CD40L-expressing recombinant vaccinia virus (rVV40L):

Generation of central memory CD8+ T cells and induction of tumor cell death

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Abbreviations

mAbs, monoclonal antibodies

qRT-PCR, quantitative Real-Time-Polymerase Chain Reaction

GAPDH, glyceraldheide-3-posphate dehydrogenase

GM-CSF, granulocyte macrophage-colony stimulating factor

HLA, human leukocyte antigen

MHC, major histocompatibility complex

PBMCs, peripheral blood mononuclear cells

APC, antigen presenting cell

CTL, cytotoxic T cell

T_{CM}, central memory T cell

T_{EM}, effector memory T cell

MOI, multiplicity of infection

VV, vaccinia virus

VV WT, wild-type strain of Vaccinia Virus

rVV40L, CD40L-expressing recombinant vaccinia virus

s40L, soluble CD40L recombinant protein

1. INTRODUCTION

1.1. Cancer and Immune Intervention

Cancer represents the second leading cause of death worldwide as indicated by the over eight millions of deaths registered in 2012 (NIH/NCI). In the last decades, several immunotherapeutic strategies have been investigated in pre-clinical and clinical studies in order to develop new platforms for treatment of tumor-bearing patients. The rationale supporting the clinical evaluation of different strategies aiming to induce, amplify or skew antitumor immunity is based on the potential capacity of the immune system to mediate tumor eradication and most importantly, on the necessity to overcome the limited effectiveness of current standard anti-cancer treatments. In particular, pioneering studies performed in animal models have initially shown the ability of the immune system, and, in particular, of CD8+ T cells, to mediate tumor clearance. These encouraging results have been recently confirmed by the characterization of local and systemic immune responses in cancer patients. Indeed, increased percentages of tumor specific T cells in peripheral blood and high tumor infiltration by CD8+ T cells in tumor deposits have been associated with significantly increased overall or progression free survival at least in melanoma, renal, ovarian, lung and gastrointestinal cancer patients[1,2]. However, although therapeutic potential of CD8+ T cell has been extensively reported, cumulative results obtained in animal models and clinical evidences have also indicated that intrinsic alterations as well as active immune resistance of malignant cells represent major limitations preventing the generation of protective CD8+ T cell responses mediating cancer elimination[3,4].

Tumor cells express antigens potentially recognized by naturally arising CD8+ T cells. Currently, two distinct categories of tumor-associated antigens (TAAs) have been defined according to their pattern of expression: (i) shared TAAs and (ii) unique TAAs. In particular, unique TAAs are represented by viral antigens, derived from infectious agents responsible of neoplastic transformation, and antigens that results from mutations, deletions and recombination of specific gene sequences (neo-antigens). In contrast, shared TAAs include antigens that are overexpressed (overexpressed antigens) or expressed at similar levels by

transformed cells and by their normal counterparts (differentiation antigens) or in germline cells (cancer-testis antigens)[5].

Interestingly, results obtained in pre-clinical and clinical studies, addressing the therapeutic potential of vaccination strategies, have indicated that the therapeutic

effectiveness of targeting of CD8+ T cells against major histocompatibility complex class I (MHC class I) restricted peptide (pMHC complexes) derived from neo-antigens, may be limited by heterogeneous expression among malignant cells. Furthermore tolerance might prevent the induction of immune responses against antigens of predominantly self-origin. Indeed, with the exception of viral antigens derived from human papilloma virus that have shown clinical effectiveness in prevention and treatment of cervical carcinoma, a major limitation for cancer immunotherapies strategies targeting differentiation and overexpressed TAAs, is represented by the low-affinity nature of the circulating T cell receptors specific for self/tumor antigens. In this respect, studies have shown that curative potential of low-affinity tumor-reactive CD8+ T cells may be limited either as a consequence of a reduced clonal expansion of antigen-specific precursor or by a limited implementation of effector functions upon recognition of target cells[6]. Indeed, among the different factors that synergistically contribute to cancer immune evasion, a critical determinant is represented by the ability of tumor cells to prevent the formation of a productive immunological synapse with tumor-reactive CD8+ T cells. Furthermore it has been extensively reported, in particular for solid tumors, that reduced expression of pMHC complexes by tumor cells results in a defective activation of low-affinity tumor-specific CD8+ T cells due to a reduced TCR occupancy[7-9].

In addition to the intrinsic features of tumor antigens, curative potential of tumor-reactive CD8+ T cells, regardless of the affinity of TCR expressed, is also limited by the activation in transformed cells of distinct mechanisms that synergistically promote the immunological tolerance of cancer. In this respect, it has been extensively reported that anti-tumor activity of CD8+ T cells can be limited by the expression, on cellular surfaces of transformed cells, of inhibitory ligands[10]. Furthermore, the infiltration and/or the activity of tumor-reactive CD8+ T cells may also be regulated by the activation of genes encoding for enzymes and soluble factors directly or through the recruitment of immunosuppressive cells including regulatory T cells (Tregs) and myeloid-derived suppressor cells (MSDC)[11-14].

In this scenario, the harnessing of CD8+ T cells against cancer as a successful immunotherapy may appear as a difficult challenge. However, the increased overall survival

and/or objective cancer regression evaluated according to the RECIST criteria (Response Evaluation Criteria in Solid Tumors) reported by several clinical trials where cancer patients were administered antibodies mediating immunological checkpoints blockade, adoptive cellular therapy (ACT) and different formulations of cancer vaccines clearly demonstrate the therapeutic potential of immunotherapies strategies aimed at promoting the generation and the antitumor activity of CD8+ T cells. Furthermore, clinical evidence obtained from treated cancer patients indicates that strategies aiming at promoting antitumor CD8-mediated immune responses may result in more durable clinical benefits as compared to standard chemotherapy and radiotherapy treatments.

1.2. Cancer Immunotherapy strategies.

Passive cancer immunotherapy strategies are based on the administration of therapeutic antibodies or tumor- reactive T lymphocytes. In contrast, active strategies aim at promoting the in vivo generation or boosting of the immune system against tumor cells, based on the administration of different vaccine formulations. Although with distinct mode of actions, both strategies share a common denominator, namely they rely on tumor-specific T cell responses.

1.2.1 Passive Immunotherapeutic Approaches.

Administration of antibodies targeting TAAs or immunological checkpoints blockade and adoptive transfer of in vitro expanded tumor-infiltrating lymphocytes (TILs) or genetically engineered T cells, represent successful examples of passive immunotherapeutic approaches. Several of these strategies have been recently approved by US Food and Drug Administration (FDA) as well as European Medicines Agency (EMA) as first line therapy for solid tumors and hematological malignancies.

1.2.1.1 Antibodies---against Immune Checkpoints Blockade.

Therapeutic potential of monoclonal antibodies (mAbs) for cancer treatment is primarily related to their unique capacity to recognize specific cell surface antigens. In

addition to this marked specificity, mAbs are also able to promote through their Fragment crystallizable region (Fcr), the elimination of target cell by activating innate immune cells. According to the definition of "magic bullets" provided by P. Ehrich, mAbs have been successfully used in the last two decades as successful treatments for solid tumors and hematological malignancies. In particular, initial strategies were based on the direct antineoplastic activity of mAbs targeting specific markers expressed by tumor and associated stromal and endothelial cells[15]. In contrast, in the last years a novel target for mAbs have been extensively evaluated in clinical setting. In particular mAbs targeting lymphocytes inhibitor receptors or their cognate ligands (Immune Checkpoints) has been developed in order to bypass the poor cellular immunogenicity as well as the active immune-evasion of tumor cells.

Activation of T cells is triggered by T cell receptor (TCR) mediated antigen recognition. However effective generation of a protective immune response is tightly regulated by the balance of co-stimulatory and inhibitory signals received by antigen specific T cells during cellular immune responses. In this regard, Cytotoxic T-lymphocyte Associated Antigen 4 (CTLA-4, CD152) and Programmed Death protein 1 (PD-1, CD279) have been extensively indicated as the master regulators of T cell responses. Initial studies suggested distinct patterns of immunomodulation mediated by these immune-checkpoint receptors. In particular, CTLA4 receptor has been traditionally indicated as a critical inhibitor of early activation of T cells in secondary lymphoid organs whereas PD1 receptor has been mostly associated to maintenance of self-tolerance in peripheral tissues.

This initial dichotomy has been recently revised. Indeed, our current understanding of immune regulation mediated by CTLA-4 and PD-1 receptors is in line with a synergistic inhibitory activity of these two receptors in secondary lymphoid organs as well as in peripheral tissues.

1.2.1.1.1 CTLA---4 pathway.

CTLA-4 belongs to the CD28 family. Its inhibitory activity is mostly related to the down modulation of CD28 costimulatory receptor activity. CTLA-4 inhibitor receptor is characterized by a higher affinity in comparison to CD28, for the co-stimulatory surfaces

molecules CD80 (B7.1) and CD86 (B7.2) expressed by professional antigen presenting cells (APCs)[16]. Due to its high affinity for the ligands, CTLA-4 receptor can abrogate early phase of T cell activation by preventing CD28-CD80/CD86 interaction. CTLA-4 receptor can directly recruit and activate distinct intracellular phosphatases which, in turn, "switch off" the activation signals delivered by TCR and CD28. These inhibitory activities synergistically abrogate the acquisition of effector functions and proliferative potential by T cell upon antigen recognition. In preclinical studies, a pivotal role of CTLA-4 : CD80/CD86 inhibitory pathway, in preventing the generation of a stable T cell conjugation with APCs (Schneider, 2006), has also been documented. Notably, inhibition of T cell responses mediated by CTLA-4 receptor is further supported by a direct activity on APCs via the induction of the indolamine-2,3-dioxigenase (IDO). The induction of this inhibitory enzyme in APCs, has been reported as a consequence of backward signals transduced by CD86 molecules upon interaction with CTLA-4-expressing T cells[17].

CTLA-4 receptor is normally stored in the cytoplasm of resting naïve and memory T cells and it is rapidly translocated on the cellular surface upon TCR engagement. Due to its inhibitory mode of action and further supported by the rapid expression on T cell surface upon antigen recognition, CTLA-4 receptor has been extensively indicated as a critical regulator of the early phase of T cell activation[18,19]. In this regard, experimental studies performed in animal models confirmed the pivotal role of CTLA-4 receptor. Indeed, the insurgence of lethal lymphoproliferative disorders characterized by the massive activation of auto-reactive CD8+ and CD4+ T cells in secondary lymphoid tissues was extensively reported in gene deficient or anti-CTLA-4 mAbs treated mice[20,21]. Notably, preclinical models also support a critical role for CTLA-4 receptor in modulating T cell activity in peripheral tissues by mediating the immunosuppressive function of regulatory T cells (Tregs). Fully humanized mAbs targeting CTLA-4 inhibitory receptor have been generated for cancer treatment in view of the critical role of this immune checkpoint in tolerance maintenance. Indeed, limited clonal expansion of tumor-reactive CD8+ T cells bearing "low affinity" TCRs and high infiltration of Tregs within tumor tissues represent two major limitations in the establishment of immunological control of several solid tumors[6,11].

In 2011, Ipilimumab a fully humanized (IgG1) monoclonal antibody targeting CTLA-4 receptor has been approved by US Food and Drug Adiministration (FDA) as well as European Medicine Agency (EMA) as first line of therapy for advanced melanoma patients in virtue of the results obtained in two randomized double blind phase III clinical trials. In

particular, in 2010 Hody and co-workers[22] initially reported an increased overall survival in refractory-metastatic melanoma patients receiving Ipilimumab alone (10.1 months; 137 patients) or in combination with gp100 specific vaccination (10.0 months; 437 patients) in comparison to those receiving only gp100 vaccine (6.4 months; 136 patients). Therapeutic potential of Ipilimumab was promptly confirmed in 2011 by the results obtained by Robert and collaborators on a cohort of 502 patients with previously untreated stage III/IV metastatic melanoma[23]. In particular a higher survival rates at three years, was observed in patients receiving Ipilimumab plus dacarbazine (20.8%) as compared to those treated with dacarbazine plus placebo (12.2%). Finally, clinical benefits associated to ipilimumab-based therapy have been recently restated by the cumulative results obtained from the follow up of more than 1800 patients enrolled in phase II/III clinical trials. Indeed, durable survival (in some case extended to 10 years) has been observed in almost 20% of Ipilimumab-treated patients[19,24].

Despite the objective and durable clinical responses observed in cancer patients upon Ipilimumab administration, critical considerations are now emerging regarding CTLA-4 blockade strategies. In particular, antitumor activity of anti-CTLA-4 mAbs is critically affected by their specific isotype. Indeed Tremelimumab, a fully humanized IgG2 anti-CTLA-4 antibody, despite encouraging results in early melanoma trials failed to induce a statistical significant survival advantage as compare to standard-of-care chemotherapy in first line treatment of patients with metastatic melanoma[25]. In this regard, a possible explanation for this negative result can be formulated in view of some preclinical studies. In particular results obtained in animal models, clearly underline how the antitumor effect of anti-CTLA-4 treatment is also dependent by the ability to induce Fc Receptor © (FcR©)-mediated intratumoral Tregs depletion possible due to antibody-dependent cell-mediated cytotoxicity (ADCC). According to these results, it is possible to speculate that the reduced antitumor activity of Tremelimumab (IgG2) in comparison to Ipilimumab (IgG1), is associated to a reduced ability of anti-CTLA-4 IgG2 monoclonal antibody (Tremelimumab) to modulate immune system as a consequence of reduced ligation of FcR©. In addition to this "technical issue", anti-CTLA-4-blockade therapy based on the administration of Ipilimumab, has shown two major limitations represented by the limited antitumor activity in non-melanoma cancers and by the insurgence of a new category of potentially lethal side effects indicated as
immune-related adverse events (irAEs)[22,23,25].

In this regard, clinical benefits observed in melanoma patients have not been confirmed in patients bearing non-melanoma tumors such as renal (RCC), lung and metastatic

castration-resistant prostate cancer (mCRPC) in response to Ipilimumab-based therapy. The observed therapeutic discrepancy of anti-CTLA-4 blockade it is nowadays correlated by the relative higher immunogenicity of melanomas as compared to other solid tumors. Indeed, in melanoma patients the ability of the immune system to spontaneously generate tumor-reactive CD8+ T cells recognizing well-known antigens including MelanA/MART-1, Tyrosinase and gp100 has been extensively reported. In addition recent technological advances in whole-exomic sequencing clearly indicate that this already high immunogenicity of melanoma cells is further increased by high frequency of non-synonymous mutation in transformed melanocytes resulting in the expression of a broad range of tumor-specific mutated antigens (neo-antigens). In this scenario, as indicated by a detailed characterization of melanoma-specific CD8+ T cell repertoire during Ipilimumab-based therapy, antitumor effects associated with CTLA-4 blockade appear to be mostly associated to the rapid appearance of new tumor-specific CD8+ T cell reactivities against patient specific neo-antigens[26-28]. Unfortunately, therapeutic potential of CTLA-4 blockade is also limited in melanoma patients by the severe immunotoxicity observed in 15-30% of treated patients. In particular, side effects commonly observed mostly affect skin, gut, liver and endocrine system. Notably, the insurgence of these immune-related adverse events (irAEs) may be considered as an intrinsic effect of CTLA-4 blockade strategy, tightly associated to therapeutic potential of Ipilimumab-based therapy. Indeed, abrogation of inhibitory signals in secondary lymphoid organs and in peripheral tissue by partial depletion of Tregs, is obviously associated with the activation and acquisition of effector functions by auto-reactive CD8+ T cells. Furthermore, the severity of collateral damages to normal tissues during anti-CTLA-4 therapy can be further exacerbated by direct activity of auto-reactive T cells recognizing self-antigens overexpressed by malignant cells. In this scenario, its tempting to speculate that severe side effects observed in cancer patients receiving anti-CTLA4 monoclonal antibodies may arise as a consequence of cross-reactivity or/and as a consequence of bystander activation of self-reactive CD8+ T cells.

1.2.1.1.2 PD1 : PD---L1/2 pathway.

Programmed cell Death protein 1 (PD-1; CD279) is an inhibitory receptor that belongs to CD28/CTLA-4 family of T cell co-receptors and it is critically involved in the modulation of T cell activity. Although PD-1 and CTLA-4 receptors display a similar pattern of expression, these two immunological checkpoints receptors are differentlially regulated.

Indeed, similar to CTLA-4, PD-1 receptor is constitutively expressed at high level on cellular surface of Tregs and also detectable on naïve and memory T cells following TCR-mediated activation. However, PD-1 expression on activated T cells is regulated at transcriptional level, thus its expression on cellular surface is delayed (12hours) as compared to that of CTLA-4 receptor[10,18,19]. Interestingly, engagement of PD-1 receptor on different T cell subsets produces distinct effects. Indeed, PD-1 signaling has a pivotal role in promoting the survival and the immunosuppressive function of CD4+ Tregs through the up-regulation of phosphatase and tensin homolog (PTEN) molecule and by sustaining the expression of forkhead-box-protein p3 (FoxP3) transcription factor[29]. In contrast, engagement of PD-1 receptor expressed by activated T cells results in their progressively reduced proliferative capacity and effector cytokines production (T cell exhaustion) and might ultimately lead to clonal deletion of specific T cell [30,31]. Furthermore, progressive reduction of T cell effector functions by PD-1 receptor engagement is associated to the recruitment on its intracellular domain of inhibitor phosphatases (SHP-2, PP2A), which in turn abrogates kinases signals derived by TCR and CD28 co-stimulatory receptor triggering[19,32]. In contrast, PD-1-mediated induction of T cell apoptosis has been correlated to direct inhibitory effects on the expression of anti-apoptotic molecules such as BCL-$_{XL}$[19,32-34]

So far, two PD-1 ligands, PD1-ligand 1 (PD-L1; CD274, B7-H1) and PD1-ligand 2 (PD-L2; CD273, B7-DC) have been identified. These two ligands belong to B7 family and arise from gene duplication as suggested by their 37% sequence homology. Nevertheless, PD-L1 and PD-L2 display a distinct spectrum of expressions and regulation. In particular, PD-L1 is expressed on cellular surface of hematopoietic, stromal and endothelial cells in response to interferon-© (IFN-©) produced by activated T cells. In contrast, PD-L2 is prevalently detected on antigen presenting cells (APCs) and its expression is regulated by IFN-© and to a much greater extent by interleukin-4 (IL-4). In view of the broad distribution of the ligands and to the relatively delayed expression of PD-1 on activated T cell as compared to CTLA-4 receptor, PD-1 : PD-L1/PD-L2 pathway has been indicated as the major regulator of T cell

activity in peripheral tissues during inflammatory responses as suggested by the insurgence of milder autoimmune disease in PD-1 deficient mice[10,35].

Among the different immune suppressive mechanisms promoting tumor escape, dis-regulation of PD-1 : PD-L1/PD-L2 inhibitory axis has been extensively indicated as a critical determinant promoting cancer progression. In particular, expression of PD-1 receptor on cellular surface of a significant fraction of tumor-infiltrating T-lymphocytes (TILs) has been

reported in several solid tumors and in particular for melanoma patients[7]. Expression of PD-1 receptor on TILs is related to intrinsic regulation patterns and further promoted by the immunosuppressive tumor microenvironment (TME). Indeed, the extent of PD-1 receptor expression and thus its immunosuppressive activity is directly associated to the chronic low affinity recognition by tumor-reactive T cells of their cognate antigens but also by the marked dominance of immunosuppressive factors in the tumor microenvironment (TME) including transforming growth-factor ® (TGF-®) and interleukin-10 (IL-10)[36-38]. In addition, cancer progression as a consequence of a dis-regulation of PD1 : PD-L1/PD-L2 pathway, is also associated to the extensive expression of PD-1 ligands on cellular surface of malignant and tumor infiltrating immune suppressive cells. In particular, up-regulation of PD-L1 and PD-L2 on tumor cells surface has emerged as an intrinsic as well as adaptive mechanism underlying immune resistance to endogenous tumor-specific immune responses. Intrinsic immune resistance is referred to the up-regulation of both ligands on cellular surface of malignant cells because of genetic instability of cancers. Indeed, the constitutive expression of PD-L1 and/or PD-L2 has been reported in different solid tumors and hematological malignancies as a consequence of the activation of specific signaling pathways associated to neoplastic transformation and/or chromosomal re-arrangement. In contrast, adaptive immune resistance is referred to the active expression of PD-1 ligands and particularly PD-L1, on cellular surface of tumor cells in response to IFN-© production by infiltrating NK, activated CD4+ T helper
cells and CD8+ T cells[38-40]. Based on this background and further stimulated by the objective clinical responses observed in Ipilimumab-based treatment and by the milder and less frequent autoimmune side effects observed in preclinical studies, generation and evaluation of therapeutic potential of monoclonal antibodies targeting PD-1 : PD-L1/PD-L2 pathway in cancer patients has been recently investigated.

Two fully human IgG4 monoclonal antibodies targeting PD-1 receptor, Nivolumab (BMS-936558; Bristol-Myers Squibb, ONO Pharmaceuticals) and Pembrolizumab (MK-3475; Merck) have been recently FDA-approved as first-line of therapy for advanced unresectable melanoma and non-small-cell lung cancer [41-44]. The FDA approval of these two anti-PD-1 monoclonal antibodies for cancer treatment is based on the striking results observed in randomized phase III clinical trials. In particular, Nivolumab-based therapy resulted in an objective response rate of 40% and 20%, respectively, for advanced melanoma and NSCLC patients whereas only in 13.9% and 9% of melanoma and lung cancer patients dacarbazine or docetaxel based therapies resulted in objective clinical responses[41,42]. Therapeutic potential of

anti PD-1 monoclonal antibodies for unresectable stage III-IV melanoma has been recently

confirmed by the results obtained by Robert C. and co-workers[44]. Notably, PD-1 inhibition resulted in a significantly prolonged progression free-survival as compared to CTLA-4 blockade. Indeed, the estimated 6 months progression free-survival rates were 47.3% and 46.4% for those patients receiving Pembrelizumab every 2 or 3 weeks respectively, whereas Ipilimumab based therapy resulted effective only in 26.5% of treated patients[44].

The marked clinical efficacy of monoclonal antibodies targeting PD-1 inhibitor receptor has represented a critical element promoting the further evaluation of antitumor activity and immune correlates in different cancer types of drugs interfering with PD1: PD-L1 inhibitory axis. In this regard, encouraging results have been recently reported from clinical trials designed in order to evaluate therapeutic potential in different epithelial cancers such as head and neck squamous carcinoma, renal, lung, ovarian, gastric and colorectal cancer of monoclonal antibodies targeting PD-L1 [42,45,46].

In addition to their antitumor activity and in line with results obtained in animal models, monoclonal antibodies targeting PD-1 inhibitory pathway are also characterized by a reduced incidence of drug-related adverse events. A parallel clinical evaluation of antitumor activity and side effects associated to Ipilimumab and Pembrelizumab (Robert C NEJM 2015) treatments in advanced melanoma patients clearly indicates a reduced insurgence of fatigue, nausea and pruritus as treatment-related adverse events of grade 3-5 severity in Pembrelizumab group (10.1%) as compared to patients receiving Ipilimumab (19.9%). Furthermore, safety of Pembrelizumab based therapy was also reinforced by the specific evaluation of immune related adverse events (irAe) associated to the distinct treatments. In this regard, although intravenous administration of Pembrelizumab or Ipilimumab resulted in a different pattern of irAe probably reflecting a different mode of action, both monoclonal antibodies resulted in the insurgence of colitis. However, only 2.5% of Pembrelizumab-treated patients experienced immune related colitis whereas the insurgence of this side effect was detected in about 7% of advanced melanoma patients receiving intravenous administration of Ipilimumab[44]. Insurgence of milder toxicity as a consequence of therapeutic targeting of PD-1 inhibitory pathway has been recently confirmed by clinical data obtained in anti-PD-L1 monoclonal antibodies based therapy. In particular, cumulative resulted obtained in different phase I dose escalating clinical trials showing that adverse events associated to intravenous administration of MPDL-3280A, were mostly limited to the first cycle of therapy and did not require medical treatment[42,45,47].

The immune characterization of patients receiving monoclonal antibodies targeting PD-1: PD-L1 pathway critically contributes to increase our knowledge on the role of this inhibitory axis during cancer progression. In particular, initial studies reported discordant observations particularly concerning tumor PD-L1 status and patient prognosis. Indeed, in different studies immunohistochemical analysis of PD-L1 expression within tumor masses was initially reported as having no impact on the survival of tumor bearing patients. However, recently published observational and clinical studies on the prognostic role of PD-L1 in cancer patients are in line with the inflammatory tumor model. In this regard, PD-L1 overexpression on tumor cells appears to be associated to a high infiltration by IFN-© producing T cells. However, clinical efficacy of anti-PD-L1 based therapy correlates to ligand overexpression on recruited immune cells including myeloid derived suppressor and canonical dendritic cells. In view of this specific pattern of expression, it is tempting to speculate that PD-1 : PD-L1 pathway promotes tumor escape by modulating at different levels tumor specific cellular responses. In particular, PD-L1 overexpression by tumor cells may represent a first line of defense against initial host protective immune response whereas ligand expression on immune cells may be considered as an additional layer of local
immunosuppression exploited by solid tumors in order to bypass T cell responses[10,38,39,48].

1.2.1.1.3 Immune Checkpoints Blockade: Conclusions

Clinical benefits observed in tumor bearing patients receiving monoclonal antibodies (mAbs) targeting immune checkpoint receptors and ligands, have opened a new era for cancer immunotherapy and, most importantly, further confirmed how immune system harnessing against cancer represent more than an attractive idea. However, critical considerations also arise from the recent clinical and preclinical studies. Indeed, the insurgence of considerable immune related adverse events (irAEs) particularly in cancer patients receiving CTLA-4 blocking antibodies and objective therapeutic effects only against solid tumors characterized by high mutational load (Restifo and Rosenberg C.Cell 2015) still represent major limitations of immune checkpoint blockade based therapies. In this regard, a detailed characterization of the immune effects in cancer patients receiving mAbs targeting CTLA-4 receptor and PD-1 inhibitory axis represents a critical step for a further exploitation of this immunotherapeutic strategy. Interesting, recent clinical evidences have clearly pointed out how survival benefits

observed in cancer patients responding to CTLA-4 blockade based therapy are related to “de-

novo" generation of tumor specific T cell responses whereas clinical benefits as a consequence of PD-1 : PD-L1 pathway inhibition are associated to the re-activation of a pre-existing antitumor immunity. Based on these observations, the rationale supporting the initial clinical evaluation of monoclonal antibodies targeting immune checkpoints has been recently revised. In particular, it is now becoming evident that clinical efficacy of cancer therapies based on intravenous administration of mAbs targeting CTLA-4 receptor or PD-1 pathway is not associated to a general activation of T cell responses but is related to their ability to promote respectively the priming of naïve or the restoration of memory activity only of tumor-reactive CD8+ T cells. In line with these distinct effects on naïve and memory CTLs, in patients receiving anti CTLA-4 mAbs, tumor shrinkage is usually delayed and often preceded by an increase of tumor mass whereas in patients receiving mAbs inhibiting PD-1 : PD-L1 pathway tumor regression is commonly rapid and mostly detectable already at the first therapy response assessment. These observations confirm, one more time, the ability of the immune system to generate potentially protective T cell responses against tumor. Furthermore, in view of the durable clinical responses observed in responding patients receiving mAbs targeting immune checkpoints and in particular PD-1 receptor, a critical determinant dictating the outcome of mAbs based therapies is represented by the generation and maintenance of long-lasting (memory) tumor-reactive CD8+ T cells[26,28,45,46].

1.2.1.2 Adoptive cellular therapy (ACT) for cancer treatement.

Identification of interleukin-2 (IL-2) as a T cell growth factor, and its therapeutic effect observed upon intravenous administration in tumor bearing mouse can be considered as the first indirect proof of T cells capacity to mediate tumor-regression. This initial observation was then reinforced by the results obtained in pioneering studies of adoptive cell transfer (ACT) performed in animal models. In particular, the effectiveness of in vitro IL-2 expanded tumor-infiltrating lymphocytes (TILs) to mediate, upon re-infusion in singenic mice, regression of established tumors was formally demonstrated. In the last decades, these findings in murine models have been successfully confirmed also in cancer patients. Indeed, the clinical efficacy of adoptive cellular therapies based on re-infusion of large numbers of in vitro-expanded TILs has been consistently reported [49-51].

Initial evidence of therapeutic potential of ACT strategies for cancer treatment has been provided in 1994 when the results obtained from 86 metastatic melanoma patients enrolled in a clinical trial were reported. In particular, TILs used in this clinical study were obtained by enzymatic digestion of tumor specimens and in vitro expanded by sequential serial passage in 6000 IU/ml of IL-2 until an average of $1x10^{11}$ of lymphocytes was obtained. TILs generated with this protocol were then intravenously administered to cancer patients in combination with high dose of IL-2 (720.000 IU/kg). A relatively low responses rate (34%), comparable to studies in which dacarbazine and IL-2 cytokine were administered alone or in combination to metastatic melanoma patients, was observed in this first trial. However, this initial study clearly indicates also the clinical effectiveness of immunotherapies strategies based on adoptive transfer of tumor-reactive T cells[51,52]. Furthermore a retrospective analysis, comparing responder versus non-responder patients enrolled in this clinical trial, has provided crucial informations influencing the design of subsequent anti-cancer adoptive therapies. In this regard, objective clinical responses were preferentially observed in those patients receiving TILs generated from subcutaneous lesions (49%) as compared to melanoma patients receiving TILs obtained from lymph nodes (17%), thereby suggesting that suitable tumor-reactive T cells were localized, mostly, within tumor mass. In addition, anti-cancer efficacy of TILs was inversely correlated with the time required for their in vitro manufacturing and resulting in a reduced survival and expansion of infused cells. Based on these initial findings, different protocols aiming to identify suitable target antigens as well as the generation of TILs displaying higher persistence and reduced senescence have been extensively evaluated.

An initial attempt performed in order to select effective anti-tumor reactivity within the bulk of TILs cultures was represented by the introduction of a selective screening of in vitro expanded lymphocytes before re-infusion. In particular, after initial expansion of 21-36 days in presence of high doses of IL-2, individual TILs cultures, upon enrichment for CD8+ T cells, were selected for large-scale production according to their capacity to produce IFN-© upon co-culture with autologous or HLA-matched established melanoma cell lines. Notably, the introduction of an enzyme-linked immunosorbent assay (ELISA) for the selection of tumor-specific CD8+ T cells represents the basis of clinical success of "selected TILs protocol" as a cancer immunotherapy for metastatic melanoma. Indeed, cumulative results

from different trials indicate how re-infusion of selected TILs resulted in objective and durable clinical responses in 50% of metastatic melanoma patients[51]. A major limitation of ACT therapies based on selected TILs protocol is represented by the complexity of the

methodology resulting in the generation of sufficient numbers of TILs only in a limited

percentage of patients undergoing through surgical excision of melanoma lesions. In addition, a limited reproducibility of the in vitro expansion of autologous lymphocyte cultures obtained from tumors of different origin still represents a limitation preventing the widespread application of ACT for treatment of other cancers. Based on these observations and with the aim to expanding clinical efficacy of ACT-therapies to a broaden cohort of melanoma and also other cancer patients, different techniques have been developed. In particular, genetic re-targeting of peripheral blood lymphocytes and protocols based on a reduced in vitro manipulation of TILs ("young TILs") have been extensively explored[35,53].

The introduction, through lenti or retroviral vectors, of genes encoding for tumor-reactive conventional alpha-beta TCR or chimeric antigen receptors (CARs) in autologous T lymphocytes has extensively been indicated as a powerful resource for cancer treatment based on ACT. The first clinical evidence on the effectiveness of adoptive cell therapy based on the re-infusion of engineered T cells was obtained from the induction of objective clinical responses with manageable toxicity in two out of 17 metastatic melanoma patients receiving autologous T lymphocytes expressing a α/β TCR recognizing MelanA/Mart-1 melanoma differentiation antigen cloned from a melanoma patients enrolled in a previous trial[54]. In line with this observation, in a subsequent clinical study objective clinical responses were observed in 30% (6/20) and 19% (3/16) of metastatic melanoma patients receiving respectively engineered T cells targeting with high avidity MelanA/Mart-1 or gp100 HLA-A0201-restricted epitopes. However, unwanted and most importantly non-negligible side effects related to the infusion of engineered T cells recognizing with high avidity tumor shared antigens were also observed in treated patients. In particular, destruction of normal melanocytes in the skin, eyes and inner ear was directly correlated with the insurgence of vitiligo, eye toxicity and hearing loss on treated patients[55]. The insurgence of toxicity related to the recognition of minimal amount of cognate antigen on cellular surface of normal cells (on-target toxicity) has been reported, to different extents, in several clinical trials and is widely considered as a formal proof of anti-tumor efficacy of adoptive cellular therapies. However, the onset of life-threatening side effects has been also described in patients receiving engineered T cells recognizing with high avidity non-mutated self-antigens expressed also on normal tissue[56-58]. In line with this observation, the needs for improved methods in the selection of suitable target antigens have been extensively indicated as a critical determinant dictating the outcome of adoptive cellular therapies. In line with this consideration, autologous T cells have then been re-targeted against cancer-testis antigens (CTA) and/or mutated antigens selectively expressed by malignant cells. In this respect,

controversial results have been obtained. In particular, adoptive cellular therapies based on autologous lymphocytes expressing conventional α/β TCR recognizing with high affinity cancer testis antigens (CTA) have shown objective clinical responses in metastatic melanoma (5/10 OR) but resulted also in the death of four out 11 treated patients. Remarkably, patient death was attributed to neurotoxicity related to the, initially unrevealed, expression in the grey matter of genes belonging to MAGE family of cancer-testis antigens. In addition to on-target toxicities against normal cells, the insurgence of fatal cardiogenic shock has been also reported and associated to an unexpected cross-reactivity of affinity-enhanced TCR against HLA-A01-restricted MAGE-A3 with an unrelated peptide derived from titin protein expressed in cardiac muscle cells (off-target toxicity)[56-58]. In line with these observations, similar results were obtained from cancer patients receiving antitumor re-targeted autologous T lymphocytes through the introduction of chimeric antigen receptors (CARs). A CAR structure is composed of a single-chain variable fragment (scFv) of a monoclonal antibody fused with a spacer domain and intracellular T cell signaling domains derived by CD3-zeta chain, CD28 and CD137 (4-1BB). The rationale supporting the development of CARs was represented by the attractive idea to simultaneously confer an antibody-like tumor-specificity to transduced T cells and to further promote their production of effector and cytotoxic molecules. Technological advantage of CARs was based on their capacity to allow the recognition of target antigens in an MHC class I unrestricted manner thereby overcoming tumor immunoevasion associated mechanisms such as downregulation of peptide-HLA complexes and/or to alteration of antigen presenting machinery of tumor cells and, most importantly, to the possibility to apply ACT to all cancer types[59-61]. Clinical effectiveness of ACT based on anti-CD19 CAR has been extensively reported and resulted in 2010 in the FDA approval for treatment of B-lymphomas[53,62,63]. Although in vitro studies and pre-clinical studies in murine models have indicated a marked antitumor activity for T cells expressing chimeric receptors targeting antigen overexpressed by transformed cells, so far, a limited clinical effectiveness has been observed in patients bearing solid tumors. In addition, clinical application of CARs technology for treatment of solid tumor is further restrained by the objective difficulty in the identification of target antigens selectively express on tumor cells or alternatively detectable only in nonessential tissues. Indeed, the insurgence of severe side effects related to on-target activity of CAR-T cells against normal cells has been described particularly in colorectal and renal cell carcinoma patients receiving transduced lymphocytes expressing chimeric receptor targeting tumor shared antigens such as carcynoembrionic

antigen (CEA), receptor tyrosine-protein kinase ERBB2 antigen or carbonic anhydrase 9 antigen[57,64].

The insurgence of severe side effects associated either to on-target and/or off-target toxicities still represent a major limitation restraining clinical application of engineered T cells for treatment of solid tumors. Different strategies have been studied in order to prevent the insurgence of ACT-related immunopathology. In particular, selective expression, on/in engineered lymphocytes of CD20, truncated EGFR and thymidine kinase derived from herpes simplex virus has shown promising results by rendering infused cells sensible to cytotoxic effects of clinically approved monoclonal antibodies and anti-viral therapies[65].

An alternative strategy that has been extensively explored in order to reduce the high dropout rate associated to standard selected TILs protocol for melanoma treatment is represented by the "young TILs" methodology[35,66,67]. Generation of younger lymphocytes has been initially prompted by the necessity to simplify the laboratory procedures for the generation of selected TILs. In this regard, failure in the generation of autologous tumor cell line and limited availability of established melanoma cell lines expressing less frequent HLA alleles have been traditionally ascribed as critical limitations of selected TILs protocol. Furthermore, initial clinical evidences suggested that extended culture times might potentially restrain their antitumor efficacy by reducing their survival upon in vivo re-infusion. In this scenario, clinical effectiveness of unscreened, minimally cultured TILs has been extensively reported. In particular, cumulative results obtained from different trials indicate that young TILs protocol resulted in the eligibility of nearly 90% patients undergoing through surgical excision of melanoma lesions and resulted, upon re-infusion, in the induction of objective and durable clinical response in almost 50% of treated patients. Furthermore, clinical experience based on young TILs protocols has contributed to the identification of critical determinants regulating antitumor potential of ACT strategies. In this respect, clinical relevance of neo-antigens and the impact of differentiation status of re-infused TILs have been initially suggested by the results obtained in different trials in which melanoma patients received younger lymphocytes[35,66,67].

The suggestion that tumor specific mutations might be suitable targets for cancer therapies has initially been provided by the inherent genetic instability of transformed cells and further restated by the increased immunogenicity of tumor cells displaying a higher mutation rate. In this respect, an increased interest for nonsynonymous mutations has been recently registered among cancer immunologists. Indeed, emerging data from exomic

sequencing of different tumor types have indicated either a high frequency of nonsynonymus mutations in epithelial cancers and, most importantly, that these exomic alteration might results in the creation of new epitopes recognized with high avidity by naturally arising T lymphocytes. Initial evidences suggesting a correlation between recognition of neo-antigens and induction of cancer regression have been provided by some trials in which tumor reactivity of in vitro expanded autologous T lymphocytes was evaluated in response to the recognition of autologous melanoma cell lines and/or HLA-matched cell lines expressing defined differentiation antigens and/or cancer-testis antigens CTA. In particular, the selective production of IFN-© in response to autologous tumor cells and not against established cell lines observed in a consistent fraction of TILs, obtained from responder patients, suggested that cancer regression can be driven by the recognition of patients specific tumor mutated antigens. The formal proof that nonsynonymous mutations, more than epigenetic changes triggering the expression of CTA, might be targets of T recognition of tumor cells, resulting in the induction of objective and durable clinical responses has recently been provided. Indeed, it has been shown that clinical efficacy of TILs re-infused in melanoma patients is mostly

associated to the recognition of random somatic mutations in the cancer[27,67,68]. Despite identification of nonsynonymous mutation has restated the therapeutic potential of tumor-reactive T cells and might potentially overcome the insurgence of side effects commonly observed by targeting shared antigens, critical theoretic and pratical considerations still prevent the widespread application of ACT strategies against neo-antigens for cancer treatment. Indeed, high frequency of mutations has been identified only in specific cancer types including melanomas, renal and lung cancer. Furthermore, whole-exomic sequencing of melanoma tumor has revealed that, only a limited number of nonsynonymous mutations can efficiently result in the generation of immunogenic peptides presented in the contest of MHC class I and class II molecules. In addition, despite considerable efforts by the scientific community, the identification of clinically relevant mutations to target during ACT therapies is still complicated. Indeed, algorithms predicting peptide binding to patient specific MHC molecules are not yet reliable in particular for less frequent MHC alleles. In contrast, generation of strings of minigenes encoding each mutated amino acid flanked by 10-12 amino acids has been indicated as an efficient approach allowing the precise screening of patient specific TILs reactivity regardless of their HLA restriction. However, generation of these DNA constructs still appear far from routine application and has so far been clinically evaluated only in melanoma patients[27,50,69].

In addition to critical information concerning tumor-reactivity, clinical experience with "young TILs protocol" has further contributed to underline the relevance of intrinsic properties of infused CD8+ T cells in dictating the clinical success of ACT-based strategies. Adoptive cell therapies have been extensively defined as "living" treatment based on the capacity of infused TILs to expand and, most importantly, to long-term survival. Objective clinical responses have been extensively correlated to TILs persistence one month after transfer and durable clinical benefits associated to their persistence at later timepoints. In this respect, clinical effectiveness of current ACT protocols has been significantly associated to patient pre-conditioning through administration of lymphodepleting nonmyeloablative chemotherapy (NMC) and total-body irradiation. The rationale supporting the pre-conditioning of the host is represented by the necessity to overcome the global immunosuppression of late stages cancer patients. However, immunodepleting chemotherapy with cycloposhamide (60mg/kg) and fludarabine (25 mg/m^2) alone or in combination with 200 or 1200 centigray total-body irradiation before the adoptive transfer of highly selected tumor reactive CD8+ T cells resulted in an increased, but not significant, overall response rate[51,70]. These clinical evidences indicate that although lymphodepleting preparative regimens may enhance antitumor efficacy by promoting the depletion of immunosuppressive cells (Tregs, MDSCs), augmenting the availability of specific homeostatic cytokine (IL-15), induction of durable responses is also affected by functional properties of infused T cells. In line with this consideration, it has been reported in different trials in which patients received TILs in vitro expanded either with the standard or young protocols, that objective and, most importantly, durable clinical responses were observed in patients receiving less differentiated tumor-reactive lymphocytes. Indeed, durable clinical benefits were correlated with the percentages of CD8+CD27+ and length of telomeres of infused TILs and associated with their long-term persistence[49,51,70,71].

1.2.2 Active Immunotherapeutic strategies: Cancer Vaccines

Active immunotherapies strategies rely on different vaccine formulations designed to amplify pre-existing, or, alternatively, prime tumor-reactive CD8+ T cells mediating elimination of transformed cells, and CD4+ T cells sustaining, through the production of cytokines, expansion and acquisition of effector functions by cytotoxic T lymphocytes.

Anti-cancer vaccines have traditionally been classified as therapeutic and preventive. Therapeutic vaccines are administered in order to promote regression of existing cancer whereas the clinical relevance of preventive vaccines is related to their capacity to prevent tumor occurrence [72-75].

1.2.2.1 Preventive cancer vaccines

In line with the clinical effectiveness against infectious agents, prophylactic vaccines preventing cancers of viral origin such as liver, hepatocellular carcinoma (hepatitis B virus; HBV) and cervical cancer (human papilloma virus; HPV) have entered routine clinical practice. In particular, therapeutic potential of all the licensed preventive cancer vaccines targeting oncoviruses relies on their unique capacity to promote the generation of virus-specific humoral and cellular responses preventing HPV and HBV infections[73].

1.2.2.2 Therapeutic cancer vaccines

In addition to protective potential in preventive regimens, vaccines targeting human papilloma virus have also shown a marked clinical efficacy as therapeutic approach for cancer treatment. In particular, sub-cutaneous immunization with a mix of synthetic long-peptide

encompassing different MHC-class I and class II restricted epitopes derived from early HPV-E6 and -E7 antigens, may result in objective and long-lasting cancer regression[76]. Since viruses represents the underlying cause in approximately only 10% of all cases of cancer[73], the latter observation has critically contributed to generate a renewed interest for the design of cancer vaccines aiming to instruct CD8+ T cells to specifically target non-viral tumor associated and/or specific antigens. In particular, different strategies based on (i) peptides, (ii) proteins, (iii) whole cells and (iiii) viral vectors have been developed with the aim of promoting antitumor immunity by modulating the antigen presenting capacity of dendritic cells[74,75]. In the last decade encouraging and objective clinical responses have been observed in cancer patients, leading in 2010 to FDA approval of Sipuleucel-T (Provenge) for treatment of metastatic castration-resistant prostate cancer (mCRPC)[77].

Initial attempts to promote the in vivo generation of a specific immune response against cancer were based on the administration of MHC-class I restricted peptides derived from identified tumor antigens. In this respect, initial results obtained upon clinical application of epitope-based cancer vaccines indicated limited therapeutic benefits in treated patients and infrequent expansion of CD8+ T cells recognizing the selected tumor antigens. The limited effectiveness of these initial trials has been related to our poor understanding of the biology of dendritic cells. Indeed, administration of peptide-based cancer vaccines in these pioneering studies was often performed without an effective dendritic cell-activating adjuvant and therefore immunization with peptides was mostly associated to the induction of tolerance more than cellular immunity against selected epitopes[74,78,79]. Based on these initial observations, considerable efforts have been done to overcome this initial limitation of immunization protocols and different adjuvants increasing either the half-life of administered free-peptides as well as the activation of dendritic cells have been identified for clinical application. Therapeutic potential of peptide-based cancer vaccines has been recently restated by the results obtained in a multicenter phase III clinical trial involving 185 patients with stage IV or locally advanced stage III cutaneous melanoma. In this study melanoma patients were randomly assigned to receive standard IL-2 therapy alone or in combination with a therapeutic vaccine composed of $gp100_{209\text{-}217}$ short peptide plus incomplete Freund's adjuvant. Notably, co-administration of vaccine plus IL-2 resulted, as compared to IL-2 monotherapy, in an increased overall response rate (16% vs 6%) as well as an increased overall survival (17.8 months vs 11 months)[80]. Although initial limitations, associated to poor immunization induced by single epitope, were overcome by the introduction of adjuvants as core element of these therapeutic vaccines, critical determinants prevents the widespread

application of these methodology for cancer treatment. In particular, the application of epitope-based vaccines is mostly limited to subsets of patients expressing frequent HLA-alleles and further restrained among these groups by the ability of transformed cells to prevent CD8+ mediated immune recognition through antigen mutation and loss. In addition, single short-peptide based vaccines are unable to promote the activation of CD4+ T cells, required for the establishment of anticancer long-lasting immunity. Therefore, different immunization protocols, based on the administration of proteins, tumor cells (or their lysat), in vitro pulsed dendritic cells and viral vector, have been extensively evaluated for cancer treatment. The use of full-length proteins as targets for cancer vaccines has initially been promoted by the necessity to provide, in a HLA-independent manner, a broad panel of epitopes that might be presented, in the contest of MHC class I and class II molecules, on cellular surface of dendritic cells. However, only a limited clinical effectiveness has been traditionally observed in treated patients. Indeed, different phase III clinical trials involving non-small cell lung cancer or B-lymphoma bearing patients receiving respectively full MAGE-3 and individual idiotype protein have failed to reveal a clinical benefit associated to the vaccination[74].

An alternative strategy of similar immunotherapy is represented by the use of cell-based vaccines. In this regard, immunization protocols based on the sub-cutaneous injection of autologous or allogeneic tumor cells or alternatively in vitro antigen loaded dendritic cells have shown opposite results. Therapeutic potential of whole tumor cells as a cancer vaccine was initially associated to the possibility to simultaneously elicit the immune response CD4+ and CD8+ mediated against undetermined but patient's specific antigens. In this respect, pioneering studies have shown a significant immune response in pancreatic and colorectal cancer patients receiving respectively GM-CSF-transduced or irradiated autologous tumor cells. In particular, in two small trials, administration of whole cell based vaccines has been associated to the induction of tumor-reactive CD8+ T cells in 3 out of 14 pancreatic cancer[74,81]. In addition to crucial information concerning the immune-related effects of tumor-cell based vaccines, their clinical relevance has been stated in a phase III trial (ONCOVAX) involving 254 colorectal cancer patients receiving, following cancer resection, irradiated autologous tumor cells demonstrating a 61% risk reduction for recurrence and a significantly longer recurrence free period[82]. Based on these promising results and the difficulty to obtain autologous tumor cells for immunization protocols, allogeneic tumor cell lines have been clinically evaluated as therapeutic cancer vaccines. However, evaluation of this therapeutic approach has not revealed, so far, the induction of clinical benefits in treated patients. In particular initial phase I/II trials enrolling almost 200 prostate cancer patients indicate an

encouraging therapeutic potential for an allogeneic vaccine composed by a mixture of irradiated and GM-CSF expressing LNCap and PC3 established cancer cell lines (GVAX).Based on this background, two subsequent phase III trials were initiated but were interrupted respectively as a consequence of discouraging predictive analysis and for safety reasons[74,83].

In sharp contrast, a significantly increased overall survival has been reported for metastatic castration-resistant prostate cancer (mCRPC) patients receiving cell-based therapeutic vaccine termed Sipuleucel T (Provenge). Sipuleucel T vaccine is composed by autologous antigen presenting cells (APCs) cultured with a chimeric protein resulting from the fusion of GM-CSF with differentiation antigen prostatic acid phosphatase (PAP; GM-CSF-PAP). Currently, Sipuleucel T represents the first, and so far the only, FDA approved active immunotherapeutic approach for advanced prostate cancer patients. In particular, the license for Sipuleucel T as treatment of cancer derives from the results obtained in a double blind, multicenter phase III clinical trial involving 512 mCRPC patients. These patients were randomly assigned in a 2:1 ratio to receive either Sipuleucel T or placebo intravenously every two weeks for a total of three infusions and resulting in significant reduction of risk of death and increased median overall survival of treated patients. Indeed, for Sipuleucel T group were reported a 22% relative reduction of risk of death and a median survival of 25.8 months versus 21.7 months in the placebo group[77]. However, cancer immunologists have extensively questioned the FDA approval of Sipuleucel-T vaccine. In particular, the lack of a precise definition of infused vaccine, the use of an inadequate placebo control, limited immunological characterization of treated patients and the failure of the trial to report evidence of tumor regression or delay in disease progression have been criticized. To this respect, it must be underlined that majority of clinical trials performed are, to different extent, affected by limiting factors influencing the production of standardize products. Furthermore, the lack of tumor shrinkage is not an unusual result for immunotherapeutic approaches for cancer and it has extensively related to delayed antitumor activity of induced immune responses or alternatively to their capacity to keep malignant cells under constant restrain. These observations have promoted the recent reformulation of criteria adopted in defining effectiveness of immunotherapies strategies and have led to the restatement of overall survival as the unique arbiter of clinical success for therapeutic approaches.

Finally, an alternative strategy that has been heavily exploited for the generation of efficient therapeutic cancer vaccines is represented by the generation of recombinant viral

vectors expressing tumor-associated antigens alone or in combination with immunomodulatory molecules. In addition to logistic considerations such as reduced cost for a relative easy production, several evidences obtained in pre-clinical studies have initially prompted the use of viral vector for cancer treatment. A high immunogenicity has been traditionally ascribed for viruses and related to their recognition through toll-like receptors (TLRs) expressed by host-immune cells and resulting in the generation of a pro-inflammatory environment. In addition, in different studies it has been reported how infection of dendritic cell with recombinant viral vectors expressing a transgene encoding a tumor associated antigen resulted in an increased expansion of cognate CD8+ T cells leading to the elimination of malignant cells expressing the tumor antigen encoded by the viral vector[79,84] . In line with these pre-clinical evidences, it has been extensively indicated that transgenes expressed by a viral vector are more immunogenic than protein administered with adjuvant[79,84]. Nevertheless, the production of neutralizing antibodies and the expansion of vector-reactive CD8+ T cell have initially limited the clinical effectiveness of virus-based vaccines. Indeed, it has been initially reported how the suitable induction of tumor-reactive CD8+ T cells upon vaccination in murine models and cancer patients with recombinant vaccinia virus expressing the cognate tumor associated antigen was significantly reduced. These evidences have led to the design of the heterologous prime-boost approach for clinical application of viral vector-based cancer vaccines. In the prime boost approach, tumor- specific CD8+ T cells are initially primed with a recombinant viral vector expressing the cognate antigen whereas their further expansion is guaranteed by multiple booster vaccinations induced by a different recombinant viral vector expressing the same tumor antigen or alternatively peptides and DNA constructs thereby limiting the host-neutralizing and virus-specific cellular immunity[79].

Among different viral vectors, therapeutic cancer vaccines composed by recombinant modified vaccinia virus Ankara (MAV) has shown a remarkable clinical effectiveness for treatment of advanced prostate cancer patients. In particular, in a multicenter phase II clinical trials in which patients were randomly assigned in a 2:1 ratio to receive either a recombinant vaccinia virus carrying different transgenes encoding for prostate specific antigen (PSA) along with three immunostimulatory molecules (B7.1, ICAM-1 and LFA-3; TRICOM ; PSA-PROSTVAC V) in combination with GM-CSF or an empty vector plus saline injection. After priming with PSA-PROSTVAC V patients, tumor-reactive immune responses were further stimulated with six fowlpox-based vector boosts (PSA-PROSTVAC). Despite the absence of significant differences between the two groups in terms of progression free survival, PROSTVAC VF vaccine resulted in a better overall survival (30% vs 17%) and a longer

median survival by 8.5 months (25.1 vs 16.6). Therefore, and to a larger extent than Sipuleucel T, a viral vector has been significantly associated with objective clinical benefits for treated patients. Indeed, PROSTVAC VF vaccine, as compared to cell-based vaccine, has produced in concurrent studies involving 32 mCRPC patients and in a pilot study enrolling 25 metastatic carcinoma patients, clinical evidences of post-vaccination increased T cell responses targeting respectively PSA antigen, carcynoembrionic antigen (CEA) and mucin-1 (MUC-1) and clinical benefits[85-87].

1.2.2.3 ncer vaccines; where do we stand.

In the last 60 years, the attractive idea to immunize patients against cancer has been heavily exploited and resulted in the establishment of different platforms of cancer vaccines. Despite extensive efforts by cancer immunologists and encouraging results in animal models, clinical applications of different active immunotherapies strategies including peptides, proteins, whole cells and viral-vectors based vaccines have shown, so far, a limited antitumor effectiveness. A major limitation affecting the impact of different active immunotherapeutic strategies is represented by the late time of intervention. Indeed, late-stages cancer patients have been mostly involved in clinical trials. In these patients, a global immunosuppression characterized by the exhaustion of tumor-reactive T cells and further exacerbate by the establishment of a corrupted tumor microenvironment have been associated to the failure of therapeutic vaccines in clinical settings. In this scenario, despite hundreds of vaccine strategies evaluated, only few have shown clinical efficacy in phase II/III trials. The better/increased overall survival observed in cancer patients receiving respectively Sipuleucel-T and PROSTAVAC VF vaccines represent an objective achievement and underline the clinical effectiveness of active immunotherapeutic strategies.

In this regard, further studies are urgently required to elucidate the induction and the infiltration of tumor reactive CD8+ T cells within tumor mass in vaccinated patients in order to formally correlate therapeutic potential of cancer vaccines with their capacity to instruct the immune system to eliminate malignant cells. Beside practical advantages and reduced invasiveness/morbidity in comparison to passive strategies, cancer vaccines can potentially results in the generation of effective and long-lasting protection against cancer by promoting the generation of effector and memory-tumor reactive CD8+ T cells.

1.3. Primary and Memory CD8+ T cell responses

Generation of antigen specific $CD8^+$ T cells is crucial for the control of a variety of bacterial and/or viral infections and mostly for the eradication of malignancies. As a consequence of an infection or upon vaccination, the immune system responds by generating a primary immune response with the aim to control the infectious agent. Classically, CD8-mediated primary immune response consist of three distinguishable phases: a) expansion phase b) contraction phase c) memory maintenance phase (**Fig.1**)[88].

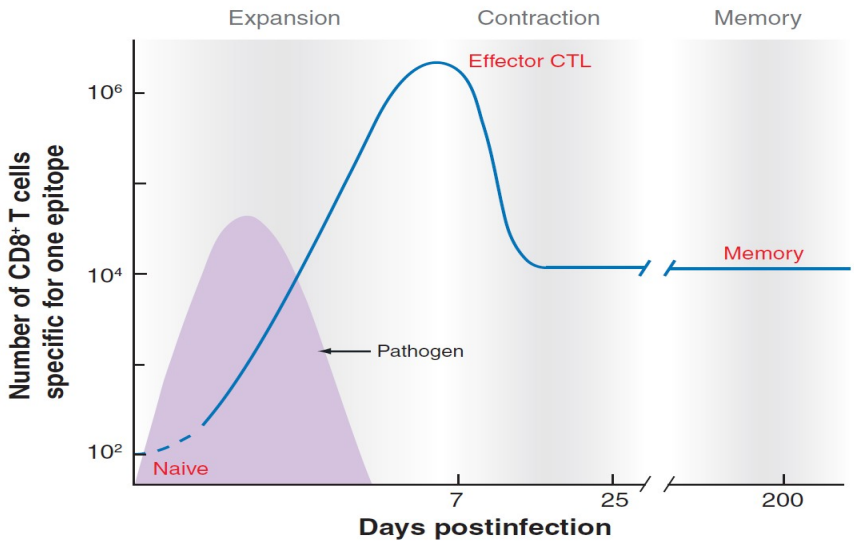

Figure 1. Kinetic of CD8-mediated primary immune response. Upon antigen recognition, antigen specific naïve $CD8^+$ T cells originate a burst of effector cells. After pathogen clearance, only a minor fraction of effector $CD8^+$ T cells (5-10%) survive and differentiate into memory cells conferring a long lasting protection to the host upon re-exposure to the same pathogen.

(modified from Effector and Memory CTL differentiation. Williams M.A. and Bevan M.J. Annu.Rev.Immunol.2007. 25: 171-92.

The frequency of naïve $CD8^+$ T cell specific for a given antigen has been reported to be in the range of 1 in 100.000. However, upon appropriate antigen recognition, these antigen reactive $CD8^+$ T cells undergo a massive clonal expansion resulting in the generation of 50.000 daughter cells. Remarkably, during expansion, naïve $CD8^+$ T cells differentiate into effector cytotoxic T-lymphocytes (CTLs) able to migrate to infected sites where, through the production of cytotoxic molecules (perforin and granzymes) and cytokines (IFN-γ and TNF-α), they can mediate the elimination of infected cells. Once the elimination of the pathogen/antigen source is completed, the population of antigen specific CTLs undergo a rapid contraction phase and the large majority of effector cells (90-95%) die by apoptosis. Only a small fraction of CTLs (5-10%) survive to this contraction phase and further mature

into memory $CD8^+$ T cells. This memory $CD8^+$ T cells are then maintained in antigen-independent but cytokines dependent manner. To this respect, a pivotal role for the common-γ-chain cytokines such as IL-7 and IL-15 in promoting memory $CD8^+$ T cells survival and proliferation, has been extensively reported[88-90].

The generation of immunological memory during a primary immune response is the hallmark of the adaptative immune system and leads, over many decades, to the host protection in case of re-exposure to the same infectious agent. Indeed, memory $CD8^+$ T cells in comparison to naïve T cells display a higher efficacy to control the secondary exposure to a pathogen. Notably, the superior host protection mediated by memory $CD8^+$ T cells is related to their higher frequency, increased capacity to proliferate and to their enhanced capacity to rapidly acquire effector functions and to generate a secondary burst of effector CD8+ T cells upon antigen recognition [88,91,92].

1.3.1 Heterogeneity of memory CD8+ T cell compartment.

Identification of memory $CD8^+$ T cells was initially based on the selective expression of specific surface molecules including CD27, LFA-1 (CD11a), LFA-3 (CD58) and the low molecular weight protein product of splice variants of CD45 gene, CD45RO [93-95].

Today, it is widely accepted that memory pool is composed by different subsets of $CD8^+$ T cells that can be distinguished according to the expression of unique combination of surface and intracellular markers associated to distinct cellular functions as well as distinct anatomic localizations [96-98].

The initial evidences on the heterogeneity of CD8 memory compartment were provided by *Sallusto et al.*[99] and unequivocally revealed the existence of two distinct subsets. These two subsets were identified according to the selective expression of homing and chemokine receptors by $CD8^+CD45RO^+$ T cells[99]. Notably, one subset of memory $CD8^+$ T cells similar to naïve T cells, express both the lymph-node homing receptor CD62L (L-Selectin; SELL) and the T-cell zone homing CC-chemokine receptor 7 (CCR7; CD197)[100] This $CD45RO^+CD62L^+CCR7^+$ subset was subsequently defined as central memory T cells (T_{CM}) to indicate their potential to home to secondary lymphoid organs. On the other hand, the memory subset lacking on cellular surface either CCR7 or/and CD62L was instead termed

as effector memory T cells (T_{EM}) because of their preferential localization in non-lymphoid tissues[99,101].

A more detailed analysis of T_{CM} and T_{EM} further revealed how the different phenotypic profile and anatomic location of these two subsets of memory $CD8^+$ T cells also reflected distinct cellular functions. Indeed, upon *in vitro* stimulation and/or in vivo re-challenge, $CD8^+$ T_{CM} displays are characterized by a marked proliferative response supported also by the production of IL-2 but not to the release of substantial amount of effector cytokines (e.g. TNF-α and IFN-γ) and/or cytotoxic molecules (Perforin and Granzymes). In contrast, $CD8^+$ T_{EM} display a reduced proliferative response but can promptly mediates inflammatory reaction and cytotoxicity as indicated by their rapid and robust secretion of IFN-γ, TNF-α and granules containing perforin and granzymes[99,102,103].

This initial classification of memory $CD8^+$ T cells, as initially suggested by *Sallusto et al.*, was then further divided using new surface markers, leading to the characterization of additional subsets of memory $CD8^+$ T cells endowed with specific phenotypic and functional properties.

In particular, in combination with CD45RO and lymph-node homing receptors CD62L/CCR7, the selective expression of the member of tumor necrosis factor (TNF) superfamily of receptors CD95 (APO-1/Fas)[104] and CD28[105] allows the identification of three further discrete subsets of memory $CD8^+$ T cells named as stem cell-like memory cells (T_{SCM}), transitional memory (T_{TM}) and terminal effector memory $CD8^+$ T cells (T_{TE} or T_{EMRA}^+) [94,106,107] (**Fig.2**). Interestingly, the observation of a progressive reduction in telomerase length, the content of T cell receptor (TCR) excision circles (TRECs) and a parallel increased in the effector-associated genes (Granzymes, Perforin and IFN-γ) from $T_{SCM} > T_{CM} > T_{TM} > T_{TE}$, a precursor-product relationship has been proposed for these different subsets of memory $CD8^+$ T cells [103,108-110].

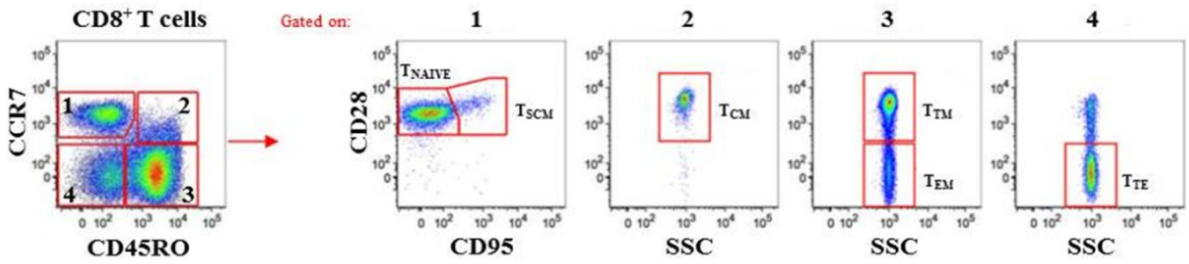

Figure 2. Heterogeneity of memory CD8 compartment. Polychromatic flow cytometry characterization of $CD8^+$ T cells isolated from peripheral blood of healthy donor. Identification of multiple subsets of memory $CD8^+$ T cells according to the selective expression of CD45RO, CCR7, CD95 and CD28.

(modified from The who's who of T cell differentiation: Human Memory T-cell subsets. Mahnke YD1, Brodie TM, Sallusto F, Roederer M, Lugli E. Eur J Immunol. 2013, 43(11):2797-809.

1.3.2. Lifespan protection: maintenance of memory CD8+ T cells.

Long-term maintenance of antigen-experienced T cells has been traditionally associated to the homeostatic signals induced by common- γ-chain cytokines. Indeed, different model of acute infection showed how survival of memory CD8+ T cells does not require a continue exposure to the cognate antigen but is mostly associated to the presence of IL-7 whereas exposure to IL-15 has been associated to the homeostatic turnover of memory T cells. Interestingly, this initial though has been recently extended in virtue of the identification of memory CD8+ T cells with stem cell-like qualities (T_{SCM}). In this scenario, it has been suggested the T_{SCM} cells can be consider like the conventional hematopoietic stem cells (HSCs), as quiescent and undifferentiated progenitors. In this regard, it has been proposed how this small subset of memory $CD8^+$ T cells, characterized by the unique capacity to self-renew, can also generate a differentiated progeny (T_{CM}, T_{EM} and T_{TE}) in response to homeostatic stimuli (IL-7, IL-15) and TCR stimulation[108,109,111].

Despite the remarkable attractiveness, the existence of a subset of memory $CD8^+$ T cells with stem-like attributes is still debated and mostly complicated by the absence of a clear anatomical relationship among memory T cell subsets[109]. The initial identification of stem cell-like memory $CD8^+$ T cells was based on the overexpression of ATP-binding cassette (ABC)-superfamily multidrug efflux protein (ABC-B1). The interest for ABC-B1 transporter was related to its ability to confer protection from toxic xenobiotics (e.g. chemotherapeutic agents) and endogenous metabolites, to canonical hematopoietic stem cells. The analysis of

ABC-B1 in memory $CD8^+$ T cells from acute myeloid leukemia (AML) patients after repeated cycles of chemotherapy, allowed the identification of a small subpopulation of T_{CM} and T_{EM} further characterized by the selective expression of CD161 (KLRB1; NKRP1A), the α-chain of IL-18 receptor (IL-18Rα) and high levels of c-kit. Interestingly, a fraction of these subpopulations of stem cell-like memory cells, specific for immunodominant epitopes derived from Cytomegalovirus (CMV), Epstein Barr Virus (EBV) and Influenza virus, were readily detectable in peripheral blood of AML patients after chemotherapy. The latter observation clearly suggested how these subsets of stem cell-like memory $CD8^+$ T cells are able to mediate host-protection against re-activation of CMV and EBV and, most importantly, their ability to mediate immune reconstitution of the memory T cell pool [112,113]. However, the stemness of this chemotherapy-resistant population of $CD8^+$ T cells has been then questioned as a consequence of their further characterization. Indeed, it was then reported that these $CD161^+$ IL-$18R\alpha^+$ memory cells also express characteristic features of terminally differentiated $CD8^+$ T cells such as KLRG1, Blimp-1, shorter telomeres and reduced telomerase activity [114]. In addition, the analysis of TCR repertoire revealed a marked abundance of the T cell receptor chain $V\alpha\ 7.2^+$ indicating that these cells are mostly mucosal associated invariant T cells (MAITs) rather than canonical T cells with stem cell-like properties[108].

This initial failure in the identification of T_{SCM} has been apparently overcome by the work of *Gattinoni et al.*[108] describing, in the peripheral blood of healthy donors, the existence of discrete population of memory T cells that, *in vitro*, was shown to be self-renewing and multipotent. In particular, this discrete subset of memory $CD8^+$ T cells was defined by a specific phenotypic profile (CD45RA, CD62L, CCR7, CXCR3, CD122, CD127, CD95), which also reflected a distinct gene expression profile characterized by low levels of transcripts associated with T cell senescence (T-bet, KLRG1, Granzyme A and Perforin). Interestingly, *Gattinoni et al.* also reported how the generation of this stem cell-like population of memory T cells is a consequence of the triggering of Wnt-pathway during the *in vitro* priming of sorted naïve $CD4^+$ and $CD8^+$ T cells whereas studies performed in non-human primates (NHPs) indicate that antigen-specific T_{SCM} naturally arise upon infection with simian immunodeficiency virus (SIV) and persist for long-term in an antigen-independent manner [115].

Despite a plethora of studies, performed in human and animal models, confirmed the existence of a subset of T_{SCM}, their stemness (self-renew and multipotency) has been always

demonstrated by *in vitro* assays and never at single cell level. In this regard, the development of cellular barcoding technologies [116,117] and the adoptive transfer of a single $CD8^+$ T cell have recently questioned the existence of T_{SCM}. Indeed, it was reported in mouse model, 500 days post-infection (500 p.i.) with Listeria Monocytogenes (LM), the long-term persistent memory pool was composed only by canonical T_{CM} ($CD44^{hi}$ $CD62L^+$ $CXCR3^+$ $CD122^+$) and T_{EM} ($CD44^{hi}$ $CD62L^-$ $CXCR3^+$ $CD122^+$) subsets. No antigen-specific $CD8^+$ T cells, with a stem-cell like phenotype, were detected. In addition, using an elegant serial transfer of single $CD8^+$ T_{CM}, the authors unequivocally demonstrated the stemness capacity of central memory CD8+ T cells. Indeed, a single primary T_{CM} upon adoptive transfer and subsequent LM-challenge, was able to confer protection to the host by generating an entire heterogeneous progeny of antigen specific $CD8^+$ T cells including secondary T_{CM}. Moreover, similarly to primary T_{CM}, a single secondary T_{CM} was equally efficient in conferring protection to immunocompetent or immunocompromised host upon LM infection[118].

In view of the latter observation the characterization of adult stem cells in the immune system remain to be clarified. Nevertheless, analysis of the clonogenic potential of individual T cell reinforces the therapeutic potential of T_{CM} according to their self-renewal and multilineage differentiation capacity.

1.4. The origin of long-lived memory CD8+ T cells.

Generation of long-lived antigen specific CD8+ T cells has always represented the aim of the different immunization protocols for infectious disease and malignancies. However, the underlying mechanisms regulating the formation of immunological memory and the lineage relationship between memory and effector CD8+ T cells are still poorly defined [96,119-121].

1.4.1. Identification of effector and memory CD8+ T cells.

In view of the natural history of CD8-mediated immune response, the initial key question was about the selection of the effector cells able to survive to the contraction phase, and to further mature in memory $CD8^+$ T cells. This process of selection of memory cells among the effector $CD8^+$ T cells was originally proposed to be completely random. In this scenario, all effector cells are equipotent and their further maturation into memory $CD8^+$ T cells is related to the encounter, during and after the peak of primary immune response, with survival factors and/or, depending on their high TCR avidity[6,103,109,122-124].

This initial interpretation on the formation of immunological memory referred as the ON-OFF-ON model, also imply that the effector cells can be consider the progenitors of long-lasting memory $CD8^+$ T cells. In support of this model, the use of genetic tagging systems, controlled by effector-associated genes, clearly indicate how also memory $CD8^+$ T cells precursors, during primary immune response, are highly activated and transiently display effector functions but do not lose the ability to home to secondary lymphoid organs and to persist in an antigen independent fashion and to self-renew [125,126].

In sharp contrast with the ON-OFF-ON model, the "Developmental Model" can alternatively explain the origin of memory CD8+ T cells. In the latter model, memory cells do not arise from effector cells but directly from activated naïve T cells, furthermore these memory precursor cells does not experience a proper effector state during the primary immune response[109]. This model is based on the observations that following stimulation, memory $CD8^+$ T cells proliferated less as compared to effector cells, as indicated by the increased telomerase activity and telomeres length, and that upon *in vitro* restimulation

memory CD8$^+$ T cells can arise from memory cells but not from effector cells [94,107,120,127,128] (**Fig.3**).

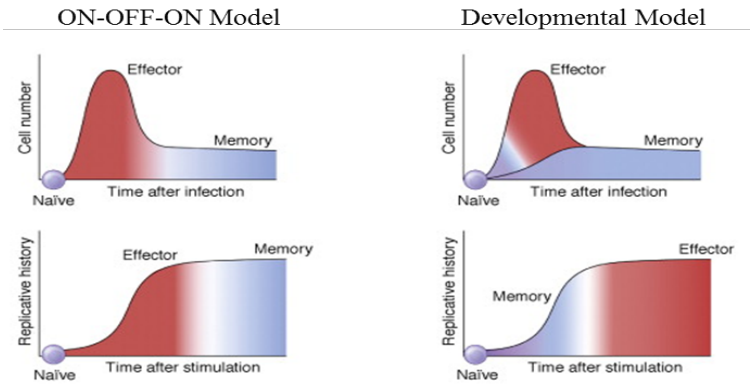

Figure 3. Heterogeneity of memory CD8 compartment. Two different model have been proposed to explain the acquisition of memory qualities by antigen-specific CD8+ T cells. The on-off-on model postulates that effector CD8+ T cells can acquire memory qualities after resolution of infection. In contrast, according to the developmental model, memory CD8+ T cells precursors are originated during primary immune response from antigen specific naïve precursor that does not acquire enhanced effector functions and do not undergo through several rounds of divisions.

(modified from Lineage relationship of effector and memory T cells. Gattinoni L. and Restifo N.P. Current Opinion in Immunology (2013) 25 (556-563) .

The Developmental model is further supported by the *ex-vivo* characterization of antigen specific CD8$^+$ T cells at the peak of primary immune response. In this regard, several evidences clearly pointed out how, like the memory compartment, the pool of effector cells is quite heterogeneous and different subsets of CD8$^+$ T cells can be identified according to selective expression of genes and surface molecules, proliferative capacity and long-term survival. In this scenario, a population of short-lived effector cells (SLEC) mediates pathogen removal, during the primary immune response, by the production of cytotoxic molecules (perforin and granzymes) and effector cytokines (IFN-γ and TNF-α) and then rapidly undergo apoptosis.

In sharp contrast with SLECs, a subset of memory precursor effector cells (MPEC) was also identified. In particular, MPECs are characterized by a reduced effector functions, a marked production of IL-2 upon antigen recognition and are able to further mature in long-lasting memory CD8$^+$ T cells. Identification of SLECs and MPECs is based on the selective expression of killer lectin-like receptor 1 (KLRG1) and α-chain of the IL-7R (IL-7Rα;

CD127) respectively [129,130]. As expected, these phenotypic differences are associated with different gene expression programs orchestrated by dedicated transcription factors. In this regard, the commitment of naïve $CD8^+$ T cells toward SLEC lineage is tightly regulated by high levels of T-bet (T-box transcription factor TBX21) and the transcriptional repressor Blimp-1 (B-lymphocyte-induced-maturation protein 1). These two transcriptional factors promote, in a synergistic manner, the generation of SLECs by activating the expression of effector molecules (IFN-γ, perforin and granzymes) and inhibiting the expression of factors associated with generation of MPECs.

On the contrary, differentiation of activated naïve $CD8^+$ T cells toward MPEC lineage is initially promoted by the transcription factor eomesodermin (EOMES; T-box brain protein 2) and then maintained by Id3 (inhibitor of DNA binding 3) and TCF-1 (T cell Factor-1). In addition, according to their marked memory potential and long-term survival, MPECs are also characterized by the expression of anti-apoptotic members of the Bcl-2 (B-cell lymphoma 2) family such as Bcl-2, $Bcl\text{-}_{XL}$ and Bcl-6 [128-132].

The identification and characterization of MPECs and SLECs as distinct and stable populations of effector $CD8^+$ T cells, do not exclude the existence of transitional subsets of effector cells with intermediate phenotypes and functional attributes. In this regard, the differentiation state of intermediate subsets of effector $CD8^+$ T cells has been reported to be associated with the selective expression of several surface markers. In particular, in combination with KLRG1 and IL-7Rα, the selective expression of CD62L, CXC-chemokine receptor 3 (CXCR3; CD183) and CD27 allow the identification of effector cells with marked memory potential [128,133].

1.4.2. Mechanisms of memory CD8+ T cell formation.

Despite the development of widely applicable technologies allowing the identification of memory $CD8^+$ T precursors cells within the effector pool, there is still a debate about the origin of these memory precursors. In this regard, four different models have been proposed to explain the simultaneous generation of effector and memory $CD8^+$ T cells during a primary immune response (**Fig.4**) [116,128,129].

The initial thought regarding the origin of immunological memory, was consistent with the *one naïve cell, one fate* model. According to this model, before the recognition of the cognate antigen, a naïve $CD8^+$ T cell is already committed to differentiate either into an effector cell or alternatively into a memory $CD8^+$ T cell, but not both [116,128]. In support of this pre-established commitment of a naïve $CD8^+$ T cell, it was initially reported how T_{CM} and T_{EM}, isolated from peripheral blood of healthy donors, were composed by distinct and stable clonotypes [116,128,134]. However, the recent development DNA barcode-based lineage tracing technology, that allows the characterization of the progeny arising from individual clonal precursor, clearly indicate that individual naïve $CD8^+$ T cell, with the same antigen specificity, can generate either a short-lived progeny or a long-lived effector cells (**Fig.4A**) [135].

Notably, this initial model disproved, at single cell level, the plasticity of naïve $CD8^+$ T cells as indicated by their capacity to generate all the different subsets of effector and memory $CD8^+$ T cells [116,117,128]. In sharp contrast with the *one naïve cell, one fate* model, the current understanding of memory cells origin is consistent with one *naïve cell, multiple fates* which postulated that effector cells and all the different subsets of memory T cells can arise from the same naïve $CD8^+$ T cell precursor [116,128]. In this scenario, three different mechanisms have been proposed and distinguished depending on the fact that the fate decision is either taken before the first cell division of primed naïve $CD8^+$ T cell, or at later stages of the primary immune response as a consequence of multiple rounds of interaction with antigen-bearing dendritic cells and in response to pro-inflammatory cytokines.

The first model proposed is the signal-strength model. According to this model, differentiation of a naïve $CD8^+$ T cell toward T_{SCM}, T_{CM}, T_{TM}, T_{EM} and effector T cells appears to be tightly regulated by the overall strength of the signals derived from the antigen (signal-1), co-stimulation (signal-2) and inflammatory cytokines (signal-3) during the priming of naïve $CD8^+$ T cells. In view of the signal-strength model, the fate of naïve $CD8^+$ T cell is programmed before the first cell division. According to this model, "minimally strong" signals are required to promote differentiation of naïve cells into memory cells. However, if these signals are stronger excess, they induce the generation of effector $CD8^+$ T cells destined to die during the contraction phase of primary immune response (**Fig.4B**)[116,128,136-138].

Similar to the signal strength model, the "decreasing-potential model" describes that the fate of primed naïve $CD8^+$ T cells is defined by the overall strength of the signal received during the priming. However, in this model, differentiation of naïve $CD8^+$ T cells is not

regulated by the intensity of the signals received before their first division but is rather modulated by the cumulative effect of successive round of stimulation during the primary immune response. In animal models, it has been shown how a decreased inflammation, consequence of antibiotic treatment or delayed transfer of naive specific T-cells, is associated with an increased differentiation of naïve cells into memory $CD8^+$ T cells (**Fig.4C**) [116,128,136,139-141].

Finally, the cell fate specification was also proposed to be defined through the asymmetric segregation of critical determinants in the daughter cells of a primed naïve $CD8^+$ T cell [142]. Interestingly, in the asymmetric cell fate model, the unequal inheritance of specific factors is realized before the first cell division of the clonal precursor and is orchestrated by the protein kinase C-ζ (PKC-ζ). Indeed, this ancestral regulator of asymmetric division allows the early identification of daughter cell committed to memory differentiation[143]. In this regard, it has been reported how, in pre-mitotic activated naïve $CD8^+$ T cells, PKC-ζ is preferentially accumulated at the opposite side of immunological synapse (distal pole) and identifies the distal daughter cell further characterized by preferential accumulation of TCF-1, IL-7Rα and CD62L. In sharp contrast, the pole where T cell-APC occurs (proximal pole; proximal daughter cell), was described as the segregation location of several determinants such as LFA-1, CD8, α-chain of the IL-2 receptor (IL-2Rα; CD25), IFN-γ receptor (IFNγR) and granzyme B. This asymmetric partitioning of distinct factors into the daughter cells as a critical determinant for fate specification, was reported to formally promote the differentiation of proximal cell and distal cell into short-lived and memory precursor effector cells respectively (**Fig.4D**) [127,128,142,143].

All these different models, proposed for the generation of a heterogeneous pool of effector $CD8^+$ T cells, including subsets with a marked memory cell potential and longevity, are not mutually exclusive. In addition, for all the suggested models, initial(s) stimulation of antigen-specific CD8+ T cells represents a critical step in the definition of memory potential of naïve T cells.

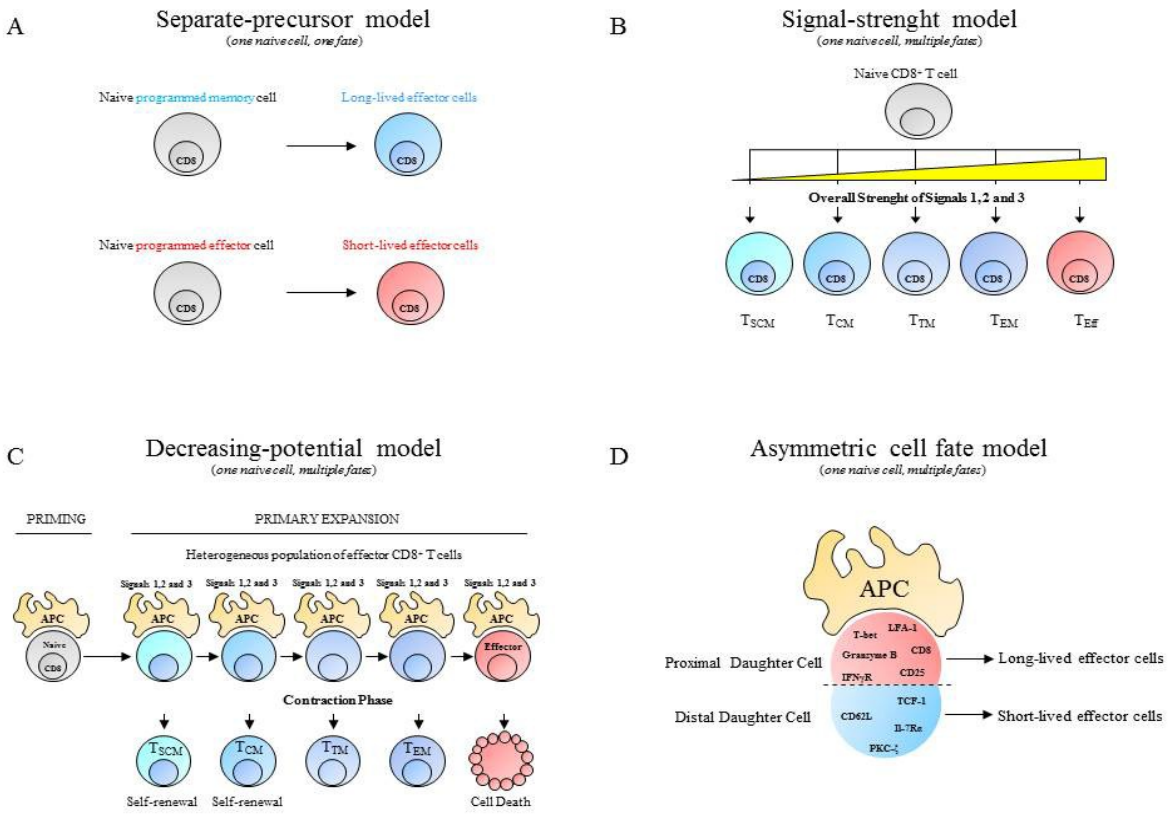

Figure 4. Heterogeneity of memory CD8 compartment. Different model have been proposed in order to explain origin and heterogeneity of memory CD8+ T cells. The separate precursor model postulates that commitment of naïve CD8+ T cells to effector or memory lineage is defined during thimic selection. In contrast, signal strenght model, decreasing potential model and asymmetric cell fate model postulate that lineage commitment of antigen specific naïve CD8+ T cell is defined during initial stimulation.

1.5. Priming of CD8+ T cell responses: The first step on the path to memory.

Generation of CD8+ T cell responses is a regulated process and several factors have been reported to affect both clonal expansion and, most importantly, the differentiation of antigen-specific naïve precursors into effector and memory T lymphocytes. In this respect, it has been extensively shown how antigen dose and its persistence might affect activation of CD8+ T cells and acquisition effector functions. In particular, low amount of antigen may impair the ability of the immune system to generate CD8+ T cells responses (antigen ignorance)[144]. In contrast, as indicated in several preclinical and clinical studies of chronically infected and cancer patients, antigen persistence is normally associated to the acquisition by reactive CD8+ T cells of an exhausted phenotype characterized by an impaired proliferative capacity and reduced production of effector cytokines upon stimulation[7,30,31]. Furthermore, the molecular structure of the antigen has been also shown to regulate the activation of reactive T lymphocytes. Indeed, several studies aiming to elucidate crucial aspects of antigen recognition by T cell receptor (TCR) have shown that activation, differentiation and survival of reactive CD8+ T is primarily regulated by the capacity of antigen derived peptide to be loaded in the groove of MHC class I molecules (pMHC) and by the capacity of reactive T cells to recognize with high avidity cognate pMHC complexes express on target cells[6].

Although the primary stimulus promoting CD8+ T cell activation is associated to engagement of TCR, different studies underlined that differentiation of antigen specific naïve precursors into effector and memory T cells is also critically regulated by other signals integrated by responding T cells. Our current understanding is consistent with the "three signals model" postulating how activation and differentiation of naïve CD8+ T cells are regulated by the composition and the strength of signals associated to antigen recognition (signal-1), co-stimulatory receptors (signal-2) and pro-inflammatory cytokines (signal-3)[145].It has been extensively shown that clonal expansion of antigen specific naïve CD8+ T cells is initiated upon T cell receptor (TCR) mediated recognition of antigen-MHC class I complexes. However, in particular in situations of low TCR occupancy and/or affinity, activation of naïve CD8+ T cells is decisively sustained by signals derived by co-stimulatory receptors, predominantly CD28, interacting with their cognate ligands. Indeed, in naïve CD8+ T cells, CD28 signaling results in the activation of different transcription factors, including nuclear factor kB (NF-kB) and nuclear factor of activated T cells (NFAT), regulating cell proliferation and differentiation of responding T lymphocytes[6,32,146]. Furthermore, execution

of cellular programs leading to the acquisition of effector functions and most importantly to the differentiation of antigen-specific naïve precursors into long-lasting memory cells has also been extensively associated to the cytokine milieu experienced by responding CD8+ T cells during the priming. Studies have shown that specific pro-inflammatory cytokines such as IL-12 and IFNs-type I, are critical determinants defining the memory potential of CD8+ T cells[147-151].

In view of this model and according to their ability to provide all the signals required for activation of antigen-specific naïve precursors, a pivotal role for antigen presenting cells (APCs) has been repeatedly demonstrated in the generation of protective and long-lasting CD8+ T cells responses.

1.5.1 Role of Antigen Presenting cells and cytokines

Antigen Presenting Cells (APCs) are a heterogeneous group of immune cells able to initiate and/or sustain cellular immune responses by processing and presenting antigens for recognition by T lymphocytes. In this regard, it has been extensively reported that the capacity of APCs to mediate the activation of CD8+ T cells is related to the specific subset interacting with T-lymphocytes and further regulated by their activation status. Among the different cells endowed with antigen presenting capacity, dendritic cells (DC) have been traditionally described as the main inducers of CD8+ T cell responses: the "professional" APC[152,153].

The pivotal role of DCs, in orchestrating cellular immune responses, has been shown to correlate with their ability to migrate from periphery to secondary lymphoid organs, upon antigen capture, where they mediate initiation of CD8+ T cell responses. Most importantly, together with other factors, activation/ maturation status of DCs represent a critical determinant dictating the outcome of CD8+ T cell responses towards tolerance or protective immunity. In particular, in a number of studies, activated (mature) DCs have been shown to more effectively promote, clonal expansion and acquisition of effector functions by naïve CD8+ T cells as compared to resting (immature) DC. Remarkably, the effectiveness of activated (mature) DCs has been associated to the high expression on cellular surfaces, along to antigen/MHC-class I complexes, of a panel of co-stimulatory ligands including CD80

(B7.1) and CD86 molecules (B7.2) that amplify, upon engagement of CD28 co-receptor, the strength of the signal-1 in responding T lymphocytes[146]. Furthermore, it has been recently showed that activated DCs can also shape the memory potential of responding CD8+ T cells due to their capacity to secrete, upon activation, high levels of a broad array of different cytokines. In this respect, it has been repeatedly shown that specific pro-inflammatory cytokines such as IL-12, IFN-α and IFN-β (IFNs-type I) promote the expansion and the acquisition of effector functions by activated CD8+ T cells[150,154].

Interestingly, in the last years, a crucial role for these pro-inflammatory cytokines has also been revealed in the effective generation of memory CD8+ T cells. In particular, in different studies performed in animal models, it has been observed that abrogation of IL-12 and IFN-α signaling on naïve CD8+ T cells affects their differentiation into memory cells, long-term survival and their ability to mount strong proliferative response upon secondary stimulation[155,156]. Interestingly, the ability of IL-12 and IFN-α to shape the memory potential of naïve CD8+ T cells has been associated to their capacity to sustain chromatin remodeling initiated by TCR and CD28 associated signals. In fact, direct signaling of IL-12 and IFNs-type I prevents the decline, over the time, of mRNA levels of genes regulating effector functions (IFN-γ, granzymes, perforin) but also survival and differentiation (Bcl-3; Bcl-6; IL-7Rα; T-bet and Eomesodermin) of responding CD8+ T cells[155,156]. A critical relevance of IL-12 and, to a lesser extent, IFN-α, in promoting survival and acquisition of memory qualities by antigen-specific naïve precursors cells, has been shown in a set of experimental studies based on the adoptive transfer of CD8+ T cells lacking expression of functional receptors for IL-12 and/or IFNs-type I[155,156]. However in some other reports, a dispensable role or detrimental effect of these pro-inflammatory cytokines in defining the memory potential of CD8+ T cells has also been suggested. In particular, in distinct infectious models, it has been underlined a potential role for IL-12 and/or IFNs-type I in promoting the generation of KLRG1+ IL-7Rα- short-lived effector cells (SLECs). In these studies, commitment to SLEC lineage is associated with the induction of transcription repressor Blimp-1 and, most importantly, T-bet transcription factor[157-159]. Indeed, differentiation of naïve CD8+ T cells into SLECs has been associated to the capacity of T-bet and Blimp-1 to regulate the expression of genes mediating the acquisition of effector functions (IFN-γ, Granzymes, Perforin and CD178 (FAS-L)) and to further avoid the generation of memory-precursors effector cells (MPECs) by abolishing the expression of different genes encoding factors, including Bcl-6 and IL-7Rα, promoting long-term survival[124,160].

Remarkably, different hypothesis have been formulated in order to explain these contradictory observations. In particular, differences in the experimental settings such as the use of specific infectious agents, different doses and transgenic mice may partially account for the opposite results reported. In addition to these technical considerations, several evidences suggest that mostly the timing and the intensity of pro-inflammatory cytokines signaling on naïve CD8+ T cells are critical determinants, dictating their differentiation toward SLECs or MPECs lineage[141]. In this regard, IL-12 and, to a lesser extent, IFN-α signaling on CD8+ T cells is crucial during the priming for the definition of memory potential and does not result in the up-regulation of KLRG1 senescent marker on their cellular surfaces[156]. In line with this observation, it has been recently shown that during clonal expansion, before the acquisition of phenotypic signature of SLEC or MPEC lineage, the majority of antigen-specific naïve CD8+ T cells acquire a phenotype, termed of early effector cells (EEC), characterized by the lack of KLRG1 as well as IL-7Rα. Interestingly, hierarchical characterization of antigen-specific EEC CD8+ T cells has revealed that although this population is programmed to differentiate into SLECs or MPECs based on the intensity of early inflammatory signals received during the priming, it is also susceptible to additional inflammatory signals that can alter the lineage commitment[161]. Hence, it is reasonable to assume that paracrine production of pro-inflammatory cytokines, mostly IL-12 and IFNs-type I, by antigen-bearing DCs during priming is crucially involved in the definition of memory potential of CD8+ T cells whereas inflammatory signals derived by other cells may potentially drive the differentiation of antigen specific precursor toward SLEC lineage. In addition, although animal models have shown that strong and persistent inflammatory signals favor the generation of SLECs in a T-bet dependent manner[157], clinical evidences observed from acute or chronic infected individuals indicates that, more than the expression of T-bet, it is the ratio T-bet/Eomesodermin which defines the capacity of CD8+ T cells to mediate long-term viral control. Indeed, in HIV chronic infected individuals and in patients experiencing CMV opportunistic infection upon solid organ transplantation a reduced T-bet/Eomesodermin ratio have been correlated respectively with an in vivo exhausted phenotype and an impaired proliferative response of virus-specific CD8+ T cells[161-163].

In this scenario, the pivotal role of dendritic cells in shaping the magnitude and also the memory potential of CD8+ T cell responses has induced considerable efforts by the scientific community in order to modulate the immunostimulatory potential of dendritic cells. In this respect, it has been demonstrated that the activation of dendritic cells can be mediated by (i) innate immune system derived inflammatory cytokines, (ii) upon recognition, via pathogen

recognition receptors (PRRs), of pathogen associated or damage associated molecular patterns (PAMPs and DAMPs respectively) and, most importantly, (iii) by CD4+ T cells.

1.5.2 CD4+ T helper cells.

CD4+ T lymphocytes can be considered as critical regulators of the immune system. In particular, the pivotal role of CD4+ T cells in different processes of innate and adaptive immune responses has been traditionally ascribed to the heterogeneity and the marked plasticity of the members of this T lymphocytes lineage. So far, different subsets of CD4+ T cells with specialized functions, reflecting the selective expression of cellular surface antigens, transcription factors and cytokines production profile, have been identified[164]. Among the different subsets, CD4+ T helper 1 (Th1) have been repeatedly shown to support generation of effective memory CD8+ T cells against intracellular pathogens and solid tumors. However, despite considerable research efforts, several aspects regarding the helper activity of Th1 are not completely defined yet. In particular, definition of the time window for CD4+ T cell requirement as well as the molecular mechanism(s) supporting the generation of protective and long-lasting CD8+ T cell responses are still debated[165].

Initial results obtained in different immunization models suggested a critical role for Th1 cells during the priming of antigen specific CD8+ T cell responses (Programming model)[165,166]. Interestingly, this initial interpretation has been later extended. Indeed, it has been reported that CD4+ T helper cells may also promote survival and proliferative response, upon re-challenge, of memory CD8+ T cells (Maintenance Model)[165,167]. Notably, the existence of two different models concerning the intervention of CD4+ T helper cells can be explained in virtue of the distinct mechanisms by which help can be delivered during CD8+ T cell responses.

Th1 cells have been repeatedly shown to provide help basically through the paracrine production of high amounts of cytokines, most importantly, interleukin-2 (IL-2) and by "licensing" antigen-presenting cells (APCs Licensing) to optimally stimulate naïve CD8+ T cells. In view of the pivotal role of DCs in shaping the memory potential of antigen-specific naïve precursor, collective results obtained by different studies suggested that cytokines production, including IL-2, by bystander CD4+ Th1 cells is mostly associated to survival of

memory CD8+ T cells after resolution of primary infection and/or in promoting their proliferative response upon re-challenge. Indeed, it has been shown that IL-2 can act as a surrogate of CD4+ T helper cells during maintenance phase or recall but not during the priming of naïve CD8+ T cells. Furthermore, it has also been shown that in vivo administration of IL-2 during initial stimulation of CD8+ T cells could potentially lead to the generation of SLECs[160,165,168].

1.5.3. Shaping memory potential of naïve CD8+ T cells: CD4+ T cells and APC Licensing.

The initial evidence on the pivotal role of CD4+ T helper 1 cells in modulating antigen presenting capacity of dendritic cells was provided by pioneering studies aiming at elucidating T cell help dependence of CD8-mediated immune responses in different vaccination/infectious models. In particular, these initial studies clearly underlined a differential requirement for Th1 cells, mostly dictated by the biology of the immunization setting, in promoting clonal expansion and acquisition of effector functions by CD8+ T cells (Primary Response). Indeed, strong CD8+ T cell responses, in absence of CD4+ Th1 cells, were extensively described in animal models upon infection with lymphocytic choriomeningitis virus (LCMV) vesicular stomatitis virus (VSV), vaccinia virus (VV) and listeria monocytogenes (LM). In sharp contrast, presence of CD4+ T cells was critically required in order to promote CD8-mediated primary immune responses against non-infectious antigens such as minor histocompatibility antigens, protein antigens and tumor-associated antigens[165,168]. Initial interpretation of these results was based on the exclusive capacity of different viral and bacterial pathogens, as compared to cellular antigens, to activate antigen-presenting cells (APCs) indirectly by inducing innate immune system-derived inflammatory cytokines and directly by recognition via pathogen recognition receptors (PRRs), expressed by APCs, of pathogen associated molecular patterns (PAMPs). Interestingly, these stimuli have been associated to different phenotypic and gene expression profiles. Indeed, it has been shown that up-regulation of maturation markers (MHC class II; CD80; CD86) on cellular surfaces can be observed as a consequence of the exposure of DCs to inflammatory cytokines, including IFNs type I and TNF-α, produced by other cell types. However, the capacity of DCs to prime naïve T cells and to promote their differentiation in effector cells was critically dependent on the direct recognition of infectious agents. In this respect, different categories of

PRRs, most importantly Toll-like receptors (TLRs), have shown to initiate an intracellular signaling in DCs, leading to the de-novo transcription of genes encoding pro-inflammatory cytokines including IFN-α/β and IL-12 [169,170].

Remarkably, this initial model on CD4+ T cell helper dependence of CD8-mediated immune responses has then been revised by different studies in which functionality of resulting memory CD8+ T cells generated in absence ("helpless") or presence ("helped") of CD4+ T cells was evaluated. Collectively, these studies have shown that, although primary CD8+ T responses generated in response to different viral or bacterial infections can in fact be also CD4+T helper 1 cells independent, memory CD8+ T cells generated in helpless conditions were unable to mount productive recall responses. Indeed, in sharp contrast with helped, helpless memory CD8+ T cells have been reported to be functional impaired, thus conferring a limited protection upon re-challenge[166,171,172]. In particular, memory CD8+ T cells generated under these conditions were reported to be characterized by reduced survival, production of a limited array of effector cytokines and defective proliferative response due to up-regulation of inhibitory receptor programmed cell death-1 (PD1)[173], impaired IL-2[174] production and/or TNF-related apoptosis-inducing ligand (TRAIL) mediated apoptosis upon secondary stimulation[166]. However, these observations have been challenged by different studies. Indeed, some reports have described the capacity of the immune system, in different model of CD4+ deficient mice, to generate protective and long-lasting CD8+ T cells in particular against viral and bacterial pathogens. Indeed, memory CD8+ T cells generated in helpless condition were able to confer host protection, upon secondary exposure to the pathogen based, on their marked proliferative response and acquisition of effector function upon antigen recognition. Intriguingly, this apparent discrepancy has been potentially resolved by the observation, in specific experimental settings, of the capacity of some pathogens to induce "per se" a high production of IL-12 and IFNs-type I during host infection, thereby potentially bypassing the requirement of CD4+ T helper cells during the priming of antigen-specific naïve precursors[175].

Based on this background, our current understanding of the interplay between dendritic cells (DCs), CD4+ and CD8+ T cell in the generation of immunological memory is still consistent with the "APC-Licensing" model postulating that CD4+ T cells orchestrate the generation of memory CD8+ T cells predominantly by promoting the activation of dendritic cells through CD40 ligand (CD40L; CD154)/CD40 receptor pathway[176,177].

1.6. CD40 ligand-CD40 receptor pathway.

CD40 receptor is a 48 kDa type I transmembrane protein that belongs to Tumor Necrosis Factor receptors (TNFR) superfamily. Remarkably, engagement of CD40 receptor by its cognate ligand results in the formation of homotrimeric complexes promoting the recruitment, on different cytoplasmic domains, of adapter proteins termed TNFR-associated factors (TRAFs). The TRAFs family included six different members (TRAF-1,2,3,4,5 and 6), and all of them have been shown to mediate the intracellular signaling of the members of TNFR family upon interaction with the cognate ligands. Interestingly, CD40 ligation has been associated to the activation of a wide spectrum of molecular processes. In particular, CD40 receptor initiated signals are associated to the activation, through TRAFs family members, of distinct intracellular signaling pathways associated to nuclear factor-kB (NF-kB), mitogen activated protein kinase (MAPKs), phoshoinositide 3-kinase (PI3K) and pospholipase Cγ (PLCγ). In addition to the capacity to promote the activation of distinct signaling pathways, engagement of CD40 receptor resulted in the modulation of different cellular functions also as a consequence of its broad pattern of expression. Indeed, CD40 receptor is found on different cell types including epithelial, endothelial, stromal cells and, most importantly on cellular surface of cells endowed with antigen presenting capacity such as B cells, dendritic cells, macrophages, monocytes and possibly on T cells[178-182].

CD40 ligand (CD40L; CD154; gp39) is a 32-33 kDa type II transmembrane glycoprotein that belongs to TNF superfamily expressed on cellular surface of natural killer cells (NK), natural killer T cells (NKT), CD8+ and, most importantly, CD4+ T cells. In addition to the transmembrane form, two shorter version (31 and 18kDa) of CD40L protein have been described. These shorter CD40L forms are generated after proteolytic cleavage and are mostly secreted by activated platelets and T cells[178-182].

Although soluble and membrane bound CD40L possess an equal ability to form trimers, the presence of these two different isoforms is associated with different biological effects. Indeed, elevated serum levels of s40L has been described in cancer and chronic inflammation and associated to survival, expansion and enhanced immunosuppressive activity of myeloid derived suppressor cells (MSDC)[14] and Tregs[183]. In contrast, membrane-bound CD40L expressed, mostly, by activated CD4+ T cells is clearly associated with the activation of humoral and cellular immune responses. The different effects induced by membrane bound

and soluble CD40L isoforms might reflect the different environment and molecular characteristic of the ligation of CD40 receptor ligation on different cell types, thereby resulting in the modulation of different cellular functions. To this respect, it has been also shown that biological responses induced by the ligation of CD40 receptor are correlated to the extent as well as the strenght of stimulation on target cells. To this respect, different cellular consequences initiated by CD40 receptor triggering have been associated to the selective recruitment of different TRAFs molecules and/or to the activation of distinct intracellular signaling pathways[184,185]

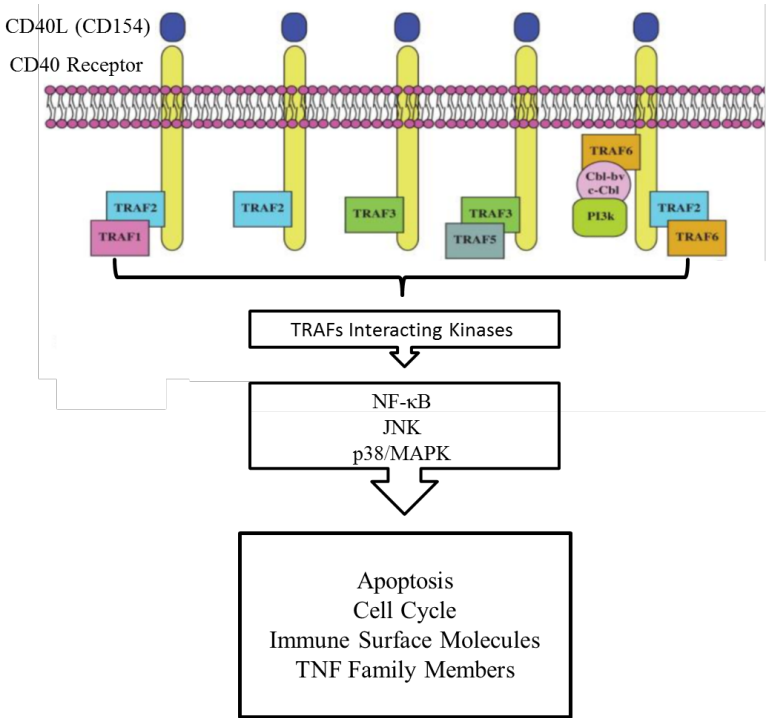

Figure 5. Multiple effects of CD40:CD40L pathway. Ligation of CD40 receptror has been associated to the modulation of different cellular responses. The multiple effects associated to CD40: CD40L pathway are related to the broad pattern of expression of CD40 receptor and to several intracellular signaling cascades initiated in response upon CD40L engagement. In particular, different TNF-receptor associated factors (TRAFs) have been identified as critical mediators of CD40 receptor initiated signals. TRAF proteins are recruited on different cytoplasmic domains of CD40 receptor and can mediate the activation of NF-kB, JNK and p38/MAPK pathway resulting, according to the cell type as well the strength and the extent of the stimulus, in apoptotic or prosurvival effects. Furthermore, ligation of CD40 receptor on immune cells such as dendritic cella and B-lymphocytes has been associated to the up-regulation of costimulatory ligand on their cellular surface and secretion of pro-inflammatory cytokines.

(adapted from: Molecular mechanisms and funcrion of CD40/CD40L engagement in the immune system. Elgueta R., Benson M.J.,deVries VC et al. Immunol Rev.2009 May ; 229 (1) :152-172.)

1.6.1 CD40 ligand-CD40 receptor and Immune System.

CD40/CD40L pathway has been initially described as a critical regulator of humoral immune responses. Studies with mutation of the CD40 receptor and/or CD40L genes resulted in impaired germinal centre (GC) formation, immunoglobulin (Ig) isotype switching, generation of long-lived plasma cells and memory B-cells differentiation. In addition, CD40/CD40L pathway is also associated with the induction of T cell response. Indeed, CD4+ T cells, activated by TCR mediated recognition of cognate antigen-MHC class II complexes, rapidly express membrane bound CD40L on their cellular surface. Hence, activated CD4+ CD40L+ T cell can promote the activation of CD40-expressing dendritic cells. Indeed, CD40 receptor activity on DCs resulted in the delivery of prosurvival signals (Bcl-2), resistence to FAS-L induced apotosis, up-regulation of a broad panel of surfaces molecules involved in the generation of immunolgical synapse. Indeed, the engagement of CD40 receptor resulted in the an increased expression of MHC class I/II molecules and in the up-regulation of adhesion and costimulatory molecules including CD80 (B7.1), CD86 (B7.2), CD58 (LFA-3), CD54 (ICAM-1). Furthermore, CD40-stimulated dendritic cells acquire the capability to secrete pro-inflammatory and effector cytokines including TNF-α, MIP-1α, GM-CSF, IFNs-type I, IL-6, IL-1β and IL-12. Interestingly, in response to paracrine production of IL-12, responding T-cell can further increase the expression on cellular surfaces of CD40L, thereby further sustaining activation of dendritic cells[179,181].

Different studies have also underlined how stimulation by microbial stimuli and selective TLR-agonists (CpG, poly I:C and LPS) can potentially overcome blockade of CD40/CD40L pathway[186,187]. However, experiments in animal models have demonstrated that microbial products cannot replace the signals initiated by CD40 receptor. Indeed, in a set of studies performed to evaluate CD8+ T cells priming capability of CD40-stimulated or TLRs-stimulated dendritic cells, abrogation of CD40 signaling resulted in a marked reduction in expansion, cytokines production and acquisition of memory qualities by transgenic and antigen-specific endogenous CD8+ T cells[188,189]. Notably, the relative reduced immunostimulatory potential of TLR-stimulated dendritic cells appears to be associated to different capacity of TLR-agonists to induce up-regulation of co-stimulatory ligands and of selective pro-inflammatory cytokines. Interestingly, TLR-initiated signals, most importantly through the nuclear factor kB (NF-kB), resulted in the up-regulation of co-stimulatory CD40 receptor on cellular surfaces of dendritic cells recognizing microbial products. Thereby,

despite a marked stimulatory potential of TLR-agonists, only CD40 induction appears associated to the full activation of dendritic cells[170,189]. In support of this hypothesis, it has been observed how, in response to autocrine or paracrine production of pro-inflammatory cytokines, TLR-stimulated DCs may acquire the capacity to optimally prime naïve CD8+ T cells. Interestingly, the increased antigen presentation capacity of dendritic cells, observed under these conditions, is correlated to the ability of IFN-α receptor-initiated signals to sustain nuclear translocation of NF-kB, resulting in increased cytokines productions by stimulated DCs. Furthermore, exposure to IFN-α resulted in up-regulation and/or maintenance of high expression of CD40 co-stimulatory receptor on cellular surfaces of DCs. Based on these findings, it is reasonable to assume that T-cell help-independence, described in the generation of effective memory CD8+ T cells against specific infectious agents, might be related to the synergistic effects associated to the recognition of certain pathogen associated molecular patterns (PAMP) further sustained by the autocrine and/or paracrine production of pro-inflammatory cytokines. Nevertheless, also in these experimental settings, a crucial role for CD40L-expressing activated CD4+ T cells is indirectly suggested by the up-regulation of CD40 receptor associated to IFNα receptor initiated signals alone or in combination with TLR agonists stimulation[169,170,190]. In particular, it is tempting to speculate that engagement of CD40 receptor might result in the modulation of the quantity and quality of signal 2 and 3 delivered by dendritic cells to responding CD8+ T cells. In addition, though some TLR agonists have shown a potential capacity to promote DCs-mediated cross-presentation of exogenous antigens to CD8+ T cells, it must be underlined how this latter functional capacity has been extensively associated to the ligation of CD40 receptor. In this scenario, stimulation of CD40 receptor on cellular surface of antigen presenting cells, most importantly DCs, by CD40L-expressing CD4+ T cells represents a critical step in regulating the induction of protective CD8+ T cell responses[177,191,192].

The identification of CD40/CD40L pathway has also contributed to reconcile critical considerations on the capacity of CD4+ T cells to promote activation as well as differentiation of naïve CD8+ T cells. Initial interpretation of "helper activity" was based on the simultaneous recognition, on the same DC, of cognate antigens by CD4+ and CD8+ T cells. In particular, the CD4+ T help dependence of CD8-mediated immune responses was related to the CD4+ paracrine production of high amounts of IL-2, supporting the clonal expansion and acquisition of effector functions by antigen specific naïve precursors. A major limitation of this model is the low probability that two rare antigen-specific T-cells were simultaneously engaged on the same DCs[176].

T-cell help dependence of CD8+ T cell responses has been then revised by the pionering studies of P. Matzinger[177] and collaborator leading to the definition of Antigen Presenting cells Licensing model (APCs licensing) suggesting a sequential interaction of CD4+ and CD8+ T cells with antigen-bearing dendritic cells. In particular, interaction of CD4+ T cells with an antigen presenting cells, via CD40/CD40L pathway, induces a sustained activation of the second resulting in an efficient priming of CD8+ T cells even after dissociation of CD4+-APC interaction[176,177].

Interestingly, based on recent technological advance, the generation of a "three cell cluster" involving DC, CD4+ and CD8+ T cell was shown to occur in vivo. Indeed, in vivo imaging studies have shown that antigen-driven interaction of T cells and DC in lymph-nodes can last for hours[193]. Based on these findings, it was proposed that a responding CD8+ T cell might encounter, in T cell areas, a pre-existing two cell cluster composed by CD4+ T cells and antigen-bearing DC. Noteworthy, it has been also shown how both CD4+ T cells and activated DCs can actively recruit responding CD8+ T cells through the production of different chemokines. In particular, CCL3 and CCL4 chemokines which play a pivotal role in recruiting antigen-specific naïve CD8+ T cell expressing CCR5 receptor, thereby potentially orchestrating the simultaneous engagement of dendritic cells by both T subsets during priming[194,195].

In view of the latter observation and according to studies showing the capacity of murine CD8+ T cells to express, upon antigen recognition, detectable levels of CD40 receptor, different studies have been performed to further dissect the molecular basis of CD40/CD40L pathway in promoting the generation of CD8+ T cell responses[196,197]. The data showed that the formation of a three cell cluster could potentially also promote the direct interaction of CD4+ T cells and naïve CD8+ T cells and the deliver, through CD40/CD40L pathway, of signals promoting the differentiation of the latter into memory cells. These reports [196,197] also showed that, activation of CD40 receptor-deficient naïve CD8+ T cells did not resulted in significative defects in their capacity to mount a protective primary immune response. Nevertheless inhibition of CD40-initiated signals clearly affected the capacity of responding CD8+ T cells to re-express IL-7R⟨ and, most importantly, resulted in the generation of "lethargic" memory cells. Indeed, mouse CD40 deficient CD8+ T cells were characterized by reduced proliferative capacity and cytokines production, particularly IFN-γ and IL-2, upon in vivo antigenic re-challenge or in vitro stimulation[196-198]. The critical relevance of CD40 receptor associated signals in defining memory potential of antigen

specific naïve precursors was further consolidated by several evidences. In particular, studies showing the inhability of CD4+ T cells to restore the functionality of memory CD40-deficient CD8+ T cells during maintenance phase and/or upon antigenic re-challenge[196]. Based on these findings, it was then proposed that CD40 signaling on dendritic cells and CD8+ T cells has different effects on the generation of CD8+ T cell responses. In this regard, stimulation of CD40 receptor on dendritic cells has been associated to the clonal expansion and acquisition of effector functions by antigen-specific naïve precursors. On the other hand, CD40 expression on CD8+ T cells appeared as a crucial element mediating the execution of cellular programs leading to their differentiation into memory cells. Indeed, in mouse model, ligation of CD40 receptor expressed by activated CD8+ T cells has been associated, through the activation of different intracellular signalling pathway to the acquisition of effector functions (FAS-L; Perforin and Granzymes) and the expression of homeostatic cytokines receptor genes such as IL-7Rα, IL-15Rα, and IL-21Rα thereby resulting in a fine modulation either of CD8+ primary and memory responses[196-198].

Identification of CD40 receptor on CD8+ T cells, as a crucial and non-reduntant element supporting their differentiation into memory cells, has been extensively reported, in mouse model, in response to cellular antigens [196-198]. In contrast, the putative impact of this co-stimulatory receptor in mediating the generation of effective memory CD8+ T cells against infectious agents has not yet fully elucidated[199]. Indeed, discrepant results have been reported. Some studies indicate that T-helper activity during priming of antigen specific may be dispensable in view of the capacity of some pathogens to promote the direct activation of dendritic cells and also to induce on their cellular surfaces CD40L expression thereby potentially capable to activate either neighbor antigen presenting cells and responding CD8+ T cells[200]. However, other studies reported the generation of fully functional memory CD8+ T cells in response to infectious agents even in the absence of CD40 receptor expression on their cellular sufaces[199]. Interestingly, these latter results might be associated to the ability of different pathogens, to induce a massive production of IFN-α. To this respect, it must be underlined that molecular characterization of CD40 and IFN-α receptor associated signals resulted in convergent transcriptional outcomes in dendritic cells, thereby it is tempting to speculate that these two pathways can be potentially redundant in CD8+ T cells[201,202]. In this regard, further studies, especially in human system, are urgently required in order to characterize the gene expression profile and functional consequence of CD40 activity on CD8+ T cells activation and differentiation. Nevertheless, the latter observation represents also indirect evidence confirming either the role of certain pro-inflammatory cytokines and

most importantly the critical relevance of APC-licensing in shaping memory potential of primed CD8+ T cells[201-203].

1.6.2 CD40 ligand-CD40 receptor; a pathway to enhance anti-tumor CD8+ T cells.

It is nowadays widely accepted that memory potential of naïve CD8+ T cells is defined alongside the induction of protective primary immune response. A plethora of studies have shown how the initial stimulation of antigen-specific naive precursors can affect either their clonal expansion and acquisition of effector functions but also the size, phenotypic and functional attributes of the resulting pool of antigen-specific memory CD8+ T cells[166,172,174].

Among different factors regulating the generation of effective anti-tumor CD8+ T cell responses, CD4+ T cells have been consistently shown to play a pivotal role[166,172,174]. Although different mechanisms have been proposed, similar results obtained in studies performed in CD40L- and CD4-deficient mice receiving agonistic monoclonal or recombinant protein targeting CD40 receptor, provided the direct correlation between the requirement of CD4+ T cells and CD40 receptor/CD40 ligand pathway in the generation of CD8-mediated anti-tumor immune responses[191,192]. In particular, helper activity of CD4+ T cells is extensively associated to the ligation of CD40 receptor expressed on cellular surfaces of dendritic cells[203]. In addition, studies also showed a direct help of CD40L+ CD4+ T cells to naïve CD8+ T cells expressing, upon antigen recognition, detectable levels of CD40 receptor on their cellular surfaces[196]. One must underlined that, although the actual presence/role of CD40 receptor activity on CD8+ T cells is still controversial, CD40L-mediated activation of antigen presenting cells, has been consistently reported as a fundamental step promoting antitumor immunity[177,191,192,203]. In this regard, therapeutic potential of CD40-stimulated dendritic cells has been associated to their potential capacity to promote activation and differentiation of tumor-reactive CD8+ T cells expressing low-affinity T cell receptor (TCR) recognizing the cognate antigen in non-inflammatory conditions[6,204].

In line with these observations, initial studies performed in animal models to evaluate the therapeutic potential of different strategies targeting tumor-reactive CD8+ T cells expressing low-affinity TCR have been extensively criticized. In particular, immunization protocols based on MHC-class I epitope vaccines resulted in a limited expansion of tumor-

reactive CD8+ T cells characterized by a marked impairment in cytotoxic activity and proliferative response upon, antigenic re-challenge. Interestingly, according to the results obtained in different studies, the limited effectiveness of this vaccine formulation in the generation of effective anti-tumor CD8+ T cell responses has been consistently associated to the inefficient priming of antigen-specific naïve precursors. Indeed, different observations obtained in murine models have shown that reduced TCR initiated signals might potentially results in the generation of effector and memory CD8+ T cells. In this regard, studies performed by using altered peptide ligands (APLs) and transgenic lymphocytes displaying mutation in the TCR, have indicated that also a weak stimulation of naïve CD8+ T cells is sufficient to promote their differentiation into functional effector and memory cells. Although major differences were not observed between CD8+ T cells expressing low and high affinity TCRs in the acquisition of effector functions and in the capacity to mount memory responses, a weak TCR signaling has been associated to a significant reduction in the accumulation of stimulated cells[6,205]. In particular, upon antigen recognition, a limited clonal expansion and reduced survival have been described for CD8+ cells bearing a low-affinity TCR[6,205]. In view of the latter observation and further reinforced by different studies in which the role of accessory signals, during the priming of tumor-specific naïve precursors, underlined the potential role of CD40/CD40L for cancer immunotherapy. Indeed, enhanced co-stimulation and production of pro-inflammatory cytokines by CD40-stimulated dendritic cells might instruct tumor-reactive CD8+ T cells for effective short and long-term immune responses. Up-regulation of co-stimulatory CD80 (B7.1) and CD86 (B7.2) ligands has been shown to promote the effective activation of antigen-specific naïve precursor upon interaction with co-stimulatory CD28 receptor expressed on their cellular surfaces. Indeed, CD28 receptor initiated signals resulted in a clonal expansion of responding CD8+ T cells through the amplification of TCR-signaling and also by directly inducing the secretion of IL-2. In addition, the marked efficacy of CD40-stimulated dendritic cells to promote the generation of effective antitumor CD8+ T cells responses has been further reinforced based on their ability to produce high levels of inflammatory cytokines. Inflammation is known to promote the generation of high numbers of functional effector CD8+ T cells regardless of the strength of TCR stimulus. In this regard, studies performed in animal models and clinical evidences have indicated a crucial role for IL-12 and IFNs-type I in cancer immunotherapy. In particular, direct signaling of IL-12, produced by CD40L-activated dendritic cells, has been associated to efficient priming and survival of self/tumor-reactive CD8+ T cells. In particular, an increased expression of the high-affinity IL-2 receptor α-chain (CD25) on cellular surfaces of IL-12

conditioned CD8+ T cells was indicated as a critical determinant promoting their clonal expansion of primed T cells[206]. Furthermore, IL-12 initiated signals on tumor-reactive CD8+ T cells is also associated to the execution of an effector program, as indicated by the increased expression of genes encoding for products mediating cytotoxic effector functions[155,156,206,207].

In addition to a pivotal role in promoting the generation of high numbers of functional effector cells, CD40-stimulated dendritic cells have shown a marked efficacy in preventing cancer recurrence. In particular, generation of long-lasting tumor-reactive immune responses has been extensively associated to helper activity, CD40L-mediated, of CD4+ T cells to license antigen presenting cells to optimally stimulate tumor-reactive CD8+ T cells. To this respect, the acquisition of memory qualities by naïve precursor is also associated to the high production of IL-12 and IFNs-type I cytokines produced by CD40-stimulated dendritic cells. In particular, IL-12, signaling on CD8+ T cells resulted in the up-regulation, in a STAT-4 dependent manner, of anti-apoptotic factor such as Bcl-2 as well as Bcl-3 while restraining the protein level of pro-apoptotic protein BIM[208]. In addition, studies in which the relative contribution of inflammation in the generation of memory CD8+ T cells pool has been evaluated, described a critical role for IL-12 in promoting the differentiation of stimulated naïve CD8+ lymphocytes into memory precursor effector cells (MPECs), their ability to long-term persist in response to homeostatic cytokines such as IL-7 and IL-15 and further differentiate in different memory CD8+ T cell subsets. The impact of the level and quality of inflammatory signals, integrated during priming, by tumor-reactive CD8+ T cells appears key to regulate their differentiation toward effector or central memory subsets[175].

Taken together, these results underline how activation of antigen bearing dendritic cells through CD40/CD40L pathway might results in the generation of anti-cancer primary and memory response mediated by CD8+ T cells recognizing self/tumor antigens. However, anti-tumor activity and potential clinical effectiveness of these CD8+ T cells has been questioned mostly for their limited execution of effector function as a consequence of low avidity interaction with limited epitope-MHC class I complexes express by tumor cells. Pre-clinical and clinical studies, performed in order to evaluate therapeutic potential of agonist-anti CD40 receptor antibodies and CD40L-expressing viral vectors, have suggested that anti-tumor activity of low-affinity CD8+ T cells might potentially be sustained through indirect effects on infiltrating myeloid cells and CD40+ tumor cells. Indeed, ligation of CD40 receptor resulted either in the rescue of exhausted dendritic cells, thereby in a potential provision of inflammatory signals, and also in an increased immunogenicity of target cells. In particular,

stimulation of CD40 receptor expressed by tumor cells resulted in an increased expression of peptide-MHC complexes on their cellular surface thus potentially overcoming limitation associated to low-affinity with an increased TCRs occupancy[84,209].

1.7. Memory CD8+ T cells and Cancer.

CD8+ T cells have been extensively indicated as critical mediator of protective immunity against cancer. Initial immunotherapy strategies were aimed at promoting the in vitro expansion or in vivo generation of tumor reactive CD8+ T cells with effector (T_{eff}) or effector memory (T_{EM}) phenotype. Indeed, therapeutic potential of T_{eff} and/or T_{EM} was attributed to their preferential trafficking to peripheral tissues where they might mediate tumor eradication through their marked cytotoxic activity and antigen-driven IFN-γ secretion.

In the last years, this interpretation regarding the antitumor activity of CD8+ T cells has been revised. Indeed, several studies have underlined how therapeutic potential of tumor-reactive CD8+ T cells is also critically regulated by other functional attributes including the ability of long-term survival and, most importantly, their capacity to self-renew upon antigen recognition. In particular, tumor-reactive CD8+ T cells with stem cell-like (T_{SCM}) and/or central memory (T_{CM}) phenotypic profile has been extensively reported as superior mediators of therapeutic antitumor immunity against established cancer[49,51,111,210-212].

T_{SCM} and T_{CM} are identified according to the expression of CCR7 and CD62L homing receptors. These surface molecules have been indicated as critical markers promoting the migration of immune cells from peripheral blood to secondary lymphoid tissues such as spleen, lymph nodes (LNs) and mucosal Peyer's patches. In this respect, among other integrins and chemokine receptors, a pivotal role for CD62L has been reported. Indeed, CD62L engagement of its ligands (glycosaminoglycans; GAGs) expressed on luminal surfaces of high endothelial venules (HEV) represents a critical interaction defining the ability of T_{SCM} and T_{CM} to continuously re-circulate through lymph nodes. In addition to this preferential trafficking, it has been shown that T_{SCM} and T_{CM} can be properly activated as a consequence of the recognition of cognate antigens on cellular surfaces of professional antigen presenting cells. Activation of tumor-reactive CD8+ T cells with stem cell-like/central memory phenotype induces an enhanced proliferative response resulting in a progeny of cells

retaining T_{SCM}/T_{CM} attributes and also of daughter cells with T_{EM} and T_{eff} phenotypic and functional properties. Hence, generation of protective anti-tumor immune responses appears to be correlated not only to the ability of CD8+ T cells to differentiate into effective cytotoxic T-lymphocytes targeting tumor cells but also in the maintenance of a pool of antigen specific long-living memory cells representing a reservoir of tumor-reactive effector CD8+ T cells[108,210].

Identification of T_{SCM}/T_{CM} as superior mediator of antitumor immunity has critically challenged our initial understanding of the protective potential of different subsets of memory CD8+ T cells. In particular, according to the initial division model proposed in order to explain the heterogeneity of memory compartment, CD8+ T_{CM} have been described as mediators of host-protection against systemic challenge whereas T_{EM} function as sentinels for immediate protection from peripheral challenge such as solid tumors and some infectious agents. However, superior host-protection has been extensively reported for CD8+ T_{SCM}/T_{CM} against virus, bacteria and most importantly tumors, irrespective of immunization or infectious model and the route of antigenic challenge. This observation has obviously influenced the design of passive as well as active immunotherapy strategies. In this respect, in order to prevent terminal differentiation of in vitro expanded autologous tumor infiltrating T lymphocytes (TILs) before re-infusion in cancer patients, different culture conditions are currently under investigation. In this regard, exposure to compounds promoting the activation or inhibition of different pathways, such as homeostatic cytokines (IL-7, IL-15 and IL-21), have been shown to promote the expansion tumor-reactive CD8+T cells with stem cell-like and central memory phenotypic and functional attributes[108,111,210-212]. Although the rationale supporting passive and active CD8+ T cells strategies for cancer treatment is different, therapeutic potential of T_{CM} has been unequivocally demonstrated in either case. Indeed, antitumor efficacy of adoptively transferred tumor-reactive CD8+ T_{SCM}/T_{CM} has been observed even at limiting numbers of infused cells[211]. Furthermore in a recently published study, therapeutic potential of CD8+ T_{CM} cells has been further confirmed at single cell level in response to bacterial infection[118]. In view of the latter observation, antitumor efficacy of T_{CM} lymphocytes is not only related to the number of cells adoptively transferred but also to their intrinsic antitumor activity. Based on this background and prompted by the complex logistics of adoptive treatments, the development of immunotherapy strategies aiming at promoting the rapid in vitro or in vivo generation of tumor-reactive CD8+ lymphocytes displaying phenotypic and functional attributes of central memory T cells is urgently required.

1.8. Targeting CD40 receptor to harness the immune system against cancer.

Generation of functional memory CD8+ T has been repeatedly shown to be critically affected by the strength and the quality of signals integrated by naïve precursors[204,213]. In this regard, a critical determinant is represented by the maturation status of dendritic cells[152,181]. Indeed, it has been shown that engagement of CD40 receptor expressed on their cellular surface by CD40L-expressing activated CD4+ T cells results in the up-regulation of antigen presenting molecules (MHC class I/II), co-stimulatory ligands and cytokines production leading to optimal prime of naïve CD8+ T cells[191,192]. Based on these observations and further prompted by the necessity to overcome limitations associated to the activation of CD4+ T cells in the generation of CD8-mediated antitumor immunity, agonistic anti-CD40 receptor and recombinant viral vectors expressing the transgene encoding for CD40L have been extensively exploited in animal models and in early phase clinical trials [84,209].

Initial evidences underlying the enhanced efficacy of agonist CD40 monoclonal antibodies to promote the expansion of tumor reactive CD8+ T cells, were obtained in pioneering studies performed in animal models. Indeed, administration of anti-CD40 monoclonal antibodies has been shown to represent an efficient substitute of CD4+ T cells and resulted in the rapid generation of anti-tumor CD8+ T cell responses in response to both singenic lymphoma tumor cells and cancer vaccines targeting oncoproteins[191,192,214]. Based on these results, therapeutic potential of agonistic anti-CD40 monoclonal antibodies has been recently evaluated[209,215]. Interestingly cumulative results obtained from different phase I trials involving patients bearing lymphoma and solid tumors have reported the induction of a stable disease in treated patients (20%-50%) with a variable extent regarding the progression free-survival[209]. In addition to the evaluation of clinical responses according to the RECIST criteria, these studies have further restated the rationale supporting the exploitation of immunotherapies targeting CD40 receptor. Indeed, targeting of CD40 receptor with the administration of chimeric agonist IgG1 mAb (ChiLob 7/4) or with a fully-humanized IgG2 mAb (CP-870,893) resulted respectively in the maturation of dendritic cells, as indicated by the up-regulation of CD83 surface antigen, and in the activation of B-cells as antigen presenting cells[215,216]. Indeed, it was reported a significant up-regulation of CD86 co-stimulatory ligand along with a trend of increased expression of antigen presenting (MHC-class II) and CD54 (ICAM-1) adhesion molecule on peripheral B cells of patients receiving

CP-870,893 monoclonal antibodies and resulting, in a minor fraction of melanoma patients, in the transient expansion of MelanA/Mart-1 specific CD8+ T cells [216].

In addition to its pivotal role in promoting maturation of dendritic cells leading to the induction of effective cellular responses, the development of cancer immunotherapies strategies targeting CD40 receptor has been further prompted by the opportunity to potentially overcome several barriers promoting the establishment of immune tolerance of malignant cells[84,209,215,216]. In this regard, it has been reported how triggering of CD40 receptor expressed on endothelial cells might results in the production of pro-inflammatory cytokines and in the up-regulation, on their cellular surface, of adhesion molecules promoting T cell arrest and subsequent infiltration within inflamed tissues [217]. In addition to this enhanced recruitment of effector cells within tumor mass, targeting of CD40 receptor expressed by tumor cells and infiltrating immune cells has been also associated to a significant reduction of immunosuppressive mechanisms preventing the elimination, CD8-mediated, of transformed cells[84,209,215,216]. Indeed, CD40 receptor expression has been identified in nearly all B-cell malignancies and in almost 70% of solid tumors including melanoma, breast, lung, bladder, prostate, colorectal and pancreatic carcinomas[209]. Interesting, engagement of CD40 receptor expressed on cellular surface of transformed cells has been associated to an indirect effect supporting the activity of tumor-reactive CD8+ T cells. Indeed, a marked up-regulation of molecules promoting antigen presentation (MHC-class I/II) and promoting the formation of a functional immunological synapse between target cells and tumor-reactive CD8+ T cells. Indeed, increased expression level of CD54 adhesion molecules, co-stimulatory ligands (CD86) and production of recruiting (IL-8) and inflammatory cytokines (IL-6) supporting T cell activity has been described for in vitro CD40-stimulated tumor cells of different origin[178,209,215]. In addition to a significant effect on the immunogenicity of transformed cells, triggering of CD40 receptor has been also indicated as a critical event regulating the survival and apoptosis of transformed cells[209,218]. Indeed, an increased tumor cell survival has been associated to the in vitro stimulation of B cells derived from malignancies including NHL and CLL[209,215]. In contrast, stimulation of CD40 receptor express on cellular surface of tumor cells derived from melanomas, bladder and ovarian cancer has been associated to the inhibition of tumor growth and also potentially associated to the induction of apoptosis of malignant cells[84,209,215,218]. Interestingly, these opposite effects on CD40 receptor expressing transformed cells has been correlated to the intensity of CD40 receptor initiated signals leading to a selective recruitment of TRAFs molecules and activation of peculiar intracellular signaling pathway resulting in the expression of genes encoding for pro-apoptotic factors and the

activation of caspase 3 and 9[178,209,215,218,219]. Based on these initial evidences, different monoclonal antibodies have been engineered with the aim to mediate elimination of transformed cells as a consequence of direct signaling on target cells and by promoting, through fragment crystallizable region (Fc region), the elimination of tumor cells by activating myeloid cells (ADCC) and the complement cascade (CDC). In this regard, encouraging results have been recently obtained in pre-clinical studies and early phase trials involving patients with multiple myeloma (MM), non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukemia (CLL) receiving either a humanized agonist anti-CD40 IgG1 mAbs (Dacetuzumab; SGN-40) or a fully human agonist antibody preventing the ligation of CD40 by CD40L (Lucatumumab; HCD122) and resulting in the achievement of stable disease in a percentage between 20-65% of treated patients [215]. In line with this results underlining the critical relevance also of myeloid cells such as macrophages for tumor eradication, critical results have been also obtained in pre-clinical and clinical studies performed in pancreatic ductal adenocarcinoma (PDA) patients receiving systemic administration CP-870, 893 monoclonal antibodies. In particular the cohort of patients with surgical incurable PDA received intravenous administration of standard-care treatment gemcitabine in combination with the agonist anti-CD40 IgG2 monoclonal antibody. Remarkably, therapeutic treatment resulted in in the induction, in a significant fraction of treated patients, of objective clinical responses. Indeed, the achievement in 4/21 of partial response and 11/21 disease stabilization whit a median progression free-survival of 5.6 months on a total of 21 treated patients have been observed. Interestingly, analysis of surgically excised tumor lesions obtained from patients with partial response indicate an immune infiltrate mostly composed of macrophages and not, as expected, of T cells. Cancer regression in a T-cell independent manner has been then confirmed in murine model of PDA and a critical relevance for direct CD40 signaling on macrophages has been revealed. Indeed, systemic administration of agonist anti-CD40 mAbs (FGK45) following gemcitabine treatment resulted in murine model in the rapid activation of macrophages, as indicated by the elevated surface levels of MHC class II and CD86 molecule, and their rapid infiltration within tumors. Once in the mass of transformed cells, these CD40-stimulated macrophages were able to mediate cancer regression according to their capacity to secrete high levels of effector cytokines such as IL-12 and TNF-α and most importantly to mediate the disruption of tumor matrix by the degradation of collagen I fibers [220]. In line with the latter observation and further supported by cumulative results obtained in preclinical and clinical studies, in the last years a growing consensus has been registered concerning the multiple effects of cancer immunotherapies targeting CD40 receptor through antagonist and,

most importantly, agonist monoclonal antibodies. In particular, triggering of CD40 receptor expressed on cellular surfaces of dendritic cells has been extensively associated to the activation and programming of tumor-reactive naïve CD8+ T cells to acquire effector functions and potentially differentiate into long-lasting memory cells[191,192]. Furthermore, anti-CD40 monoclonal antibodies can efficiently sustain the anti-tumor activity of reactive CD8+ T cells either as a consequence of a direct effect on tumor cells and by modulating the activity of tumor-infiltrating myeloid cells therefore overcoming the corrupted tumor microenvironment. Unfortunately, a major limitation associated to the administration of agonist monoclonal antibodies targeting CD40 receptor is represented by the concerning regarding their potential toxicity. In particular, the insurgence of cytokine release syndrome (increased serum levels of TNF-α, IL-6, IL-1β and IL-10) and liver stress have been reported in a consistent fraction (almost 50%) of cancer patients involved in different trials and often requirement of steroids administration. The insurgence of these side effect is directly associated to the systemic administration of monoclonal antibodies resulting in the activation of immunocompetent and normal cells[215].

Recombinant viral vectors expressing a transgene encoding CD40L protein represent an alternative strategy that has been further exploited in order to target CD40 receptor for cancer treatment. In particular, CD40L-expressing recombinant adenovirus (AdCD40L) and vaccinia virus (rVV40L) have been evaluated in different studies performed in animal models and also in few early phase clinical trials. Interestingly, the initial rational supporting the generation of engineered viral vectors expressing CD40L was represented by the synergistic effects between TLRs engagement and CD40L protein encoded by the transgene in activating CD40 dendritic cells. In line with this initial observation several evidences has been reported in vitro studies and in animal models regarding the ability of infected dendritic cells to promote the expansion of tumor-reactive CD8+ T cells. In addition, clinical evaluation of cancer vaccines based on subcutaneous administration of tumor cells transduced with AdCD40L have been associated in CLL patients to a count reduction of malignant B-cells correlated to the induction of T cells responses targeting leukemic cells [218 221].

In addition to adenovirus based viral vectors, recombinant vaccinia virus encoding tumor-associated antigens along with transgene (s) encoding for different co-stimulatory ligand (s) have been extensively evaluated in pre-clinical and clinical studies. Vaccinia virus is an enveloped double-strand DNA vector that belongs to poxviridae family. Its genome length is of approximately of 190 Kbp and contain 250 genes encoding for products

regulating both cell-cycle and structural proteins. Remarkably, vaccinia virus has been extensively used in the last two centuries as a worldwide vaccine against variola virus and resulted in its official eradication in 1979[222,223]. Furthermore, specific features of vaccinia virus have prompted the use of this viral vector as a cancer vaccine. First, in sharp contrast with adenoviruses, vaccinia virus can efficiently infect mammalian cells as a consequence of a membrane fusion process and not upon engagement of specific cell surface receptors expressed by target cells. In addition, vaccinia virus replication in the host cell is also "safe and rapid". Indeed, viral replication does not require the integration in the host genome but takes place in cytoplasmic structures termed viral factories and the first viral particle is normally secreted 8 hours after infection of target cells. Finally, vaccinia virus can be easily engineered and multiple transgenes can be accommodated in the viral backbone making this viral vector as a successful toll for antigen specific cancer immunotherapeutic approaches[84,222-224]. Based on this background, in the last 15 years, we extensively evaluated the ability of recombinant vaccinia virus (rVV) to efficiently promote the generation of tumor-reactive CD8+ T cells. In particular, we have previously shown in preclinical studies how recombinant vaccinia virus (Copenaghen Strain) expressing transgenes encoding tumor-associated antigens along with co-stimulatory ligands can efficiently promote the expansion of reactive CD8+ T cells from peripheral blood of healthy donors and, most importantly, from surgically excised tumor lesions [225-228]. The enhanced efficacy of our recombinant vaccinia virus in promoting the generation antitumor CD8-mediated immune response has been then confirmed in clinical studies. Indeed, a phase I/II trial involving twenty metastatic melanoma patients has been recently conducted on metastatic melanoma patients. In particular, therapeutic treatment of cancer patients was based on the initial intradermal administration of a replication incompetent (UV-inactivated) recombinant vaccinia virus accommodating transgenes encoding for HLA-A0201-restricted epitope derived from tumor associated antigens (MelanA/MART-1_{27-35}, $gp100_{280-288}$ and $Tyrosinase_{1-9}$) along with co-stimulatory ligands such as CD80 and CD86 proteins (rVVmelB7). Furthermore, boost of vaccination represented by the administration of corresponding free-peptide along with GM-CSF were further provided to the enrolled patients. Notably, this vaccination protocol resulted in the regression of individual metastasis in three out of 11 patients whereas disease stabilization was observed in 7/11 melanoma patients. Furthermore, clinical observations indicated how clinical benefits induced in treated patients were related to the high immunogenicity of rVVmelB7. Indeed induction of CD8+ T cells targeting all the three HLA-A0210 restricted epitopes derived from tumor-associated antigens were detected in 43% of patients upon

intradermal administration of rVVmelB7. However, tumor-reactive CD8+ T cells induced resulted limited in time and critically dependent from further rVVmelB7 boost of stimulation [229]. In order to improve the magnitude and the duration of CD8-mediated cellular responses against tumor antigens we have then generated a CD40L-expressing recombinant vaccinia virus (rVV40L) that has shown in a previous in vitro study, an enhanced efficacy in promoting the maturation of dendritic cells that in turn efficiently activate peptide specific CD8+ T cells. However, phenotypic profile and functional properties of stimulated CD8+ T cells were not evaluated [230].

1.9 STUDY AIM

The aim of the current work is to evaluate the capacity of a CD40L-expressing recombinant vaccinia virus to mimic molecular basis of CD4+ T cell helper activity in the generation of antigen-specific CD8+ T cells with phenotypic and functional attributes of central memory lymphocytes.

In addition to the modulation of tumor-specific CD8+ T cell responses, we further investigated the ability of rVV40L to inhibit proliferative capacity of established tumor-cell lines upon infection or through the activation of myeloid cells.

2. MATERIALS AND METHODS.

2.1 CD40 Ligand-expressing recombinant Vaccinia Virus construction (rVV40L)

CD40L Ligand-expressing recombinant Vaccinia Virus (rVV40L) was generated as previously described[230]. Briefly, human CD40L cDNA was PCR amplified from PHA stimulated human PBMCs, cloned in a shuttle plasmid under the control of a virus early promoter and inserted into the Copenhagen Vaccinia Virus Wild-Type strain (VV WT).

In order to avoid the strong cytopathic/lytic effect of the replicating virus in in-vitro studies, viral replication was inactivated by DNA cross-linking by using psoralen (1ug/ml) and long-wave UV (365nm) irradiation. The inhibition of replication and cytopathic effect of UV-treated viruses was evaluated on infected monolayers of sensitive CV-1 cells (ATCC CCL70) and CD14+ monocytes at 24 hours post infection[230].

2.2 Cell Cultures

Peripheral blood mononuclear cells (PBMCs) from healthy donors were obtained by gradient centrifugation. $CD14^+$ monocytes and $CD8^+$ T cells were isolated by using antibody-coated magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany). Cells were then cultured in RPMI-1640 supplemented with 1% GlutaMAX-I, 1% non-essential amino acids (NEAA), 1% sodium pyruvate, HEPES, 1% Kanamycin Sulfate (Gibco-Life Technologies, Lucerne, Switzerland), thereafter referred to as complete medium (CM) and 10% Fetal Bovine Serum (FBS, Gibco-Life technologies, Lucerne, Switzerland) for monocyte activation studies or 5% pooled human AB serum (Blutspendezentrum, University Hospital Basel, Switzerland) for T-cell stimulation assays. (To add also the information relatives to cell lines). Established, verified cell lines were obtained from ATCC (Manassas, VA) and cultured in CM supplemented with 10% FBS.

2.3 Gene Expression Analysis.

Total cellular RNA was extracted from $CD14^+$ monocytes, $CD8^+$ T cells and established tumor cell lines using the RNeasyVR Mini Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's protocol. RNA was reverse transcribed using M-MLV reverse transcriptase following manufacturer's instructions (Invitrogen-Life Technologies, Lucerne, Switzerland). Human IL-12p40, IFN-γ, IFN-α, IFN-β, TNF-α, IL-10, indoleamine-2,3-Dioxygenase (IDO), TNF receptor associated factor 1 (TRAF1) and CD40 receptor gene expression was evaluated by quantitative RT-PCR (qRT-PCR) using specific primer sets (TaqMan® Assays, Applied Biosystems-Life Technologies, Lucerne, Switzerland) and normalized to human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene expression.

2.4. Flow Cytometry

Fluorochrome-labeled monoclonal antibodies (mAbs) recognizing CD1a, CD3, CD8, CD14, CD16, CD45RA, CD28, CD54 (ICAM-1), CD62L, CD95, CD69, CD40L (CD154), IL-7R⟨ (CD127), CXCR3 (CD183), PD-L1 (CD274) HLA-ABC and HLA-A0201 were obtained from Becton Dickinson (Allschwil, Switzerland). In addition, mAbs recognizing CCR7 (CD197) and CD45RO (Clone UCHL1) were obtained from BioLegend (Lucerne, Switzerland) whereas mAbs recognizing CD40 receptor (Clone 5C3) were obtained from eBioscience (San Diego, CA). In order to identify cells bearing antigen-specific T-cell receptors (TCR), $CD8^+$ T cells were stained with soluble HLA-A0201-peptide, streptavidin R-PE conjugated multimers containing L27Melan-A/MART-$1_{26–35}$, Vaccinia Virus $H3L_{184–192}$, HCMV$pp65_{495-504}$ and influenza A MP_{58-66} (ProImmune, Oxford, UK).

Cells were stained with appropriate mAbs dilutions for 30 minutes at 4°C in the dark. Following gentle washing, specific labeling was evaluated by flow cytometry (FACScalibur; Becton Dickinson Allschwil, Switzerland). Data were analyzed by FlowJo software (Tree Star, Ashland, OR).

2.5 ELISA assay

Presence of interleukin-2 (IL-2) in supernatants of cell culture from was measured three days after stimulation of memory CD8+ T cells by using an ELISA kit (Becton Dickinson, Allschwil, Switzerland), according to the manufacturer's instructions. This system uses 2 different IL-2 antibodies allowing its capture on solid surface followed by the labelling with an enzyme coupling secondary antibody.

2.6 Peptides.

HLA---A0201---restricted L27Melan---A/MART---$1_{26---35}$, Vaccinia Virus $H3L_{184---192}$, HCMV $pp65_{495---504}$ and Influenza A $MP_{58---66}$ peptides used in this study were provided by NeoMPS Laboratories (Strasbourg, France).

Purified $CD14^{+}$ cells were incubated for 4h at 37° with individual or pooled peptides at 1∝g/ml final concentration in culture medium.

2.7 Cell sorting.

Magnetically isolated CD8+ T cells were then stained (as described above) with the following mAbs: CD45RO-FITC, CD8-PE CD3-APC or CD62L-APC. $CD3^{+}CD8^{+}$ T cells and Naïve T cells ($CD8^{+}CD45RO^{-}CD62L^{+}$) were sorted using FACSAria or Influx Cell sorters (Becton-Dickinson Allschwil, Switzerland). Preparations used in this study showed a purity of at least 99%.

2.8 $CD14^{+}$ cells activation following rVV40L infection or s40L treatment.

Magnetically sorted $CD14^{+}$ cells were infected in 500ul 10% FCS RPMI 1640 CM for 1h at 37°C with Vaccinia Virus Wild Type (VV WT) or CD40L-expressing recombinant Vaccinia Virus (rVV40L) at a MOI of 5. $CD14^{+}$ cells were also activated with soluble

CD40L recombinant protein (s40L; 0.5∝g/ml Enzo Lifescience, Farmingdale, NY) alone or upon infection with VV WT (VV-WT+s40L). Following overnight incubation at 37° in FCS 10% CM supplemented with GM-CSF (10ng/ml, Laboratorio Pablo Cassarà, Buenos Aires, Argentina). The expression of surface markers and cytokine gene expression and production were evaluated by flow cytometry, qRT-PCR analysis and ELISA assays.

2.9 In vitro $CD8^+$ T cells priming.

Naive $CD8^+$ T cells (CD8+ CD45RO- CD62L+) were co-cultured in RPMI 1640 CM supplemented with 5% HS with either allogeneic or autologous peptides-pulsed $CD14^+$ monocytes at a 5:1 ratio. On day 8, primed $CD8^+$ T cells were harvested and flow cytometric analysis was performed as described above.

2.10 Activation of CD8+ T cells.

In order to evaluate the potential CD40 expression upon activation, sorted CD3+ CD8+ T cells were stimulated by plastic bound anti-CD3 (10µg/ml, eBiosciences, San Diego CA) and soluble anti-CD28 (αCD28; 0.5∝g/ml; Becton Dickinson, Allschwil, Switzerland). Alternatively, $CD8^+$ T cells were stimulated with anti-human CD3/CD28 coated beads (TC expander, Invitrogen, Basel, Switzerland) a 1:3 $CD8^+$ T cells:beads ratio. In indicated experiments activation of $CD8^+$ T cells was performed in presence of soluble CD40L recombinant protein (0.5∝g per ml; s40L). Proliferation was assessed by 3H-thymidine incorporation according to standard procedures.

2.11 CD8+ T_{CM} proliferation assays.

Proliferative capacity of $CD8^+$ T_{CM} cells was assessed, 72h after antigen specific or polyclonal re-stimulation, by carboxyfluorescein-succinimidyl-ester (CFSE, Invitrogen, Basel, Switzerland) staining dilution. Briefly, $CD8^+$ T cells were washed in PBS

supplemented with 0.1% BSA (Sigma–Aldrich, Postfach, Switzerland). Cells were then incubated with 0.5 mM CFSE for 10 min at 37°. After washing with "cold" 5% HS RPMI 1640 CM, $CD8^+$ T cells were cultured with autologous $CD14^+$ monocytes previously pulsed with indicated peptides at a 1:1 ratio of for 72h. Alternatively, proliferation was induced by using plastic bound anti CD3 (10µg/ml, eBiosciences, San Diego CA) and soluble anti CD28 mAbs (1µg/ml) (Becton Dickinson, Allschwil, Switzerland).

2.12 rVV40L infection of tumor cells.

Na8, HCT116 and Colo205 cancer cell lines were left untreated or infected with VV WT or rVV40L at MOI of 10. Furthermore, tumor cells were treated with s40L (0.5∝g/ml) alone or upon infection infection with VV WT. Proliferative capacity of Na8, HCT116 and Colo205 was evaluated at day 4 by evaluating ^{3}H-thymidine incorporation in the last 18 hours of culture. In addition, percentage of apoptotic cells in cultures under investigation was assessed by using Annexin V/ Dead cell apoptosis Kit according to the manufacturer's intructions.

Alternatively, H358 and HepG2 cell lines were left untreated or infected with VV WT or rVV40L at MOI of 10. After 24 hours cells were left alone or cultured with isolated CD14+ monocytes at the ratio of 1:1. At day 4, cell cultures were harvested and proliferative capacity of tumor cells and TNF-α gene expression in CD14+ cells were respectively evaluated by ^{3}H-thymidine incorporation and qRT-PCR.

2.13 Statistical Analysis.

Statistical analysis software SPSS (Version 14.0, SPSS Inc., Chicago, IL) was used throughout the study. Skewness, Kurtosis distribution parameters and respective standard errors were used to test normality of the concerned populations. Mann-Whitney non-parametric test, (non-Gaussian distribution of the population) was used to compare mean of gene expression in different samples. Outliers were defined using Grubbs' test. All reported P-values were considered to be statistically significant at $P \leq 0.05$.

3. RESULTS

In order to evaluate the role of CD40 ligand (CD40L; CD154)/CD40 receptor pathway in the generation of immunological memory, initial experiments were performed to assess the expression and, most importantly, functional consequences of CD40 receptor triggering on isolated antigen presenting cells (APCs) and CD8+ T cells obtained from peripheral blood of healthy donors.

3.1. Characterization of CD40 on human CD8+ T cells.

In order to evaluate the role of CD40 ligand (CD40L; CD154)/CD40 receptor pathway in the generation of immunological memory (CD8+ Tcm), initial experiments were performed to assess expression and, most importantly, functional consequences of CD40 receptor triggering on isolated antigen presenting cells (APCs) and CD8+ T cells obtained from peripheral blood of healthy donors.

Generation of fully functional memory $CD8^+$ T-cells in experimental mouse models was previously reported, in a limited numbers of studies, to be critically dependent on the engagement of CD40 receptor expressed on activated $CD8^+$ T-cells. Indeed, selective deficiency of CD40 receptor in CD8+ T cells prevented the direct delivery of help by CD40L expressing CD4+ T helper cells during the priming of cellular response and resulted in the generation of memory CD8+ T cells characterized by a reduced proliferative capacity and impaired acquisition of effector functions upon secondary stimulation. Interesting, ligation of CD40 receptor on CD8+ T cells has been shown of proven relevance in the response against cellular antigens, and, therefore, of remarkable interest for our purposes[196,197].

3.1.1 CD40 on resting or activated human CD8+ Tcells

In order to evaluate, the potential role of this mouse model mechanism in humans, CD40 receptor expression on human $CD8^+$ T-cells was assessed in resting condition or upon activation.

Similarly to murine T-cells, CD40 receptor expression was undetectable on the surface (**Fig. 1A**) or in the intra-cellular compartment (**Fig.1B**) of resting human CD8+ T-cells.

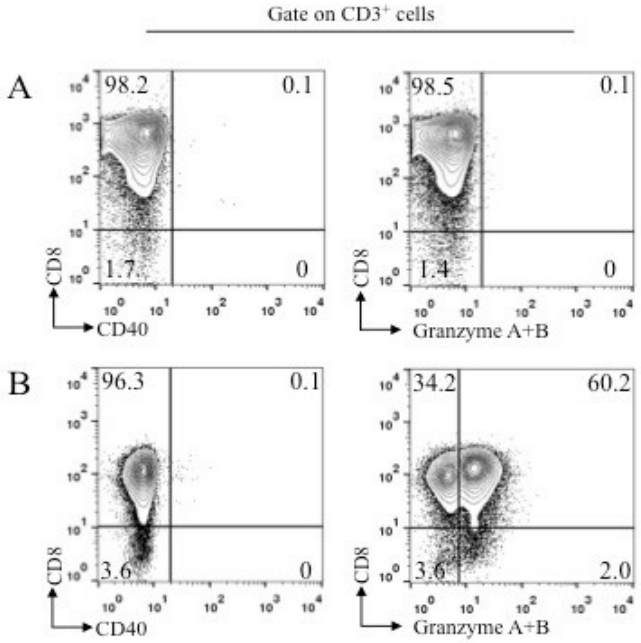

Figure 1. Resting human CD8$^+$ T cells do not express CD40 receptor. Expression of CD40 receptor and granzymes A-B on the surface **(a)** or in the intra-cellular compartment **(b)** of human peripheral blood CD8$^+$ T-cells from healthy donors was analyzed by flow-cytometry. Data refer to one representative experiment out of five performed with similar results.

In order to investigate the expression of CD40 receptor on activated CD8+ T cells, magnetically isolated CD8$^+$ T-cells were stimulated with plastic-bound anti-CD3 and soluble CD28 monoclonal antibodies or, alternatively, with anti CD3/CD28 coated beads. A clear up-regulation in the expression of CD69 and CD40L surface antigens, confirming successful activation and the potential capacity of CD8+ T cells to license APCs was observed. However, surface expression of CD40 receptor remained undetectable at any of the time points tested on stimulated T cells. (**Figs. 2A, B**). Furthermore, Real Time-PCR (RT-PCR) on sorted and activated CD3$^+$CD8$^+$ T-cells (purity>99%) also confirmed that, while IFN-γ gene expression was clearly up-regulated, mRNA level CD40 receptor was not (**Fig. 2C**).

To further assess the possible functional relevance of residual CD40 expression, escaping flow-cytometry and RT-PCR detection, CD3+ CD8+ T cells were activated by CD3/CD28 triggering in the presence of soluble CD40L recombinant protein (s40L) and proliferative capacity of activated CD8+ T cells was assessed at different time points. Notably, also in this experimental setting, the expression of CD40 receptor on activated CD3+ CD8+ T cells remained elusive. Indeed, activation of CD8+ Tcells in presence of s40L did not result in an increased proliferative capacity of stimulated T cells at any of the time points tested (**Fig. 2D).**

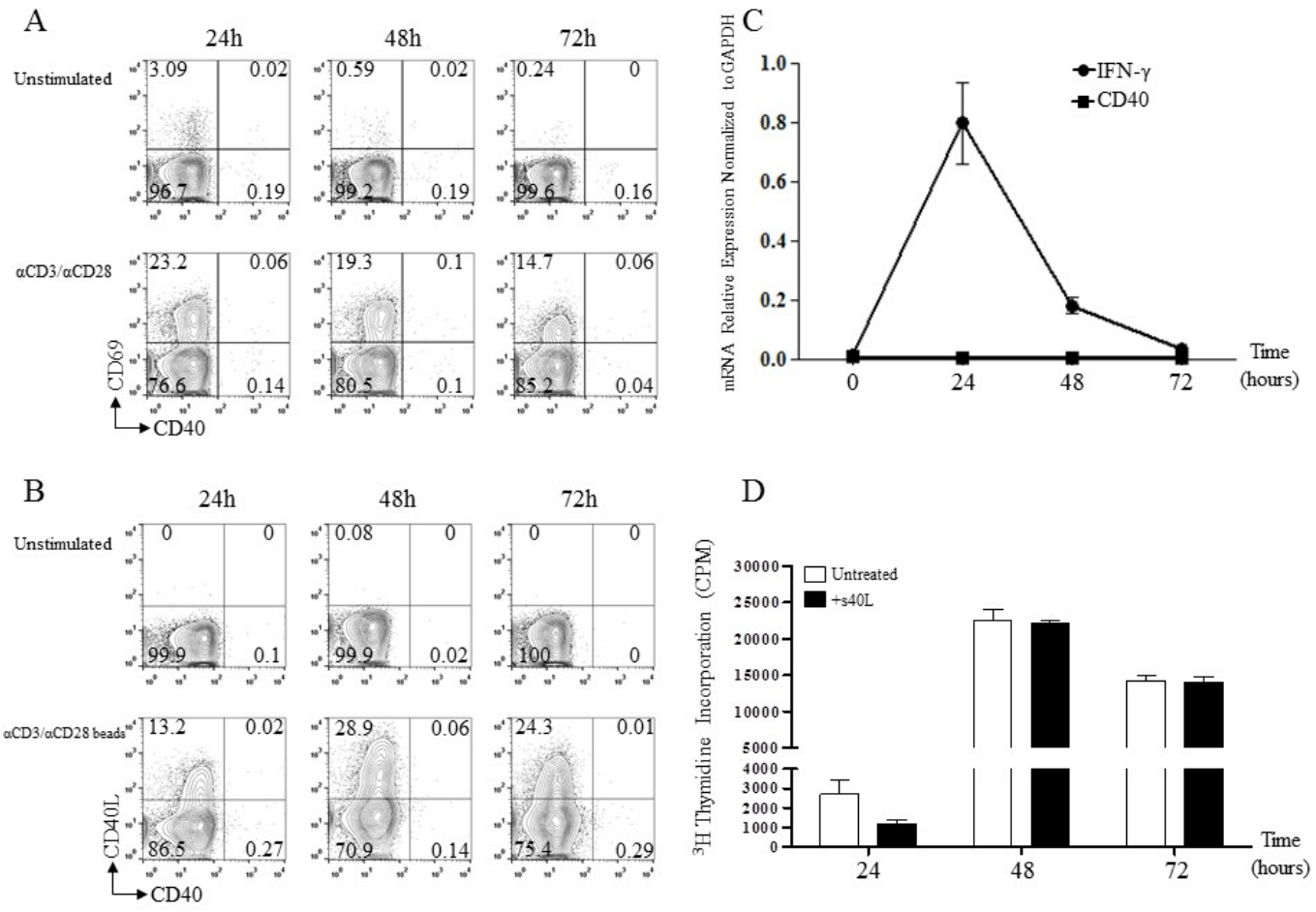

Figure 2. Activated human CD8$^+$ T cells do not express CD40 receptor in antigen presenting cells free-system. Magnetically sorted CD8$^+$ T-cells were activated **(A)** with different doses of plastic bound anti-CD3 (10 or 1 ∝g/ml) and soluble anti-CD28 (0.5 ∝g/ml) or anti CD3/CD28 coated beads (ratio beads : CD8$^+$ T-cells = 3 : 1) **(B)** respectively. At the indicated time points, cells were collected and stained with anti-CD3 mAbs, anti-CD8 mAbs and **(A)** anti-CD69 mAbs or **(B)** anti-CD40L (CD154) mAbs. The results shown here refer to one representative experiment out of five performed with identical results. **(C)** Kinetics of CD40 receptor and IFN-© gene expression analysed by RT-PCR from anti-CD3 (10ug/ml) and anti-CD28 (0.5µg/ml) stimulated CD3$^+$CD8$^+$ T-cells. **(D)** Sorted human CD3$^+$CD8$^+$ T-cells were activated with anti-CD3 (10 ∝g/ml) and anti-CD28 (0.5 ∝g/ml) in the presence (black bars) or absence (white bars) of soluble CD40L recombinant protein (s40L; 0.5µg/ml). Proliferation of CD3$^+$CD8$^+$ T cells was assessed at the indicated time points by ^{3}H-thymidine incorporation during the last 18h of culture.

It has been extensively reported that activation of CD8+ T cells is critically affected by the cytokine milieu experienced by responding cells during antigen recognition. Based on this background, we hypothesized that expression of CD40 receptor on cell surfaces of anti-CD3/anti-CD28 stimulated CD8+ T cells could be promoted by specific inflammatory

cytokines including IL-12 and IFNs-type I. Thus, we evaluated the expression of CD40 receptor on CD8+ T cells activated in presence of "conditioned medium" derived from CD14+ monocytes activated by viral infection and/or by s40L stimulation. Interestingly, levels of activation markers of CD8+ T cells in presence of culture medium derived from activated CD14+ cells, and, in particular, of infected monocytes, were clearly increased as compared to CD8+ T cells stimulated in presence of medium derived from resting CD14+ monocytes. However, despite an enhanced up-regulation of CD69 activation marker, CD40 receptor expression on activated CD8+ T cells remained undetectable in any tested condition (**Fig. 3**).

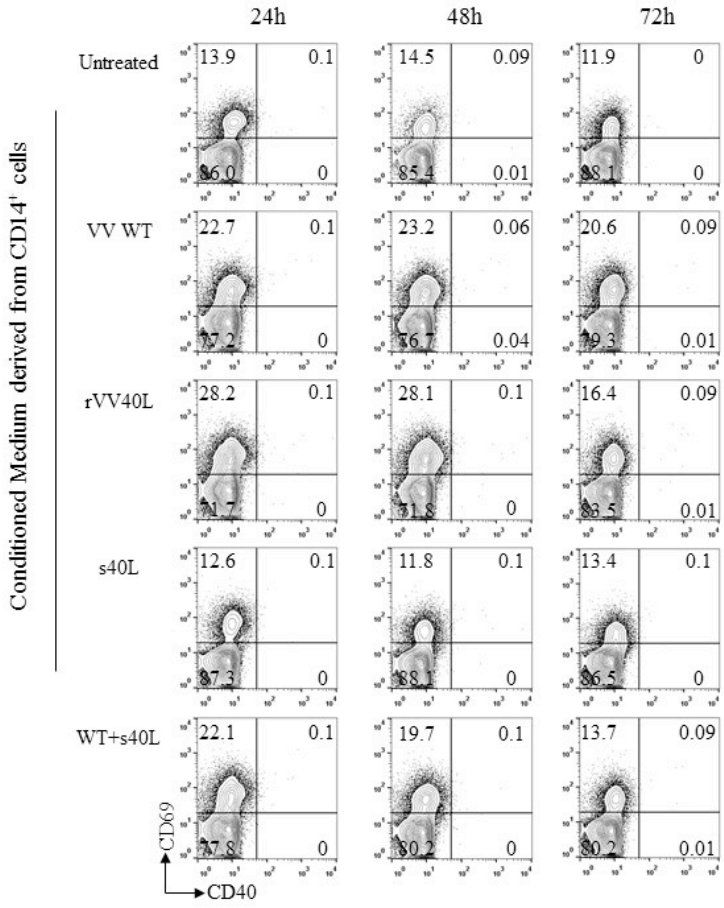

Figure 3. Human CD8$^+$ T cells activated in presence of stimulated CD14$^+$ derived medium do not express CD40 receptor. Isolated peripheral blood CD14$^+$ monocytes were left untreated, infected with rVV40L or with VV WT. Furthermore, CD14$^+$ monocytes were also treated with s40L alone or upon infection with VV WT (VV WT+s40L). After treatment, 3x10^6 CD14$^+$ monocytes were cultured for 3 days in 1 ml of RPMI-1640 CM supplemented with FCS 10%. Human CD8$^+$ T-cells were activated with plastic-bound anti-CD3 (10 µg/ml) and soluble anti-CD28 (0.5 µg/ml) in the presence of supernatants from different CD14$^+$ monocytes cultures. At the indicated time points, CD8$^+$ T cells were collected and stained with anti-CD40 mAbs and anti-CD69 mAbs. Data refer to one representative experiment out of three performed with similar results.

Taken together these data indicate that CD40 receptor is not expressed on cell surfaces or in the intra-cellular compartment of human CD8+ T cells. Furthermore, in contrast with murine counterparts, expression of CD40 receptor remains negative also on activated human CD8+ T cells. Indeed, analysis of the gene expression profile of in vitro activated CD8+ T cells did not reveal an up-regulation of CD40 receptor mRNA levels.

3.1.2 Acquisition of CD40 receptor as a consequence of intercellular trogocytosis.

Generation of adaptive immune responses is a tightly regulated process in which several cell types, endowed with different functions, are involved. Functional attributes defining the role of distinct immune cells during immune responses are frequently related to the peculiar expression of specific molecules on their cellular surfaces. Interestingly, in the last years, this scenario has been partially revised. Indeed, in several in vitro and in vivo studies, it has been reported that immune cells can overcome the limits of their transcriptome by extracting specific proteins from other cells[231-233]. This phenomenon has been named trogocytosis and different mechanisms promoting the intercellular exchange of surface molecules between immune cells have been reported. In this respect, several studies performed in animal models have revealed the capacity of T cells to acquire MHC class I/ II and co-stimulatory molecules from APC following immunological synapse formation. Interestingly, acquisition by T cells of APCs-derived proteins has been shown to be initiated by the engagement of T cell receptor (TCR) and to affect the biology of the immune system during the generation of adaptive cellular responses[231-233].

Based on this background and aiming at investigating the possible role of trogocytosis in the acquisition by human T cells of CD40 receptor expressed by APCs, we initially evaluated membrane exchange between CD14+ monocytes and CD8+ T lymphocytes in autologous and allogeneic settings. Interestingly, in accord with studies in experimental animals, we detected a limited presence of CD40 receptor on CD8+ T cells cultured with allogeneic but not with autologous CD14+ monocytes (**Fig. 4A**). In order to formally prove that detection of CD40 receptor on T lymphocytes was due to membrane exchange, we co-

cultured in a mixed leukocyte reaction (MLR) HLA-A0201- CD8+ T cells with HLA-A0201+ CD14+ monocytes and we then performed a detailed polychromatic flow cytometry analysis.

Remarkably, as shown in **Fig. 4B**, in this experimental setting, we could detect CD40 receptor expression on cellular surfaces of CD8+ T cells as a consequence of the membrane exchange with allogeneic CD14+ monocytes as confirmed by the transfer of the heterologous HLA type on CD8+. Indeed, detection of CD40 receptor on cellular surface of activated CD8+ T cells resulted in the extraction from allogeneic CD14+ monocytes also of HLA-0201 class I molecules, restricting antigen presentation. Hence, these data indicate a possible involvement of trogocytosis phenomena in the acquisition by CD8+ T cells of CD40 receptor from cellular surfaces of APCs. However, evaluation of the magnitude of membrane exchange between CD14+ monocytes and CD8+ T cells revealed that only a very limited percentage of T lymphocytes was able to acquire, over the time, CD40 receptor (**Figs 4A, B**). Moreover, as stated previously, this positivity only appeared in allogenic setting.

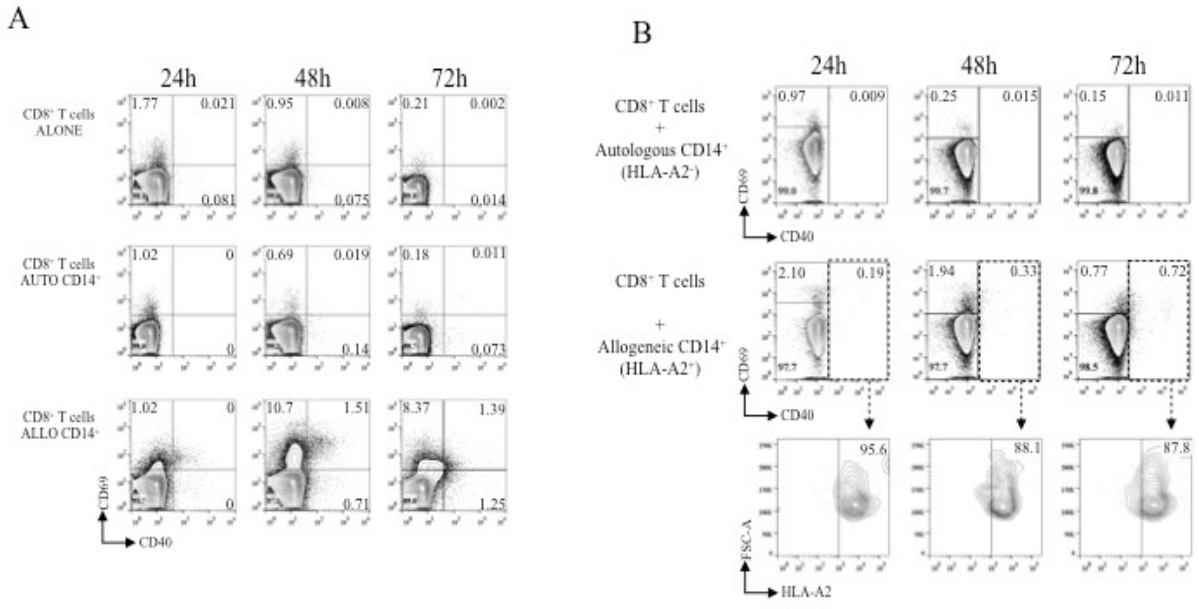

Figure 4. Activated human CD8+ T cells acquire CD40 receptor as a consequence of membrane exchange phenomenon. Isolated human CD8+ T cells were co-cultured with autologous or allogeneic CD14+ monocytes at 1:1 ratio. CD69 and CD40 receptor expression was evaluate at indicated time points on CD3+ CD8+ T cells. Stimulation with allogeneic monocytes resulted in the expression of CD40 receptor on activated (CD69+) CD8+ T cells **(A)**. Mixed leukocyte reaction involving HLA-A2- CD8+ T cells and HLA-A2+ CD14+ monocytes revealed that CD40 receptor expression on cellular surface of T-lymphocytes is associated to membrane exchange, trogocytosis, phenomenon. Indeed CD40+ CD8+ T cells also express HLA-A2 molecules derived from allogeneic CD14+ monocytes **(B)**. Data refer to one representative experiment out of three performed with similar results.

In this respect, although further investigation would enable the evaluation of the putative benefits related to the acquisition of CD40 receptor for CD8+ T cells, it is reasonable to assume that in a human setting effects of CD40L/CD40 receptor triggering in the generation of effective memory CD8+ T cells are only related, to the activation of antigen presenting cells.

3.2. CD40 receptor on human CD14+ monocytes.

Activation of cells endowed with antigen presenting capacity like dendritic cells, B cells and monocytes may be achieved following engagement of CD40 receptor expressed on their surfaces by CD40L expressed by activated T cells[176]. In this regard, we previously showed that APC infection by rVV40L promotes their activation. Indeed, rVV40L infection of in vitro generated DCs resulted in a marked up-regulation of co-stimulatory molecules' expression and IL-12 secretion[230]. Based on this background, we investigated the activation of CD14+ monocytes following treatment with rVV40L or s40L alone or in combination with vaccinia virus wild- type (VV-WT+s40L) infection. In particular, we assessed the expression of a panel of co-stimulatory and inhibitory ligands on cell surfaces of treated CD14+ monocytes and we further evaluated their cytokine gene expression profiles.

IL-12 and IFNs type I have been shown to play important roles in memory T cell generation. In particular, T cell help independence observed in the generation of effective memory cells has been attributed to the ability of some infectious pathogens to promote secretion of high amounts of IL-12 and IFN-α/β[156,207]. Furthermore the critical relevance of these pro-inflammatory cytokines, in promoting anti-tumor CD8+ T cells responses, has been extensively reported in preclinical and clinical studies (see above). Interestingly, we observed a marked and sustained up-regulation of IL-12p40, IFN-α and -β gene expression in $CD14^+$ monocytes upon rVV40L infection whereas activation of $CD14^+$ cells by s40L, alone or in combination with VV-WT, was significantly less efficient (**Fig.5**).

Remarkably, in sharp contrast to rVV40L, s40L, and, to a lower extent, VV-WT, appeared to promote the expression of interleukin 10 (IL-10) and indoleamine-2,3-dyoxigenase (IDO) genes in treated $CD14^+$ cells (**Fig. 5**). Interestingly, IL-10 has been indicated as a critical immunoregulatory cytokine abrogating the activity of innate immune

system and preventing the generation of adaptive immune responses. In particular, IL-10 production by myeloid-derived suppressor cells (MDSCs) has been indicated as a critical mediator promoting tumor tolerance. Furthermore, IL-10 cytokine has been reported, among other soluble factors, as critical mediator involved in Treg-mediated suppression in particular by promoting, through phosphorylation of STAT-3, IDO enzyme production by tumor-infiltrating MDSC[13,84]. Interestingly, expression of high levels of IDO enzyme have been also correlated in different solid cancer, with the ability of tumor cells to evade antitumor immune responses. In particular, immunosuppressive effects on CD8+ T cells activity have been associated to the ability IDO enzyme to promote local depletion of tryptophan as well as accumulation of kinurenyn [234].

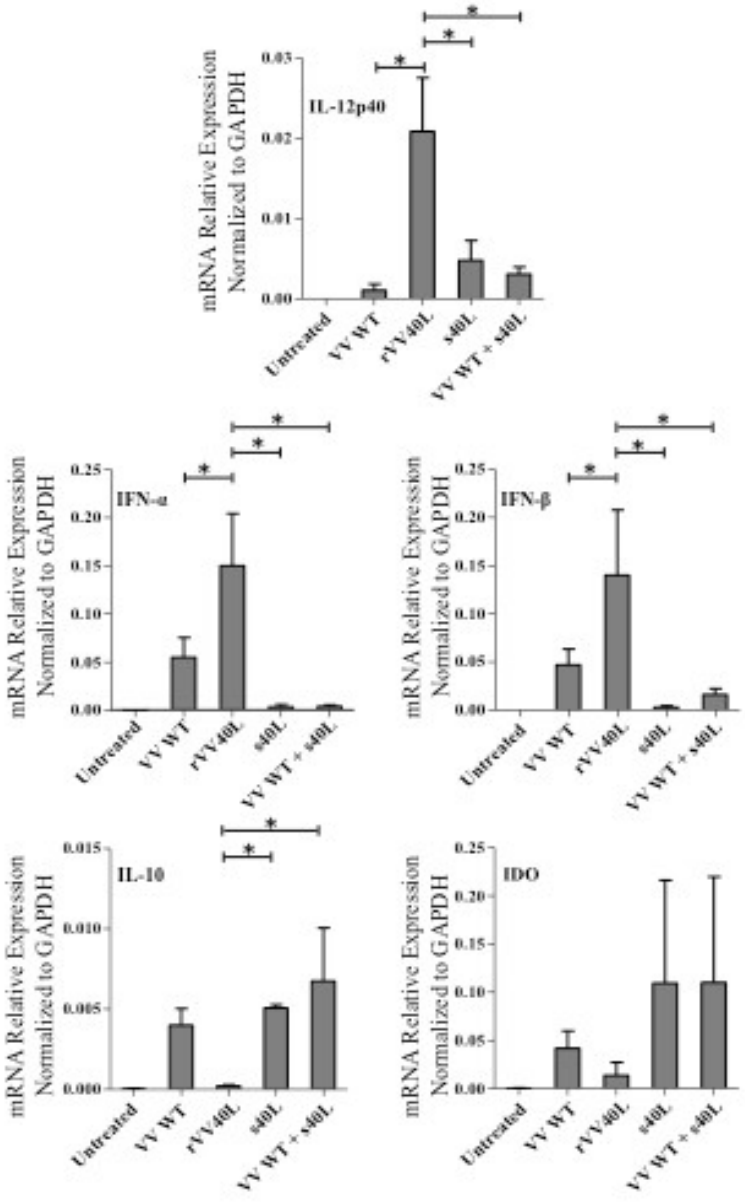

Figure 5. CD40L-expressing recombinant Vaccinia Virus (rVV40L) efficiently induces pro-inflammatory cytokines in infected CD14+ monocytes. Purified peripheral blood $CD14^+$ cells from healthy donors were left untreated, infected with rVV40L (rVV40L) or with VV-WT (VV WT) at MOI 5. Moreover, $CD14^+$ cells were also treated with soluble CD40L recombinant protein alone (s40L) or following VV WT infection (VV WT+s40L). After 24 hours of culture, $CD14^+$ cells were harvested and total cellular RNA was then extracted, reverse transcribed and **(A)** expression of IL-12p40 (n=5), IFN-α (n=4), IFN-β (n=4), Indoleamine 2,3-dioxygenase (IDO; n=3) and IL-10 (n=4) genes was analyzed by qRT-PCR, using GAPDH gene expression as reference. Values are reported as mean+/-standard error to the mean (SEM).

CD8+ T cell activation requires sustained T cell receptor (TCR) interaction with MHC-peptide complexes in the immunological synapse (IS) between T cells and antigen-presenting cells (APCs). In this regard, we assessed the expression the expression in $CD14^{+}$ cells of a panel of molecules involved in the generation of the immunological synapse with T cells. Interestingly, culture of CD14+ monocytes in the presence of VV-WT, rVV40L, s40L or both, resulted in a similar increase of MHC class I molecules expression (HLA-ABC), as compared to untreated cells whereas expression of CD54 (ICAM-1) adhesion molecule, promoting the stabilization of APC-T cell interaction, was only marginally affected (**Fig. 6**). Activation of APCs promotes the expression of a variety of markers involved in co-stimulation or inhibition of CD8+ T cell responses. Indeed, s40L induced a significant increase of the expression of CD80 co-stimulatory but also PD-L1 T-cell inhibitoryligand, as compared to untreated controls ($P<0.001$). In contrast, infection with VV-WT or rVV40L, resulted in a non-significant increases of CD80 and PD-L1 expression levels on infected monocytes (**Fig. 6**).

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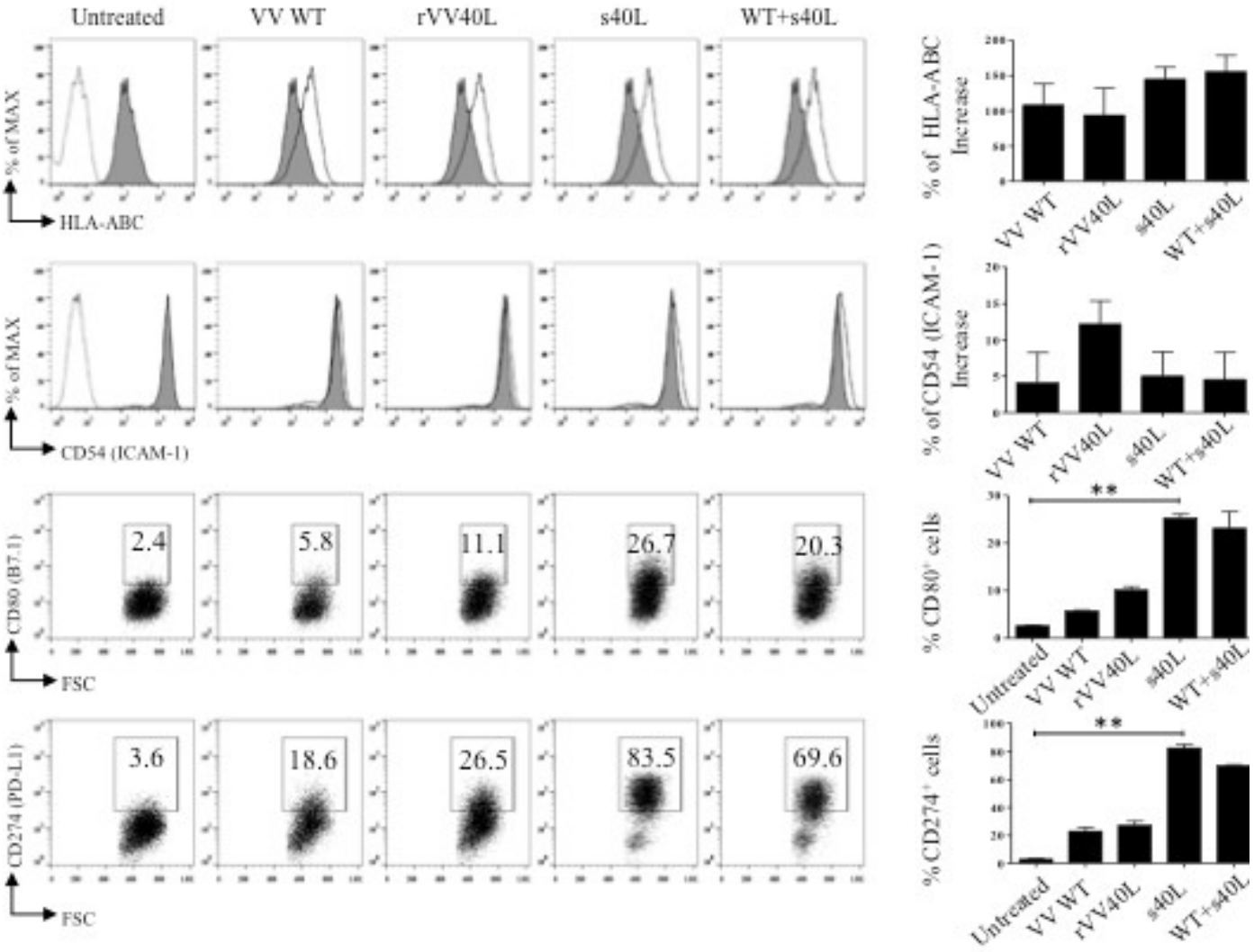


Figure 6. rVV40L-infected CD14+ monocytes do not express PD-L1 (CD274). $CD14^+$ activation was analyzed by evaluating increases in mean fluorescence intensity (MFI) of HLA-ABC and CD54 (ICAM-1) expression on differently treated monocytes (shaded histograms), as compared to untreated controls (open histograms) whereas CD80 and PD-L1 (CD274) expression was evaluated as percentage of positive cells. Data in the left panel summarize the results from three independent assays performed with cells from different donors. Similar results were obtained by flow cytometric analysis of differentially treated CD14+ monocytes. Remarkably, in contrast with s40L stimulated cells, rVV40L-infected CD14+ monocytes did not significantly up-regulate PD-L1 (CD274) expression. *: $P<0.05$, **: $P<0.01$.

CD14+ monocytes are highly plastic myeloid cells. In this respect, it has been extensively reported that activated CD14+ cells could progressively differentiate towards dendritic cell (DC) and/or macrophages (Mo). In order to evaluate the differentiation potential of viral infection and s40L treatment, we evaluated the cell surface expression of CD1a and CD16 on untreated and differentially activated CD14+ monocytes. Notably, CD16 expression, suggestive of a macrophages differentiation was not significantly affected by

activation of CD14+ monocytes. Furthermore, CD1a expression was undetectable in all culture conditions. Hence, up-regulation on cell surfaces of co-stimulatory molecules and enhanced IL-12p40, IFN-α and -β gene expression on/in rVV40L-infected CD14+ monocytes is consistent with the ability of our recombinant viral vector to promote antigen presenting capacity of CD14+ monocytes without promoting their differentiation into more specialized APCs (**Fig.7**).

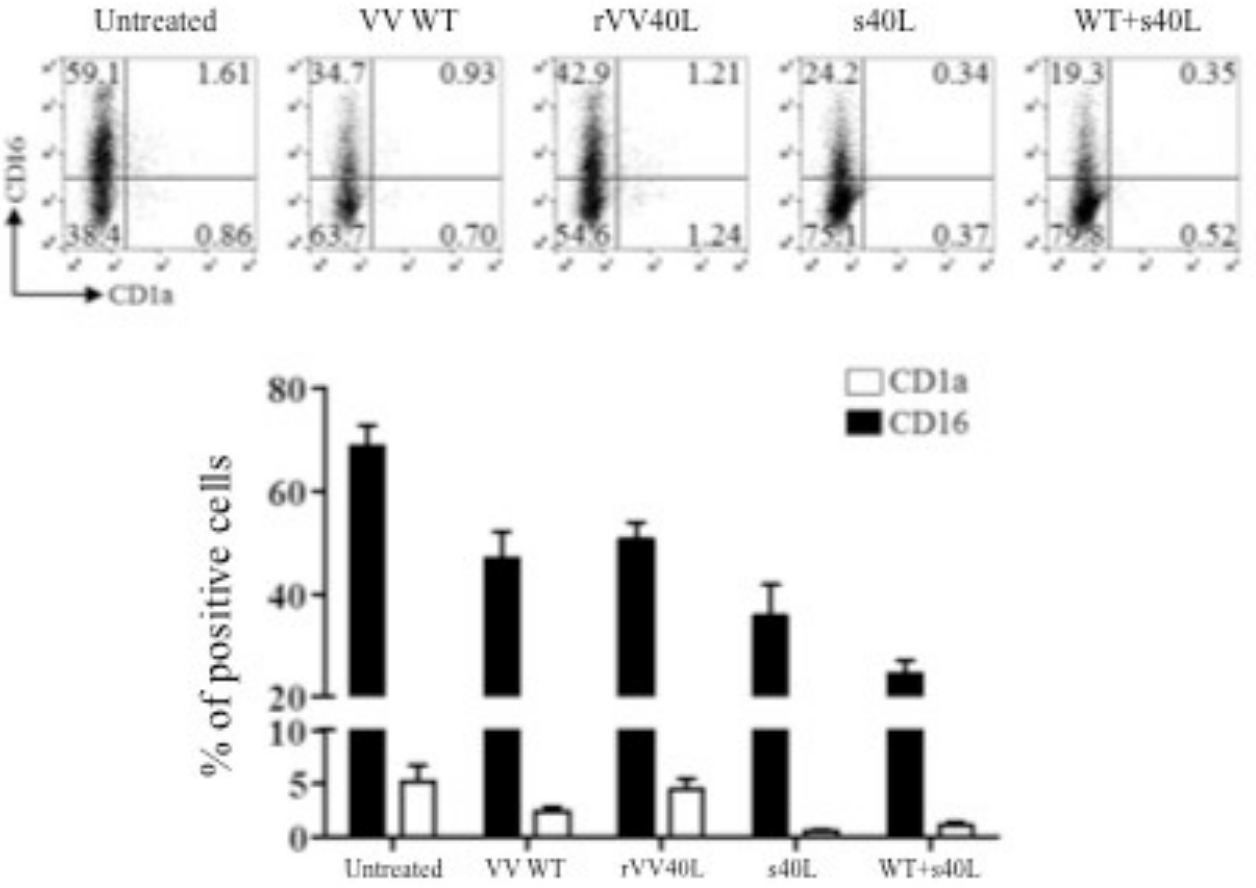


Figure 7. rVV40L-infected CD14^{+} monocytes do not differentiate into professional antigen presenting cells (CD1a+). Magnetically isolated CD14+ peripheral blood monocytes were left untreated or treated as indicated. After 24h of culture, CD14+ monocytes were harvested and stained with anti-CD16 mAbs and anti-CD1a mAbs in order to evaluate their differentiation toward specialized antigen presenting cells such as macrophages and dendritic cells. A representative experiment (upper panel) and cumulative results from four independent experiments are shown (bottom panel).

These data may also underline the different biological properties of membrane-bound CD40L, as provided by rVV40L-controlled infection, as compared to its soluble form. In this regard, it is tempting to speculate that differential pro-inflammatory cytokine gene expression observed in rVV40L- infected as compared to s40L-activated CD14+ cells possibly reflect a differential cross-linking of CD40 receptor expressed on the cellular surfaces of monocytes. Indeed, rVV40L infection, as compared to recombinant s40L protein, resulted also in a sustained expression of genes encoding for pro-inflammatory cytokines. In particular, high expression of IL-12p40, IFNs-type I genes was observed in rVV40L infected CD14+ cells but not in s40L-treated monocytes (**Fig. 8**).

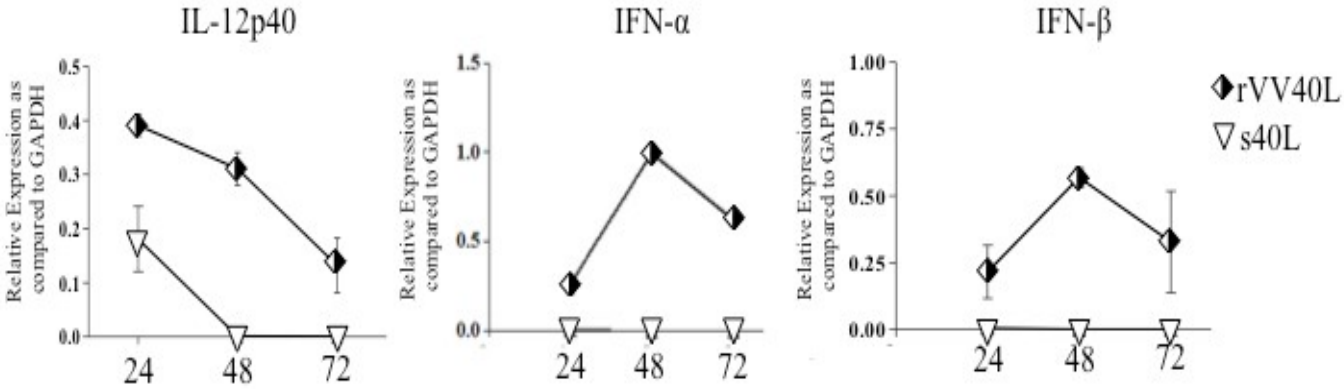


Figure 8. CD40L expressing recombinant vaccinia virus infection of CD14+ monocytes induces a sustained gene-expression of pro-inflammatory cytokines. Isolated CD14+ monocytes were left untreated or treated as previously described. At indicated time points, culture were harvested and total cellular RNA from CD14+ monocytes was then extracted, reverse transcribed and **(A)** IL-12p40 (n=3), IFN-α (n=2) and IFN-β (n=2), gene expression was analyzed by qRT-PCR. Values are reported as mean+/-standard error to the mean (SEM).

3.4 rVV40L infected CD14+ monocytes promote "in vitro" generation of alloreactive central memory-like CD8+ T cells.

Induction of $CD8^+$ memory cells and effective immune response against infectious agents and, most importantly, solid tumors have been shown to require IL-12 and /or IFN Type I[156,204,207]. Therefore, gene expression and phenotypic profiles suggested that rVV40L-infected $CD14^+$ monocytes might be highly effective APC in these regards, as indicated by the increased IL-12 and IFN-type I gene expression in the absence of IL-10 and IDO gene expression and by the fact that they minimally expressed PD-L1. Based on these observations, we evaluated the ability of rVV40L-$CD14^+$ monocytes to promote the differentiation of naïve $CD8^+$ T-cells into memory cells in different antigenic settings.

Initial experiments were performed by using allogenic $CD14^+$ cells as stimulators, to bypass the issue of the low frequency of antigen specific $CD8^+$ T cell precursors in the naïve lymphocyte compartment. In particular, sorted naive $CD8^+$ T-cells ($CD8^+$ $CD45RO^-$ $CD62L^+$) were co-cultured with allogeneic $CD14^+$ cells, previously treated as described above. Phenotypic characterization of primed $CD8^+$ T-cells performed on day 8, revealed the enhanced ability of rVV40L-infected $CD14^+$ cells to promote the differentiation of a significant fraction of alloreactive naïve $CD8^+$ T-cells into central memory-like cells as indicated by the co-expression of CD45RO and CD62L. Of note, s40L-activated $CD14^+$ cells were also able to promote the generation of central memory-like $CD8^+$ T-cells but to a significantly lower extent, as compared to rVV40L-infected monocytes. Figures **9A** and **C** show a representative experiment and collectively summarize data from eight independent assays. Remarkably, 31.4±12.5% (range 14.8-42.9%) of naïve $CD8^+$ T-cells primed with rVV40L-infected $CD14^+$ cells acquired a T_{CM} phenotype as compared to 12.1±5.2 % (range 7.1-20.2%) of central memory-like T cells obtained upon stimulation of naïve $CD8^+$ T cells with s40L-activated $CD14^+$ monocytes (P=0.0019) (**Fig. 9C**).

IL-7Rα has been reported to play a pivotal role in memory cell homeostasis and, as indicated in several studies, may be used to identify memory effector precursor $CD8^+$ T-cells (MPECs) differentiating towards long-lasting memory cells. In this regard, CXCR3 (CD183) expression has also been associated with long-term T cell memory[124,130,160]. Indeed, it has been described that effector CD8+ T cells with enhanced memory potential, longevity and proliferative capacity are characterized by the expression, among other surface antigens, of IL-7Rα and CXCR3 chemokine receptor. Therefore, to confirm the ability of rVV40L-

infected monocytes to promote the "in vitro" generation of memory $CD8^+$ T-cells, we also analyzed the expression of these markers in CD8+ cells allostimulated by differentially activated monocytes.

Confirming the first results, rVV40L-infected monocytes were able to expand percentages of CD45RO+/CD62L+/CD127+ CD8+ T cells (17.7±5.8 %, range 12.2-25.1%) significantly ($P<0.001$) higher than all other stimuli. Similar evidence was also obtained for CD45RO+/CD62L+/CXCR3+ CD8+ T cells (16±9 %, range 6-32%, $P<0.03$), or for CD45RO+/CD62L+/CD8+ cells expressing both CD127 and CXCR3 (15±9 %, range 1-28%, $P<0.05$). **Figures 9B and D** report data from one representative experiment and summarize data from eight independent experiments.

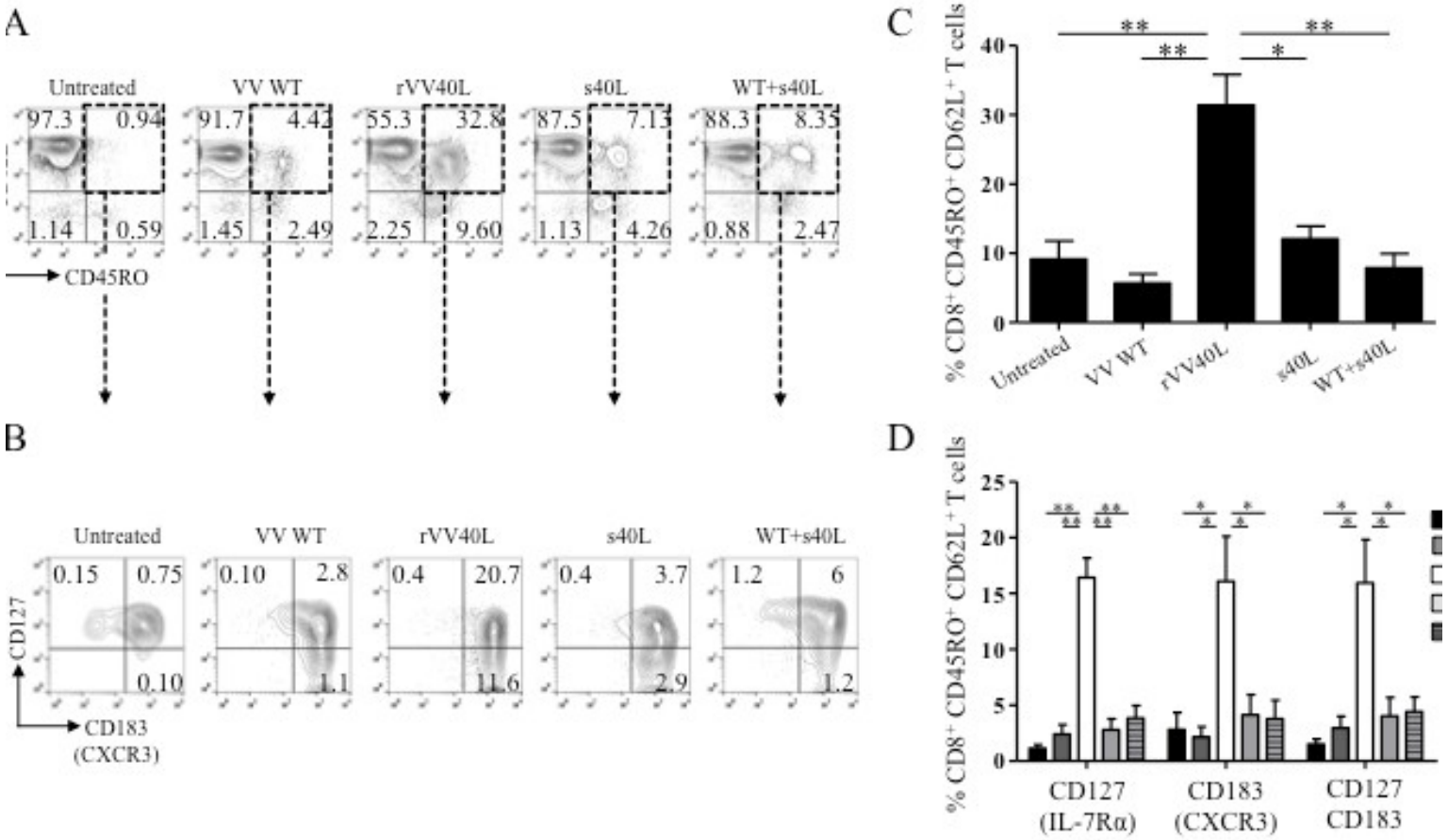


Figure 9. rVV40L infected CD14$^+$ cells promote *"in vitro"* differentiation of alloreactive naïve CD8$^+$ T cells into central memory-like cells. 1x10^6 sorted peripheral blood derived naïve CD45RO-/CD62L+ CD8$^+$ T-cells from healthy donors were co-cultured in RPMI-1640 CM supplemented with 5% HS with 2x10^5 allogeneic peripheral blood CD14$^+$ monocytes left untreated or treated with VV-WT, rVV40L, s40L or VV-WT+s40L (see "materials and methods"). On day 8, CD8$^+$ T-cells were stained with fluorochrome labelled anti-CD45RO and anti-CD62L mAbs. Data from a representative experiment are reported in panel **A**, whereas panel **C** summarizes data from 8 independent experiments. Expression of IL-7Rα (CD127) and CXCR3 (CD183) was also evaluated on gated CD45RO+/CD62L$^+$ T cells. Data in panel **B** refer to one representative experiment whereas panel **D** summarizes the results from eight independent experiments. Data in dot plot quadrants refer to percentages of total CD8+ T cells.
*: P<0.05, **:P<0.01.

3.5 rVV40L-infected CD14$^+$ monocytes promote "*in vitro*" generation of antigenic peptide-specific central memory-like lymphocytes from naïve CD8+ T cells.

The data obtained in the allogeneic setting, prompted us to evaluate the ability of rVV40L-infected CD14+ cells to shape also CD8-mediated immune responses specific for HLA-A0201-restricted antigenic peptides deriving from tumor associated antigen (TAA), such as L27MelanA/MART-1$_{26-35}$, or from viral proteins.

In the TAA-model, percentages of antigen specific CD8$^+$ T-cells expanded upon priming with rVV40L or s40L-activated CD14$^+$ monocytes, as evaluated by multimer staining on day 8 (Fig. 4A top), were similar. However, >45% of L27MelanA/Mart-1$_{26-35}$ positive CD8$^+$ T-cells primed with rVV40L-infected CD14$^+$ monocytes displayed a central memory-like phenotype, whereas APCs treated with s40L recombinant protein promoted the differentiation of a significantly lower fraction of naïve CD8$^+$ T-cells into central memory-like cells (31.0%) (**Fig. 10A**).

These data were then further reinforced by evaluating the induction of CD8+ T cell specific for a mixture of viral HLA-A0201-restricted immunodominant epitopes derived from human cytomegalovirus, vaccinia virus and influenza virus (Vaccinia Virus H3L$_{184–192}$, HCMVpp65$_{495-504}$ and Influenza A MP$_{58-66}$). In this setting, multimer staining indicated that rVV40L-infected monocytes expanded antigen specific CD8$^+$ T cells, to an extent similar to sCD40L activated CD14+ monocytes **(Fig. 10B)**. However, consistent with the TAA-specific and allogenic stimulation experiments, a preferential induction of central memory-like CD8$^+$ T-cells was also observed upon priming with rVV40L-infected CD14$^+$ cells as compared to s40L-stimulated CD14$^+$ monocytes (**Fig.10B**).

Cumulative results obtained from five independent experiments performed in order to evaluate the ability of rVV40L-infected CD14+ monocytes to promote the induction of peptide-specific CD8+ T cells are reported in figure **10C** and **D**. In particular, as reported in figure **10C**, rVV40L-infected CD14+ monocytes display a significantly higher efficacy in promoting the expansion of tetramer positive cells upon naïve CD8+ T cell stimulation. Most importantly, rVV40L-infected monocytes were significantly more efficient than all other APC under investigation, and, in particular, s40L stimulated monocytes (rVV40L: 35.8±17.9%, range 19-62.5%, vs. s40L: 13.5±11.1%, range 3.5-31%, P=0.03) in promoting the generation of tetramer positive (CD45RO+/CD62L+) T_{CM} from naïve CD8+ T cells (**10D**). Furthermore,

in line with the results obtained in the allogeneic setting, a significant percentage of these peptide-specific CD8+ T_{CM} cells also express IL-7Rα (CD127) (**Fig.10D**).

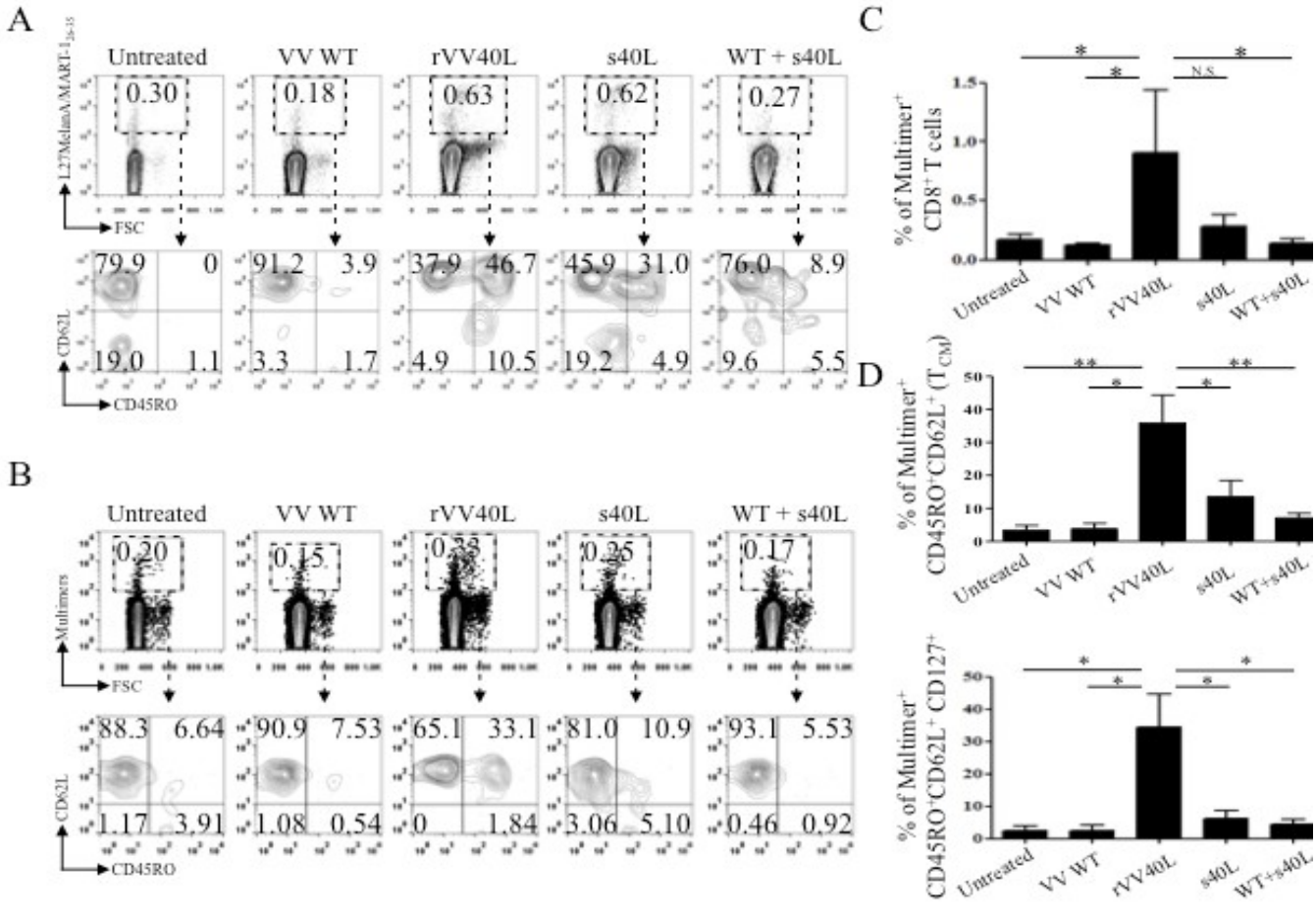


Figure 10. rVV40L-infected CD14+ cells promote *"in vitro"* generation of $CD8^+$ T cells with a central memory-like phenotype recognizing antigenic peptides from tumor associated or viral antigens. $2x10^5$ magnetically sorted, peripheral blood $CD14^+$ cells from HLA-A0201 positive healthy donors, treated as indicated, were incubated for 4 hours with (panel **A**) L27MART-1/$MelanA_{26-35}$ peptide (1μg per ml) or (panel **B)** with a mixture of Vaccinia Virus $H3L_{184–192}$, $HCMVpp65_{495-504}$ and Influenza A MP_{58-66} (1μg per ml of each) HLA-0201-restricted antigenic peptides. Cells were then washed and used to prime $1x10^6$ autologous sorted peripheral blood naïve CD45RO-/CD62L+ $CD8^+$ T cells. On day 8, primed $CD8^+$ T-cells were stained with **(A)** L27 Melan-A/$MART\text{-}1_{26–35}$ HLA-A0201 multimers. Percentages of total CD8+ T cells are reported in the dot plots. Gated, multimer specific CD8+ T cells were then stained with CD45RO/CD62L specific, fluorochrome labelled mAbs. Percentages reported within dot plots' quadrants are referred to total multimer positive cells. Data refer to one representative experiment of two performed with similar results. Cultures stimulated with APC pulsed with viral peptides were similarly (as in panel **A**) stained with a mixture of corresponding multimers (panel **B**) on day eight. Data refer to one representative experiment out of three performed with similar results. **Panel C** summarizes data regarding the expansion of multimer specific CD8+ T cells as induced by the differentially treated APC under investigation in the five independent experiments. Percentages of multimer specific CD8+ T cells expressing CD45RO/CD62L T_{CM} phenotype and IL-7Rα (CD127) from the five independent experiments are reported in **panel D**. *: $P<0.05$, **:$P<0.01$.

Remarkably, the acquisition of the phenotypic signature of central memory cells by rVV40L-CD14 primed naïve cells does not only reflect the ability of infected monocytes to significantly promote the expansion of antigen-specific CD8+ T cells. Indeed, evaluation of the Ratio, T_{CM} / Naïve + TEM + T_{eff} on multimer + cells, underlines the unique capacity of rVV40L-infected monocytes to modulate not only the magnitude but also, most importantly, the quality of CD8-mediated immune responses (**Fig. 11**). Indeed, among the different APCs under investigation, CD40L-expressing recombinant vaccinia virus infected CD14+ monocytes, display a superior ability in promoting the differentiation of naïve CD8+ T cells into T_{CM}.

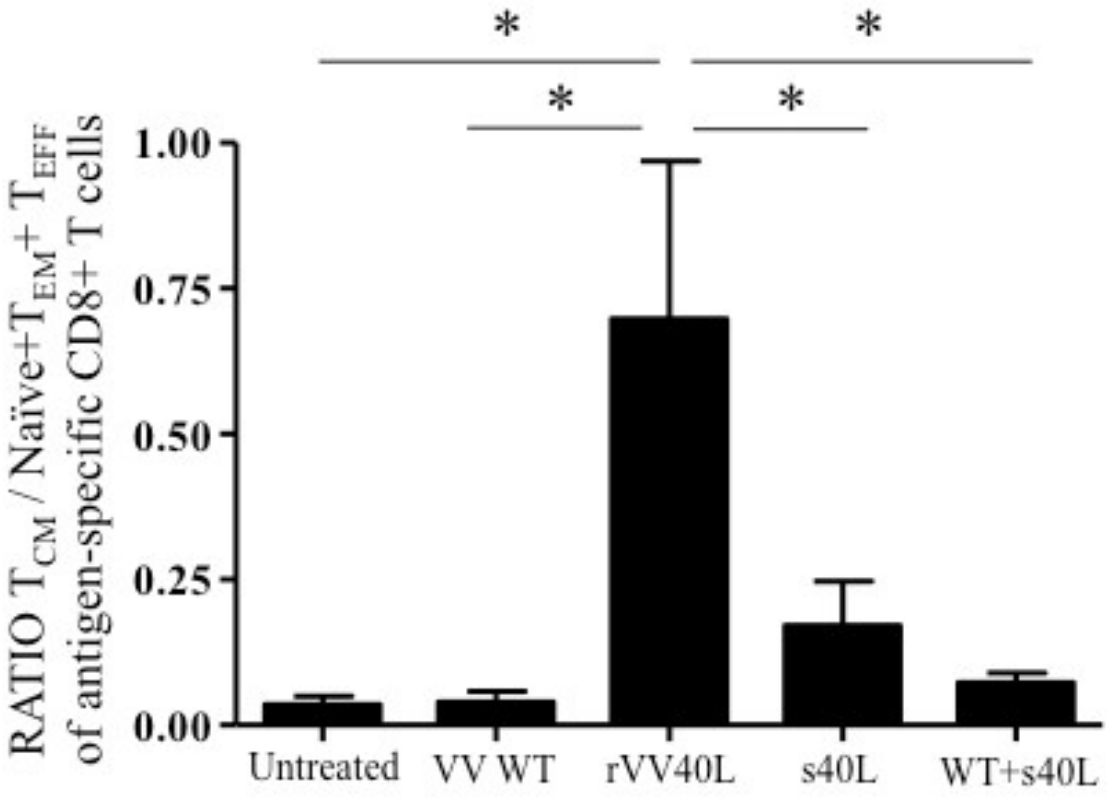


Figure 11. CD40L expressing recombinant vaccinia virus efficiently shape the quality of CD8 mediated immune responses. CD8+ T cells from HLA-A0201+ donors were stimulated by autologous APC pulsed with antigenic HLA-A0201-restricted peptides following the indicated treatments, as shown in figure 10. In order to better discriminate between quantitative and qualitative CD8+ T cell responses induced by rVV40L-infected monocytes the RATIO T_{CM} / Naïve + T_{EM}+ T_{eff} was analyzed as the percentage of multimer+ CD8+ T cells with a specific phenotypic profile identified according to the expression of CD45RO and CD62L surface antigens. *: P<0.05, **:P<0.01. The figure reports cumulative data from five independent experiments.

3.6 Priming of "truly" naïve or restimulation of stem cell-like memory CD8+ T cells.

Our current understanding of lifespan maintenance of immunological memory is compatible with a stem cell-like memory T cells (T_{SCM}) model postulating the existence or a rare population of T-lymphocytes capable to self-renew and to differentiate, upon antigen recognition, in memory and effector T cells subsets[108]. Despite considerable efforts, phenotypic characterization of T_{SCM} is still elusive. T_{SCM} have been initially identified as a distinct subset of CD8+ lymphocytes according to the expression of a panel of surface molecules normally detected on naïve precursors including CD45RA, CCR7 (CD197), CD62L and CD28 along with high levels of surface antigens such as CD95, CD122 and

CD183 that, intriguingly are known to be normally expressed by memory and effector CD8+ T cells[108].

In order to evaluate the percentage of T_{SCM} within sorted CD45RO-CD62L+ naïve population used in our experiments, magnetically isolated CD8+ T cells derived from peripheral blood of healthy donors were characterized for the expression of specific antigens on their cellular surfaces. In particular, identification of antigen-experienced CD8+ T cells with stem cell-like qualities has been based on the expression of CD45RA, CCR7 (CD197), CD95 and CD62L[235] (**Fig. 12A**) or CD28[103] (**Fig. 12B**). Cumulative results obtained from twelve (n:12) independent experiments revealed, in line with other studies, a marked paucity of CD8+ lymphocytes with a stem cell-like phenotype in peripheral blood of healthy donors. Indeed, based on the expression of CD45RA, CCR7, CD95 and CD62L or CD28, T_{SCM} represent 0.67% +/- 0.37% (Range: 1.33% - 0.17%) or 0.57% +/- 0.39% (Range 1.23% - 0.04%), respectively, of total naïve CD8+ T cells (**Fig. 12C**).

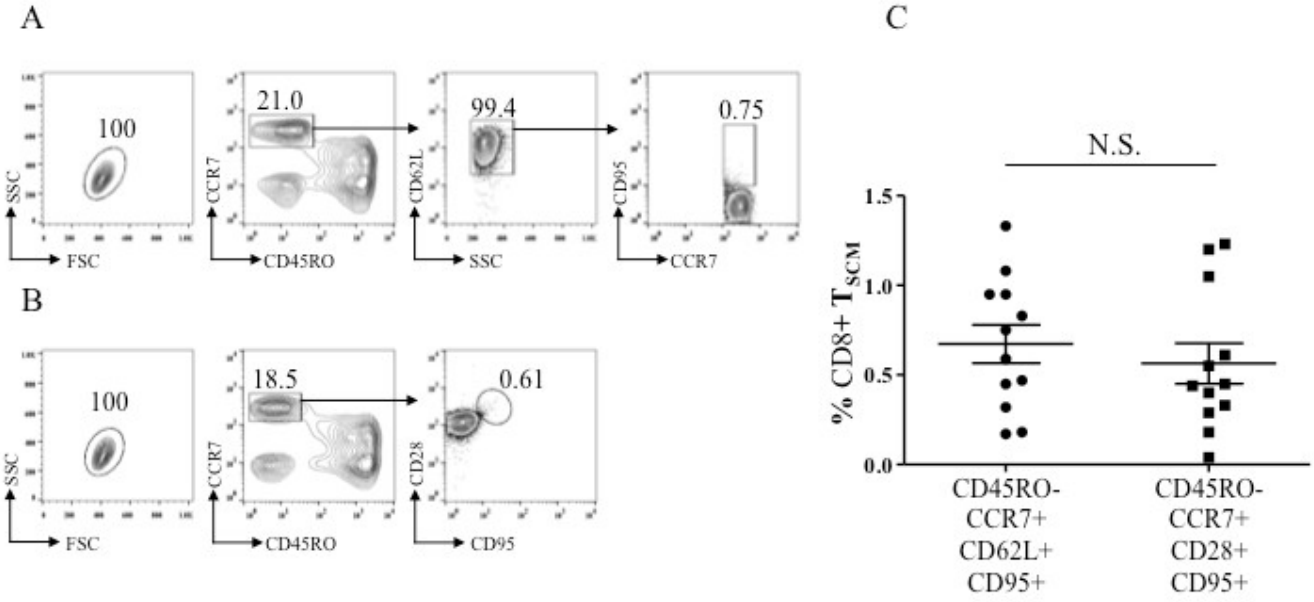


Figure 12. Evaluation of CD8+ T cells with stem cell-like phenotype (T_{SCM}). CD8+ T_{SCM} were identified according to the expression of CD45RO, CCR7, CD95 and CD62L (**A**) or CD28 (**B**) surface antigens, as shown in representative experiments. Cumulative results obtained from the analysis performed on CD8+ T cells isolated from twelve different healthy donors are reported in panel **C.**

This initial phenotypic characterization of memory stem cell-like T cells has been later challenged by pre-clinical and clinical studies aiming at specifically targeting this population to further define its contribution to immune reconstitution in patients receiving haploidentical hematopoietic stem cell transplantation (HSCT). Collectively these studies have demonstrated that differentiation of naïve CD8+ lymphocytes in T_{SCM} is also promoted by homeostatic cytokines in vitro and in vivo. However, in contrast to pharmacologically instructed T_{SCM}, antigen-experienced CD8+ T cells with stem cell-like qualities generated in presence of IL-7 and IL-15 display a phenotypic profile resembling conventional T_{CM}. Indeed, under these culture conditions, T_{SCM} were defined based on the expression on cell surfaces of CCR7, CD62L, IL-7Rα, CD95, CD45RA but also CD45RO. In view of these observations, and considering the gating strategy that we designed to isolate naïve (CD45RO- CD62L+) CD8+ T cells, it is tempting to speculate that the presence of putative T_{SCM} defined as CD45RA+ T cells within sorted naïve population may represent a major limitation in our experiments.

Aiming at further confirming the enhanced capacity of rVV40L-infected CD14+ monocytes to promote the rapid differentiation of antigen-specific naïve precursors into T_{CM}, we performed a set of in vitro experiments in which generation of central memory CD8+ T cells was evaluated in healthy donors that had not received vaccinia virus vaccination. Therefore, in these donors, vaccinia virus specific CD8+ T cells should be comprised only in truly naïve compartment. In line with these considerations, phenotypic characterization of CD8+ T cells performed in order to evaluate the possible contamination of T_{SCM} within tetramer positive lymphocytes clearly indicated the absence of vaccinia virus specific CD8+ T lymphocytes with stem cell-like qualities (CD45RO- CD62L+ CD95+; **Fig.13A left panel**). Nevertheless, also in these not-vaccinated donors the presence of T_{SCM} was comparable with the frequency detected in vaccinated donors (**Fig.13A middle panel**). Cumulative results obtained from four independent donors, indicate, within vaccinia virus specific cells an irrelevant contamination of naïve T cells also expressing CD95 (0.013% +/- 0.0078%; Range 0.002%-0.0078%) whereas within total CD8+ T cells, T_{SCM} represented 0.2% +/- 0.2% of analyzed cells (Range 0.042%-0.3%) (**Fig.13A right panel**). Based on this background, we evaluated the capacity of differently treated autologous CD14+ monocytes to prime vaccinia virus specific CD8+ T cells. Notably, phenotypic characterization performed at day 8, of naïve CD8+ T stimulated with CD14+ that were left untreated or alternatively infected with VV WT or rVV40L indicate a significant increase in the percentage of vaccinia virus-specific CD8+ T cells only upon priming with rVV40L-infected CD14+ monocytes (**Fig.13B upper panel**). Furthermore, phenotypic characterization of tetramer positive CD8+ T cells clearly

indicate the preferential differentiation of vaccinia virus specific naïve precursors into T_{CM} defined according to the expression of CD45RO and CD62L on their cellular surfaces. Indeed, cumulative results from four independent experiments indicate that priming of naïve cells with rVV40L-CD14 resulted in the generation of 51.8% +/- 20.2% (Range 71.4%-25.5%) of vaccinia virus specific CD8+ T cells with a T_{CM} phenotype. In contrast, only 16.2% +/- 11.9% of tetramer positive cells with a central memory phenotype were observed in cell cultures primed with VV WT-CD14+ monocytes (**Fig.13B bottom panel**).

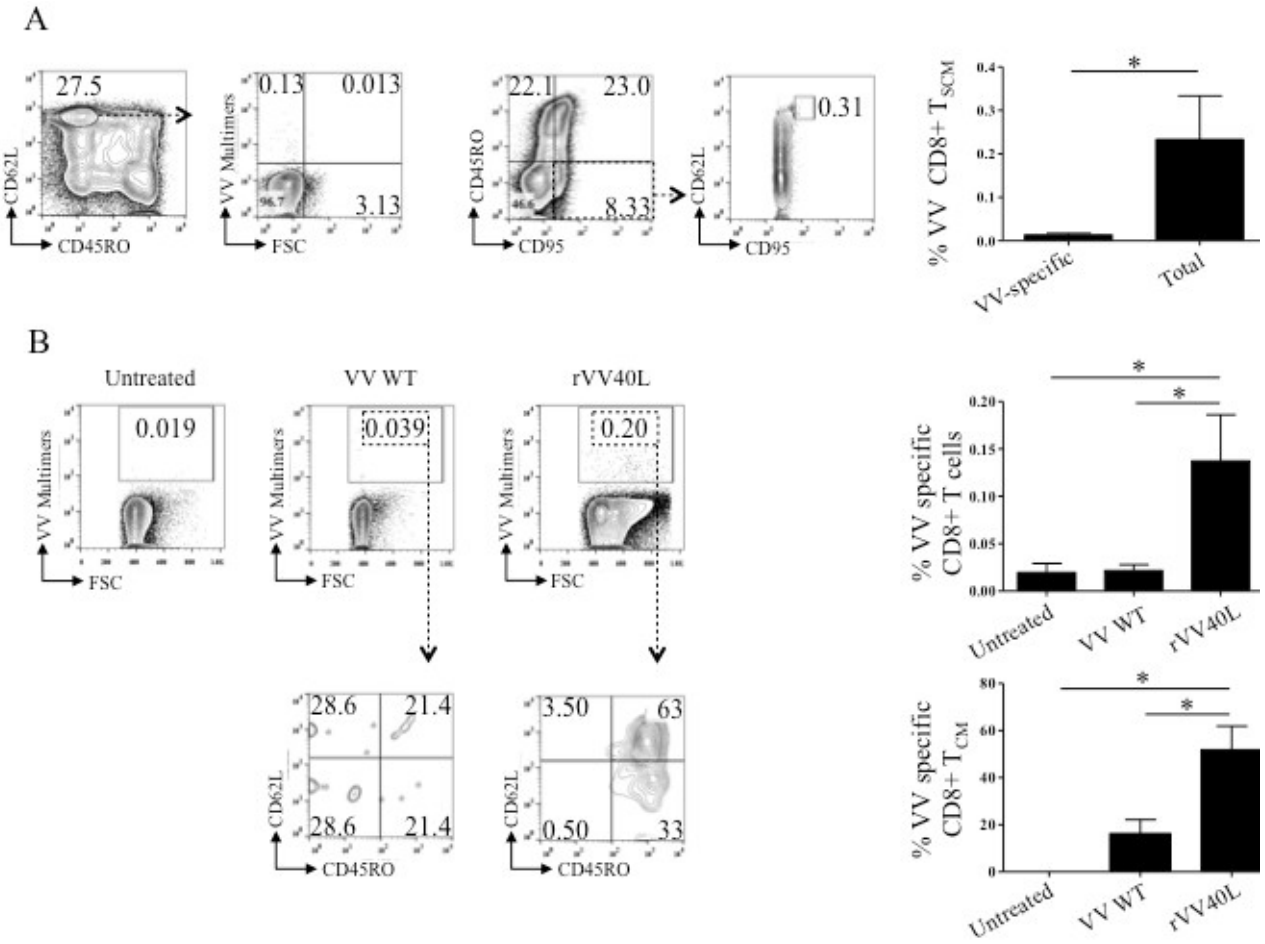


Figure 13. rVV40L-infected CD14+ cells promote *"in vitro"* differentiation of functionally naïve $CD8^+$ T cells into T_{CM}. (A) CD8+ T cells were isolated from HLA-A0201+ healthy donors not vaccinated against small-pox virus were stimulated with APC infected with VV WT or rVV40L. On day eight cells were tained with a mixture of Vaccinia Virus HLA-0201-multimers, anti-CD45RO mAbsand anti-CD62L mAbs fluorochrome-labeled mAbs. Percentages of multimer positive cells and phenotypes of multimer positive cells were then evaluated. A representative experiment and cumulative results from four independent experiments are shown. *: P<0.05, **:P<0.01.

Taken together, these data indicate that rVV40L infection of CD14+ monocytes resulted in the expansion and acquisition of central memory phenotype of truly naïve CD8+ T cells and not as a consequence of the expansion of putative pre-existing antigen specific T_{SCM}.

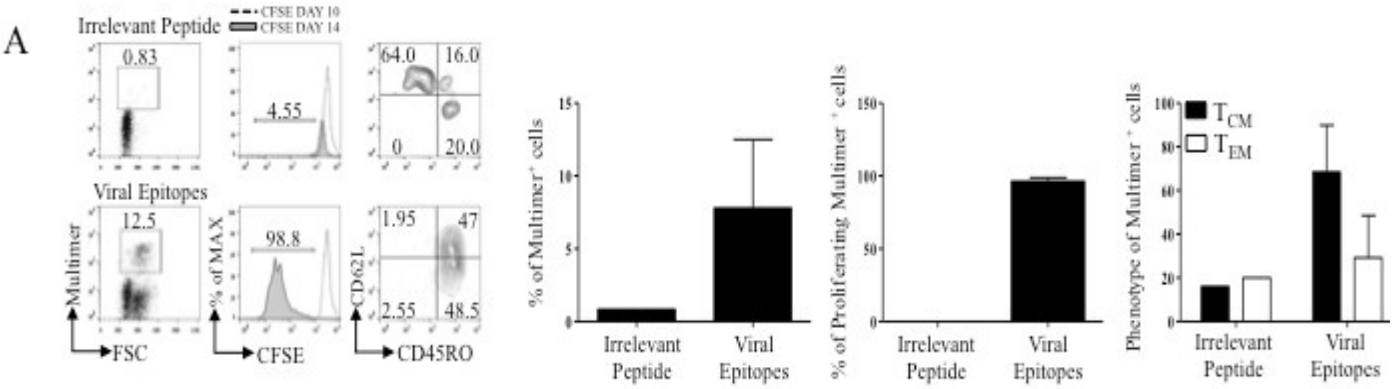
3.7. Functional analysis of central memory-like CD8+ T cells induced by rVV40L-infected CD14+ monocytes.

$CD8^+$ T-cells generated upon stimulation by rVV40L-infected $CD14^+$ cells were characterized by the expression of the typical T_{CM} phenotypic profile. Based on these findings, we sought to evaluate also their functional properties. In particular, we addressed the proliferative potential, differentiation into effector memory cells and IL-2 production upon T cell receptor triggering.

In order to assess the proliferative and differentiation capacity of our rVV40L generated T_{CM}, naïve $CD8^+$ T-cells were stimulated with a pool of three HLA-A0201-restricted immunodominant viral peptides derived from human cytomegalovirus, vaccinia virus and influenza virus (Vaccinia Virus $H3L_{184–192}$, $HCMVpp65_{495-504}$ and Influenza A MP_{58-66}) using rVV40L-infected $CD14^+$ cells as antigen presenting cells. Cells were then re-stimulated on day 10 with peptide-pulsed untreated $CD14^+$ monocytes and characterized for phenotypic and functional properties three days later. In this experimental setting, >90% of tetramer positive cells showed evidence of CFSE dilution, whereas stimulation by irrelevant peptide was completely ineffective. Importantly, in half of the proliferating cells CD62L expression appeared to be down-regulated, consistent with the acquisition of a putative effector-memory phenotype, while the rest of proliferating tetramer positive cells, retained their T_{CM} phenotype (**Fig.14A**).

A putative limitation of this experimental setting is represented by the inability to formally exclude that, within proliferating cell population detected at day three after re-stimulation, residual naïve CD8+ T cells eventually unresponsive to primary stimulation.

In this regard, we observed a significant lower percentage of peptide-specific CD8+ T cells retaining a (CD45RA+/CD62L+) naïve phenotype at day 8 after priming with rVV40L-infected CD14+ monocytes as compared to all other culture conditions under investigation (**Fig14B**).



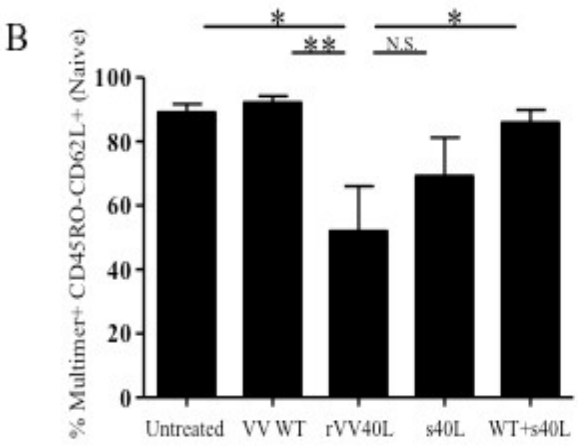


Figure 14. rVV40L-CD14 promote *"in vitro"* generation of functional central memory precursor $CD8^+$ T cells specific for human Cytomegalovirus, Vaccinia Virus and Influenza Virus. (A) HLA-A0201+ $CD8^+$ T-cells primed for eight days in the presence of rVV40L-treated monocytes pulsed with a mixture of Vaccinia Virus $H3L_{184–192}$, $HCMVpp65_{495-504}$ and Influenza A MP_{58-66} peptides (1µg/ml of each) were harvested, CFSE labelled and stimulated for three days in the presence of autologous untreated monocytes pulsed with the specific or irrelevant peptides. Cells were then stained with antigen specific HLA-A0201 multimer, anti-CD45RO and anti-CD62L mAbs. Percentages of cells showing evidence of specific staining or CFSE dilution were then analyzed. Data refer to one representative experiment and summarize the results of two independent assays. **(B)** Percentages of multimer positive CD8+ T cells still retaining a naïve phenotype (CD45RO-CD62L+) at day 8 after priming with peptide pulsed CD14+treated as indicated or left untreated were analyzed. The panel shows cumulative results from five independent experiments.
*: $P<0.05$, **:$P<0.01$.

Nevertheless, to address this issue, we performed additional experiments based on the re-stimulation with anti CD3/CD28 of CD8+ T cells sorted according to CD45RO/CD62L expression rVV40L-Naïve (CD45RO-CD62L+) and rVV40L-T_{CM} CD8+ T cells generated following an initial allostimulation by rVV40L infected monocytes in primary cultures. In this setting, <10% rVV40L Naïve CD8+ T cells showed evidence of CFSE dilution contrast as compared to >90% of rVV40L-T_{CM} CD8+ T cells (**Fig.15A**). Interestingly, proliferative capacity of rVV40L- T_{CM} CD8+ T cells resulted also in the generation of consistent population of CD62L- effector-memory cells was detectable upon secondary stimulation. In addition, rVV40L-T_{CM} CD8+ T cells were also able to produce detectable amounts of IL-2 in mAb stimulated secondary cultures (**Fig. 15B**).

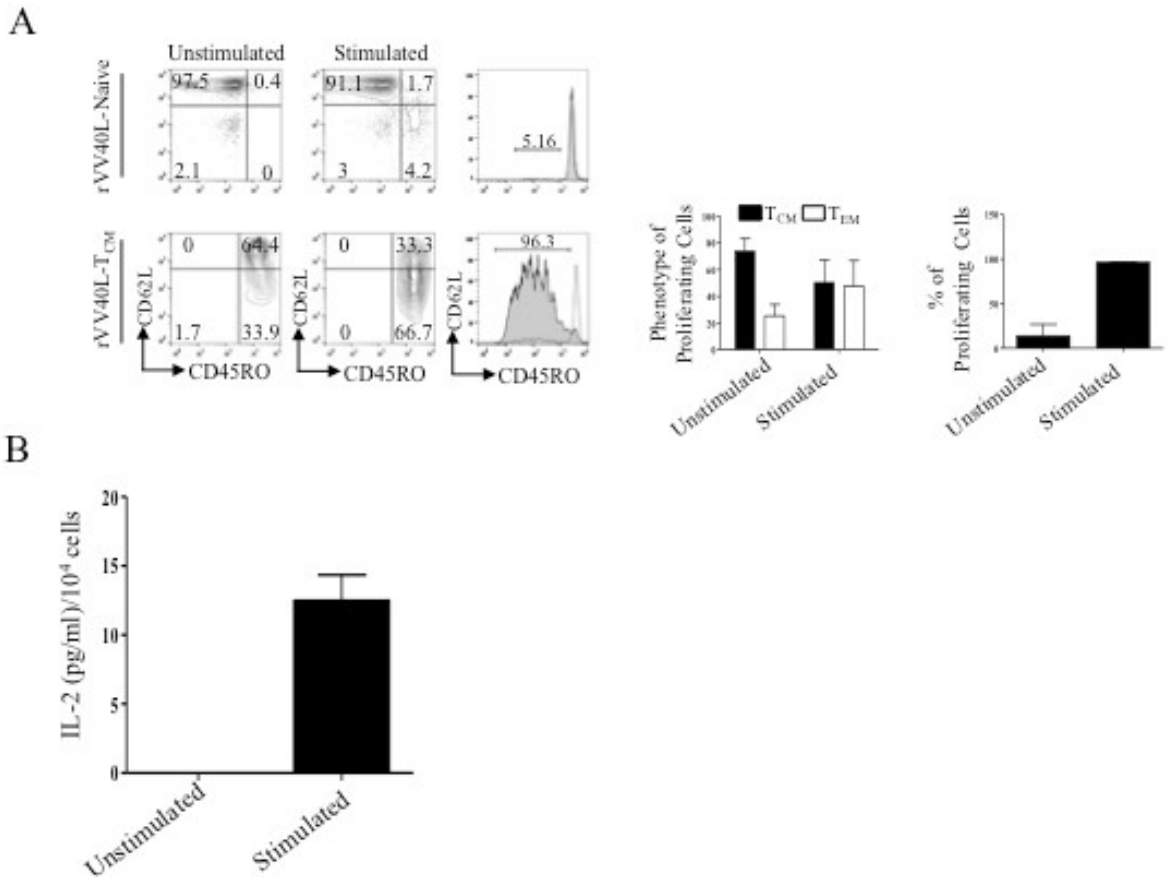


Figure 15. Functional characterization of T_{CM} induced by rVV40L-treated monocytes. CD45RO-/CD62L+ naïve CD8+ T cells were stimulated for eight days with allogenic rVV40L-treated monocytes. At day 8, Naïve and T_{CM} CD8+ T cells were then sorted based on CD45RO and CD62L expression, CFSE stained and re-stimulated with anti CD3 and anti CD28 mAbs. Following a three days culture, cells were harvested, and stained with anti CD45RO and anti CD62L mAbs. CFSE dilution and marker expression were then analyzed. Data refer to one representative experiment and summarize the results of two independent assays. **(B)** IL-2 production by sorted rVV40L induced T_{CM} following a 3days anti CD3 and anti CD28 mAbs stimulation. Data summarize three independent experiments.

In conclusion, our data indicate a remarkable efficacy of our CD40L-recombinant vaccinia virus to promote the generation of CD8+ T cells with functional characteristics of T_{CM}.

3.8 rVV40L infection produces in cytostatic and cytotoxic effects on tumor cells.

The expression of CD40 receptor has been reported in nearly all B-cell malignancies and approximately in 70% of solid tumors. Furthermore, triggering of CD40 expressed on cell surfaces of transformed cells has been associated either to pro-survival effects or to inhibition of tumor cells proliferation and/or apoptosis of targeted cells. Interestingly, these differential effects have been associated to the extent of CD40 receptor expression as well as to the specific signal transduction chain integrated by transformed cells[184,209].

In order to evaluate the biological responses induced by our replication incompetent CD40L-expressing recombinant vaccinia virus (rVV40L), we selected a panel of established tumor cell lines. In particular, Na8 melanoma cell line (CD40+), HCT116 (CD40+) and Colo205 (CD40-) colorectal cancer cell lines were used in order to evaluate the effects of rVV40L infection and s40L treatment. As expected, rVV40L infection resulted in the up-regulation of CD40L in a similar fraction of tumor cells regardless of their CD40 receptor status. Furthermore, membrane-bound CD40L provided by controlled viral infection resulted, in both Na8 and HCT116 cell lines, in a reduction of CD40 receptor expression on their cellular surfaces. In sharp contrast, tumor cell lines cultured in presence of soluble CD40L recombinant protein display levels of CD40 receptor comparable to untreated tumor cells (**Fig.16**).

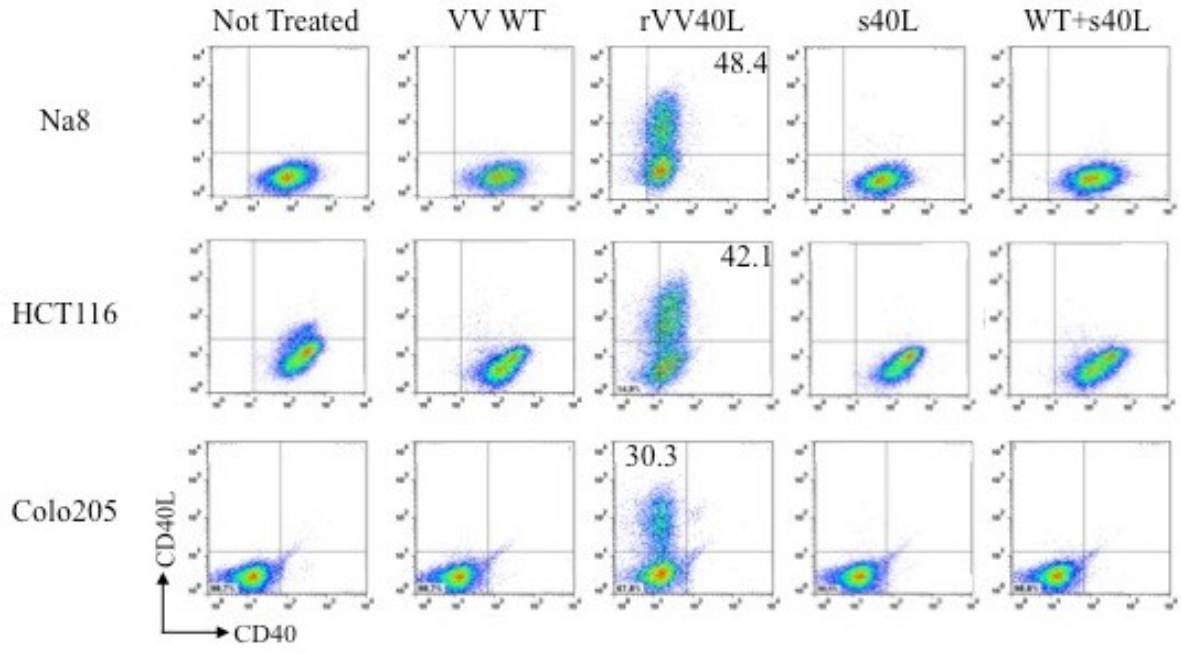


Figure 16. rVV40L infection of established tumor cell lines. 1x10^6 cells from Na8 melanoma and HCT116 and Colo205 colorectal cancer cell lines were left untreated or infected with VV-WT or rVV40L at MOI 10. Moreover, cells from established tumor cells lines were also treated with soluble CD40L recombinant protein alone (s40L) or following VV WT infection (VV WT+s40L). After 24 hours of culture cell were harvested and CD40 receptor and CD40L expression on cellular surfaces were evaluated by flow cytometry analysis. Data refer to one representative experiment out of five performed with similar results.

In line with this observation, suggesting different biological properties of membrane bound CD40L as compared to its soluble form, rVV40L infection of Na8 (CD40+) melanoma cell line resulted in 30% proliferation inhibition and in a significant increase in the percentage of apoptotic cells as compared to untreated tumor cells. In contrast, s40L-stimulation of Na8 (CD40+) melanoma cell line did not resulted in cytostatic or cytotoxic effects. Remarkably, ligation of CD40 receptor expressed on cellular surface of HCT116 (CD40+) did not affect their proliferative capacity or their survival. Indeed, similar to Colo205 (CD40-), both rVV40L infection and s40L-treatment failed to abrogate in vitro expansion of treated HCT116 (CD40+) colorectal cancer cell line and did not resulted in an increased percentage of apoptotic cells (**Figs.17A, B**).

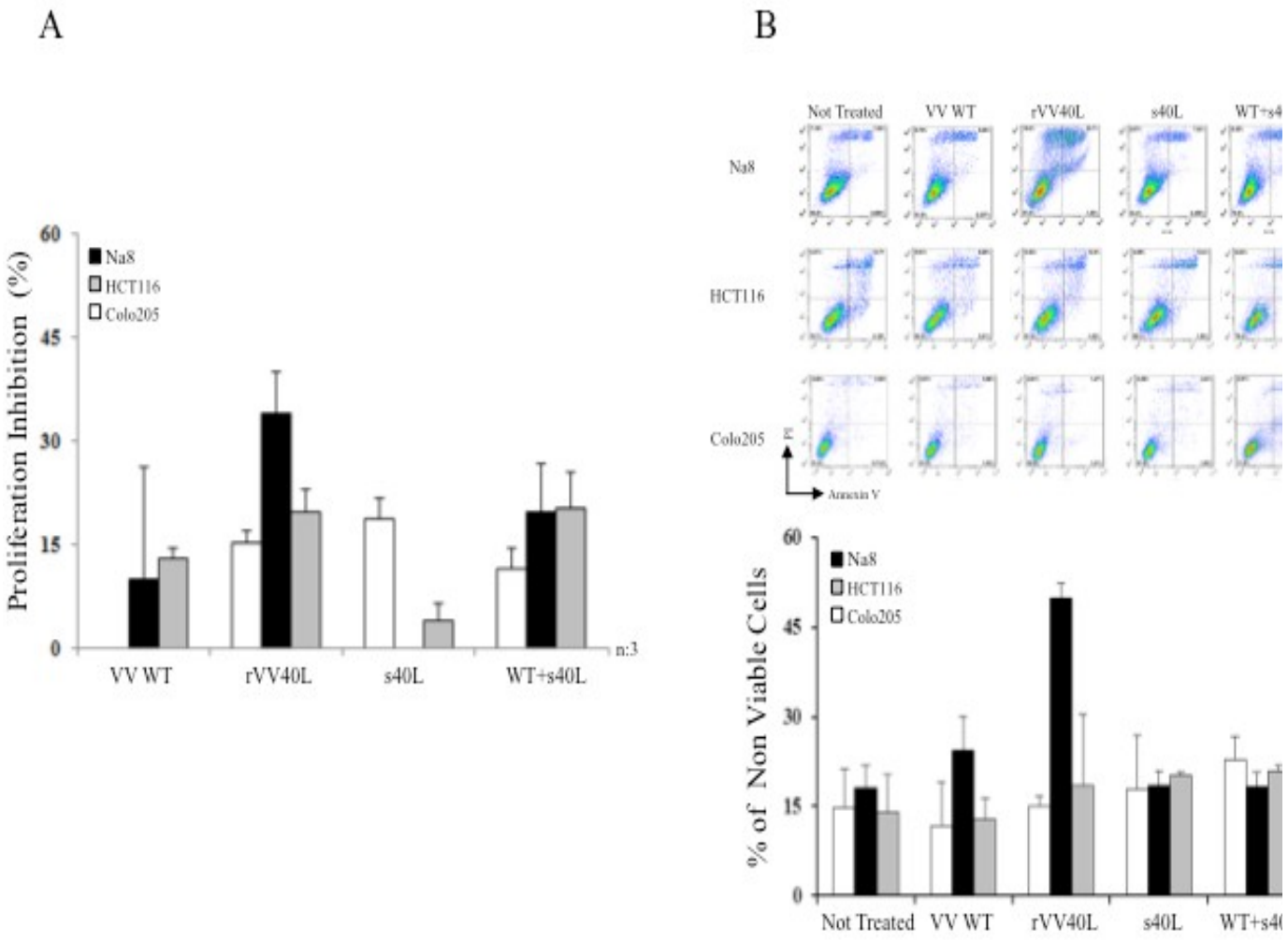


Figure 17. rVV40L infection of established tumor cell lines resulted in the induction of cytostatic and cytotoxic effects

Na8 melanoma cell line, HCT116 and Colo205 colorectal cancer cell lines were left untreated or treated as indicated and cultured for 4 days. (**A**) Proliferation of tumor cell lines was assessed by ^{3}H-thymidine incorporation during the last 18h of culture and expressed as % of proliferation inhibition as compared to untreated cultures. Data summarize the results obtained in three independent experiments. (**B**) After 4 days of culture, untreated and differently treated tumor cell lines were harvested and percentages of non-viable cells was defined as the sum of annexin V+/PI-, annexin V+/PI+ and annexin V-/PI+ cells. A representative experiment (panel B upper dot plots) and cumulative results obtained from three independent experiments (lower histograms) are reported.

It has been extensively reported that ligation of CD40 receptor by CD40L results in the clustering of the receptor that, in turn, induces the recruitment, to its cytoplasmic domain, of TNF-receptor associated factors (TRAFs) mediating the activation of different intracellular signaling pathways. Six different adapter proteins compose the TRAFs family. TRAF-1 has been shown to be regulated at transcription level in response to CD40 receptor initiated signals and to play a pivotal role in regulating the activity of the other TRAF proteins [182,236]. In this scenario, we evaluated whether the absence of cytostatic and cytotoxic effects on HCT116 (CD40+) colorectal cancer cell line might be related to an impaired and/or reduced intracellular signaling initiated by CD40L-mediated ligation of CD40 receptor expressed on their cellular surfaces. Interestingly, we observed a marked up-regulation of TRAF-1 gene expression in Na8 (CD40+) melanoma cell line upon rVV40L infection whereas s40L, alone or in combination with VV-WT, was significantly less efficient. In sharp contrast, triggering of CD40 receptor expressed on cellular surfaces of HCT116 (CD40+) did not result in the up-regulation of TRAF-1 gene-expression levels (**Fig.18**).

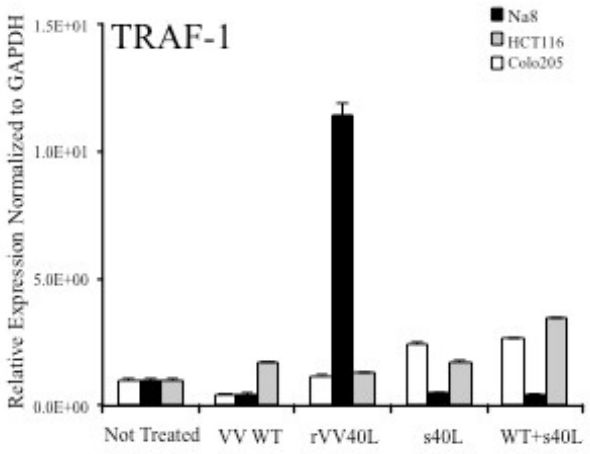


Figure 18. Cytostatic and cytotoxic effects on tumor cells induced by rVV40L infection are associated to the up-regulation of TRAF1. Na8 melanoma cell line, HCT116 and Colo205 colorectal cancer cell lines were infected with VV-WT or with rVV40L at MOI of 10 or left untreated. Furthermore, tumor cell lines were also treated with soluble CD40L recombinant protein (s40L) alone or in combination with VV WT infection (WT+s40L). At day 4, tumor cells from different cultures were harvested and total cellular RNA was then extracted, reverse transcribed and TRAF-1 gene expression was analyzed by qRT-PCR, using GAPDH expression as reference. Values are reported as mean+/-standard error to the mean (SEM) and summarize the results obtained from three independent experiments.

Taken together, these data indicate underline the enhanced efficacy of our replication incompetent CD40L-expressing recombinant vaccinia virus as compare to soluble CD40L recombinant protein to mediate, upon infection, cytostatic and cytotoxic effects on established tumor cell lines. Furthermore, we also reported how abscence of these effects, on CD40 receptor expressing tumor cells, might be potentially explained as a consequence of an impaired intracellular signaling resulting in a defective up-regulation of TRAF-1 adapter protein.

3.9 rVV40L-infection promotes tumoricidal activitiy of CD14+ monocytes.

The rationale supporting the initial development of cancer immunotherapies strategies targeting CD40 receptor was represented by the activation of antigen presenting cells, most importantly dendritic cells, in order to promote the generation of effective antitumor T cell responses. Furthermore, studies performed in animal models and evidences obtained in clinical setting have indicated that tumor regression might also be achieved in a T-cell independent manner. In this regard, a pivotal role has been described for myeloid cells of monocytes/macrophages lineage. Indeed, CD40-stimulated macrophages have shown to efficiently acquire tumoricidal activity based on their capacity to produce reactive nitrogen intermediates and effector cytokines including TNF-α [84,209,220].

In order to evaluate the capacity of our CD40L-expressing recombinant vaccinia virus to promote tumor regression through the activation of myeloid cells, we performed a set of in vitro experiments in which established tumor cell lines expressing CD40 receptor were left untreated and infected either with vaccinia virus wild type (VV WT) or rVV40L and then cultured in absence or presence of allogeneic CD14+ monocytes isolated from peripheral blood of healthy donors. Interestingly, we observed a marked reduction of proliferative capacity of H358 (CD40+) non-small-cell lung cancer cell line upon direct infection with rVV40L whereas in vitro expansion of HepG2 (CD40+) hepatocellular cell line was not affected. Indeed, a similar percentage of proliferating cells were observed upon infection with VV WT and rVV40L. Nevertheless, a considerable reduction in proliferative capacity of rVV40L infected HepG2 (CD40+) cells was observed upon three days co-culture with

allogeneic CD14+ monocytes (**Fig.19A**), correlating with the ability of CD40L-expressing recombinant vaccinia virus to induce an enhanced TNF-α gene expression (**Fig.19B**).

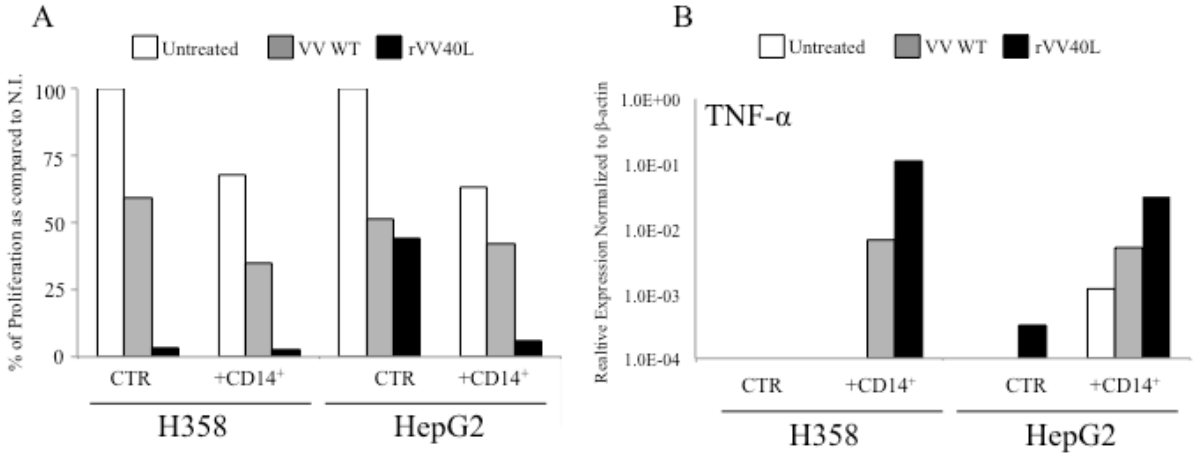


Figure 19. rVV40L infection of tumor cells promotes the activation of CD14+ monocytes. $1x10^6$ H358 NSCLC derived cell line and HepG2 hepatocellular carcinoma derived cell line were left untreated and infected with VV-WT and rVV40L at MOI 10. After 24 hours cells were harvested and cultured in presence of absence of CD14+ monocytes at 1:1 ratio. (**A**) Proliferation of tumor cell lines was evaluated at day 4 by ^{3}H-thymidine incorporation during the last 18h of culture and expressed as % of proliferation as compared to cultures that were left untreated whereas induction TNF-α gene expression was evaluated by qRT-PCR. Data are referred to a representative experiment out of three performed with similar results.

Collectively these results indicate that replication incompetent CD40L-expressing recombinant vaccinia virus might efficiently mediate antiproliferative effects on in vitro established tumor cell line either by the triggering of CD40 receptor expressed on their cellular surface or by promoting the antitumor activity of CD14+ monocytes.

4. DISCUSSION

In the last years, therapeutic potential of different cancer immunotherapy strategies has been restated by the objective and durable clinical responses observed in treated patients enrolled in different trials. In this regard, an increased attention has recently been focused on strategies targeting CD40 receptor. Indeed, it has been reported that administration of agonist anti-CD40 receptor monoclonal antibodies or CD40L-expressing viral vectors might result in vigorous and multifaceted antitumor effects[84,209]. In particular, it has been shown that ligation of CD40 receptor expressed on cellular surface of malignant cells might result in direct cytostatic and cytotoxic effects. Furthermore, objective cancer regression might be achieved following CD40 receptor induced immune activation. Indeed, signaling via CD40 receptor expressed on cellular surfaces of myeloid cells has been shown to result in the acquisition of direct antitumor activity and in an enhanced ability, particularly for dendritic cells, to promote the generation of tumor-specific CD8+ T cells potentially eliminating, upon recognition, target malignant cells.

Antitumor efficacy of CD8+ T cells has traditionally been associated to their enhanced production of effector cytokines and cytotoxic molecules initiated by the recognition of cognate antigen. In the last decade, this initial interpretation regarding the anticancer efficacy of CD8+ T cells has been revised. Indeed, it has been reported that clinical efficacy of CD8+ T cells is also affected by their differentiation status. In particular, it is nowadays widely accepted how generation and expansion of tumor-reactive CD8+ T cells with phenotypic and functional profiles of central memory lymphocytes represent key clinical priorities for successful cancer treatment[118,210-212].

An important role in the generation of effective memory $CD8^+$ T-cells is played by the conditions of the initial stimulation and $CD4^+$ T-cell help has repeatedly been shown to be of essential relevance [237-239]. $CD8^+$ T lymphocytes primed in "helpless" conditions might still be able to kill targets expressing appropriate antigens and MHC, and to produce a limited array of cytokines, but they would fail to respond to specific TCR triggering with proliferation and IL-2 production [237]. "Helper" functions for $CD8^+$ T-cell activation are basically elicited through two main mechanisms. First, stimulation by MHC class II restricted antigenic epitopes induces, in $CD4^+$ cells, the paracrine production of high amounts of cytokines, most importantly IL-2, supporting $CD8^+$ T-cell expansion. Furthermore, activated $CD4^+$

lymphocytes are also able to "license" APCs to optimally present MHC class I restricted antigens through CD40 triggering by CD40 ligand (CD154) [240,241].

Clinical application of these concepts suffers from a number of limitations. For instance, the use of TAA-derived HLA-class II restricted immunogenic peptides promoting $CD4^+$ T cells activation in active specific cancer immunotherapy would imply a strict selection of eligible patients based on both HLA-class I and II typing. On the other hand, the administration of exogenous cytokines is limited by their inherent toxicity [242,243]. In this regard, agonistic anti-CD40 therapeutic monoclonal antibodies have been successfully utilized, but systemic administration raises concerns due to CD40 expression in platelets and endothelial cells and its potential role in auto-immune / inflammatory processes.

Based on this background and further prompted by the clinical experience acquired in the last fifteen years[227,228,230,244], we have generated a non-replicating recombinant Vaccinia Virus encoding human CD40L. We previously demonstrated that infection of $CD14^+$ monocytes or DCs, with rVV40L led to their activation as indicated by the up-regulation of co-stimulatory ligands and further confirmed by the increased expression of genes encoding for pro-inflammatory and effector cytokines [230]. In this study we have then carefully analyzed the quality of immune responses induced upon naïve $CD8^+$ T-cells stimulation by CD14+ monocytes expressing CD40L following rVV40L infection.

Notably, experimental "in vivo" studies suggest that CD40 receptor expression on activated $CD8^+$ T-cells, plays a crucial role in promoting their differentiation into functional memory cells[196,197]. However, these models hardly mirror human memory T cell formation since we observed that human resting or activated $CD8^+$ T cells do not express CD40 receptor, although they might exogenously acquire some from adjacent APCs as a consequence of intercellular trogocytosis[231-233]. These observations underlined the critical role provided by APCs or more precisely by signals delivered by co-stimulatory ligands and soluble factors to CD8+ T cells, in dictating their differentiation toward memory lineage.

In this regard, our data indicate that CD14 infection by rVV40L induces the expression of IL-12p40 and IFN-α and –β genes, encoding cytokines of essential relevance for T cell memory induction[155,156,207]. Indeed, these molecules have been shown to activate APC and to directly promote the development of memory CD8+ T cells by preventing activation induced apoptosis by improving their viability and proliferation potential[155,156,207]. Furthermore, at difference with s40L-stimulated monocytes, rVV40L-activated $CD14^+$ cells

do not express IL-10 and IDO genes, thereby suggesting a highly effective capacity of activating T cells. In addition, rVV40L infection does not induce a marked up-regulation of PD-L1 on non-professional APCs, at difference with CD14+ monocytes treated with VV WT or s40L, thus suggesting that these cells are unlikely to induce exhaustion in activated T cells. In this respect our data extend previous findings showing that s40L, which is also detectable in sera from cancer bearing patients[14], may promote PD-1 expression in activated T cells. Furthermore, these observations underline the differential biological activity of CD40L in surface expressed or soluble form in modulating the activity of APCs and possibly related to the ability of the former to efficiently cross-link CD40 receptor[184,185].

In line with this observation, rVV40L-activated $CD14^+$ cells proved highly efficient APCs for naïve $CD8^+$ T-cells priming in a variety of experimental settings, including, allostimulation, and response to TAA-derived epitopes or viral antigens, although they did not appear to differentiate towards professional APCs, since they failed to express CD1a upon infection. Most importantly however, they were able to preferentially promote the generation of antigen specific cells with a T_{CM}–like phenotype, whereas s40L-activated $CD14^+$ monocytes appeared to promote the progression of naïve $CD8^+$ T-cells towards more terminally differentiated stages (T_{EM} /T_{EMRA})[103]. Strikingly, a single "in vitro" stimulation of naïve $CD8^+$ T- cells appeared to suffice for effective induction of antigen specific T_{CM} cells.

A variety of factors and multiple mechanisms are known to contribute to memory $CD8^+$ T-cell generation. They include antigen specific features, such as nature and concentration of the immune-stimulating materials [245] or T cell intrinsic characteristics, such as antigen precursor frequency [246]. In addition, APCs intrinsic features, including levels of co-stimulatory molecules and inhibitory ligands expressed and, most importantly, cytokines released by APCs are widely recognized to play a critical role in memory T cell induction. In this respect, the increased expression of IL-12 and IFNs-type I detected in rVV40L-infected as compared to s40L-tretated monocytes suggest that these cytokines might be of critical relevance in promoting the differentiation of antigen-specific naïve precursors into CD8+ T cells with phenotypic and functional attributes of central memory lymphocytes. In this regard, it has been reported that precursors of long lasting $CD8^+$ T-cells may be identified based on their selective expression of surface markers. Detection of a specific phenotypic profile on rVV40L primed $CD8^+$ T-cells confirms their activated state, as witnessed by CD45RO and CXCR3 expression, their potential ability to localize in secondary lymphoid organs, as indicated by CD62L expression, and their responsiveness to homeostatic stimuli, consistent

with IL-7Rα (CD127) expression[130]. Furthermore, besides phenotypic profiles, functional data provide striking evidence of the nature and potential clinical relevance of central memory $CD8^+$ T-cells elicited upon rVV40L infection. Indeed, these lymphocytes are characterized by a high proliferative potential in response to stimulation by non-professional APCs, leading to 4-5 cell divisions in a large majority of cells. In addition, advanced differentiation towards effector T cells was also detectable. In view of these latter observations, it must be underlined that despite the "in vitro" nature of this study, phenotypic and, most importantly, functional characterization of "8-days" T_{CM} generated upon stimulation of naïve CD8+ lymphocytes with rVV40L infected CD14+ monocytes closely resembles features of ex-vivo sampled human T_{CM} and and also emerging from a variety of experimental models[210-212]. Therefore, these cells generated through rVV40L infection of CD14+ cells may be considered as "bona fide" T_{CM}. In this respect, further investigation are warranted in order to clarify the molecular basis and the precise differentiation model underlying the significant expansion of CD8+T_{CM} in cell cultures primed with CD40L-expressing recombinant vaccinia virus.

In addition to its remarkable efficacy in the modulation of quality and intensity of different signals delivered from APC shaping CD8+ T cell responses, the ability of rVV40L to inhibit tumor cells proliferation upon infection or through the activation of myeloid cells of monocyte/macrophage lineage was also evaluated. In this regard, preliminary data underline the remarkable effectiveness of rVV40L, as compared to s40L-treatment, to promote cell death of a panel of established tumor cell lines "in vitro". Furthermore, CD40L-expression on cell surfaces of infected tumor cells was sufficient to provide effective targets for TNF-α mediated cytotoxicity elicited, by CD14+ monocytes[84,209,220].

Notably, these effects are generated by a replication inactivated CD40L-expressing recombinant vaccinia virus, thus suggesting that its "in vivo" administration would be associated with minimal potential adverse effects. It is tempting to speculate that similar reagents might therefore be of high relevance for vaccination purposes. Indeed, a critical limitation of different cancer vaccine formulations to induce objective anti-tumor T cell responses is represented by the insufficient delivery, processing and presentation of the chosen tumor associated antigen to/by dendritic cells[74].On the other hand, in several tumor types such as melanomas and lung cancers, it has been reported that majority of tumor-infiltrating lymphocytes (TILs) recognize tumor specific neo-antigens arising from non-synonymous mutations accumulated in malignant cells during tumor progression[27,28]. Identification of neo-antigens may represent the beginning of a new era for active, antigen-

specific cancer immunotherapy strategies. However clinical application of these concepts is still limited by the complexity of procedures required for the molecular identification of MHC-class I/II restricted tumor antigens arising from non-synonymous mutations. In this scenario, thanks to its cytotoxic activity on transformed cells, intra tumoral injection of replication incompetent CD40L-expressing recombinant vaccinia virus might favor the release on tumor specific antigens promoting the generation of effective and long-lasting CD8+ T cell responses through the modulation of antigen presenting capacity of tumor-infiltrating myeloid cells[84,209,247].

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