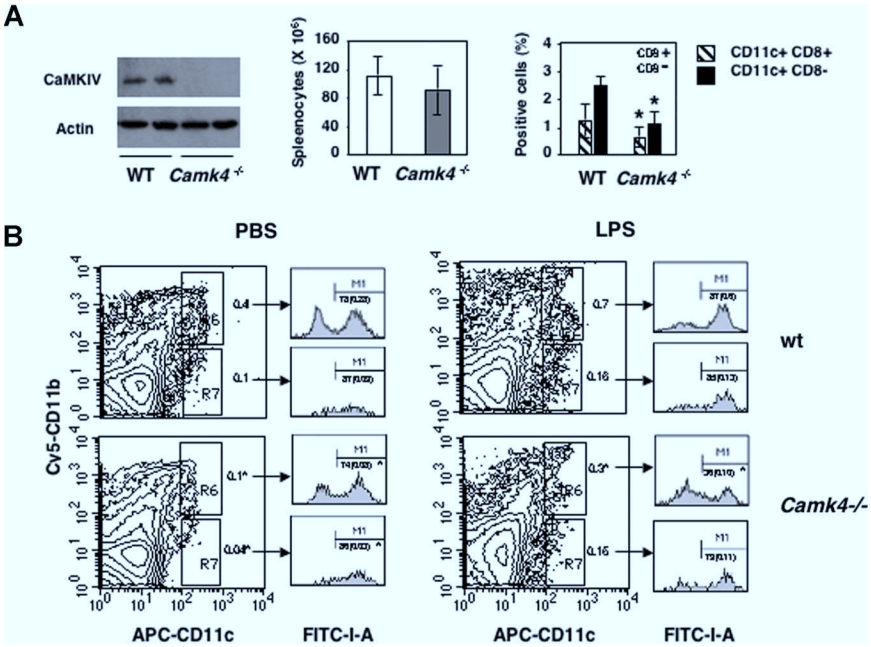

Figure 4. The number of splenic mature DCs is reduced in *Camk4*$^{-/-}$ mice. Splenocytes from normal or *Camk4*$^{-/-}$ mice were counted and stained for CD11c and CD8. (A) Left panel: immunoblots show CaMKIV and actin expression in splenocytes isolated from 2 mice from each genotype. Middle: bar graph reports mean (± SD) of the total number of splenocytes (n = 15 mice per genotype). Right: percentage of WT and *Camk4*$^{-/-}$ CD11c + subsets. Bars graphs show mean (± SD) representing 15 mice per genotype. **P* < .01. (B) LPS-induced mature DC accumulation is impaired in *Camk4*$^{-/-}$ mice in vivo. LPS or phosphate-buffered saline (PBS) was injected into *Camk4*$^{-/-}$ and control WT mice. Eighteen hours later, splenocytes were isolated and triple-stained with anti-CD11b, -CD11c and -I-A antibodies. For the typical dot plot profiles, the inset values show the percentage of cells in the R6/R7 gates (CD11b^{high}/CD11c^{high} and CD11b^{low}/CD11c^{high}, respectively). FACS profile histograms show I-A expression. Inset values refer to the percentage of I-A^{high} cells in the R6/R7 gates. Values in parenthesis display the percentage of CD11b^{high}/CD11c^{high}/I-A^{high} and CD11b^{low}/CD11c^{high}/I-A^{high} in whole splenocytes. **P* < .01.

CaMKIV-K71M, nor control virus interfered with LPS-induced increases in the surface level of CD86 or CD83 (Figure 3).

The effect of ectopic CaMKIV expression on DC survival was evaluated at 24 hours by both annexin-V staining and the trypan blue-exclusion assay. As shown in Figure 3, in mock-infected DC, LPS treatment led to a significant decrease in the percentage of annexin-V-positive cells and induced a parallel increase in the percentage of viable cells (trypan blue unstained cells), compared with DCs cultured in regular medium. Overexpression of CaMKIV-WT in DCs induced a detectable antiapoptotic effect (Figure 3). Contrariwise, CaMKIV-K71M did not affect viability of untreated DCs but abrogated the antiapoptotic effect induced by LPS, suggesting that the kinase-inactive protein might play a dominant/negative role in this instance. These results clearly implicate CaMKIV in survival of human monocyte–derived DC and suggest that the absence of CaMKIV in mice should negatively impact the number of DC cells.

Camk4$^{-/-}$ mice contain a decreased number of DC

To evaluate the hypothesis, we analyzed spleen-derived DCs in WT and *Camk4*$^{-/-}$ mice. Splenocytes from *Camk4*$^{-/-}$ and WT mice were isolated, counted, and stained with anti-CD11c and CD8α antibodies. Immunoblots were performed to measure CaMKIV expression (Figure 4A left). *Camk4*$^{-/-}$ and WT mice contained comparable numbers of splenocytes (Figure 4A middle), but the former genotype showed a significant decrease in the percentage of both CD11c^{+} CD8α^{+} and CD8α^{-} subsets (Figure 4A right). Similar results were found on analysis of CD11c^{+} cells present in the lymph nodes of WT versus CaMKIV null mice (data not shown).

The injection of LPSs in WT resulted in a significant increase in the percentage of cells with a phenotype typical of mature myeloid DC: CD11c^{high}/CD11b^{high}/I-A^{high} (0.28 ± 0.05 vs 0.65 ± 0.07, untreated vs LPS-treated; Figure 4B right). Otherwise, this treatment did not induce similar changes in *Camk4*$^{-/-}$: the CD11c^{high}/CD11b^{high}/I-A^{high} population failed to accumulate in response to LPSs and only 37% of the CD11c^{high}/CD11b^{high} subset, compared with the 87% detected in WT mice, expressed high levels of I-A molecules. Therefore, genetic ablation of CaMKIV led to a marked defect in the accumulation of cells showing typical markers of mature myeloid DC in response to LPSs.

The CD11c^{high}/CD11b^{low} population contains a mixture of DCs at different stages of differentiation, including DC precursors (DCp), immature DC (iDC), and plasmacytoid DC (pDC), which display different abilities to replicate and differentiate in basal condition as well as in response to LPSs.[32] Our data show a significant decrease in the percentage of CD11c^{high}/CD11b^{low} cells in untreated *Camk4*$^{-/-}$ mice (0.1% vs 0.04%, WT and *Camk4*$^{-/-}$, respectively). However, although LPSs did not induce significant changes in the percentage of CD11c^{high}/CD11b^{low} cells, this did occur in *Camk4*$^{-/-}$ mice (Figure 4B). Thus, in WT, the CD11c^{high}/CD11b^{low} population seems to be made up predominantly of LPS-unresponsive DC subsets (ie, pDC). However, in *Camk4*$^{-/-}$ mice, the CD11c^{high}/CD11b^{low} cells appear to be mainly derived from LPS-responsive DC subsets (ie, DCp, iDC). These data suggest that CaMKIV may be involved in either the developmental program of other DC subsets (ie, pDC) or in the control of the proliferative capacity of DC precursors.

Genetic ablation of CaMKIV does not prevent the ability of DC to differentiate and secrete cytokines in response to LPSs

The involvement of CaMKIV in LPS signaling was evaluated in vitro using purified DCs. To this end, CD11c^{+} cells were positively selected from splenocytes of *Camk4*$^{-/-}$ and WT mice before being cultured in the presence or absence of LPSs (10 μg/mL). After 24 hours, we measured the cell surface expression of I-A and CD86 by flow cytometry as well as the intracellular levels of TNF-α and IL-6 by immunocytochemistry (Figure 5A,B, respectively; Table 2). LPS treatment induced a comparable increase of I-A and CD86 expression in WT and *Camk4*$^{-/-}$ DC (Figure 5A). Moreover, cells from both genotypes accumulated comparable levels of IL-6 and TNF-α in response to LPSs (Figure 5B). Therefore, we conclude that CaMKIV is largely dispensable for this branch of the LPS signaling pathway.

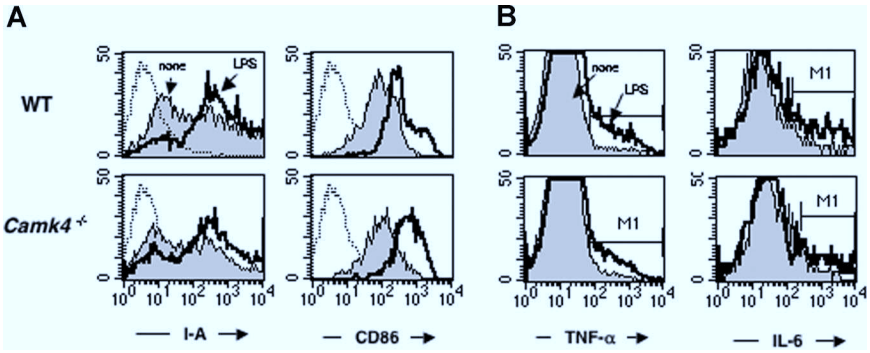

Figure 5. CaMKIV is not required for terminal differentiation and cytokine synthesis induced by LPS. Isolated CD11c$^+$ splenic DCs from WT and *Camk4*$^{-/-}$ were exposed to LPS (10 μg/mL) or left untreated (none). After 16 hours, cells were recovered and double-stained for I-A and CD86 (A). Aliquots of cells were stained for the presence of intracellular TNF-α and IL-6 (A,B).

DCs from CaMKIV null mice fail to increase CREB phosphorylation in response to LPSs

To investigate the role of CaMKIV in the early events induced by LPS signaling, we compared the levels of pCREB and pAKT in DCs isolated from WT and *Camk4*$^{-/-}$ mice cultured for 1 hour in the presence or absence of the bacterial endotoxin. Although a comparable up-regulation in the levels of pAKT was observed in DCs isolated from both genotypes, the ablation of CaMKIV prevented the increase in pCREB in response to LPSs (Figure 6; Table 3). This finding suggests a role for CaMKIV in the CREB-dependent pathway by which TLR4 regulates DC survival.

CaMKIV regulates survival of DC

To evaluate whether CaMKIV plays a direct role in regulating the survival of DCs, we isolated CD11c$^+$ from *Camk4*$^{-/-}$ and WT mice and measured their ability to survive in vitro in the absence or presence of LPSs. At different time points, cell viability was tested by trypan blue exclusion (Figure 7A). The number of viable DCs remaining in the culture in the absence of treatment decreased progressively as a function of days in culture and the time course was similar in WT and *Camk4*$^{-/-}$ cells (Figure 6). On the other hand, whereas LPS clearly increased viability of WT cells, it failed to alter the lifespan of *Camk4*$^{-/-}$ DC (Figure 7A).

To begin to evaluate the mechanism by which CaMKIV might participate in LPS-initiated signaling, we quantified the expression of Bcl-2 family proteins. CD11c$^+$ cells were isolated by positive selection from spleens of WT and *Camk4*$^{-/-}$ mice. Freshly isolated WT and *Camk4*$^{-/-}$ DCs expressed comparable amounts of Bcl-2 but undetectable levels of Bcl-xL (Figure 7B). Although the amount of Bcl-2 decreased similarly in cells of both genotypes cultured for 24 hours, LPS prevented the decrease in WT but not *Camk4*$^{-/-}$ DCs. In addition, LPS induced accumulation of Bcl-xL in WT cells, and this effect was markedly decreased in *Camk4*$^{-/-}$ DCs (Figure 7B).

Transgenic expression of Bcl-2 reverses the ability of CaMKIV-null DC to survive

To analyze the contribution of the decreased amount of Bcl-2 to survival, we generated *BCL-2*$^{tg/tg}$/Camk4$^{-/-}$ mice by crossing BCL-2$^{tg/tg}$ mice with *Camk4*$^{-/-}$ mice to generate *BCL-2*$^{tg/tg}$/Camk4$^{-/-}$ hybrid mice that overexpress Bcl-2 in a CaMKIV-null background. The immunoblot in Figure 7C shows a typical result obtained in mice carrying the 4 different genotypes. CD11c$^+$ cells were recovered by positive selection from spleens of *BCL-2*$^{tg/tg}$ and *BCL-2*$^{tg/tg}$/Camk4$^{-/-}$ mice, cultured in the presence or absence of LPSs and analyzed for viability as described previously (Figure 7D). DCs from *BCL-2*$^{tg/tg}$ and *BCL-2*$^{tg/tg}$/Camk4$^{-/-}$ mice cultured in regular medium show a comparable and prolonged lifespan. Furthermore, regardless of genotypes, the presence of LPS in the culture medium did not result in a significant increase in the number of viable cells (Figure 7D).

The immunoblot in Figure 7E shows the typical expression of total Bcl-2 and Bcl-XL in these transgenic mouse strains. Regardless of CaMKIV expression but correlated with the presence of the human Bcl-2 transgene, mice carrying the hybrid genotypes show a high level of total Bcl-2 protein that was barely altered by LPS treatment (Figure 7E). Because most of the effect exerted by the LPS-CaMKIV pathway on Bcl-2 was at the transcriptional level (data not shown), we reasoned that the ectopic promoter of the *BCL-2* transgene would require a different set of transcription factors compared with the endogenous mouse gene and, in turn, be less dependent on the presence of CaMKIV. On the other hand, CaMKIV was still required in *BCL-2*$^{tg/tg}$ hybrid mice to link the LPS–mediated pathway with

Table 2. Effects of LPS on *CamK4*$^{-/-}$ DC (n = 6)

Markers	*wt*	*Camk4*$^{-/-}$	*P*
Regular medium			
I-A (MFI)	658 ± 107	538 ± 97	.09
CD86 (MFI)	150 ± 57	128 ± 50	.54
TNF-α , %	2.3 ± 0.5	2.6 ± 0.6	.46
IL-6, %	2.6 ± 0.5	3.0 ± 0.4	.18
LPS			
I-A (MFI)	1241 ± 289	938 ± 190	.08
CD86 (MFI)	657 ± 159	830 ± 127	.08
TNF-α , %	11 ± 3	15 ± 3	.49
IL-6, %	13 ± 3	15 ± 6	.48

Means are plus or minus SD (n = 6).

I-A indicates major histocompatibility complex class II molecules; and IL-6, interleukin-6.

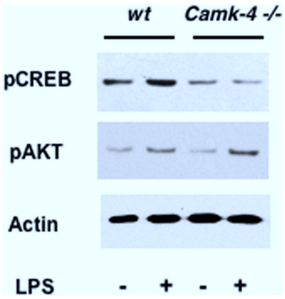

Figure 6. CaMKIV is required to link TLR4 signaling with pCREB accumulation. CD11c + DCs were isolated from spleens of WT or *Camk4*$^{-/-}$ mice and cultured in the presence or absence of LPS (10 μg/mL) for 1 hour. Whole lysates were separated by SDS-PAGE and immunoblotted with the reported antibodies (pCREB, pAKT, actin, LPS). A typical immunoblot analysis is shown.

Table 3. TLR4 signaling in *CamK4*$^{-/-}$ DC

	wt	*Camk4*$^{-/-}$
Regular medium		
pCREB	0.32 ± 0.1	0.19 ± 0.05*
pAKT	0.09 ± 0.04	0.07 ± 0.06
LPS		
pCREB	0.51 ± 0.12	0.15 ± 0.03*
pAKT	0.17 ± 0.05	0.23 ±0.06

Means are plus or minus SD of the optical density measurements expressed as the ratio between pCREB or pAKT.

* indicates statistical significance (n = 3); $P < .01$.

Bcl-xL expression (Figure 7D). This finding provides an additional evidence for a role for CaMKIV in the pathway responsible for Bcl-xL expression and also documents the dominant role played by Bcl-2 in modulating the lifespan of LPS-activated DC in a manner that involves CaMKIV signaling.

Discussion

Stimulation of TLR4 has been associated with the initiation of both apoptotic and antiapoptotic pathways, the balance of which determines the outcome of innate and adaptive immune responses.[4-6] Here we describe a novel CaMK cascade-dependent antiapoptotic pathway responsible for the survival of LPS-activated DCs. The results obtained, using pharmacologic inhibition of CaMKs, ectopic expression of CaMKIV, and a CaMKIV kinase-inactive mutant as well as mice null for CaMKIV, demonstrate that a CaMKIV signaling cascade controls the phosphorylation of CREB and accumulation of Bcl-2 necessary to support the antiapoptotic branch of the TLR4 pathway.

The multifunctional CaMK family proteins are involved in the control of differentiation and survival of several cell types, including neurons and hematopoietic stem cells.[26,27] Analysis of mouse embryos

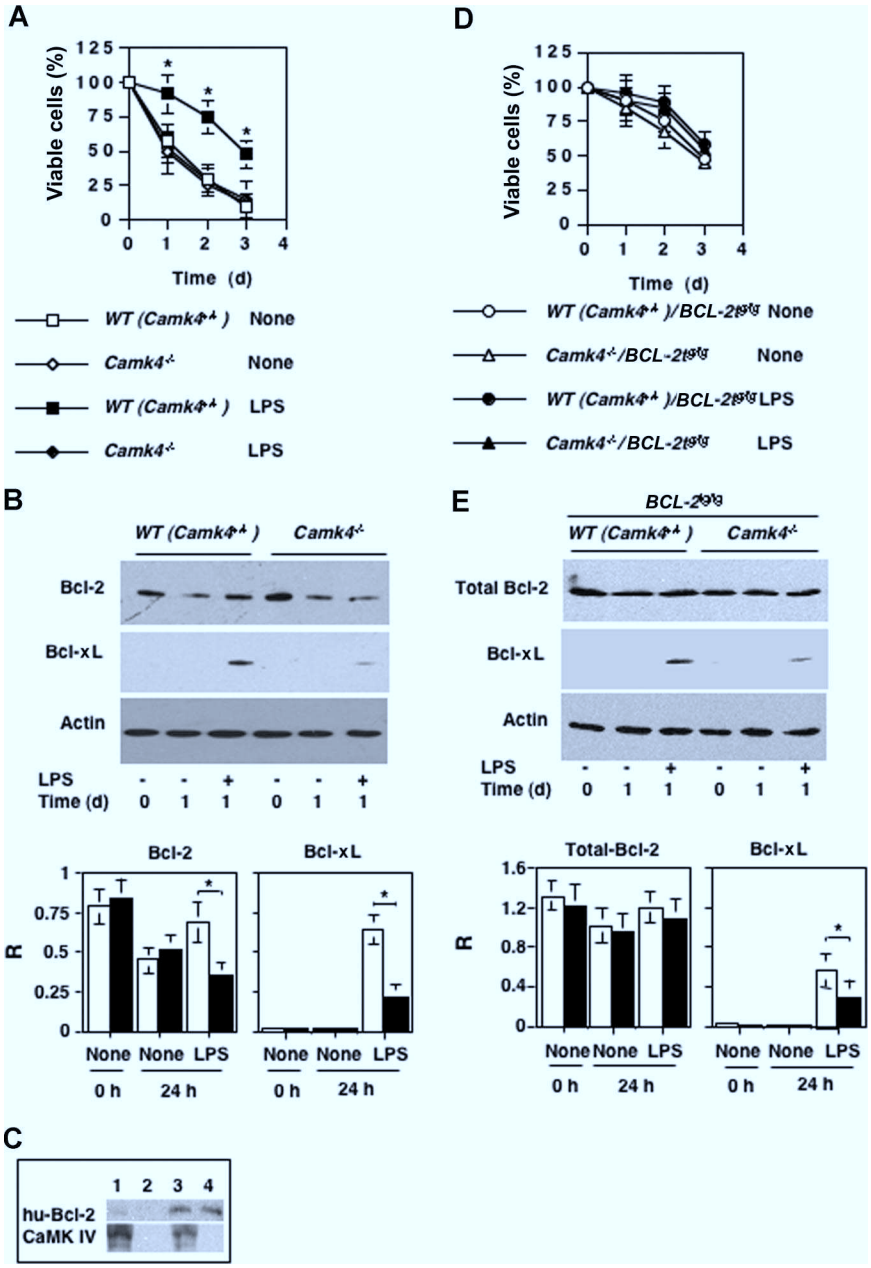

Figure 7. CaMKIV regulates lifespan and Bcl-2 family protein accumulation. CD11c + DCs were positively selected from WT (*Camk4*$^{+/+}$), *Camk4*$^{-/-}$, *BCL-2*$^{tg/tg}$ transgenic, and *Camk4*$^{-/-}$/*BCL-2*$^{tg/tg}$ hybrid mice cultured in the presence or absence of LPS (10 μg/mL). (A,D) Viability was assayed by trypan blue exclusion at daily intervals. The results represent mean and SD of 6 independent experiments. (B,E) Typical results obtained by immunoblot analysis. Bar graphs show mean (± SD) of the optical density measurements expressed as the ratio between Bcl-2 or Bcl-xL and actin bands (n = 6). (C) Immunoblot shows the typical expression of CaMKIV and hu-Bcl-2 detected in WT (*Camk4*$^{+/+}$), *Camk4*$^{-/-}$, *BCL-2*$^{tg/tg}$ transgenic, and *Camk4*$^{-/-}$/*BCL-2*$^{tg/tg}$ hybrid mice (lanes 1, 2, 3, and 4, respectively). *$P < .01$.

revealed expression of CaMKIV mRNA in the developing nervous system as well as in the hematopoietic-related tissues.[25,26] These developmental patterns coincide temporally with periods of significant cellular differentiation in the nervous system, axonal migration and neuron survival. Correlation of CaMKIV expression with differentiation is also evident in adult animals as *Camk4*$^{-/-}$ mice show major defects in maintenance of hematopoietic stem cells, postnatal maturation of Purkinje cells, thymopoiesis, ovulation, and terminal differentiation of spermatozoa.[26,33-39] Here we show that CaMKIV expression is tightly regulated during the developmental program of human monocyte–derived DCs, a well-characterized model of myeloid cell differentiation, and is also expressed in murine mature DCs isolated from secondary lymphoid tissues.

Extensive gene expression analyses performed using microarray or SAGE technologies failed to identify *Camk4* among the mRNAs that were altered during the monocyte-derived DC differentiation process.[40-42] In agreement with these findings, we show comparable *Camk4* mRNA levels in monocyte and monocyte-derived DCs. However, we provide evidence for a cytokine-dependent, rapid accumulation of CaMKIV in differentiating DCs that is overcome by a calpain-dependent mechanism that keeps CaMKIV levels low in the absence of stimulation. Calpain is a cysteine protease activated by an increase in intracellular Ca^{2+}[43,44] that influences normal signal transduction pathways by cleaving cytoskeletal proteins, membrane proteins, and enzymes normally involved in cell survival.[45] The susceptibility of CaMKIV to calpain has been documented in cerebellar granule cell neurons.[31] More recently, a role for calpain has been proposed in the mechanism regulating podosome turnover and composition in murine DCs.[46] Here, we show that inhibition of calpain activity leads to accumulation of CaMKIV in the perinuclear region of monocytes cultured in regular medium. However, our data also reveal that stabilization of CaMKIV by calpain inhibition is not sufficient to promote the nuclear translocation of CaMKIV that occurs in response to GM-CSF and IL-4. Our findings provide novel evidence to suggest that differentiating cytokines may inhibit the degradation of CaMKIV and stimulate the entry of this enzyme into the nucleus where it participates in the regulation of genes, such as Bcl-2, that are necessary to support the survival of DCs.

Recently, it has been reported that the selective inhibition of another multifunctional CaMK, CaMKII, interferes with terminal differentiation of monocyte-derived DCs by preventing up-regulation of costimulatory and MHC II molecules as well as secretion of cytokines induced by TLR4 agonists.[47] The findings described in the present study indicate that CaMKIV selectively regulates survival of stimulated DCs without interfering with their differentiation. Thus, in DC, as in neuronal cells, the coordinated activation of CaMKII and CaMKIV seems to be required to orchestrate the differentiation and survival programs.[33]

Isolated DCs are prone to apoptosis that can be modulated by a variety of bioactive molecules, including cytokines, CD40 agonists, and TLR ligands, which share the ability to regulate the levels of Bcl-2 family proteins.[8] TLR agonists seem to promote DC survival mainly by controlling the timing of the accumulation of Bcl-2 family proteins,[8] leading to the idea that Bcl-2 acts as a "molecular timer" to set the lifespan of DCs and the magnitude of the adaptive immune response.[8] The crucial role of Bcl-2 in the regulation of DC lifespan has been confirmed in vivo using transgenic mice expressing the human *BCL-2* gene under the control of the murine CD11c promoter as well as by testing the ability of Bcl-2 null DCs to survive.[8,46] In agreement with these findings, we show here that the number of viable $CD11c^+$ cells progressively decreases during culture, a phenomenon that is associated with a parallel decline in the level of Bcl-2. Stimulation of TLR4 triggers accumulation of proapoptotic Bcl-2 family proteins and induces the progressive temporal decline in the level of Bcl-2. The timing of these events sets the lifespan of DCs and our results support this contention. That is, freshly isolated DCs (time 0) contain higher amounts of Bcl-2 relative to DC cultured for 24 hours in the presence of LPS. However, a different conclusion can be drawn when taking into account the spontaneous loss of Bcl-2 expression observed in DCs cultured in absence of any stimuli coupled with the net accumulation of Bcl-2 that occurs in cells cultured with LPS for 24 hours. Therefore, while isolated DCs activate the "Bcl-2 molecular timer" and undergo spontaneous apoptosis, signals transduced by TLR4 modify the loss of Bcl-2 and thus prolong the lifespan of activated DCs.

Our results reveal that genetic ablation of CaMKIV results in a decrease in the number of mature DC present in lymphoid tissues of adult mice. Moreover, isolated DCs derived from *Camk4*$^{-/-}$ genotype show a marked defect in their capability to prolong lifespan in response to LPS, a phenomenon that is associated with the failure of TLR4 signaling to prevent the temporal decline in Bcl-2 and accumulation of Bcl-xL. However, the analysis of CaMKIV-null DC overexpressing transgenic Bcl-2 provide support for a dominant role Bcl-2 in regulating the lifespan of LPS-activated DCs and demonstrate that one of the crucial roles of the CaMKIV cascade in activated DC might be the regulation of the temporal accumulation of Bcl-2.

CaMKIV regulates survival and differentiation of several cell types, including hematopoietic progenitors, neurons, thymocytes, and osteoblasts; one common mechanism is by activating transcription by stimulating pCREB.[26,27,34,48] We show that pharmacologic inhibition of CaMKs as well as the genetic ablation of the CaMKIV gene affects the early events triggered by TLR4 stimulation by preventing accumulation of pCREB. Recently, it has been shown that a CREB-dependent pathway inhibits the pathogen-induced apoptosis of bone marrow–derived macrophages.[12] Our data confirm the relevance of pCREB in the survival program and document its involvement in the molecular mechanisms regulating the lifespan of LPS-activated DCs. Furthermore, they identify the CaMKIV/CREB signaling cascade as a novel pathway that is essential in the antiapoptotic branch of the TLR4 signaling pathway.

Genetic or microenvironmental factors may control the lifespan of activated DCs and in turn regulate the adaptive immune response by promoting the eradication of pathogens or the development of immune-mediated diseases. In this context, our results may contribute to a better understanding of the mechanisms used by pathogens to control the lifespan of antigen-presenting cells, and may inform novel perspectives to manipulate the immune response by targeting components of the CaMKIV cascade in DCs.

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Authorship

Contribution: M.I. designed and performed research and drafted the manuscript; M.G., L.V., A.M.M., F.B., E.C., D.A., A.C., V.C., and G.M. performed research on human monocyte–derived DCs; L.M.G.-T., U.S., and T.J.R. performed research on murine models; L.P. designed research using lentivaral vectors; A.R.M. interpreted data and drafted the manuscript; G.R. and M.V. interpreted data and drafted the manuscript; and L.R. designed and performed research, analyzed and interpreted data, and drafted the manuscript.

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Dendritic cells/Natural Killer cross-talk: a novel target for Human Immunodeficiency Type-1 Protease Inhibitors.

Running title

HIV-1 Protease Inhibitors and DC

by

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Abstract

HIV-1 protease inhibitors (PIs) are designed to specifically inhibit the aspartic protease of HIV-1, but several evidences shown that are also able to exert various effects on immune cells. Here we investigate the capability of HIV-1 PIs to interfere with Monocyte-Derived DCs (MDCs) lifecycle. We shown that prolonged exposure of PIs decrease the yield of MDCs; decrease the expression of typical MDCs differentiation markers; decrease ability to secrete IL-15 and IL-12 p40 in response to bacterial endotoxin stimulus. In turn, these cells fail to prime the proliferation, interferon gamma secretion and cytotoxicity of autologous NK cells. In addition, MDCs generated in the presence of PIs are more prone to NK-dependent lysis. These findings reveal DC-NK as a novel target of the immunomodulatory activity of PIs opening novel therapeutical perspective for using this category of drug to manipulate the immune response.

Introduction

Dendritic cells (DC) are well described as sentinels of the immune system, able to recognize danger signals mainly via Toll-Like Receptors, undergo maturation process and efficiently prime naïve T cells, inducing the adaptive immune response (Steinman RM 2006). Actually, there is a growing body of evidences about the interplay of DC and Natural Killer (NK)-cells (Walzer T et al. 2005). NK-cells can induce terminal differentiation of DC, and, reciprocally, DC can secrete NK-cells activating cytokines (IL-12, IL-15, type I IFNs), leading to proliferation, IFN-gamma secretion and increase of cytotoxic activity. High NK - DC (DC) ratio, results in the killing of DC which express low levels of MHC I molecules and/or human leukocyte antigen-E molecule. This "switch-off" mechanism of the immune response is mediated by triggering NKp30 NKAR and CD40L on NK cells and TNF-related apoptosis-inducing ligand (TRAIL) death receptors on DC (Walzer et al. 2005, Moretta L et al. 2006).

HIV-1 Protease Inhibitors (PIs), that are included in the Highly Active Antiretroviral Therapy (HAART) of AIDS, are specifically designed to inhibit HIV-1 aspartic protease and have significantly improved the clinical management of HIV-1 infected patients (Mastrolorenzo et al. 2007, Dash C et al. 2003). However, a number of evidences proved that HIV-1 PIs are able to inhibit mitochondrial-mediated apoptosis in uninfected T lymphocytes, in an antiviral-independent fashion. This effect can be directly mediated by inhibition of mitochondrial-membrane permeabilization, or by neutralization of pro-apoptotic effects of HIV-1 proteins (Vpr) (Hisatomi T et al. 2008). While there are more evidences about the antiviral-unrelated effects of HIV-1 PIs on T lymphocytes, poor is known about uninfected DC. Previous study report that exposure to different HIV-1 PIs (Saquinavir, Ritonavir, Indinavir, Nelfinavir) during DC differentiation, induce decrease ability to undergo maturation process and to sustain T lymphocyte proliferation (Gruber et al.2001). In addition, it is reported that HIV-1 PI Ritonavir can impairs proteasome-activity in cell lines, leading to defects in antigen processing and presentation (Schmidtke G et al. 1999). In light of this findings, we looked more extensively at the effects of HIV-1 PIs on DC differentiation and function. We describe that DC generated in the presence of Ritonavir show a marked decrease in their ability to activate NK-cells and are more prone to NK-cells mediated lysis. These results reveal for the first time that HIV-1 PIs can impairs DC-NK cells cross-talk, shedding light on their immunomodulatory activity in HIV-1 infection and opening novel therapeutical perspectives to manipulate the immune response.

Materials and Methods

Media and Reagents. The regular medium used throughout was RPMI 1640 (Invitrogen) supplemented with 2 mM L-glutamine, 50 ng/ml streptomycin, 50 units/ml penicillin, and 10% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, UT). Granulocytes monocytes-colony stimulating factor (GM-CSF) was purchased from Schering-Plough (Kenilworth, NJ) and used at a concentration of 50 ng/ml. Interleukin-4

(IL-4) was obtained from ImmunoTools and used at 1000U/ml. Interleukin-2 (IL-2) was purchased from Roche and used at a concentration of 200 UI/ml .

Saquinavir, Ritonavir, Nelfinavir, Indinavir, Amprenavir were dissolved in ethanol and added at cells cultures at a concentration of 20mM. Saquinavir, Ritonavir, Nelfinavir, Indinavir sulfate, Amprenavir were obtained through the NIH AIDS Research and Reference Reagent Program, Division af AIDS, NIAID, NIH.

Isolation and culture of NK cells and generation of MDDCs. PBMCs were isolated from healthy donors by density gradient on Ficoll Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). NK cells were freshly isolated by negative selection (StemCell Technologies Inc.). Purified NK cells contained ≤3% contamination with other PBMC subsets as determined by the expression of CD3, TCR-α/β, TCR-γ/δ, CD19, or CD14. Polyclonal NK cells and NK cell subsets were activated in vitro with recombinant IL-2 (rIL-2; Roche) at 200 UI/m for 6 d .

To generate iDCs, monocytes were purified by positive selection with anti-CD14 conjugated magnetic microbeads (Miltenyi Biotec, Bologna, Italy). $CD14^+$ cells were than cultured at a concentration of 0.5–1 x 10^6 cells/ml in regular medium supplemented with GM-CSF (50 ng/ml) and IL-4 (1000U/ml) for 4–5 days to obtain cells with typical phenotype of iDCs. To generate mDCs, iDCs were further cultured for 24–48 h in the presence of 1 μg/ml LPS (Sigma-Aldrich).

All cell culture was conducted at 37°C in humidified 5 % CO_2 atmosphere.

Flow Cytometry. Phenotype of DC were analysed by flow cytometry by using the following monoclonal antibodies conjugated: anti-HLA-I and anti-CD14 from Sigma; anti-CD1a, anti-CD86, anti-CD80, anti-CD83, anti-CD40, anti-HLA-DR, anti-HLA-ABC, anti-CD11c, anti-CD36, anti-CD54 from BD Biosciences, anti DC-SIGN from NIH research and reference reagent program.

For intracellular cytokine detection, Brefeldin A (5 μg/ml; Sigma) was added to the culture medium. Cells were then fixed and permeabilized by using a cytokine staining kit following the manufacturer's instructions (Caltag Laboratories, Burlingame, CA). Antibodies against TNF- , IL-12, IL-10, IFN-γ and IL-4 were purchased from BD Biosciences. FACSCalibur cytometer and Cellquest software were used for these analyses (BD Biosciences).

Human NK cells analysis was performed with: anti–TCR-α/β (IgG1), anti–TCR-γ/δ (IgG1), anti-CD19 (IgG1), FITC-anti-CD14, PE-anti-CD107a purchased from Becton Dickinson, USA, FITC anti -CD3/PE-Cy5 –anti-CD56 purchased from Beckman-Coulter-Immunotech, Marseille, France.

Data were collected using a FACSCAlibur flow cytometer (Becton Dickinson, USA) and analyzed using FlowJo v6.3.3 (Treestar, Palo Alto, CA, USA).

Proliferation Assay. Freshly purified NK cells were cryopreserved until required as responders. Experiments were performed in triplicate in 96-well round plates with complete medium. NK cells were cocultured at a constant concentration of 2 x 10^5 NK cells/well with autologous mDCs (stimulators) in serial dilutions (10–1.50 x 10^3 cells/well). [^{3}H]Thymidine (0.037 Mbq per well; PerkinElmer Life Sciences) was added 18 h before harvest cell cultures, and incorporation of [3H]thymidine into the cells was quantified using a b-counter.

Analysis of NK-cell cytotoxicity by chromium release. After 6 d of activation with rIL-2, NK cells were tested for cytolytic activity in a 4-h ^{51}Cr release assay. A total of 1×10^6 target cells (K562 or autologous DC) were labeled with 1mCi of $Na^{51}CrO_4$ for 1 h at 37°C.

Cells were then washed twice with complete medium and incubated with effector cells at an E:T ratio of 20:1. After incubation for 4 h at 37°C, a sample of supernatant was counted on a Microbeta Trilux Scintillation counter (PerkinElmer). Percentage of cytotoxicity was calculated using the formula (experimental-spons)/(maximum-spons) ×100%, where spons = release from targets incubated with medium alone and maximum = release from targets induced by 10% SDS (Sigma-Aldrich).

Saturating concentrations (10 μg/ml) of specific mAbs blocking NK cell receptors were added for the masking experiments performed with autologous DCs.

NK-DC cocolture. NK-DCs were cocoltured at 1:1 ratio (2X 10^5 /well) in presence of LPS (10 μg/ml) in 48-well cell culture plates. After 16-h incubation, cell culture supernatants were collected and stored at -20° until analyzed for cytokine production and NK cells were collected and analyzed for CD107a degranulation assay.

CD107a degranulation assay. r-IL2–activated purified NK cells were cocultered alone (no target control) or with K562 target cells at a 1:1 E:T ratio (2×10^5 effector cells: 2×10^5 target cells in a volume of 200 μl) in the presence of 20μl of PE-CD107a mAb for 3 h at 37°C in total. After the first 1 h 5 μl of the secretion inhibitor 2mM monensin (Sigma Aldrich, Munich, Germany) in 100% ethanol was added. At the end of coincubation, cells were washed in PBS and stained with mAbs (CD56, CD3) for flow cytometric analysis. NK cells were gated by $CD56^+/CD3^-$ staining, and CD107a expression was determined based on background level of staining exhibited by no target control cells.

Cytokine secretion. The levels of IL-12p70 (IL-12) and IL-15 secreted by mDCs were measured from cell culture supernatant by ELISA (R&D Systems and Biosource International). To detect the production of IFN-γ, freshly purified NK cells were cryopreserved until required and cocultured with autologous

LPS matured DCs in 96-well round-bottom plates with complete medium . The mDC/NK cell ratio was 1:10. The supernatant of the cultures was collected after 24 h and assayed by ELISA (BD Biosciences).

Results

HIV-1 protease inhibitor treatment affects the immune phenotype and LPS-induced terminal differentiation of DC.

To gain novel insights into the impact of HIV-1 PIs on the differentiation program and immunostimulatory properties of human DCs, we investigated whether this class of drugs influences the maturation and cytokine production of DCs. Therefore, freshly isolated CD14+ monocytes were cultured for 7 days in optimal amount of GM-CSF an IL-4 to obtain iDC, and in the presence or absence of 20uM of Saquinavir (SQV), Ritonavir (RTV), Indinavir (IDV), Amprenavir (APV) or Nelfinavir (NFV). At this time point, the expression of typical differentiation markers of iDC was evaluated by flow cytometry. We found that SQV and RTV exerted the strongest effect on iDC immunophenotype, leading to low expression of CD1a and CD86. Moreover, SQV and IDV treatment decreased CD36 expression, while APV exposure did not affect any of the markers analyzed. Notably, all the drugs tested, with the exception of APV, significantly decreased CD209 (DC-SIGN) expression (Figure 1 A). Overall, this results demonstrated that the majority of HIV-1PIs tested are able to impair iDC differentiation.

Next, we examined the terminal maturation of DC following PIs exposure. To this end iDC generated in presence or absence of 20uM of Saquinavir (SQV), Ritonavir (RTV), Indinavir (IDV), Amprenavir (APV) or Nelfinavir (NFV) were collected, washed and exposed for additional 24 hours to TLR-4 agonist, namely Lipopolysaccharide (LPS). As expected, LPS induced a marked increase in double positive CD86/CD83 percentage in DC growth in regular medium. On the contrary, and in line with the immunophenotype findings, the cells that had been exposed to PIs were unable to up-regulate CD86 and CD83, whereas the other drugs did not alter the maturation markers analyzed (Figure 1 B).

This finding seems to missmatch with the previous study by Gruber et al. in which they did not observe any effect on iDC differentiation. The discordance can be due to the different protocol applied to generate DC. Indeed, conversely to CD14+monocyte-derived DC, plastic-adherent PBMC-derived DC contain lymphocytes populations which could impair the outcome of the differentiation process. Overall, the results we obtained demonstrated that the majority of HIV-1PIs tested are able to impair iDC differentiation.

HIV-1 protease inhibitor treatment affects the cytokine production of LPS-induced terminal differentiated DC.

To further investigate the effect of the HIV-PIs on terminal differentiation of DC, we examined the amount of pro-inflammatory cytokines (TNF-alpha, IL-12, IL-15) produced in response to LPS by ELISA. We focused my study on RTV rather than on SQV, considering that the latter is less used in clinical practice, because of the plethora of adverse effects and the low biodisponibility. Furthermore, we compared RTV effects to APV, that was the less effective drug in my experimental system. Whereas LPS-dependent TNF-

alpha and IL-12p40 induction were both unchanged, RTV treatment completely blocked the secretion of bioactive IL-12p70 and IL-15 (Figure 2). These findings further substantiate the results of previous studies demonstrating that, for example, ritonavir and saquinavir inhibit the production and/or release of inflammatory cytokines and chemokines including TNF-alpha, IL-6, and IL-8, by both peripheral-blood mononuclear cells and endothelial cells (Pati et al. 2002). This effect of HIV-PIs on inflammatory cytokines has been confirmed in treated patients, as PI-HAART has also been shown to inhibit TNF-alpha, IL-2 and IFN-γ production by peripheral-blood mononuclear cells from uninfected individuals who were treated with HIV-PIs for prophylactic intervention without acquiring HIV infection (Tovo 2000). Because of the great importance of DC in the control of the inflammatory response, it was conceivable that HIV-PIs might exert their anti-inflammatory activity by impairing the immunostimulatory properties of this cell type.

Inhibitory effects of Ritonavir on the capacity of mDC to activate autologous NK cells.

Given the central role of 12p70 and IL-15 cytokines in the DC-dependent NK-cells activation (Moretta A 2005, Ferlazzo G et al. 2002), we asked if RTV-treated DC preserved their ability to interplay with NK cells. We first examined the ability of LPS-treated mature DC (mDC) to activate these innate effector cells by inducing proliferation of autologous NK cells and IFN-gamma secretion.Therefore, iDCs generated in presence or absence of Ritonavir or Amprenavir were washed and then left in regular medium or exposed to LPS (10μg/ml). After 6 hours, mDCs (stimulator) were cocultered with autologous fresh NK cells (responders) at different ratios. Cells were harvested after 4 d of coincubation, and the proliferation of NK cells was measured by 3[H]thymidine incorporation. Long exposure to RTV decreased of about 50% the ability of mDC to sustain NK- cells proliferation (figure 3 A). In addition iDCs generated in presence or absence of HIV-PIs were washed and then left in regular medium or exposed to LPS (10μg/ml). After 18 h, freshly purified autologous NK cells were coincubated with iDC or mDC for 24h. Supernatants were collected and INF-γ concentration was determined by ELISA. As demonstrated in figure 3 B Ritonavir but not Amprenavir significantly reduced the ability of LPS-activated DCs to improve IFN-γ production by NK cells. For the first time here we showed the effects of HIV-protease inhibitors on a novel system: the crosstalk DC-NK. In particular, data obtained highlighted that Ritonavir treated DCs lose their capacity to efficiently stimulate NK cells, confirming and extending to a novel target the anti-inflammatory effects of HIV-PIs.

Impact of Ritonavir on the ability of mDC to improve tumor-directed cytotoxicity of NK cells.

In line with precedents reports (Ferlazzo G et al. 2002), we reasoned that the lack of mDC cognate activation of NK-cells would affect their cytotoxic effect on a non-cognate susceptible cellular target. To test this hypothesis, we co-incubated mDC and NK-cells for 24 hours, and subsequently we analyzed the ability of DC-primed NK-cells to kill K562 target. CD107 cytometric analysis shows that K562 lysis is

severely decreased by priming with RTV-treated mDC (Figure 4). These data suggest that the DCs-mediated enhancement of tumoricidal potential of NK cells was also markedly by Ritonavir.

Impaired NK cell-mediated killing of Ritonavir treated mDC.

We next asked if NK-cells dependent killing of autologous iDCs were active in our system. This function depends on the engagement of NKp30 by still-undefined cellular ligands expressed by DCs. Indeed Cr 51+ release assay showes that non treated autologous iDC are efficiently killed by NK cells in an NKp30-dependent manner, as demonstrated by the ability of the specific mAb masking NKp30 (F252) but not of the specific mAb masking NKG2D (BAT221) to inhibit the NK killing activity (Figure 5 A). As expected untreated mDCs that, upregulate MHC-class I expression become essentially resistant to NK cells (Figure 5 B).

On the contrary, the treatment with Ritonavir increases the susceptibility to NK-cells mediated lysis of mDCs whereas iDC lysis was high irrespective to the treatment (Figure 5 A and B). This apparent paradox might be explained by the fact that RTV-treated DC failed to fully mature in response to LPS and even upon its stimulation RTV-treated DCs fail to express sufficient amounts of HLA-class I molecules that would protect from NK-mediated lysis (Ferlazzo G 2003).

Discussion

In our study we describe new evidences by which HIV-1 PI, targeting DCs, modulate NK cell function. We describe that Ritonavir (RTV) is able to impair MDDC differentiation and function, based on the expression of the typical markers and cytokine production. These alterations were responsible for a broad defects in NK cells activity. The proliferation, IFN-gamma production, killing ability was impaired and the same RTV-treated mDC was more susceptibile to NK cell induced-lysis.

The cross-talk DC-NK is an area of intense investigation in immunology and HIV research. DC are central players of the immune response for their ability to prime naïve T cells and to stimulate, by cytokines and/or by cell-cell contact, the majority of the cells involved in the immune response, including NK cells. On the other hand, NK cells are the first defense line again extra-cellular pathogen and virus, they can interact with DCs regulating their maturation and function, thereby linking the innate and the adaptive immune responses. Long-term RTV exposure results deleterious directly to the innate-immune response. IL-12p70 and Il-15 are multifunction cytokines that act on different cell types to induce and /or enhance inflammatory response. We focused on NK cells because they are directly dependent to both these cytokines for activation and function.

One of the major obstacles to the HIV eradication from infected patients is the persistence of reservoirs in witch the virus can survive latently and be protected by the drugs. DCs are one of the most important viral reservoirs. One of the DC-molecules involved in the process is CD209 (DC-SIGN). CD209 show ability to bind with high affinity the HIV-1 envelope protein gp120, and DC-SIGN+ DCs can become a vehicle for the *in trans* infection of CCR5 or CCR7 positive T cells (Kwon DS et al. 2002)

In our experimental system we found that, despite the strongest effect on the inhibition of iDC immuno-phenotype exerted by SQV and RTV, all the drugs tested, with the exception of APV, significantly decreased CD209 (DC-SIGN) expression. This implies that DC would be no more able to carry-on virus to lymph-nodes within their migrations and the spread of the infection may be reduced.

Moreover, long-term treatment with RTV makes mDC more susceptible to NK-induced lysis. The effect of RTV on the reservoirs equilibrium is therefore double: from one side it reduces the ability of DC to be infected by HIV, from the other side it favors the direct killing of the same reservoirs, creating a synergy, or at least an additive effect, on the reduction of the infection spread.

The impairment of DCs differentiation affects the crosstalk between the formers and NK cells.

The crosstalk between DC and NK is responsible for the correct homeostasis of the innate immune response compartment and for a correct inflammatory response. Our study offers a better understanding of the well-known anti-inflammatory activity of the HIV-PIs and reveals DC-NK functional units as a novel potential target for this class of antiviral drug. This might open new fields in drug discovery aimed at the synthesis of more specific molecules with a different anti-viral and anti-inflammatory activity.

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Figure Legends

Figure 1. Influence of HIV-PIs on immunophenotype and maturation of DCs. **(**A) iDCs generated in presence or absence of HIV-1 PIs were analyzed by flow cytometry. The histograms represent the percentage of cells positive for the indicated molecule. (B) iDCs generated in presence or absence of HIV-1 PIs were washed and then left in regular medium or exposed to LPS (1mg/ml) for 24 hours. Subsequently the expression levels of CD86 and CD83 at the surface of DCs were determined by flow cytometry. The histograms represent the percentage of cells positive for the indicated molecule.Asterisks indicate a statistically significant difference ($p< 0.05$).

Figure 2. Impact of HIV-PIs on cytokine expression of DCs. iDCs generated in presence or absence of HIV-1 PIs were washed and then left in regular medium or exposed to LPS (1mg/ml) for 24 hours.Supernatants were collected and the levels of secreted cytokines were evaluated by ELISA. The results are presented as mean± s.e. of duplicate determinations.Asterisks indicate a statistically significant difference ($p<0.05$) between the indicated cytokines production of Ritonavir-treated DCs in comparison to untreated DCs.

Figure 3. Influence of Ritonavir on the capacity of DCs to prime autologous NK cells. (A) iDCs generated in presence or absence of Ritonavir or Amprenavir were washed and then left in regular medium or exposed to LPS (10μg/ml). After 6 hours, mDCs (stimulator) were cocultered with autologous fresh NK cells (responders) at different ratios. Cells were harvested after 4 d of coincubation, and the proliferation of NK cells was measured by 3[H]thymidine incorporation.(B) iDCs generated in presence or absence of HIV-Pi were washed and then left in regular medium or exposed to LPS (10μg/ml). After 18 h, freshly purified autologous NK cells were coincubated with iDC or mDC for 24h. Supernatants were collected and INF-γ concentration was determined by ELISA. iDCs alone and NK cells alone or IL2 activated served as negative controls. All data are presented as the mean ± SD of experiments conducted on 8 healthy donors. Asterisks indicate a statistically significant difference ($p< 0.05$).

Figure 4. Ritonavir reduces the DCs-mediated enhancement of tumoricidal potential of NK cells**.** iDCs generated in presence or absence of Ritonavir or Amprenavir were washed and cultured with purified CD56+CD3- NK cells in the presence of LPS (10μg/ml). After 18 h, NK cells were separeted from adherent DCs and cocultured in round-bottom 96-well plates with K-562 target cells at an E/T ratio of 1:1 in presence of anti-CD107a mAb. After 4 h of culture, cells were stained with the two other indicated mAbs and analyzed by multiparametric flow cytometry. The histograms represent the percentage of $CD56^{+}CD3^{-}$ -gated NK cells staining positive for CD107 in the presence of targets following coincubation with treated or untreated mDC compared with control cultures incubated in the absence of mDC. All data are presented as the mean ± SD of experiments conducted on 8 healthy donors. Asterisks indicate a statistically significant difference ($p< 0.05$).

Figure 5. Impaired NK cell-mediated killing of Ritonavir treated mDC. (A) Autologous iDC cytolysis exerted by rIL-2–activated NK cells purified from a healthy donor . (B) Autologous mDC cytolysis exerted by rIL-2–activated NK cells purified from a healthy donor NK cells were incubated either in the absence (baseline lysis) or presence of a specific mAb masking NKp30 (F252) or NKG2D (BAT221). The NK cell/DC ratio in all experiments was 10:1. All data are presented as the mean ± SD of experiments conducted on 8 healthy donors. Asterisks indicate a statistically significant difference ($p< 0.05$).

Figures

Figure 1

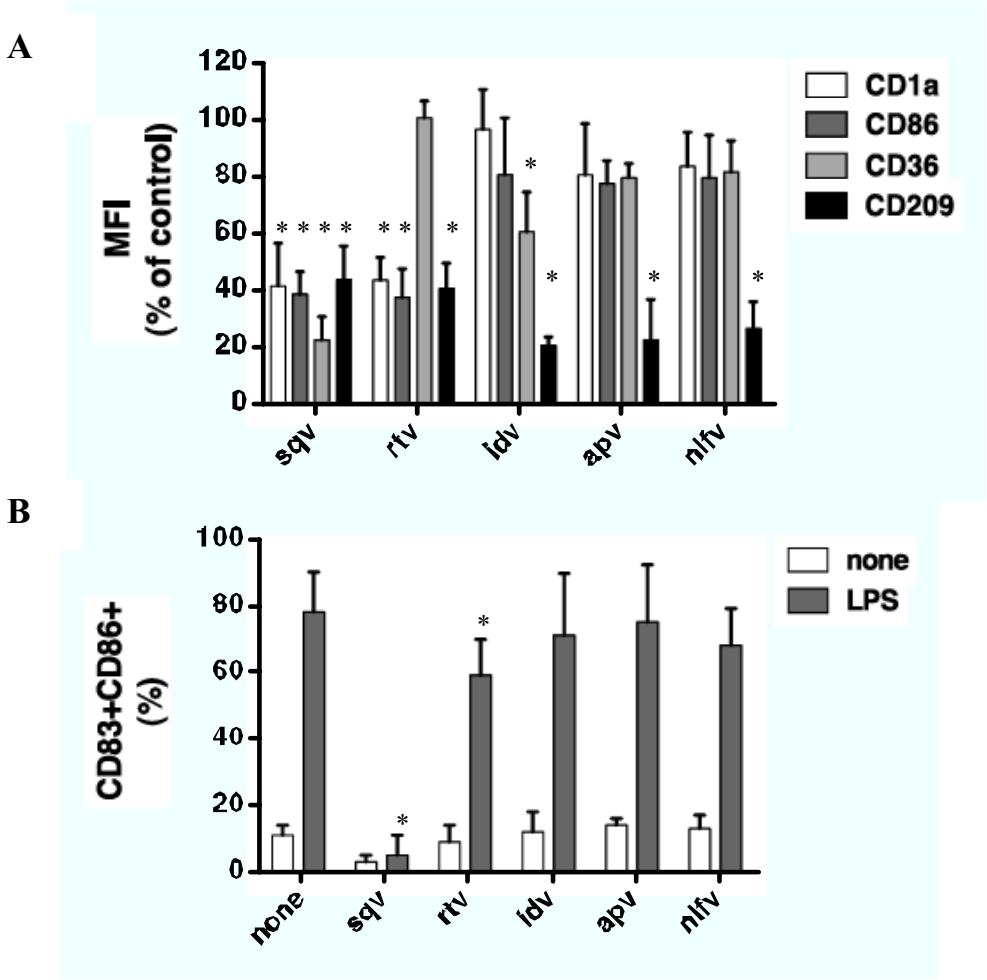

Figure 2

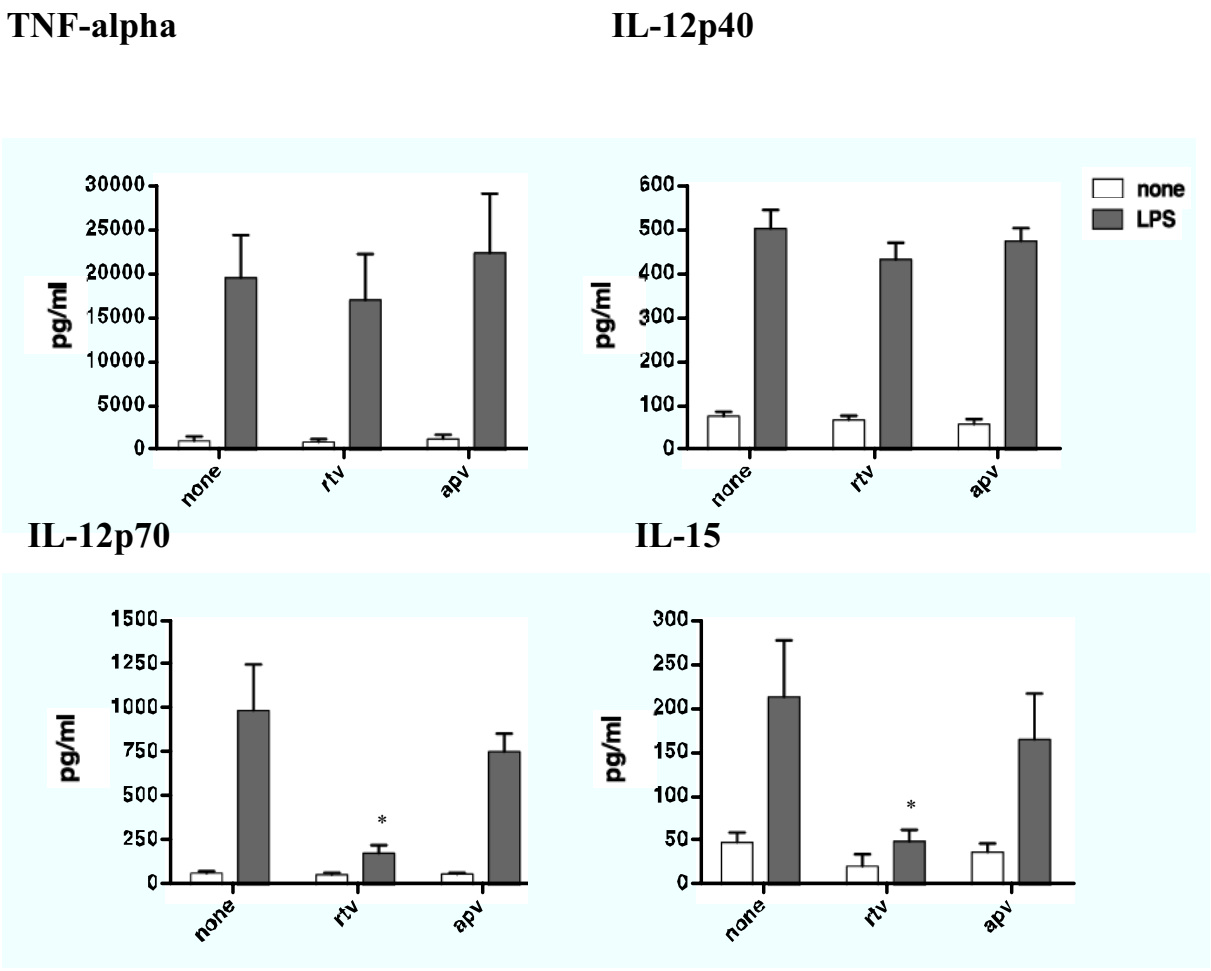

Figure 3

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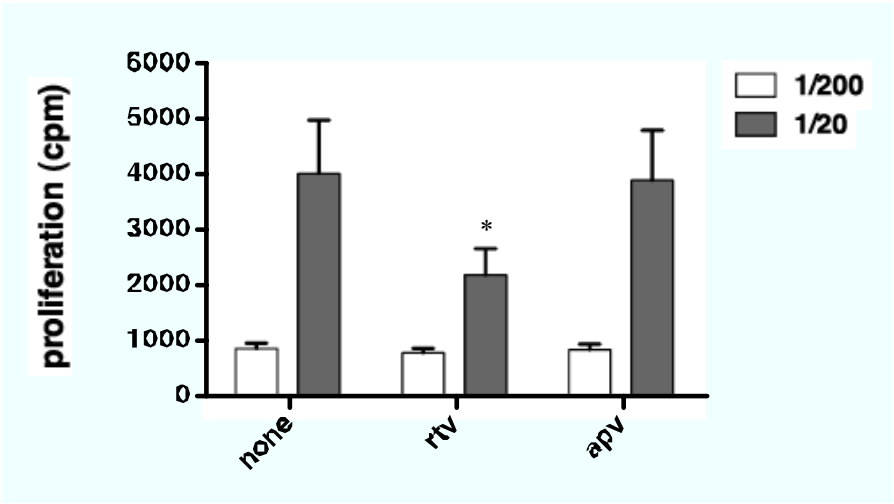
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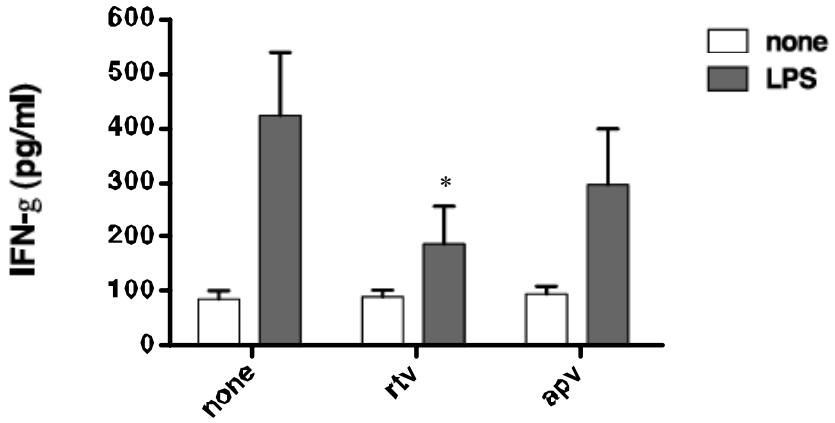
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Figure 4

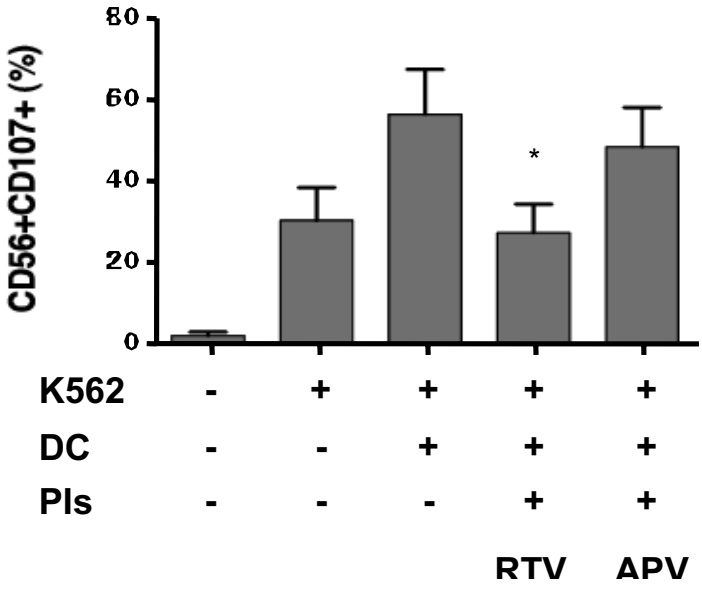
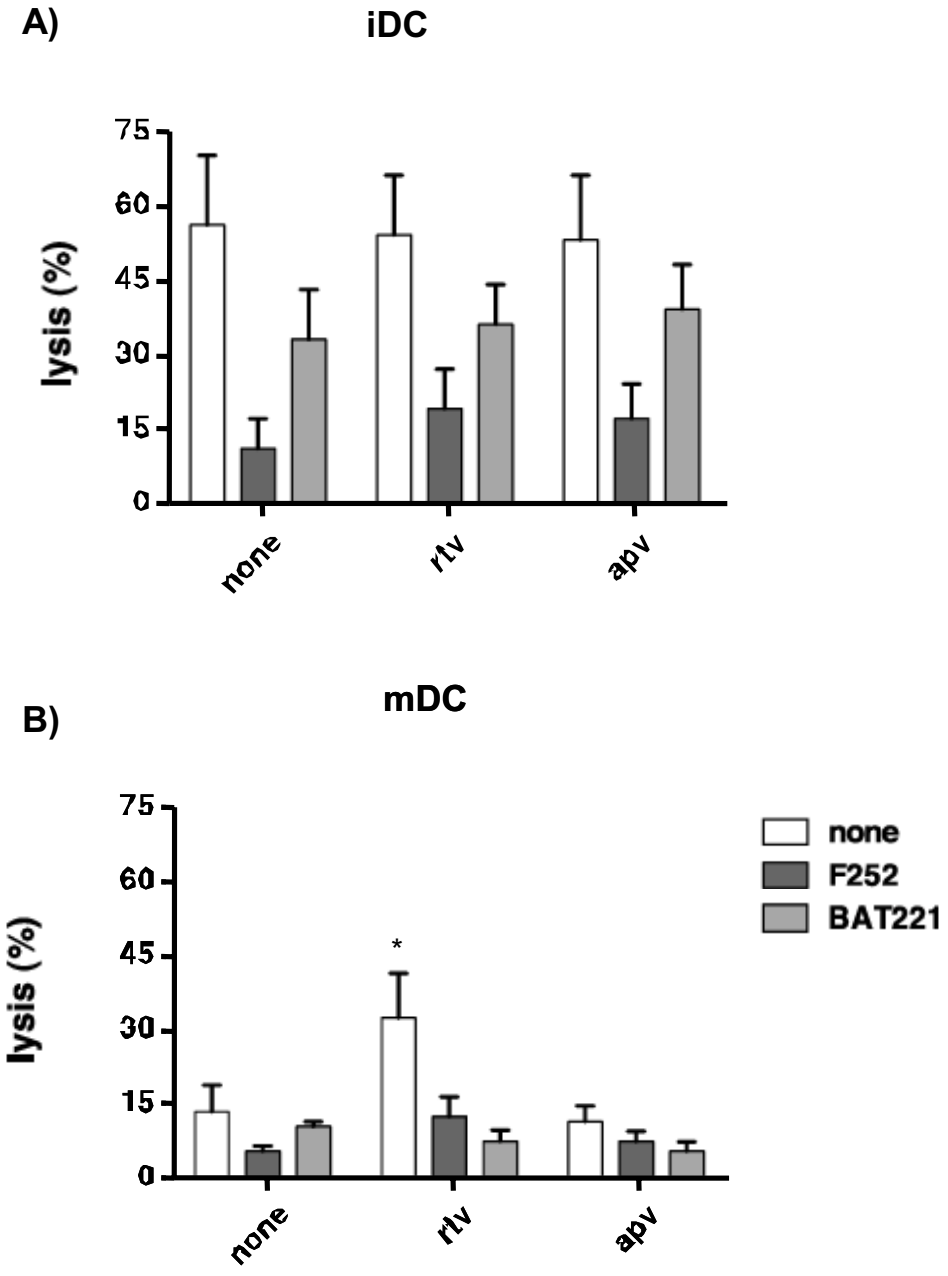


Figure 5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