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Lymphocytes genetically modified to express tumor antigens target dendritic cells *in vivo* and induce antitumor immunity

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CONTENTS

Abstract

Cancer immunotherapy attempts to harness the exquisite power and specificity of the immune system for the treatment of malignancy. Although cancer cells are less immunogenic than pathogens, the immune system is clearly capable of recognizing and eliminating tumor cells. However, tumors frequently interfere with the development and function of immune responses. Thus, the challenge for immunotherapy is to use advances in cellular and molecular immunology to develop strategies that effectively and safely augment antitumor responses.

An important strategy for mobilizing an anti-tumor response depends on optimal dendritic cells (DCs) activation, antigen presenting cells (APCs) normally charged with the task of ingesting infectious agents and others antigen bearing particles (including tumor cells) throughout the body's tissues and then rushing back to nearby draining lymph nodes where T cell priming occurs.

In this contest, he exploitation of the physiologic processing and presenting machinery of DCs by *in vivo* loading with tumor-associated antigens, may improve the immunogenic potential and clinical efficacy of DC-based cancer vaccines. In this experimental work, T lymphocytes genetically modified to express self/tumor antigens, acting as antigen carriers, efficiently target DCs *in vivo* in tumor-bearing mice. The infusion of TRP-2-transduced lymphocytes induces the establishment of protective immunity and long-term memory in tumor-bearing mice. The analysis of the mechanism responsible for the induction of such an immune response allowed to demonstrate that cross-presentation of the antigen mediated by the $CD11c^+$ $CD8\alpha^+$ DCs subset had occurred. Furthermore, results demonstrate *in vivo* and *in vitro* that DCs undergo activation upon phagocytosis of genetically modified lymphocytes (GML), a process mediated by a cell-to-cell contact mechanism independent of CD40 triggering. Targeting and activation of secondary lymphoid organ-resident DCs endowes antigen-specific T-cells with full effector functions, which ultimately increases tumor growth control and animal survival in a therapeutic tumor setting. These data clarly demonstrate that transduced T lymphocytes represent an efficient way for *in vivo* loading of tumor-associated antigens on DCs.

Introduction

Cancer immunotherapy: an overview.

The idea that the immune system can recognize and respond to tumours was formed in the late 19th century when William Coley, (surgeon at the Memorial Sloan–Kettering Cancer Center in New York) noted that rare events of spontaneous tumour regression were often preceded by infectious episodes[1]. However, attempts by Coley and his colleagues to stimulate systemic immunity directed against tumours with bacterial extracts met with limited success. The modern era of tumour immunology began several decades later with the demonstration that inbred mice could be immunized against carcinogen induced transplantable tumours and that the tumour-rejection antigens were by and large tumour-specific[2,3]. Thomas[4] and Burnet[5] then introduced the famous concept of "immune surveillance of cancer" to describe a mechanism that protects immunocompetent hosts against tumours, and the development of cancer vaccines for the human population seemed to be within reach.

The early promise did not work out, in fact results of clinical trials were disappointing[6]. Moreover, naturally induced tumours in rodents were found to be largely "non-immunogenic"[7].

The immune-surveillance hypothesis was discredited because of findings that tumour incidence in athymic nude mice was not different from that in wild-type mice[8,9].

Cumulatively, these observations contributed to the notion that tumours are not sufficiently distinct from normal tissue to activate the immune system, that is, tumours do not encode tumour antigens and this led to the conclusion that immunological intervention in cancer would be futile.

The turning point was a landmark study published in 1982 by A. van Pel and T. Boon[10]. This study showed that, contrary to the prevailing view at that time[7], it was possible to induce protective immunity against strictly defined non-immunogenic tumours, provided that the tumour cells used to immunize the mice were mutagenized, that is, exposed to radiation or chemical agents that cause DNA damage.

They showed that the primary reason for the lack of tumour immunogenicity is the inability of the growing tumour to activate the immune system, not the absence of tumour-rejection antigens. In today's 'immuno-speak', the tumour cells are antigenic,

but not immunogenic. If we can artificially activate the immune system against the tumour, otherwise called vaccination, we should be able to eradicate the tumour.

The cancer immune surveillance hypothesis has also been resurrected in recent studies that have shown that deficiencies in the components of the adaptive and/or innate arm of the immune system in mice are associated with a subtle, yet statistically significant, increased incidence of cancer[11–13]. The important observation that tumours arising in immunodeficient mice were more immunogenic[14] indicated that tumours that grow in immunocompetent mice are selected for reduced immunogenicity. The important implication is that the naturally occurring immune system can slow, but not eliminate, tumour growth, so providing a satisfactory, if not definitive, explanation for the apparent paradox of tumour formation in immunologically intact individuals. Indeed, tumour progression in patients with cancer is often associated with the secretion of immunosuppressive factors such as transforming growth factor-β (TGF-β) or downregulation of the various components of the antigen-presentation pathway[15,16].

In conclusion, there is mounting evidence to support a role for the immune system in controlling tumour progression. The impact, however, is limited; the immune system seems to slow, but not prevent, tumour progression.

The weak or non-existent inflammatory response that is elicited by the growing tumour is not conducive to the effective activation/maturation of local DCs or function of T cells. Consequently the antitumour immune response is weak and the genetically unstable tumour can adapt by natural selection (immuno-editing)[14].

The lack of immunogenicity of naturally occurring tumours in mice (and conceivably in humans) can now be understood in terms of a suboptimal condition in the tumour microenvironment to generate protective immunity and not due to the lack of tumour antigens. If so, why are carcinogen-induced tumours immunogenic? In naturally occurring tumours, the immune response is directed predominantly against normal tissue-specific products. As tolerance mechanisms have eliminated the bulk of the corresponding high-avidity T cells from the circulation, activation of the remaining low-avidity T cells is an uphill battle, resulting in a weak immune response that has no impact on tumour growth. In carcinogen induced tumours, the predominant antigenic targets correspond to *de novo* mutations that are induced by the carcinogen, which did not trigger tolerance. So, high-avidity T cells will exist in the circulation and their activation can inhibit tumour growth.

As discussed above, the tumour microenvironment is not conducive to the emigration and optimal activation of DCs, so the ensuing immune response is weak and ineffective.

The purpose of specific active immunotherapy is to stimulate an antitumour immune response by channelling the tumour antigens into the appropriate DC subset and providing the optimal conditions for the maturation of the DC into a potent immunostimulatory APC. There are four important issues to consider in designing effective cancer vaccines; how to identify potent tumour rejection antigens; how to stimulate an effective antitumour immune response; how to avoid autoimmune pathology; and how to prevent immune evasion.

Cancer immunosurveillance and immunoediting

Accumulating evidence indicates that a dynamic cross-talk between tumors and the immune system can regulate tumor growth and metastasis.

Increased understanding of the biochemical nature of tumor antigens and the molecular mechanisms responsible for innate and adaptive immune cell activation has revolutionized the fields of tumor immunology and immunotherapy. Both the protective effects of the immune system against tumor cells (immunosurveillance) and the evasion of tumor cells from immune attack (tumor-immune escape) have led to the concept of cancer immunoediting, a proposal which infers that a bidirectional interaction between tumor and inflammatory/regulatory cells is ultimately responsible for orchestrating the immunosuppressive network at the tumor site.

In this context, a major challenge is the potentiation or redirection of tumor antigen-specific immune responses. The success in reaching this goal is highly dependent on an improved understanding of the interactions and mechanisms operating during the different phases of the cancer immunoediting process.

Cancer immunoediting can be considered a dynamic process consisting of three phases: elimination (i.e., cancer immunosurveillance), equilibrium, and escape, (figure 1).

Elimination represents the classical concept of cancer immunosurveillance, equilibrium is the period of immune-mediated latency after incomplete tumor destruction in the elimination phase, and escape refers to the final out-growth of tumors that have outstripped immunological restraints of the equilibrium phase.

The most significant clinical implication of this hypothesis is that most, if not all, tumors that develop in humans may have undergone immunologic sculpting as a result of a cancer immunoediting process, with the most dramatic consequences of the process probably occurring before the tumor is clinically detectable. Ultimately, if the cancer immunoediting process indeed emerges as one of the leitmotifs of cancer progression, then an improved understanding of the immunobiology of cancer immunoediting and a molecular definition of how tumors are shaped by this process will undoubtedly bring closer to tumor immunology's capstone: the use of immunotherapy to control and/or eradicate neoplastic disease in the human cancer patient.

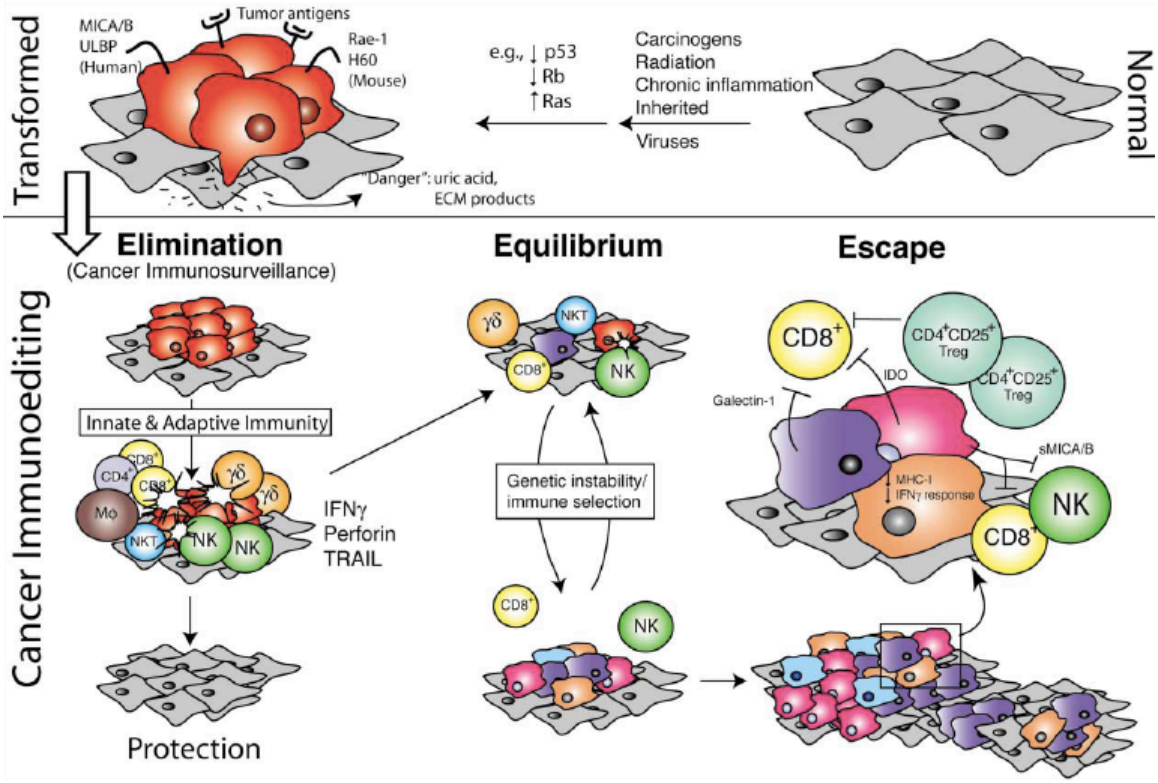

Figure 1.
The three phases of the cancer immunoediting process. Normal cells (gray) subject to common oncogenic stimuli ultimately undergo transformation and become tumor cells (red) (top). Even at early stages of tumorigenesis, these cells may express distinct tumor-specific markers and generate proinflammatory "danger" signals that initiate the cancer immunoediting process (bottom). In the first phase of elimination, cells and molecules of innate and adaptive immunity, which comprise the cancer immunosurveillance network, may eradicate the developing tumor and protect the host from tumor formation. However, if this process is not successful, the tumor cells may enter the equilibrium phase where they may be either maintained chronically or immunologically sculpted by immune "editors" to produce new populations of tumor variants. These variants may eventually evade the immune system by a variety of mechanisms and become clinically detectable in the escape phase. *(The immunobiology of cancer immunosurveillance and immunoediting, R.D. Schreiber, Immunity, Vol. 21, 137–148, August, 2004, review).*

The dendritic cell network

DCs are present in peripheral tissues in an immature state that is specialized for sampling the environment using various endocytic mechanisms, but is characterized by low levels of expression of MHC molecules and T-cell co-stimulatory molecules. Immature DCs are well equipped with a series of receptors for pathogen-associated molecular patterns and for secondary inflammatory compounds, such as Toll-like receptors (TLRs)[17,18] nucleotide-binding oligomerization domain (NOD) proteins[19], RIG-I-like receptors 6, C-type lectin receptors[20,21] cytokine receptors[22] and chemokine receptors[23]. Signalling through these receptors triggers DC migration towards the secondary lymphoid organs. On reaching these organs, DCs develop into a mature state, which is characterized by high levels of expression of MHC and T-cell co-stimulatory molecules, and the ability to present antigen captured in the periphery to T cells[17].

Heterogeneity of the DC network

There are two main categories of DCs: plasmacytoid DCs and conventional DCs. Plasmacytoid DCs circulate through the blood and lymphoid tissues and only acquire the typical DC morphology after activation, which is accompanied by the release of type I interferons (IFNs). The role of plasmacytoid DCs in antigen presentation and T-cell priming is unclear, as in fact is their categorization as DCs; this is why they are sometimes referred to as 'IFN-producing cells'[26]. Conventional DCs can be divided in three different categories. The first one corresponds to the migratory DCs, which develop from earlier precursors in peripheral tissues and travel through the afferent lymphatics to reach the local draining lymph nodes[27], where they constitute approximately 50% of all lymph-node DCs. This group of DCs is largely absent from the spleen and thymus because these organs do not receive afferent lymph. There are two subtypes of migratory DCs. The first subtype is found in all lymph nodes and corresponds to the interstitial DCs (Table 1).

Features	Lymphoid-organ-resident DC subsets			Migratory DC subsets		Monocyte derived
	$CD4^+$ DCs	$CD8^+$ DCs	DN DCs	Interstitial DCs	Langerhans cells	
Location						
Spleen	Yes	Yes	Yes	No	No	Sites of inflammation
Subcutaneous lymph nodes	Yes	Yes	Yes	Yes	Yes	
Visceral lymph nodes	Yes	Yes	Yes	Yes	No	
Thymus	Yes	Yes	Yes	No*	No	
Surface markers						
CD11c	+++	+++	+++	+++	+++	+++
CD4	+	–	–	–	–	–
CD8	–	++	–	–	–/+	–
CD205	–	++	–/+	+	+++	–/+
CD11b	++	–	++	++‡	++	++
Langerin	–	+	–	–	+++	–
CD24	+	++	+	ND	ND	ND
SIRPα	+	–	+	+	+	ND
Functional features in the steady state						
Maturity	Immature	Immature	Immature	Mature	Mature	N/A
Co-stimulatory§	+	+	+	++	++	N/A
Antigen processing and presentation‖	+++	+++	+++	+/–	+/–	N/A
MHC class II	++	++	++	+++	+++	N/A
In vitro *equivalent*						
	Bone-marrow precursors plus FLT3L	Bone-marrow precursors plus FLT3L	Bone-marrow precursors plus FLT3L	Bone-marrow precursors plus GM-CSF, TNF and TGFβ	Bone-marrow precursors plus GM-CSF, TNF and TGFβ	Bone-marrow, spleen or blood precursors plus GM-CSF

Table 1 Mouse Dendritic cells substes, *Nature Reviews Immunology Vol. 7 July 2007*

The subcutaneous lymph nodes also contain a second population of migratory DCs, namely the Langerhans cells, which migrate from the skin epidermis. Migratory DCs follow the life cycle described by the Langerhans cell paradigm: they traffic from peripheral tissues to the lymph nodes, where they exhibit a mature phenotype[24]. The second major category of lymphoid-organ DCs are the blood-derived or resident DCs, which constitute the second half of lymph-node DCs and all the splenic and thymic DCs[24]. They can be subdivided into three types that are distinguished by their expression of CD4 and CD8: $CD4^+$ DCs, $CD8^+$ DCs and $CD4^-$ $CD8^-$ (double negative) DCs (Table 1). The lymphoid-organ-resident DCs do not conform to the Langerhans cell paradigm; they develop from bone-marrow precursors within the lymphoid organs without previously trafficking through peripheral tissues[25,28–30]. Furthermore, in the absence of infection, the resident DCs maintain an immature phenotype throughout their entire lifespan[31], so they can be distinguished from migratory DCs in the lymph nodes by their lower cell-surface expression of MHC class II and T-cell co-stimulatory molecules[31,32]. Therefore, almost all splenic DCs and

approximately half of the lymph-node DCs are immature under steady-state conditions. This is true not only in mice but also in humans[33] lymphoid-organ-resident DCs spontaneously acquire a mature phenotype that is similar to that of the migratory DC populations that have reached the lymph nodes[31,34]. Such maturation is also induced in situ in mice or humans in response to pathogen-associated stimuli that enter the lymphoid organs[31,33,35–38], or in individuals who suffer multiple traumas[33]; in the case of traumas, maturation probably results from resident DCs responding to endogenous inflammatory molecules that are released from damaged tissues.

Monocyte-derived DCs: precursors of migratory DCs or 'emergency DCs'?

Monocytes can be differentiated into DCs *in vitro*[25], and this is the most common DC type used in studies of mouse and human DC biology, and for immunotherapy However, it is unclear whether the monocyte-derived DCs correspond to any of the DC subsets contained in the lymphoid organs in the steady state (Table 1).

It has been suggested that in these conditions monocytes are precursors of migratory DCs, but this is still a controversial notion[39]. Monocyte-derived DCs represent only one of the several types of DC that constitute the DC network *in vivo*, and that the conclusions of functional studies that are based on this DC subset may not be applicable to the other DC types.

Antigen-presenting functions of DC subtypes

If there is one functional feature that defines DCs, it is their high capacity to capture, process and present antigens, a prerequisite for T-cell priming. However, not all DCs have equivalent antigen-presenting roles *in vivo*. On a first approximation, all DCs efficiently present peptide antigens on their MHC class I and II molecules because peptide-ligand binding is, with exceptions, a requirement for cell-surface expression of these molecules[17], and all DC subsets express high levels of MHC class I and II molecules, especially when mature. Therefore, the question is not whether the DC types differ in their capacity to generate peptide–MHC complexes, but whether they differ in their ability to incorporate peptides that are derived from a given antigen into their presentation pathways. To be presented, the antigens have to access the

compartments where proteolytic degradation generates peptide ligands for MHC class I or II molecules. The peptides presented by MHC class I molecules are derived from proteins degraded mainly in the cytosol by the proteasome, whereas MHC class II molecules present peptides that are derived from proteins degraded in endosomal compartments by the cathepsins and other hydrolytic enzymes[17].

DCs continually present peptides that are derived from endogenous proteins on MHC class I molecules, Similarly, endogenous proteins that access the endosomal compartments of DCs are efficiently presented on their own MHC class II molecules. Therefore, all DCs constitutively present peptides that are derived from their own components on MHC class I and II molecules[40,42]. The presentation of exogenous antigens is more complicated than the presentation of endogenous antigens because it relies on the ability of cells to deliver the antigens to the correct processing compartments. These antigens must first be endocytosed by pinocytosis, phagocytosis or receptor-mediated endocytosis[17]. The internalized antigens thus become readily accessible to endosomal proteases and so can be presented by MHC class II molecules. In addition, some cells can present these antigens via MHC class I molecules, a process known as cross-presentation. This pathway is of particular relevance in DCs because they appear to be the main cell population that can cross-present antigens *in vivo*[43], and this enables them to play important roles in tolerance induction and in antiviral and antitumour immunity[44]. Among the lymphoid-organ-resident DCs, the $CD8^+$ DCs are the most efficient at phagocytosing dead cells and, consequently, at MHC class II presentation and MHC class I cross-presentation of cellular antigens[46–51] (Table 2).

Antigen	$CD8^+$ DCs			$CD8^-$ DCs			References
	Uptake	MHC class I	MHC class II	Uptake	MHC class I	MHC class II	
Endogenous							
	N/A	++	++	N/A	++	++	49–51
Exogenous: phagocytosed							
Cells	++	++	+	+/–	–	+/–	59–64
Beads	++	++	+/–	++	–	++	64
Exogenous: pinocytosed (soluble)							
	++	++	++	++	+/-	++	64,77–79,81
Exogenous: receptor-mediated endocytosed							
CD205	++	++	+	Not expressed	N/A	N/A	68,69,80
DCIR2	Not expressed	N/A	N/A	++	–	++	68
Dectin-1	Not expressed	N/A	N/A	++	–	++	69
TLR11	++	?	++	Not expressed	N/A	N/A	74,75
FcR	++	++	+	++	++	++	108

Table 2 Uptake, MHC class I and MHC class II presentation of several model antigens by $CD8^+$ and $CD8^-$ dendritic cells. *Nature Reviews Immunology Vol. 7 July 2007*

This is probably due to the differential expression of as-yet-unidentified receptors for dead cells rather than to differences in phagocytic capacity because all DCs phagocytose latex beads or bacteria[47,48,51,52]. Among the lymphoid-organresident DCs, $CD8^+$ DCs are by far the most efficient at cross-presenting cellular[46], soluble[57] or latex-beadassociated antigens[51], or antigens captured by C-type lectin receptors[53,54] (Table 2). $CD8^-$ DCs are also inefficient at cross-presenting antigens that are equally captured by the two subsets or that only they can capture By contrast, $CD8^-$ DCs seem to be more efficient than $CD8^+$ DCs at presenting exogenous antigens by MHC class II molecules (Table 2). This is particularly true for phagocytosed antigens[51] and for antigens captured by C-type lectin receptors[53,54,58], although less so for pinocytosed soluble antigens[51,56,57,59] (Table 2). One potential explanation for this dichotomy is that the MHC class I and II pathways are in general more efficient in $CD8^+$ and $CD8^-$ DCs, respectively.

An alternative explanation for the differential abilities of $CD8^+$ and $CD8^-$ DCs is based on three premises (Figure 2). The first is that the MHC class I and II presentation pathways are fully operational in both DC subsets. The second is that cross-presentation requires specialized machinery that is present in $CD8^+$ DCs but largely absent in $CD8^-$ DCs[51], so $CD8^-$ DCs can present exogenous antigens only through the MHC class II pathway (Figure 2). The third premise is that $CD8^+$ DCs can deliver exogenous antigens to either the MHC class II or the MHC class I cross-presentation pathways, but how much of a given antigen is delivered to each pathway depends on the mechanism involved in its uptake[55]. Therefore, pinocytosed soluble antigens efficiently access both pathways, antigens captured by phagocytosis or CD205 are delivered preferentially to the cross-presentation pathway, and antigens captured by the mannose receptor hardly access the MHC class II pathway at all[55].

It is plausible that cross-presentation requires the delivery of antigen to a specialized compartment (or secondary pathway with multiple compartments) that is found only in $CD8^+$ DCs[55] (Figure 2).

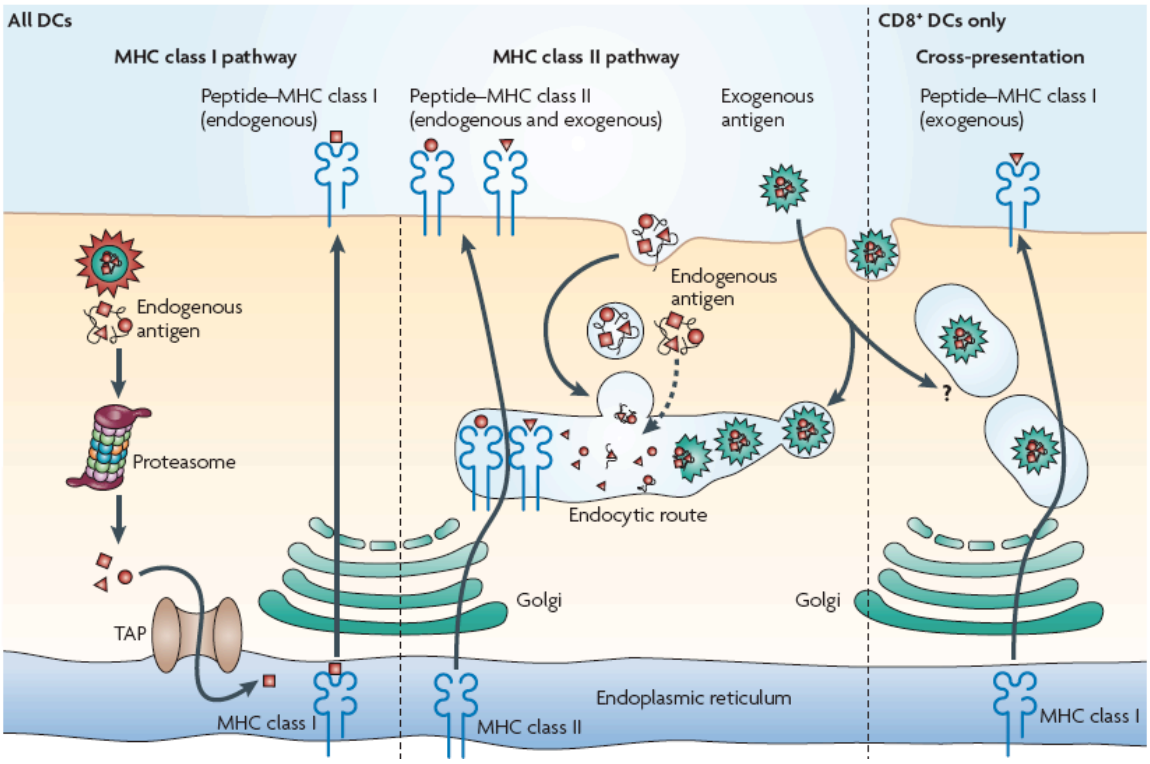

Figure 2 The antigen-presentation pathways in dendritic cells. All dendritic cells (DCs) have functional MHC class I and MHC class II presentation pathways. MHC class I molecules present peptides that are derived from proteins degraded mainly in the cytosol, which in most DC types comprise almost exclusively endogenous proteins (synthesized by the cell itself). MHC class II molecules acquire peptide cargo that is generated by proteolytic degradation in endosomal compartments. The precursor proteins of these peptides include exogenous material that is endocytosed from the extracellular environment, and also endogenous components, such as plasma membrane proteins, components of the endocytic pathway and cytosolic proteins that access the endosomes by autophagy. CD8+ DCs have a unique ability to deliver exogenous antigens to the MHC class I (cross-presentation) pathway, although the mechanisms involved in this pathway are still poorly understood. The bifurcated arrow indicates that the MHC class II and the MHC class I crosspresentation pathways may 'compete' for exogenous antigens in CD8+ DCs, or that the endocytic mechanism involved in internalization of a given antigen may determine whether it is preferentially delivered to the MHC class II pathway or the MHC class I cross-presentation pathway. TAP, transporter associated with antigen processing. *Nature Reviews Immunology Vol. 7 July 2007.*

It remains unclear which are the specific features of this putative compartment that enable cross-presentation[45]. They might include unique protease activities[61]; differential acidification[60]; a mechanism of antigen transfer to the cytosol directly[62,63] or after passage through the endoplasmic reticulum[64]; the ability to fuse with the endoplasmic reticulum to generate hybrid compartments[65–67]; or the destination of a unique MHC class I trafficking pathway[68].

Tumor specific antigens

Immunotherapy of cancer has become a more promising approach in the past decade. Developments in both basic immunology and tumor biology have increased our knowledge of the interactions between the tumor cells and the immune system.
The molecular identification of tumor-associated antigens that can be recognized by autologous T lymphocytes has cleared the way for development of different strategies for anti-tumor vaccines.
Some of these antigens appear to be tumor specific, whereas others are also present on normal tissues. Two main genetic mechanisms produce tumor-specific antigens: (*a*) Point mutations occurring in tumor cells can generate antigenic peptides either by enabling them to bind to the groove of MHC molecules or by generating new epitopes on these peptides. (*b*) In tumor cells, a gene is expressed that is silent in normal cells. On human melanoma, autologous T cells recognize tumor-specific antigens produced by both mechanisms: the antigens caused by mutational events and those encoded by cancer-germline genes. Somewhat surprisingly, in view of what is expected of natural tolerance, it is also possible to observe or generate autologous T cell responses against differentiation antigens common to melanoma and normal melanocytes.

- **Antigens encoded by cancer-germline genes.**

A small number of gene families are expressed in male germline cells and in many tumors, but not in other normal adult tissues. Because male germline cells do not express human leukocyte antigen (HLA) molecules on their surface, they do not express antigens that can be recognized by T cells (69). As a result, the antigens encoded by these cancergermline genes are strictly tumor-specific T cell targets. These antigens are shared by many tumors. The prototype of the cancer-germline genes is the *melanoma antigen-encoding* (*MAGE*) gene family[70–72]. This family is composed of 24 genes that range across three subfamilies: *MAGE-A*, *-B,* and *-C*, located in three different regions of the X chromosome. The expression of *MAGE* genes in tumors appears to be triggered by the demethylation of their promoter, apparently as a consequence of the widespread demethylation process that occurs in many tumors[73]. Six additional *MAGE* gene families have been identified in the human genome[72,74]. These genes are expressed in normal tissues. However, because of their

low similarity with the sequences of the *MAGE-A*, *-B,* and *-C* family, not a single MAGE-A, -B, or -C antigenic peptide is encoded by one of the ubiquitously expressed *MAGE* genes, leaving intact the strict tumor specificity of the antigens encoded by the *MAGE-A*, *-B,* and *-C* genes.

Other important cancer-germline gene families are the *Bage*[75], *Gage*[76], *Lage/NYESO-1*[77,78], and *SSX* families[79,80].

Investigators often state that antigens encoded by cancer-germline genes represent "self" antigens and that tolerance must therefore be broken in the course of immunization against these antigens. In one sense, cancer-germline genes are indeed self in that they are normal genes of the human genome. However, the strict tumoral specificity of the antigens is hardly conveyed by the word self. The critical issue is whether there exists some measurable degree of natural immunological tolerance against these antigens. This question has not yet been answered. One ought to be able to examine this issue with the mouse *P1A* gene, which codes for a mastocytoma P815 antigen and is also a cancer-germline gene[81]. Normal DBA/2 mice mount a CD8 response to this antigen. Transgenic mice that express the *P1A* gene in all their tissues are clearly incapable of a cytotoxic T lymphocyte (CTL) response to the relevant antigens[82]. But the crucial question has not yet been answered, namely, whether knockout mice will mount anti-P1A T cell responses more readily than normal mice. It is expected for these mice to be available in the near future. For gene *P1A*, as well as for *MAGE* genes, Kyewski and coworkers[83,84] have shown that some sporadic gene expression occurs in medullary thymic epithelial cells, thereby opening the possibility that some form of thymic tolerance might exist.

- **Antigens resulting from point mutations.**

Many antigens recognized by T cells on human tumors are encoded by a sequence containing a point mutation that originated in the tumor cells[85]. Many of the point mutations that have been identified because they generated a tumor antigen appear to have an oncogenic function. One mutation found in melanoma in a cyclin-dependent kinase molecule (CDK4) was also found in a familial form of melanoma[86,87]. The antigens encoded by point mutations, which have been identified following stimulation by autologous mixed lymphocyte/tumor cell cultures (MLTC), were invariably found to result from mutations that are present on a single tumor or on very

few. Thus, these mutated antigens are strictly tumor specific but not shared. This limits their use as potential vaccines. It is remarkable that MLTC have not produced T cells recognizing antigens encoded by mutated regions that are very frequent in cancer cells, such as mutated ras or p53. However, a mutated N-ras antigen was found to be recognized by tumor-infiltrating lymphocytes (TILs)[88]. Promising candidates for shared mutated antigens are encoded by B-Raf, a member of the mitogen-activated protein (MAP) kinase cascade. The same activating mutation of B-Raf is found in more than 60% of melanomas[89]. Recently, CD4 T cells were shown to recognize a B-Raf peptide encoded by the mutated region. These CD4 T cells also recognized cells expressing the mutated B-Raf gene[90].

- **Antigens encoded by genes overexpressed in tumors.**

The gene *PRAME* was identified as coding for a melanoma antigen recognized by autologous CTL. It is expressed at a high level in almost all melanomas and in many other tumors[91,92]. Survivin, an antiapoptotic protein of the inhibitor of apoptosis protein (IAP) family, is overexpressed in tumors, and melanoma cells are lysed by antisurvivin CTL[93,94]. However, no comparison of the level of survivin in tumor cells and in proliferating normal cells has been published. Telomerase has been suggested as a good target for immunotherapy, as its presence is essential for the proliferation of tumor cells. An antigenic peptide encoded by telomerase was recognized by CTL[95]. However, other reports claimed that CTL of patients vaccinated with the antigenic peptide recognized cells pulsed with the peptide but did not recognize tumor cells expressing telomerase[96,97]. The antigens encoded by these genes are shared between many tumors, but they are not tumor specific, and the risk of vaccinating with these antigens must be weighed carefully.

- **Antigens encoded by differentiation genes.**

Melanoma patients have T lymphocytes capable of recognizing antigens encoded by normal melanocytic differentiation genes, such as those encoded by tyrosinase, Melan A/Mart-1, gp100/pMel17, tyrosinase-related protein (TRP)-1, and TRP-2[98–101]. Vaccination with such antigens can produce vitiligo, i.e., elimination of normal melanocytes in some areas of the skin[102,103].

Passive cancer immunotherapy

Adoptive T cell therapy

Over the past 50 years, two fundamentally different strategies to stimulate antitumor immunity have been tested in humans: therapeutic vaccination and passive immunization. Passive immunization, herein referred to as adoptive T cell therapy, is the transfusion of autologous or allogeneic T cells into tumor-bearing hosts, i.e., patients. Evidence that T cells can help to control tumor growth has been provided by the analysis of tumor prevalence in immunodeficient mice and humans[104,105]. The recent explosion of knowledge in the fields of T cell and cancer biology has enabled new approaches that might bring adoptive T cell transfer to the routine practice of clinical medicine. The application of recent lessons from adoptive transfer in lymphodepleted hosts[108], the ability to overcome barriers presented by Tregs[109,110], and the use of improved culture systems[111] have not yet been tested in randomized clinical trials.

- **CTL therapy**

Improved cytotoxic T lymphocytes (CTLs) cell culture technology[112] has permitted the first clinical tests of adoptive transfer of CTLs, and the approach seems to result in substantial activity in patients with melanoma; CTLs derived from PBLs were used to treat patients with refractory, metastatic melanoma, and 8 of the 20 patients had minor, mixed, or stable antitumor immune responses[113].

The *in vivo* efficacy of the infused T cell population was indicated by the destruction of normal melanocytes and outgrowth of a MART-1–negative tumor, demonstrating the selection of a tumor variant with loss of MART-1 expression[114].

However only 3 of 11 patients had clinical antitumor responses, and a selective loss of MART-1 expression in lymph node metastases in 2 of 2 evaluated patients was observed[115]. Therefore, perhaps the most worrisome issue revealed with CTL transfers is the emergence of antigen escape variants, which seems to be more common in human tumors than in mouse syngeneic tumor models[116,117]. However, preliminary results have indicated that CTL transfers in patients with melanoma might have a vaccine-like effect, inducing epitope spreading, in that the antitumor response correlated with the detection of T cell clones with higher avidity for the tumor antigen

and with a broader tumor antigen–specific repertoire than was detected before treatment[118]. Therefore, it is possible that the problem of antigen escape variants can be addressed by enhancing the immune response to include a broad tumor antigen–specific T cell repertoire, either by increasing the efficiency of epitope spreading or by infusing CTL clones with multiple antigenic specificities.

- **TIL therapy**

Adoptive transfer therapy with Tumor Infiltrating Lymphocytes (TILs) requires the isolation of T cells from fresh patient biopsy specimens and the progressive selection of tumor-specific T cells *ex vivo* using high levels of IL-2 and various cell culture approaches (Figure 3).

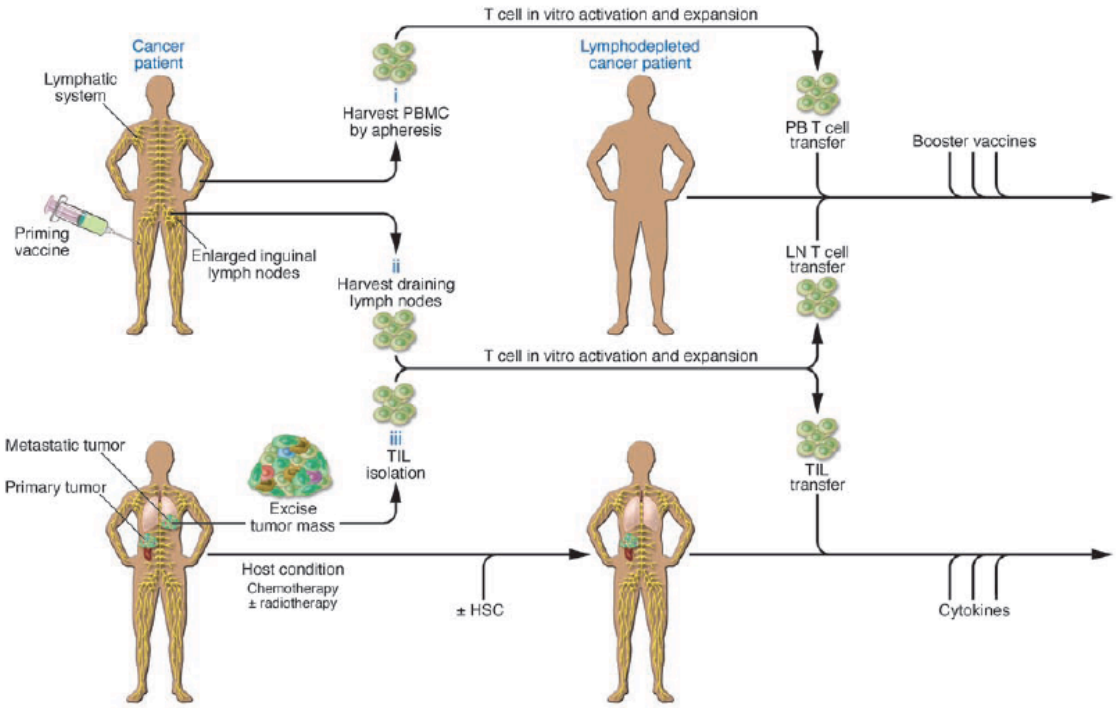

Figure 3
Schemes for adoptive transfer of autologous, vaccine-primed, *in vitro*–expanded T cells. Patients are primed with tumor vaccine followed by lymphocyte harvest. Autologous T cells are harvested from peripheral blood (i) or draining lymph nodes (ii), undergo polyclonal *in vitro* activation and expansion, and are reinfused after lymphodepleting chemotherapy. Antigen-specific immune function is measured after the administration of booster vaccines. (iii) TILs can be isolated from resected surgical specimens and expanded *in vitro* for adoptive transfer after lymphodepleting chemotherapy. Most adoptive transfer therapy approaches using TILs have involved the use of IL-2 infusion following T cell transfer in order to select tumor-specific T cells. *The Journal of Clinical Investigation, Volume 117, No 6, June 2007, 1466-1474.*

The adoptive transfer of these cells showed promise in preclinical models[119], but clinical experiences, with perhaps one exception[120], were almost uniformly

disappointing. However, recent studies at the National Cancer Institute suggest that prior host conditioning with chemotherapy increases the response to adoptive immunotherapy with TILs[108,124].

Importantly, the TILs showed prolonged engraftment compared with TILs transfused to patients without prior treatment with these chemotherapeutics, and the levels of engraftment correlated with the clinical responses.

Adverse effects in the lymphodepletion trial included opportunistic infections and the frequent induction of vitiligo and uveitis, presumably due to autoimmunity.

However, at this point, the results are difficult to interpret, as the ability to successfully generate TILs for therapy could be a predictive biomarker of a more favorable clinical outcome[106,107,125,126]. Therefore, in the absence of a randomized clinical trial it is not possible to determine how much lymphoablative chemotherapy, high-dose IL-2 administration, and TIL therapy contributed to the promising results in these recent trials[108,124]. Technical issues with producing tumor-specific T cells currently present a formidable barrier to conducting randomized clinical trials using TILs. Only 30%–40% of biopsy specimens yield satisfactory T cell populations, and the process is labor and time intensive, requiring about 6 weeks to produce the T cells for infusion[127]. Nearly all clinical experience with TILs has been with patients with melanoma because of the ready surgical availability of tumor biopsy tissue. However, should technical limitations of current tissue culture approaches be overcome, the recent studies indicating that the presence of TILs correlated positively with survival in ovarian and colorectal cancer[106,107] could extend the impact of this promising therapeutic approach to other commonly encountered epithelial cancers.

In mice, adoptive T cell therapy enhances the effects of therapeutic vaccines[128,129], and this combined approach in the setting of lymphopenia results in a further enhancement of tumor immunity compared with combined treatment in lymphoreplete hosts[130,131].

In humans with myeloma, idiotype vaccination of sibling donors with the unique tumor-specific Ig produced by the patient's myeloma cells followed by adoptive transfer in the setting of allogeneic stem cell transplantation can result in the induction of potent antitumor immunity[132]. However, although theoretically attractive, there is not yet extensive data in humans to demonstrate the efficacy of a combined vaccine and adoptive T cell transfer approach in the autologous setting. Shu and Chang have

developed an alternative vaccine adoptive transfer approach that could circumvent many of the limitations posed by adoptive transfer using TILs. They and others have shown in mice that tumor-draining lymph nodes harbor T cells that are not able to mediate tumor rejection in adoptive transfer experiments[133,134]. By contrast, if the draining lymph node cells are activated *in vitro* by various culture approaches, the cells are able to mediate tumor rejection after adoptive transfer[135]. In a further study[136], T cells were isolated from vaccine-primed lymph nodes obtained from patients with melanoma, renal cell carcinoma, and head and neck cancer. In the absence of APCs, activation with CD3- and CD28-specific antibodies greatly enhanced subsequent T cell expansion in response to IL-2 compared with activation with CD3-specific antibody alone[136]. Based on these and other preclinical data, Chang and coworkers carried out a phase I clinical trial in patients with either advanced melanoma or renal cell carcinoma[137]. Patients were vaccinated with irradiated autologous tumor cells and *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). The draining lymph nodes were harvested 7–10 days later and the vaccine- primed T cells cultured and infused (Figure 3). Among the 11 patients with melanoma, 1 had a partial tumor regression, and among the 12 patients with renal cell carcinoma, there were 2 complete and 2 partial tumor regressions[137], suggesting that there might be some clinical benefit to this adoptive transfer approach to boost the clinical efficacy of therapeutic cancer vaccines.

- **Engineered T cells**

A principal limitation of adoptive T cell therapy for some tumors is that the tumors are poorly antigenic; therefore, neither T cells with high avidity for tumor-specific antigens, nor T cells with the desired specificity remain in the patient following chemotherapy. Two strategies to overcome this limitation are now being tested in the clinic (Figure 4).

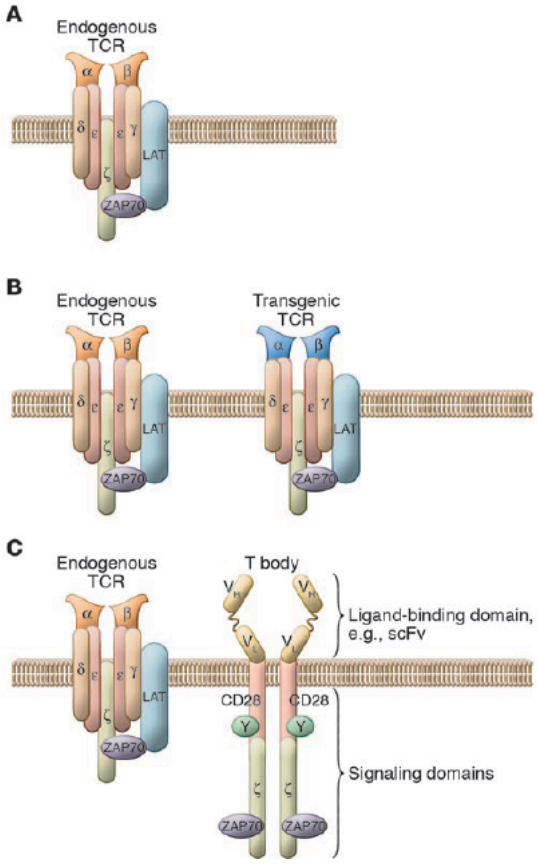

Figure 4
T cells can be engineered to have retargeted specificity for tumors. (**A**) Endogenous T cells express a single heterodimeric TCR. (**B**) Bispecific T cells are created by the introduction of genes that encode proteins that recognize antigens expressed by target tumor cells. These genes can encode natural TCRs that function in the same MHC-restricted manner as endogenous TCRs but have tumor antigen specificity. (**C**) Alternatively, these genes can encode chimeric tumor antigen–specific receptors, or T bodies, that target surface antigens in an MHCindependent fashion. T bodies express an extracellular ligand generally derived from an antibody and intracellular signaling modules derived from T cell–signaling proteins. LAT, linker for activation of T cells; ScFv, single chain variable fragment; ZAP70, ζ-chain–associated protein kinase 70 kDa. *The Journal of Clinical Investigation, Volume 117, No 6, June 2007, 1466-1474.*

One approach has been to endow T cells with novel receptors by introduction of "T bodies," chimeric receptors that have antibody-based external receptor structures and cytosolic domains that encode signal transduction modules of the T cell receptor[138]. These constructs can function to retarget T cells *in vitro* in an MHC unrestricted manner to attack the tumor while retaining MHC restricted specificity for the endogenous TCR. The targets of chimeric antigen receptors must be carefully chosen to avoid unwanted adverse effects, or that additional safety features, such as suicide switches, need to be incorporated into the vectors driving the expression of the chimeric receptor. The other major issues with the approach currently involve improving receptor design by optimizing the ligand-binding domain and by trying to

incorporate costimulatory signaling domains into the signaling module[139]. The former issue is important because the avidity of the ligand-binding domain must be tuned to afford specificity for the tumor cells and yet permit disengagement from the target so that the redirected T cells can be "serial killers." The later issue is important to ensure long-term survival of the T cells so that the proper costimulatory signals can be delivered upon tumor recognition, thereby avoiding the induction of anergy or apoptosis[140] and by potentially increasing the resistance of the T cells to the immunotoxic effects of the tumor microenvironment[141]. T cells are also being transduced to express natural TCR heterodimers of known specificity and avidity for tumor antigens[142].

In the first clinical trial using this approach, T cells were engineered to express a TCR specific for glycoprotein 100 (gp100), A concern with this approach has been that it might generate additional, novel receptor specificities by pairing of the transgenes with the endogenous TCR chains. It is encouraging that no toxicity was observed in the pilot trial, and promising persistence of the engineered T cells was observed in some of the patients. However, one issue that arose was low cell-surface levels of expression of the gp100-specific TCR, which would be expected to lower the avidity of the TCR and therefore minimize effector functions. Another general limitation of this approach for humans is that each TCR is specific for a given peptide-MHC complex, such that each vector would only be useful for patients that shared both MHC alleles and tumor antigens.

A primary issue that could limit the ultimate efficacy of adoptive engineered T cell therapy is the immunogenicity of the proteins that the T cells are engineered to express; this is likely to be a larger problem in humans than in mice because activated human T cells, unlike mouse T cells, express MHC class II molecules and have been shown to function as effective APCs[143].

Monoclonal Antibodies

The development of monoclonal antibodies by Köhler and Milstein captured the imagination of the medical community in 1975. Monoclonal antibodies, however, are just beginning to fulfill the great promise for immunotherapy inherent in their specificity, which permits their selective binding to abnormal cells[146]. Despite wide-ranging efforts, the dream of a 'magic bullet' of antibody therapy prevailing since the time of Ehrlich has proven elusive[147]. However, Levy and coworkers induced remissions in select patients with B-cell lymphoma, using immunoglobulin-specific idiotypic monoclonal antibodies[148].

A number of factors underlie the low therapeutic efficacy observed. Unmodified mouse monoclonal antibodies are immunogenic to humans, have short *in vivo* survival, and generally do not kill target cells efficiently because they do not fix human complement or elicit antibody-dependent cellular cytotoxicity (ADCC) with human mononuclear cells. Finally, in most cases the antibodies were not directed against a vital cell-surface structure such as a receptor for a growth factor that would be required for tumor cell survival and proliferation. To circumvent these problems, researchers have developed human as well as humanized antibodies[144,149]. In addition, cell-surface antigenic targets, especially receptors for cytokines, have been found to provide more effective monoclonal antibody action[145]. Furthermore, cytotoxic action of monoclonal antibodies has been augmented by arming them with toxins or radionuclides.

Such humanized monoclonal antibodies show drastically reduced immunogenicity, improved pharmacokinetics and ADCC with human mononuclear cells.

Rituximab (Rituxan) has been the first monoclonal antibody approved to treat malignancy. Using the CD20-specific antibody rituximab (Rituxan) in individuals with relapsed low-grade non-Hodgkin lymphoma, there was an overall response rate of 57% for the group of patients receiving eight weekly infusions, with 14% of these patients manifesting complete and 43% partial responses[150,151]. In addition, trastuzumab (Herceptin), a humanized antibody against the HER2/neu tyrosine kinase receptor provided an overall response rate of 15% in 222 patients with high expression of HER2/neu associated with breast cancer[152]. Both rituximab and Herceptin have been shown to improve the overall survival of appropriate patients when added to standard chemotherapy in randomized trials[152-154]. Furthermore,

remissions have been observed in one-third of patients with HTLV-1-associated adult T-cell leukemia receiving monoclonal antibody therapy directed against IL-2R[155].

A number of strategies have also been used to increase the therapeutic impact of these antibodies, including the use of genetic engineering to alter the antibody affinity for the target ligand or to modify the constant region involved in Fc receptor binding.

Although many mechanisms have been proposed to account for the antitumor activities of therapeutic antibodies (such as blockade of signaling pathways, activation of apoptosis and cytokine deprivation-mediated cell death), engagement of Fc-receptors on effector cells is a dominant component of their *in vivo* antitumor activity[156,157].

A major limitation to the use of monoclonal antibodies in the treatment of cancer is that most are poor cytocidal agents.

To address this issue, monoclonal antibodies are being linked to a cytocidal agent, such as a toxin or radionuclide, which is then targeted to the tumor cell by the antibody. The arming of antibodies with toxins has been stimulated by the approval of the first immunotoxin-armed antibody gemtuzumab ozogamicin (Mylotarg), which links the toxin calicheamicin to a CD33-specific antibody for use in the treatment of myelogenous leukemia. In addition, protein-toxin conjugates with a truncated *Pseudomonas* endotoxin genetically linked to a CD25- or CD22-specific antibody have induced remissions in patients with hairy cell leukemia[158,159].

Oncotoxins are immunogenic and manifest toxicity to normal tissues, and thus provide only a narrow therapeutic window before the development of antitoxin antibodies. Radiolabeled monoclonal antibodies have been developed as alternative cytotoxic immunoconjugates[160-166].

CD20, the major target used in the therapy of B-cell leukemia and lymphomas, has an expression limited to B cells. The unmodified antibody rituximab alone or armed with 90Y (ibritumomab tiuxetan; Zevalin) has been approved for the treatment of non-Hodgkin lymphoma[160]. The IL-2R subunit (CD25), identified by the anti-Tac monoclonal antibody, has also been used as a target for the treatment of T-cell leukemias and lymphomas[161]. The scientific basis for this choice is that IL-2R is not expressed by most resting cells, whereas it is expressed by the abnormal cells in certain forms of lymphoid neoplasia.

An important issue in designing an optimal radioimmunotherapeutic reagent is the choice of the method used to deliver the radionuclide to the tumor cell. Most clinical trials use intact monoclonal antibodies to deliver the radionuclide. Although this approach has provided meaningful efficacy, only modest tumor-to-normal-tissue radionuclide ratios are achieved. In addition, the long serum half-life of intact monoclonal antibodies prolongs radiation exposure to normal organs, which limits the radiation dose that can be safely administered. To circumvent these obstacles, various approaches, including pre-targeting strategies that separate the antibody targeting from the delivery of the radionuclide, have been developed.

In recent efforts, streptavidin has been initially targeted to the IL-2R receptor selectively expressed on the tumor-cell surface using an anti-Tac (CD25) single chain Fv-Streptavidin-fusion protein (scFvSA)[166]. This has been followed by administration of chelated biotin armed with a radionuclide.

Using this pre-targeting approach, large quantities of radioactivity were delivered to the tumor with a dramatic increase in both the tumor-to-normal-tissue ratio of radioactivity delivered and the efficacy achieved. Another component of an optimal radioimmunotherapeutic regimen to consider is the nature of the radionuclide used. Most published clinical studies used the ß-emitting radionuclides 90Y or 131I. Such ß-emitting radionuclides depend on crossfire for their action on large tumor masses. However, as the tumor mass decreases, the benefit of the crossfire effect also decreases. With various small tumors including leukemias, the therapeutic effect of high-energy ß-emitting radionuclides is limited because they yield a high dose of irradiation outside of the tumor volume as a result of the long path of the ß-irradiation. For such forms of malignancy, the development of pre-targeting approaches may focus on emitting radionuclides that could be the most effective agents for killing tumor cells without damaging adjacent normal tissue.

Active cancer immunotherapy

Cancer vaccination is the administration of tumor antigens, either in the form of inactivated tumor cells or tumor cell lysate from which the tumor antigens are taken up by antigen presenting cells (APCs) and traffic to lymphoid tissues to stimulate $CD8^+$ CTLs or $CD4^+$ helper (Th) cells of the immune system. With the idenfication of specific tumor antigens, vaccinations are more often carried out through dendritic cells (DCs) loaded with the relevant protein or peptide or DCs transfected with vector DNA or RNA. Each of these strategies will produce particular effects on the immune system (Figure 5).

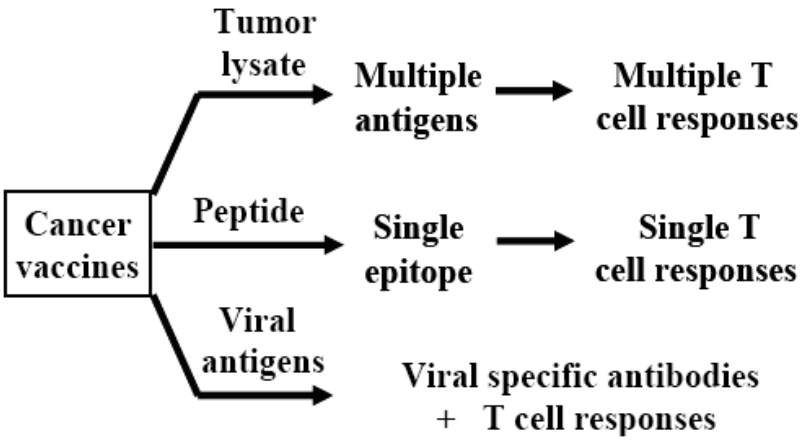

Figure 5. Cancer vaccine strategies. Different strategies for vaccination, as illustrated in the diagram, will produce different effects on the immune system, and as such have different advantages and disadvantages. *Shao-An Xue and H.J. Stauss, Enhancing Immune Responses for Cancer Therapy Cellular & Molecular Immunology. 2007;4(3):173-184.*

T cell recognized tumor antigens can be classed either as tumor-specific antigens (TSAs), where the genes encoding the TSA are found only in tumor cells and not in normal tissues, or tumor-associated antigens (TAAs), where the genes encoding the TAA are overexpressed in tumor cells but nonetheless also present at low levels in normal tissues.

- **Vaccination with tumor-specific antigens**

TSAs represent perhaps the most desirable targets for anti-cancer vaccination or adoptive therapy. Their tumor specific expression precludes any pre-existing immunological self-tolerance as might be found with antigens normally expressed, even at low levels, and thus immune responses directed against TSAs will be unlikely to damage normal tissues. Examples of TSA include the antigens of transforming viruses that cause infected cells to become cancerous, such as the gene products of human papilloma virus (HPV) or Epstein-Barr virus (EBV), and the products of mutated genes expressed only in tumor cells, such as oncogenic RAS and the BCR/ABL fusion protein.

Aside from viral proteins, malignant tissues will also present endogenous tumor-specific epitopes resulting from the mutations and novel protein expressions that contributed to the malignancy in the first place. Such proteins would not otherwise be found, and so T cells recognizing them will not be subjected to normal tolerance mechanisms, and the mutated neo-antigens would, in principle, be ideal targets for T cell based immunotherapy. However, the mutation-specific CTL responses necessary for this strategy to work are very rarely detected in cancer patients, despite tumor cells carrying up to 11,000 mutations[167]. It is possible that these mutated gene products are often invisible to CTL, due to restrictions dictated by rules governing MHC class I presentation.

Very often, peptides with specific mutations will not successfully compete with the large number of peptides derived from normal cellular proteins, and hence end up presented at too low a level for strong activation of CTL and Th cells.

- **Vaccination with tumor-associated antigens**

Given the poor presentation of tumor-specific mutated antigens as CTL targets, it turns out that the majority of peptides implicated in CTL responses in cancer patients are tumor-associated antigens. In order to reduce the risk of detrimental autoimmunity, T cell selection in the thymus and in the periphery will either remove or inactivate CTL able to recognize self-antigens with high avidity. As a consequence, autologous T cells against TAAs are primarily of low avidity. However, the problem then is that these low avidity T cells are also less sensitive to tumor growths expressing the same TAA, and offer poor tumor protection efficacy; for example, the

infusion of high avidity CTL into melanoma patients resulted in better melanoma protection compared with low avidity CTL[168]. Thus, an important goal of T cell based tumor immunotherapy is to produce high avidity responses against TAAs presented by common HLA alleles, leading to effective tumor control, but without triggering autoimmune damage in tissues expressing physiological levels of antigens recognized by CTLs. While prophylactic active vaccination with a range of TAAs has been shown to protect against tumor challenge and prevent tumor occurrence in some animal models[169-172], TAA-based vaccination has been mostly unsuccessful when deployed therapeutically. As discussed above, any high avidity autologous CTL able to respond strongly to such TAA are likely to have been deleted by central tolerance mechanisms. Moreover any high avidity CTL able to escape this may still be under tolerance mechanisms that prevent their rapid activation and expansion, which are both necessary for the inhibition of existing tumors. This may explain why vaccination against murine TSAs has been shown to be more effective than vaccination against TAAs at inhibiting the growth of existing tumors[173-175]. Nonetheless, the majority of antigen-specific vaccination strategies that have entered phase I and II clinical trials, were based on TAA vaccine preparations administered to melanoma patients. While many trials reported detectable vaccine-induced T cell responses, these response rates were low[176,177].

A recent survey of vaccination trials[178] performed on over 600 cancer patients showed an objective clinical response rate of approximately 3%. This encompassed a range of vaccination strategies, including peptides in adjuvant, viral vectors, transfected tumor cells, antigen pulsed dendritic cells and various cytokine combinations. Measurable clinical responses were absent in 97% of patients, much of which may be due to the large pre-existing tumor burdens of patients with advanced disease, as well as the development of tumor escape variants and generally low immune competence of the patients. Since the vast majority of vaccines were directed against self-antigens, it is also likely that tolerance interfered with effective immunity and that induced T cell responses were primarily of low avidity.

- **Enhancement of T cell activation: cytokines in cancer active immunotherapy:**

Active immunotherapy with cytokines represents a form of nonspecific active immune stimulation. In contrast to other biological molecules (hormones), there has been a less widespread use of the cytokines normally generated by the immune system for cell-to-cell communication. This may reflect the fact that cytokines are normally dedicated to act in a very localized microenvironment as autocrine or paracrine factors at the site of an immunological synapse. Nevertheless, there are a number of such agents that have been approved for clinical use in immunotherapy.

IFN-γ is used in osteopetrosis and chronic granulomatous disease, and IFN-ß preparations are approved for multiple sclerosis. IFN-α is used in the treatment of hairy cell leukemia, malignant melanoma, follicular lymphoma, AIDS-related Kaposi sarcoma, and hepatitis B and C.

Another application of T-cell co-stimulatory cytokines involves incorporation of their genes into viral vaccines. GM-CSF, which acts on dendritic cells, provides the broadest range of T-cell responses, including Th1, Th2 and CTL[179]. Furthermore, incorporation of the gene encoding IL-12 into DNA vaccines yielded antigen-specific responses that were predominantly Th1, whereas inclusion of IL-4 or IL-10 induced a Th2 response[179,182]. IL-2 has received approval from the US Food and Drug Administration (FDA) for use in the treatment of metastatic renal cancer and malignant melanoma, where it induced a durable complete response in 5-10% of patients[180,181]. There are, however, limitations in the use of IL-2. In terms of the immune response, in addition to its role in the initial activation of T and NK cells, IL-2 has a critical role in the maintenance of peripheral tolerance[183]. In terms of this unique function, IL-2 has a central role in activation-induced cell death (AICD), a process that leads to the elimination of self-reactive T cells[183]. As a result of this pivotal role in AICD, the T cells generated in response to tumor vaccines containing IL-2 may interpret the tumor cells as self and the tumor-reactive T cells may be killed by AICD-induced apoptosis. Furthermore, IL-2 maintains $CD4^+$ $CD25^+$ negative regulatory T cells and has been reported to terminate CD8+ memory T-cell persistence[184]. In parallel with IL-2, IL-15 is very effective in the activation of T, NK and NK-T cells[185]. In contrast to IL-2, IL-15 manifests anti-apoptotic actions, inhibits IL-2-mediated AICD and stimulates the persistence of $CD8^+$ memory cells[185,186]. In

light of these valuable characteristics, IL-15 may be superior to IL-2 in the treatment of cancer and especially as a component of vaccines where a prolonged immune response is desirable[185,186].

- **Dendritic cells – based cancer vaccines**

The discovery of tumor-associated antigens, which are either selectively or preferentially expressed by tumors, together with an improved insight in dendritic cell biology illustrating their key function in the immune system, have provided a rationale to initiate dendritic cell-based cancer immunotherapy trials. Nevertheless, dendritic cell vaccination is in an early stage, as methods for preparing tumor antigen presenting dendritic cells and improving their immunostimulatory function are continuously being optimized.

A variety of methods for generating dendritic cells, loading them with tumor antigens, and administering them to patients have been described. In recent years, a number of early phase clinical trials have been performed and have demonstrated the safety and feasibility of dendritic cell immunotherapies. A number of these trials have generated valuable preliminary data regarding the clinical and immunologic response to DC-based immunotherapy. The emphasis of dendritic cell immunotherapy research is increasingly shifting toward the development of strategies to increase the potency of dendritic cell vaccine preparations.

a. *Ex vivo* generation of DCs for cancer immunotherapy

One approach that is used by tumor immunologists is to generate *ex vivo* a population of antigen loaded DCs that stimulates robust and long-lasting $CD4^+$ and $CD8^+$ T cell responses in the patient with cancer, with the emphasis on “long-lasting”. What seems to be the rate-limiting step at present is the inability to fully recapitulate *ex vivo* the development of immunocompetent DCs, in particular the process of DC activation. In what is undoubtedly an oversimplification, DC activation can be divided into two stages (Figure 6). In the periphery, quiescent (immature) DCs undergo a maturation process in response to inflammatory stimuli originating from pathogens (pathogen-associated molecular patterns [PAMPs]) or from dying cells, collectively referred to as “danger signals” or “danger associated molecular patterns (DAMPs)”[189]. One important consequence of the maturation process is that DCs acquire the capacity to

home to lymph nodes. DCs receiving the appropriate maturation stimuli upregulate expression of CC chemokine receptor 7 (CCR7) and become responsive to CC chemokine ligand 19 (CCL19) and CCL21, chemoattractants produced in the afferent lymphatics and the lymph node. DC migration is also controlled by leukotrienes (such as LTD4 and LTE4), which act downstream of CCR7 signaling[190,191]. When reaching the lymph node, antigen-loaded mature DCs undergo an additional activation step, termed "licensing," in response to various stimuli, notably CD40 ligand (CD40L) which is expressed on cognate $CD4^+$ T cells. In addition to antigen loading, DCs need to be generated *in vitro* such that they undergo optimal maturation but not licensing because full activation of DCs *ex vivo* might be counterproductive, as discussed below. The goal, therefore, is to differentiate antigen-loaded DCs only to the point that they have acquired lymph node migratory capacity and become responsive to licensing stimuli when they reach the lymph node and encounter cognate T cells (Figure 6).

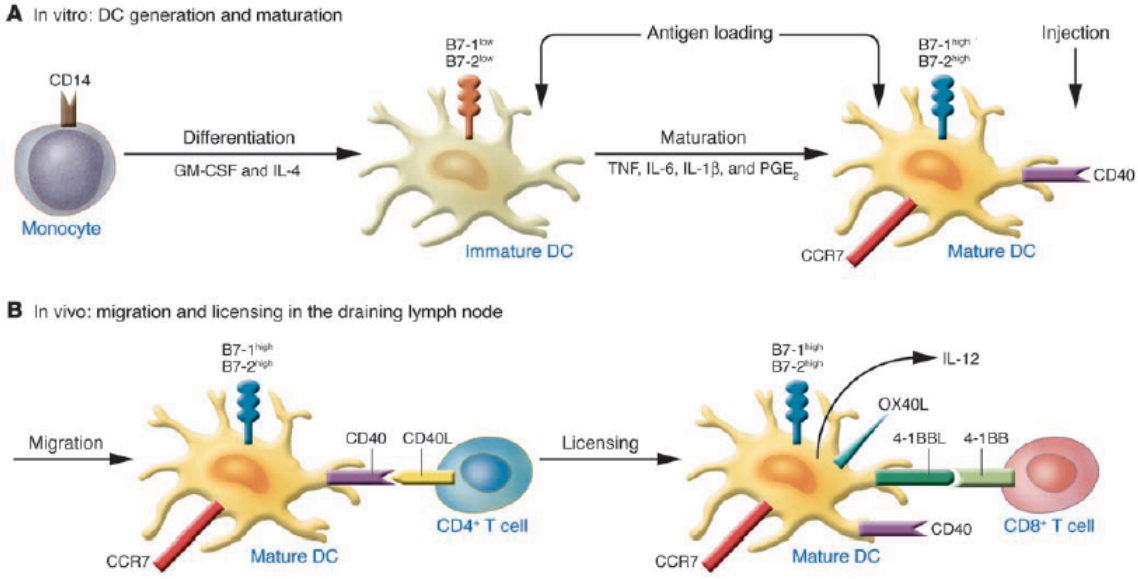

Figure 6
Ex vivo differentiation and activation of DCs for cancer immunotherapy. (**A**) The most common method used to generate DCs for clinical trials is to culture CD14+ monocytes in serum-free media in the presence of GM-CSF and IL-4. Following 5–7 days in culture, the monocytes differentiate into immature DCs, which lose CD14 expression and express moderate to low levels of CD40 and the costimulatory ligands B7-1 and B7-2. DC maturation is accomplished by culturing the immature DCs for an additional 24–48 hours in the presence of several biological agents, the most popular combination being TNF, IL-6, IL-1, and PGE2. Mature DCs further upregulate CD40, B7-1, and B7-2 and induce the de novo expression of the lymph node homing receptor CC chemokine receptor 7 (CCR7). Antigen loading occurs at either the immature or mature DC stage. (**B**) Mature antigen-loaded DCs are injected into patients subcutaneously, intradermally, or intravenously. They migrate to the

draining lymph node, where they encounter and present antigen (not shown) to cognate $CD4^+$ T cells. Cross-linking CD40 on the DCs by CD40L, which is expressed on the antigen-activated $CD4^+$ T cell, induces the mature DCs to differentiate further, a process known as licensing. Licensed DCs upregulate additional cell surface products, notably the ligands for OX40 and 4-1BB (OX40L and 4-1BBL, respectively). The licensed DCs present antigen to cognate $CD8^+$ T cells. 4-1BBL–mediated costimulation through 4-1BB on the antigen-activated $CD8^+$ T cells enhances the survival and proliferative capacity of the activated $CD8^+$ T cells. Likewise, OX40L-mediated costimulation enhances the survival and proliferation of the activated $CD4^+$ T cells (not shown). *Eli Gilboa, The Journal of Clinical Investigation Volume 117 Number 5 May 2007.*

The antigen(s) to be presented by the DCs can be provided in many forms. First, it can be added exogenously, as peptides, whole protein, tumor lysate, or apoptotic debris or complexed with antibody. Second, the DCs can be engineered to synthesize it endogenously by transfection with mRNA or cDNA encoding the antigen. Exogenous provision of short peptides corresponding to the epitopes presented by the MHC class I and MHC class II molecules used to be the favorite form of antigen to load DCs[187,188]. Such peptides are synthesized by chemical means and are readily available for clinical use. However, the logistical advantages of using peptides is offset by the need to determine the MHC haplotype of the patient (that is, the set of MHC alleles an individual expresses, which determines the MHC molecules expressed and is important because different MHC molecules bind and present different peptide repertoires), the paucity of known tumor-specific peptides (especially MHC class II restricted peptides), and the limited persistence of peptide-MHC complexes on DCs. Usually the best chice is the whole antigen because it is likely to contain peptides that can be effectively presented by most MHC molecules[187,188,192,193].

Use of protein-based antigens to load DCs, which allows peptides to be channeled into both the MHC class I and MHC class II presentation pathways, is limited by access to clinical grade reagents. A “marriage made in heaven” that lies somewhere between the peptide and protein approach is to use overlapping long (20–25 aa) peptides covering most, but not necessarily all, of the coding sequence of the tumor antigen.

This approach should provide both MHC class I and MHC class II epitopes, does not require knowledge of an individual’s MHC haplotype, and seems to be highly effective[194].

The use of antigen encoded by nucleic acid, either cDNA or mRNA, is attractive because their isolation and use in clinical settings is more straightforward than the use

of exogenously provided peptides and proteins. However, transfection of DCs with cDNA encoding antigen has not proven effective for loading DCs with the antigen[195]. By contrast, transfection of DCs with mRNA that encodes antigen has turned out to be an efficient method of loading DCs with antigen; in mice, vaccination with mRNA-transfected DCs stimulated robust CTL responses and antitumor immunity, and in phase I/II prostate cancer and renal cancer clinical trials, vaccination with mRNA-transfected DCs induced tumor antigen– specific $CD8^+$ T cell responses in the majority of patients[193]. One main drawback of transfection as an approach to expressing tumor-specific antigens in DCs is that antigen is channeled primarily into the MHC class I presentation pathway, limiting the generation of effective $CD4^+$ T cell responses[196].

Multiple clinical trials have been carried out to date targeting different cancers using different methods of generating DCs, different antigens, and different antigen-loading techniques[197]. At this early stage of clinical development, no indication or evidence has been obtained that DC vaccines represent a method of stimulating protective immunity in cancer patients that is superior to other vaccination strategies. In most studies, a fraction of patients, often half or less, exhibited immune responses against the vaccinating antigen[198]. Despite occasional correlations between immunological and clinical responses in such single-arm clinical trials[199], it is not known whether the modest clinical responses were caused by the vaccination or whether they reflect patients with better prognoses capable of mounting immune responses.

See table “Methods and antigens used for antigen loading of dendritic cells” in the next page.

Methods and antigens used for antigen loading of dendritic cells

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method	Antigen (peptide/protein)	reference
	Peptides/proteins	
Peptide pulse	PSCA and PSA	200
	MUC1	201
	hAFP	202
	PSCA, PAP, PSMA, PSA	203
	PSA, PSMA, survivin, prostein, trp-p8	204
Protein pulse	CEA	205
	PAP	206
	MUC1 (peptide and protein)	207
	RNA	
mRNA	Autologous tumor	208
	Allogeneic cells	209
	Cell lines	210
Tumor RNA		211
RNA	*In vitro* transcribed	212
	Tumor	
Tumor lysate	Allogeneic medullary thyroid carcinoma	213
	melanoma	214
	CT-26 cells	215
	MCF-7, MDA-MB-231	216
Apoptotic tumor cells	NH-1 (B-ALL)	217
	Complexes	
HSP-Ag complex	Hepatocellular carcinoma cells	218
Mannan -Ag	MUC1	219
	Virus	
adenovirus	HCV NS3	220
	αFP	221
	CEA	222
lentivirus	Sca-2, GP38, RABP1	223
	Fusion	
Tumor DC fusion	Allogeneic COLM-6 colon cancer cells	224
	Multiple-myeloma cells	225

b. *In vivo* delivery of tumor antigens to DCs

The past decade has seen a dramatic increase in the number of studies aimed at "vaccinating" against existing disease in patients, particularly cancer. Numerous tumor-associated antigens have been identified and expressed as recombinant proteins. Further, with the help of T cell epitope prediction algorithms and experimental observations, cytotoxic T cell and helper T cell epitopes have been identified for tumor antigens[226,227]. These recombinant proteins and synthetic peptides have been utilized with different adjuvants and delivery systems in preclinical studies and some in clinical studies. While numerous adjuvants have been utilized in clinical trials, Alum remains the only adjuvant licensed for use in human vaccines. Despite concerted efforts, the efficacy of clinical trials using vaccination to treat existing cancers has at best been modest. As with the more insidious or complex pathogens that have thus far eluded development of successful preventative vaccines, targeting tumor antigens directly to APCs may represent the next step forward in the development of more potent anticancer treatments.

An ideal vaccine should prime naive $CD8^+$ as well as $CD4^+$ T cells. The CD4 T helper response is important in initiating and maintaining long-term immune responses[228,229]. Dendritic cells (DCs) are key players in the immune system in that they link the innate immune response to the adaptive immune response[230]. Current dendritic cell-based vaccines use autologous DCs that are harvested from patients and then loaded *ex vivo* with antigen prior to re-administration to patients. While various strategies have been utilized to load antigen into DCs (see: *ex vivo* generation of DCs for cancer immunotherapy), by necessity all have been highly laborious. Targeting DCs *in vivo* via specific surface receptors represents a more direct and less laborious strategy and has been the subject of considerable recent investigation. A multitude of receptors have been investigated, including mannose receptor (MR), DC-SIGN, scavenger receptor (SR), DEC-205, and Toll-like receptors.

Possible targets for delivery of tumor antigens to DCs are represented by:

(1) Mannose Receptor. The mannose receptor (MR) is primarily present on DCs and macrophages, which recognize carbohydrates (mannose, fucose, glucose, GlcNAc, and maltose) on the cell walls of infectious agents (bacteria and yeast). Once binding occurs, aggregation and receptor-mediated endocytosis and phagocytosis take place.

The MR is part of the multilectin receptor family and provides a link between innate and adaptive immunity[231]. It has been demonstrated that mannan conjugated to the tumor-associated antigen MUC1 can induce strong T1 or T2- type immune responses, depending on the mode of conjugation.

In contrast to mouse studies, oxidized mannan MUC1 fusion protein induced predominantly humoral immune responses in human clinical trials. Rather than utilizing a carbohydrate-linked antigen to target the MR, a recombinant human anti-MR/tumor antigen has been used[232]. The pmel17 melanoma-associated antigen was fused to the heavy chain to produce a fully human protein, B11-pmel17. DCs treated with B11-pmel17 presented pmel17 in the context of class I and class II molecules.

CTL generated via B11-pmel17-pulsed DCs has multiple restricting elements but nevertheless only lysed HLAmatched targets.

(2) DC-SIGN. The most recently identified member of the MR family, the C-type lectin DC-SIGN, is expressed in large amounts on immature DCs, DC-SIGN, is transported by DCs into lymphoid tissues, and consequently facilitates HIV-1 infection of target $CD4^+$ T cells[233,234]. The humanized antibody hD1V1G2/G4 (hD1), directed against DC-SIGN, was recently cross-linked to KLH.38 This chimeric antibodyprotein complex (hD1-KLH) bound specifically to DCSIGN and was rapidly internalized and translocated to the lysosomal compartment of DCs.

(3) DEC-205. DEC-205 is an integral membrane protein homologous to the macrophage mannose receptor, which binds carbohydrates and mediates endocytosis[235]. Following ligand binding, DEC-205 is rapidly internalized by means of coated pits and vesicles and is delivered to multivesicular endosomal compartments that resemble the MHC class I containing vesicles implicated in antigen presentation. Extensive investigations into the molecular mechanisms of DEC-205-mediated antigen uptake and processing have now been conducted, which collectively suggest that this molecule represents a promising target for facilitation of delivery of antigens to APCs.

Despite preliminary data suggesting that DNA vaccines might provide a robust and cheap means of vaccination against a multitude of diseases, more than two decades after their initial development no human DNA vaccine has yet proved to be sufficiently effective to warrant commercialization. Strategies aimed at increasing the "general" immunogenicity of DNA vaccines such as incorporation of cytokine DNA

have led to greater immunogenicity in experimental contexts but have not achieved the ultimate aim of protective immunity. Recently, successful DEC-205 targeting via a DNA vaccine has provided evidence that targeting to relevant APCs may substantially enhance their immunogenicity and could represent the next avenue of investigation toward achieving protective immunity against disease via administration of DNA constructs in humans. Moreover, DNA encoding a single-chain antibody fragment (scFv) from NLDC-145, a monoclonal antibody against murine DEC- 205, fused with a model protein stimulated both humoral and cellular responses *in vivo*.

(4) Dendritic Cell-Associated C-Type Lectin-1 (Dectin- 1) and Lectin-2 (Dectin-2). Dectin-1 and -2 are C-type lectin receptors expressed on DCs, macrophages, neutrophils, and monocytes and are receptors for α-glucan-recognizing α1,3- and α1,6-linked glucans on yeast cell walls[236,237]. Dectin-1 can initiate inflammatory responses by the presence of an ITAM in its cytoplasmic tail. Dectins are expressed on CD8R-CD4- DCs and dermal DCs. The ability of Dectin-1 and Dectin-2 to present antigen was studied using OVA conjugated to an anti-Dectin monoclonal antibody[238,239].

Scavenger Receptor. The scavenger receptor (SR) is primarily present on macrophages and can internalize endotoxins, oxidized low-density lipoproteins, and other negatively charged proteins. Maleylated ovalbumin evidently binds to the SR, enhancing its presentation to ovalbumin specific MHC class I-restricted CTLs by macrophages and B cells[240].

Heat shock proteins (HSPs) are molecular chaperones that control folding of biosynthetic proteins. Previous studies have demonstrated that HSPs from tumor cells are capable of generating tumor specific CTL responses *in vivo*[241]. In addition, antigens covalently linked to HSPs or fused as a recombinant construct initiate MHC class I-mediated immune response. The receptor for HSP on DCs has been identified as LOX-1, a member of the scavenger receptor family. HSP did not bind other scavenger receptors, CD36, SRA-1, MARCO or CLA-1. Mice immunized with OVA conjugated to an anti-LOX-1 antibody were protected from a lethal OVA+ tumor challenge, and the therapeutic response was dependent on the presence of $CD8^+$ T cells[242].

Toll-like Receptors. Using specific Toll-like receptor (TLR) ligands which target both DCs and B cells, it has proven to be possible to induce both cellular and humoral

immune responses following immunization. It has been demonstrated that TLR7 agonists directly activate plasmacytoid DCs and TLR8 agonists directly activate myeloid DCs and monocyte-derived DCs.

FIRE and CIRE. F4/80-like receptor FIRE is expressed specifically on $CD8^-$ $CD4^+$ and $CD8^-$ $CD4^-$ DCs and weakly on monocytes and macrophages[243], CIRE is a C-type lectin receptor expressed on $CD8^-$ $CD4^+$ and $CD8^-$ $CD4^-$ DCs with no expression on macrophages or monocytes.

Fc Receptors. Fc receptors (FcR) for immunoglobulins link the humoral immune response and the cellular immune response. They also link the innate immune response to the adaptive immune response by binding to pathogens and immune complexes internalized by APCs for presenting T cell epitopes to activate T cells. There is a different FcR for each class of immunoglobulin: FcRR (IgA), Fc R (IgE), Fc*ç*R (IgG), and FcR/*i*R (IgA and IgM). Previous studies have shown that immune complexes present antigen more efficiently than antigen alone via the Fc*ç*R. Immune complexes consisting of OVA and polyclonal rabbit anti- OVA antibody were presented 10 times more effectively to T cells than noncomplexed OVA *in vivo*. All receptors other than Fc*ç*RIV are capable of uptake of immune complexes and presentation to $CD8^+$ T cells.

Bacterial Toxins. Bacterially derived toxins evidently represent attractive candidates for use as vehicles for the delivery of antigen to APCs. The level of genetic manipulation now possible can eliminate their toxicity without compromising their ligand binding or enzymatic qualities; this allows for targeted delivery of experimentally coupled antigens to APCs and subsequent intracellular processing culminating in the presentation of the antigen to the wider immune system. This avenue of research has primarily focused on induction of $CD8^+$ (cytotoxic) T cell responses via APC targeting of $CD8^+$ epitopes that are subsequently processed in the cytoplasm by proteosomes and presented on MHC class I molecules, which have proven to be protective in animal models. Recently, the bacterial toxins *Bordetella pertussis* adenylate cyclase[244] and modified anthrax toxin lethal factor[245] have led to simultaneous facilitation of both $CD4^+$ and $CD8^+$ T cell responses against artificially coupled secondary antigens.

DC-Binding Peptides. To obtain peptides that specifically bind DCs, a 12-mer phage library was panned successively on human monocytes, T cells, and B cells.

Langerhans-like DCs and unbound phage were used to pan on myeloid DCs.
Three peptides (FYPSYHSTPQRP, AYYKTASLAPAE, and SLSLLTMPGNAS) which specifically bound DCs were selected. These peptides bound distinctly different proteins on the surface of immature DCs and were internalized. DCs pulsed with a recombinant fusion protein between the peptide and the hepatitis C virus nonstructural protein 3 (NS3) activated autologous $CD4^+$ and $CD8^+$ cells from HCV infected individuals. The fusion protein primed naive $CD4^+$ and $CD8^+$ T cells in a NOD-SCID mouse xenotransplanted with PBLs and DCs from HCV subjects. Mice immunized with BMDCs pulsed with the fusion protein generated antigen specific cytokines (IFNR and TNFR) and proliferative responses.

A novel approach to target *in vivo* a specific DC subpopulation

The potent adjuvant effect of DCs capable of priming naïve $CD4^+$ and $CD8^+$ T cell responses has triggered a large number of clinical trials using *ex vivo* DC pulsed with a variety of antigens, taking advantage of this property of DCs. While it has shown promise in some instances and is a valuable research tool, harvesting DCs from potential vaccinees, pulsing them with antigen *ex vivo*, and then reintroducing them into those same recipients does not represent a practical method of preventative vaccination “en masse”. Commercial application of this strategy, should it prove to be effective in the long run, will conceivably be confined to those that already have a potentially terminal disease (such as cancer or HIV) and can afford the (necessarily very expensive) treatment regime. Furthermore, there is still debate regarding the type of human DC to use as well as the dose and route of immunization for optimal therapeutic effects. Because of the variation in mouse DC populations that are used in mouse experiments and human DCs, there is no clear extrapolation between mouse and human studies. Targeting DC populations *in vivo* with various DC specific antigens or with other approaches, may meaningfully expand on these discrepancies. The studies reported above foreshadow approaches that may give rise to clinically relevant antigen specific $CD4^+$ and/or $CD8^+$ T cell responses in humans, that could be utilized as inexpensive alternatives to *ex vivo* pulsing of DCs within large populations. Even with the various improved antigen delivery strategies, it will still be necessary to accommodate features in the novel vaccines that can overcome tumor heterogeneity, prime CD4 and CD8 responses, be suitable for multiple HLA types, and provide a

maturation stimulus. Some of these can be rectified by utilizing whole protein antigens, multiple-protein antigens, or polytope fusion proteins for immunization or potent adjuvants. However, it may also be necessary to conduct the clinical trials in patients with minimal residual disease before immune evasion strategies of the tumor are triggered.

At present, a major role in eliciting T-cell priming is conferred to secondary lymphoid organ (SLO)-resident DCs[246,247]. This suggests that the next generation of DC based vaccines should target DCs *in vivo*, thus exploiting their physiological immunostimulatory capabilities. Indeed, promising results have been obtained in murine tumor models by *in vivo* targeting of SLO-resident DCs with antigen conjugated to DC-specific mAb[248]. Defined subsets of T lymphocytes are known to be able to migrate to SLO[249], and to induce a Th-independent cytolytic T-cell response *in vitro*[250]. Additionally, our research group and others have recently shown that, in clinical settings of allogeneic bone marrow transplantation, genetically modified lymphocytes (GML) can induce a highly specific immune response against the transgene products (i.e. HSV-TK) when injected into immunocompetent patients[251,252]. This evidence represents the experimental observation at the basis of our novel vaccination approach. In murine models we showed that transduced T lymphocytes, delivering antigens to host DCs located in SLO, elicit protective immunity and long-term memory in tumor bearing mice by cross-presentation

RESULTS

Genetically modified T lymphocytes expressing the self/tumor antigen TRP-2 are able to induce tumor-specific immune responses.

Five years ago, C. Bonini and colleagues at S. Raffaele Hospital, obtained evidence that the *in vivo* administration of HSV-TK-transduced allogeneic T lymphocytes, used as donor-lymphocyte-infusions in clinical trials of allogeneic bone marrow transplantation, could specifically prime the recipient immune system in a vaccine-like mode, inducing TK-specific T-cell effectors[252,258]. This unexpected result prompted uour group to investigate whether lymphocytes genetically modified to express a self/tumor antigen (TAA) were, indeed, able to elicit TAA-specific effectors.

To address this issue, it has been used the murine self/tumor antigen TRP2, expressed by normal melanocytes and by the murine melanoma B16F1[274]. Splenocytes from wild type and $\beta 2m^{-/-}$ (MHC class I - deficient) C57Bl/6 (B6) mice were activated *in vitro* and transduced with the TRP-2-CSM retroviral vector, which codes for the TRP-2 tumor antigen and for a cell surface marker (CSM), the biologically inactive form of the low affinity nerve growth factor receptor (ΔLNGFr), for purification and in vivo tracing of the transduced cells[275] (Figure 1).

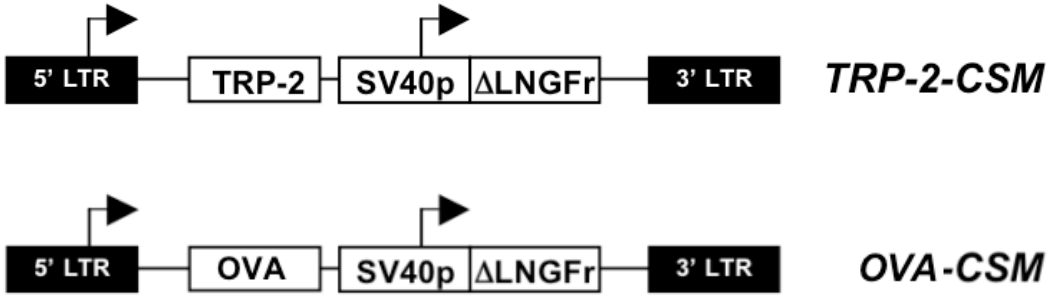

Figure 1 Structure of retroviral vectors TRP-2-CSM and OVA-CSM. The first one encoding for the murine tumor antigen TRP-2 and the ΔLNGFr surface marker and the second one encoding for the chicken ovalbumin protein and the ΔLNGFr surface marker. The 5' Viral Long Terminal Repeat sequencies (LTR), obtained from Moloney murine sarcoma virus (MoMSV), drives the TRP-2 / OVA expression while the internal promoter, obtained form Simian Virus 40 (SV40), drives the cell surface marker ΔLNGFr. Both these constructs have been used for *in vitro* transduction of B6 splenocytes.

Splenocytes obtained from B6 mice are activated and cultured *in vitro* in the presence of Concanavalin A (5 μg/ml) and IL-2 (100 U). In a large series of experiments, these culture conditions allowed to obtain homogeneous populations of $CD3^+$ T lymphocytes, transduced by 69 to 90%, evaluated as $\Delta LNGFr^+$ cells by FACS analysis at day 10 of culture (Figure 2).

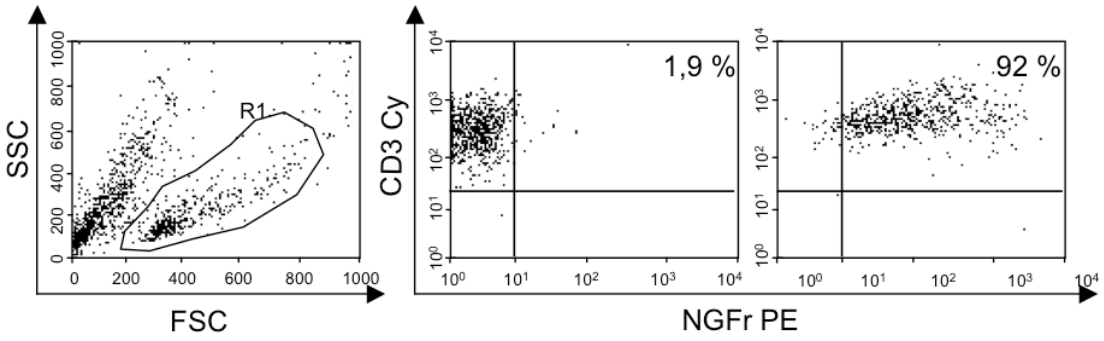

Figure 2 Flow cytometric analysis showimg transduction efficiency of B6 splenocytes measured as $\Delta LNGFr^+$ expression 10 days after colture. Ninety-five percent of the cells (R1 gate, left panel) were $CD3^+$ (middle and right panels). Ninety percent of them expressed ΔLNGFr (right panel), while mock-transduced splenocytes were negative (middle panel).

Before i.v. infusion, the rate of apoptosis in the GML populations has been evaluated. Results showed that 6% (+/- 2.4) of cells sre in early apoptosis (Annexin V^+/PI^-), 15.7% (+/- 0.9) of cells are in late apoptosis/secondary necrosis (Annexin V^+/PI^+) and only 0.45% (+/- 0.5) are in necrosis (Annexin V^-/PI^+). The vast majority of the cells (75% +/- 3.1) were Annexin V^-/PI^- viable cells (data not shown).

Transduced cells ($4x10^6$) were then injected i.v. in B6 mice, 3 times at 2 weeks interval. Two weeks after the last infusion, $5x10^4$ B16F1 melanoma cells were given s.c. in 100 μl of PBS. Mice treated with mock-GML progressively developed melanoma (squares), whereas mice vaccinated with TRP-2-GML from wild type animals (circles) controlled tumor growth as efficiently as mice treated with DCs pulsed with two TRP-2-derived peptides ($VYDFFVWL_{(181–188)}$ and $SVYDFFVWL_{(180–188)}$ diamonds) (Figure 3).

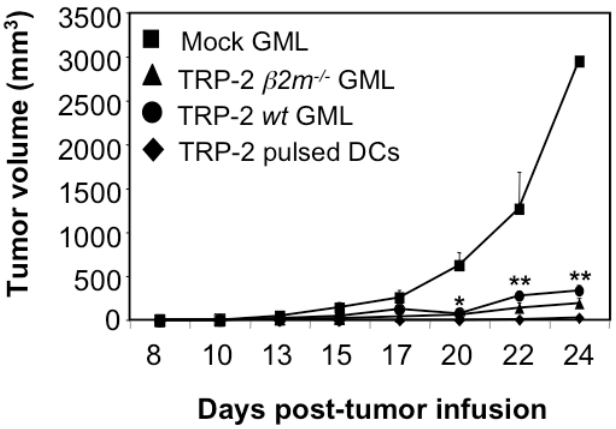

Figure 3 B6 mice were treated with Mock-transduced (squares), TRP-2-wt (circles), TRP-2-β2m$^{-/-}$ (triangles) GML or DCs pulsed with 2 TRP-2-specific peptides (diamonds). Transduced or loaded cells were injected i.v. 3 times at 2 weeks interval, and 2 weeks later $5x10^4$ B16F1 cells were implanted s.c.. TRP-2-GML from both wild type and β2m$^{-/-}$ mice were able to control tumor growth. Data are representative of two independent experiments with five mice/group. Asterisks indicate statistical significant differences (*P =0.03; **P <0.05) using the Student's t test.

The vaccination elicited T-cell effectors specific for the TRP-2 tumor antigen. Indeed, following in vitro stimulation with the TRP-2 $_{(181\text{-}188)}$ (19) and the TRP-2 $_{(180\text{-}188)}$ peptides, splenocytes from mice vaccinated with TRP-2-GML specifically recognized peptides-pulsed RMA cells (Figure 4). No such cytotoxic T cells could be established from mice treated with mock-transduced GML.

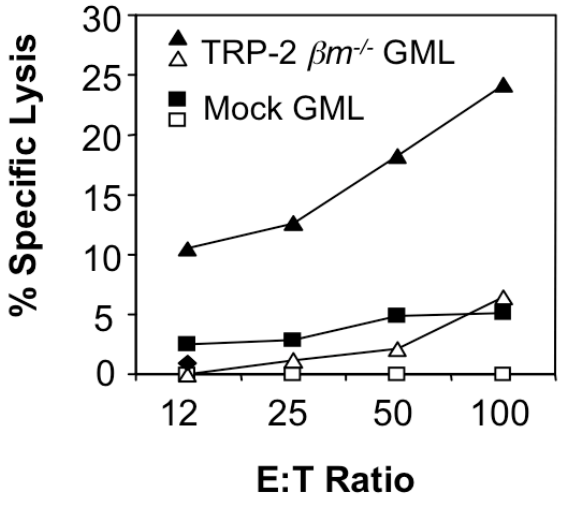

Figure 4 The infusion of TRP-2-GML elicits TRP-2-specific cytotoxic T cells. Splenocytes from animals vaccinated with TRP-2-GML from β2m$^{-/-}$ mice (triangles) or Mock-GML (squares) were restimulated in vitro with TRP-2 peptides and tested in a cytotoxicity assay against pulsed (filled symbols) or unpulsed (empty symbols) RMA cells. Data are means +/- SD of triplicates of the percentage of specific lysis at the indicated E:T ratios.

To determine whether T-cell priming was mediated by endogenous APCs phagocytosing the antigens released by GML, mice were treated with TRP-2-GML from β2m$^{-/-}$ mice (Figure 3, triangles). These animals[276] lack MHC class I expression and therefore are unable to present antigens by themselves. The injection of TRP-2-GML from β2m$^{-/-}$ mice (Figure 3) controlled melanoma growth similarly to the other conditions, indicating that the induction of the TRP-2-specific immune response would require cross-presentation by host APCs.

This vaccination approach elicited a stable memory response, since mice challenged 40 days after TRP-2-GML vaccination with B16F1 tumor cells, were able to control tumor growth (Figure 5).

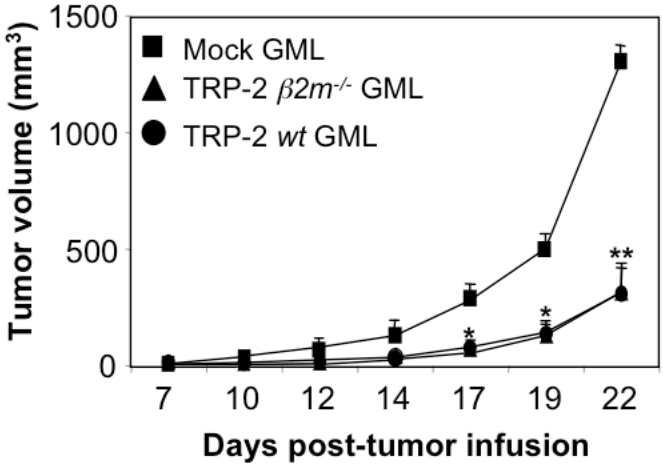

Figure 5 The infusion of TRP-2 GML induces memory antitumor responses. B6 mice were treated as described above. Forty days later, B16F1 tumor cells (5x10^4) were implanted s.c.. TRP-2-GML from both wild type and β2m$^{-/-}$ mice were able to control tumor growth. Data are representative of two independent experiments with five mice/group. Asterisks indicate a significant difference (*P <0.005; **P <0.0005) using the Student's t test.

We then asked whether the infusion of TRP-2-GML was able to effectively control tumor growth in a therapeutic setting. B6 mice bearing 3 days-established B16F1 tumors were injected i.v. 3 times at 7 days intervals with either 4x10^6 TRP-2-GML, or 10x10^6 TRP-2-GML or with 5x10^5 LPS-activated DCs pulsed with two TRP-2-derived peptides (Figure 6).

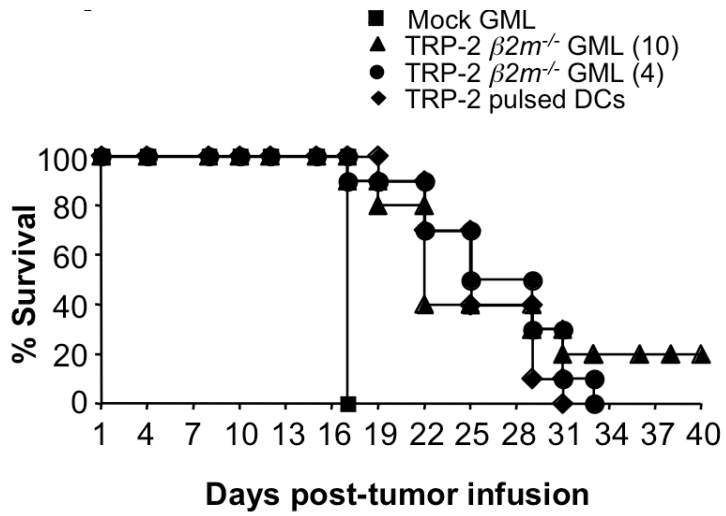

Figure 6 Kaplan-Meier survival graphs showing the results of curative experiments. B6 mice were treated i.v. with $4x10^6$ mock-transduced (squares; n=7), $4x10^6$ TRP-2-GML (circles; n=9), $10x10^6$ TRP-2-GML (triangles; n=9) from $\beta 2m^{-/-}$ mice, or with $5x10^5$ DCs pulsed with two TRP-2-specific peptides (diamonds; n=9), on day 3 after s.c. inoculation of B16F1 tumor cells ($5x10^4$). The treatments were administered 3 times at 7 days interval. Statistical comparison of the survival curves, performed by the log-rank test, gave the following results: $4x10^6$ TRP-2-GML versus mock-GML, $P<0.005$; $10x10^6$ TRP-2-GML versus mock-GML, $P<0.005$; TRP-2 pulsed DCs versus mock-GML, $P<0.005$.

We observed a statistically significant prolonged survival in mice treated with TRP-2-GML and TRP-2 pulsed DCs. Most important, we observed a superior trend of prolonged survival in mice treated with the higher dose of TRP-2-GML in respect of those treated with the optimal dose of peptide-pulsed DCs. Indeed, 20% of TRP-2-GML treated mice were still alive 40 days after tumor infusion.

***In vivo* migratory ability of genetically-modified T lymphocytes.**

Priming of immune effector cells occurs in secondary lymphoid organs (SLO)[277], where professional APCs expressing defined MHC-peptide complexes encounter and activate host naive T-cells[254]. To investigate the mechanism(s) responsible for the observed immunization, we first verified whether GML were indeed, able to reach SLO, upon i.v. infusion. GML expressing the cell surface marker ΔLNGFr were infused i.v. into B6 recipients and recovered from SLO of animals sacrificed at different times. After 24 hours, we detected 6.15% (corresponding to $7.15x10^5$ total cells) of $\Delta LNGFr^+/CD3^+$ GML within the spleen, and 0.61% (corresponding to $7.36x10^4$ total cells) in five pooled lymph nodes (LNs) (Figure 6).

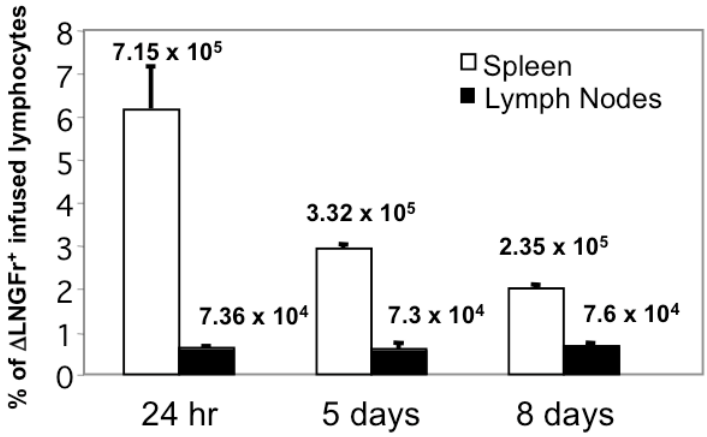

Figure 6 *In vivo* homing and persistence of genetically modified lymphocytes (GML) into secondary lymphoid organs (SLO). CFSE-labeled GML were injected into naïve B6 mice. One, 5 and 8 days after the infusion we evaluated the presence of $CFSE^+$ cells within SLO by FACS. Numbers reported on the columns indicate the absolute amount of $CFSE^+$ cells detected within SLO of treated mice. Data are representative of at least four independent experiments.

Within the spleen, the number of GML slightly decreased over time, thus suggesting that the GML had undergone cell death, a process associated with the release of antigen, and/or re-circulation through peripheral tissues. The SLO-migratory capability seems to be a feature of GML, since we were unable to detect both the lymphoid cell line RMA and the T-cell hybridoma TG40 within SLO of mice injected with comparable or higher number of CSFE-labeled RMA and TG40 tumor cells (data not shown).

To evaluate the fine localization of the GML within SLO, serial sections of spleen and LNs from mice injected with CFSE-labeled GML were analyzed. Twelve hours after the infusion, CFSE-GML were found in the periarteriolar lymphoid sheats of the

spleen and in the paracortical area of the LNs, interspersed with the endogenous $CD3^+$ cells (Figure 7).

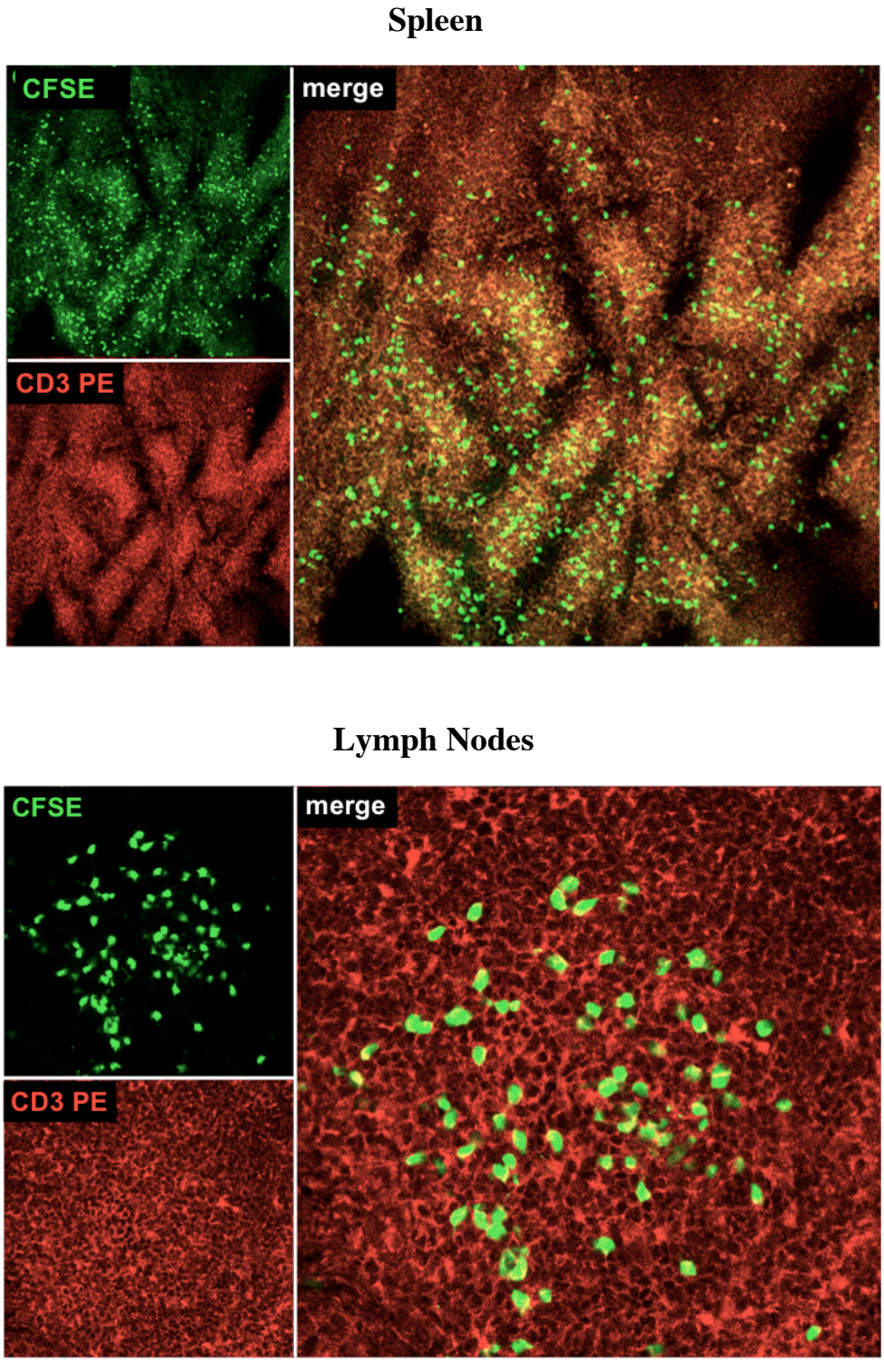

Figure 7 Serial sections of spleen and LNs were analysed by confocal microscopy 12 hours after the injection of CFSE-labeled GML. CFSE labeled cells are visualized as green staining, while CD3 is displayed as red staining. Optically merged confocal images show the co-localization, displayed as yellow staining, of the $CFSE^+$ cells with the CD3 marker in both the spleen and the LNs of treated mice (magnification spleen x20, lymph nodes x40).

These experiments clearly demonstrate that a relevant number of GML is capable of trafficking through secondary lymphoid organs.

Infusion of OVA-GML elicits OVA-specific T-cell priming.

To dissect the mechanism responsible for GML-mediated T-cell priming we used T-cells derived from the OT-I transgenic line, which carry a transgenic TCR (Vα2, Vβ5.1) isolated from a T cell clone reactive with an H-2K^b–ovalbumin octapeptide SIINFEKL (H-2K^b-pOVA$_{257-264}$), and GML transduced with a retroviral vector encoding the model antigen OVA and the cell surface marker ΔLNGFr (Figure 1, OVA-CSM vector).

As for TRP-2, splenocytes from B6 mice were transduced with the OVA-CSM retroviral vector with an efficiency of 55 to 90% (data not shown). We transferred CFSE-labeled, OVA-specific, OT-I CD8$^+$ T-cells into B6 mice and assessed their activation and phenotypic profile 6 days after the infusion of 4x10^6 OVA-transduced or mock-transduced GML from either wild type or β2m$^{-/-}$ mice. In both the experimental settings, FACS analysis of treated mice showed that OT-I T-cells had undergone multiple rounds of cell division, evaluated as CFSE dilution, both in LNs (data not shown) and spleen (Figure 8).

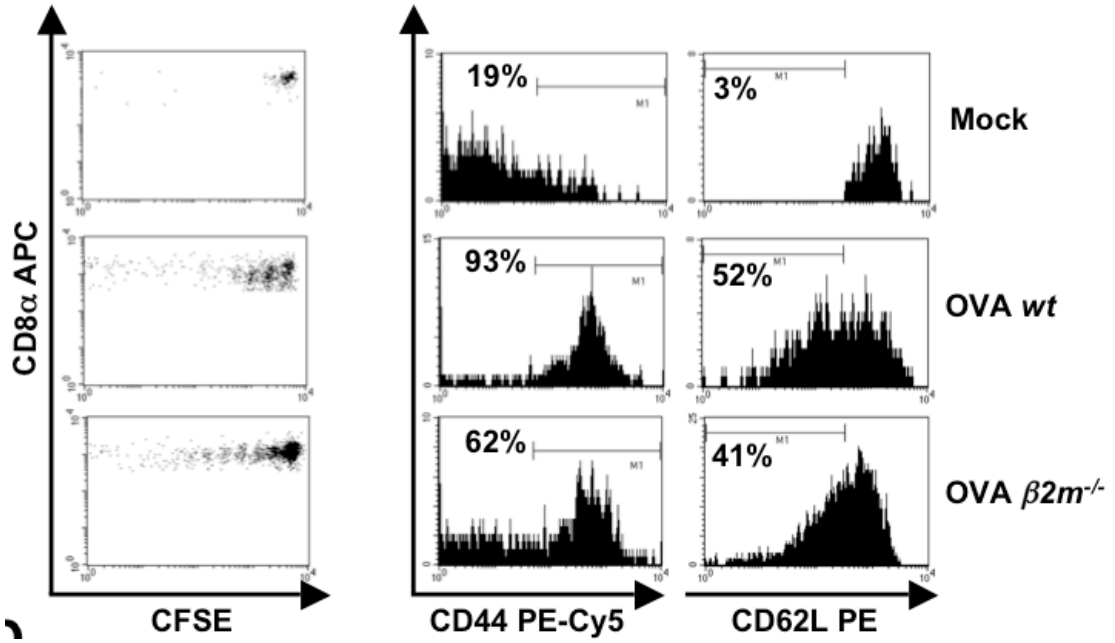

Figure 8 Adoptive transfer of CFSE labeled CD8$^+$ OT-I T-cells into B6 recipients. Twenty-four hours later mice were given mock-transduced (mock) or OVA-transduced GML from wild type (OVA-wt) or β2m$^{-/-}$ (OVA β2m$^{-/-}$) mice. Density dot plots (CFSE, left panels) show OT-I proliferation following the treatment with OVA-wt (left middle panel), OVA β2m$^{-/-}$ (left lower panel) and mock GML (negative controls, left upper panel). Histograms show the expression of CD44 and down-regulation of CD62L. Data are representative of 3 independent experiments, performed on splenocytes of two mice/group.

Furthermore, proliferating OT-I T-cells showed a marked up-regulation of CD44, a marker of T-cell activation that is regularly over-expressed upon antigen recognition[279], and down-modulation of the SLO-homing receptor CD62L (Figure 8), a function that allows activated effector cells to leave the SLO and patrol peripheral tissues[279]. Finally, we demonstrated that the induced effectors, once restimulated *ex vivo* with the $OVA_{257-264}$ peptide (SIINFEKL), specifically released IFN-γ maintaining the OVA-specific responsiveness (Figure 9), without undergoing tolerance or anergy[280].

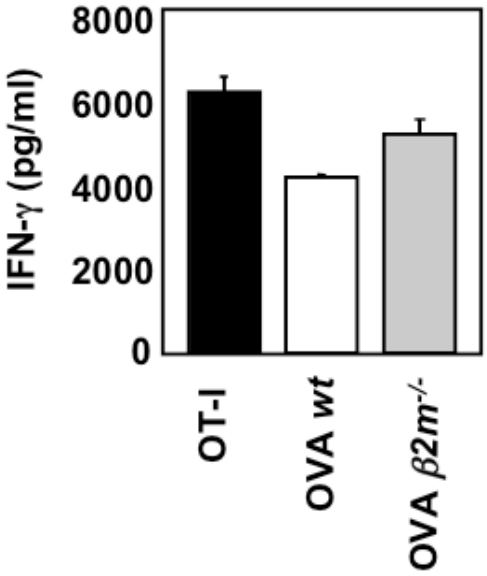

Figure 9 OT-I T cells from treated mice were restimulated in vitro with $OVA_{257-264}$-loaded splenocytes and tested against pulsed or unpulsed RMA cells. IFN-γ released by OT-I T-cells from mice treated with OVA-wt (white) or OVAβ2m$^{-/-}$ (grey) lymphocytes is shown. *In vitro* activated OT-I T cells were used as positive control (black bar).

To evaluate whether our strategy would also elicit sizeable endogenous OVA-specific T-cell effectors, mice were treated with OVA-GML 3 times at 2 weeks interval. Forty days later, spleen and LNs were harvested and stained with H-2K^{b}/SIINFEKL pentamer (Figure 10) and mAbs specific for T-cell activation and memory markers.

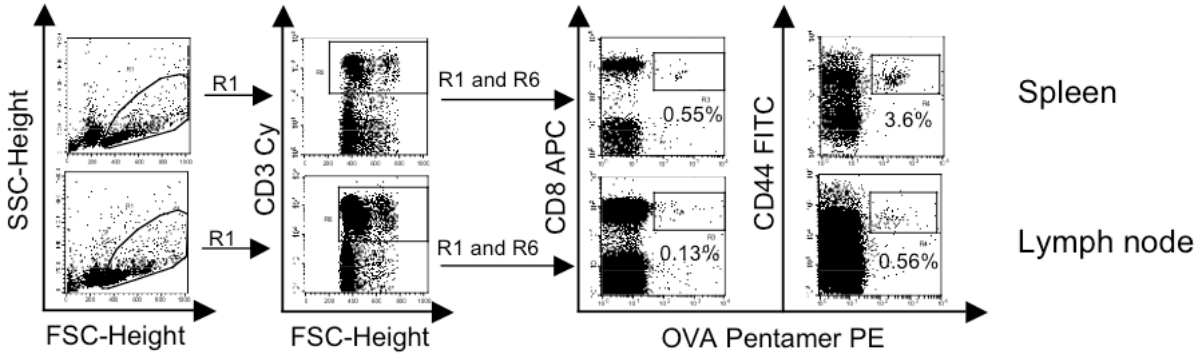

Figure 10 OVA-GML activate endogenous OVA-specific CD8$^+$ T-cells. B6 mice were treated 3 times at 2 weeks intervals with OVA-GML ($4x10^6$). Fifteen or forty days later, cells from spleen and lymph nodes were collected and stained with CD3, CD8, CD44 mAbs and with H2K^b-SIINFEKL OVA Pentamer. Analysis of CD3$^+$ cells for OVA Pentamer and either CD8 or CD44 staining is shown.

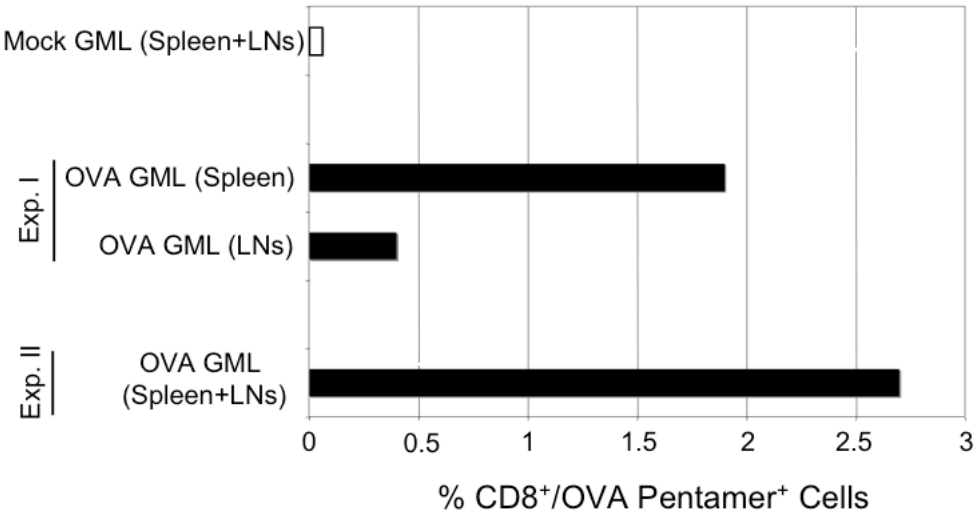

Figure 11 OVA-GML activate endogenous OVA-specific CD8$^+$ T-cells. B6 mice were treated 3 times at 2 weeks intervals with OVA-GML ($4x10^6$). Results of two independent experiments are reported, as percentage of CD8$^+$/OVA Pentamer$^+$ cells.

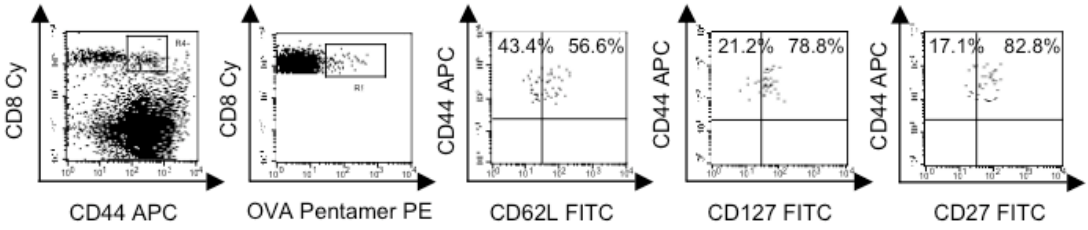

Figure 12 OVA-GML activate endogenous OVA-specific $CD8^+$ T-cells. B6 mice were treated 3 times at 2 weeks intervals with OVA-GML ($4x10^6$). $CD8^+/CD44^+$ cells were analyzed for the expression of OVA Pentamer, CD62L, CD127 and CD27 T-cell markers.

In two representative experiments OVA-GML treatment was able to significantly expand endogenous OVA-specific $CD8^+$ T-cells (Figure 11). In this experimental setting the OVA-specific $CD8^+$ T-cells showed the phenotype of memory cells (Figure 12).

We therefore evaluated whether the vaccination with OVA-GML was able to induce $CD4^+$ T-cell responses. We transferred CFSE-labeled, OT-II $CD4^+$ T-cells (obtained from OT-II transgenic mice carring a TCR specific for the OVA $peptide_{323–339}$ presented in association with MHC class II, $I\text{-}A^b$) into B6 mice and assessed their activation and phenotypic profile 6 days after the infusion of OVA-transduced or mock-transduced GML from either wild type or $\beta 2m^{-/-}$ mice (Figure 13 and data not shown).

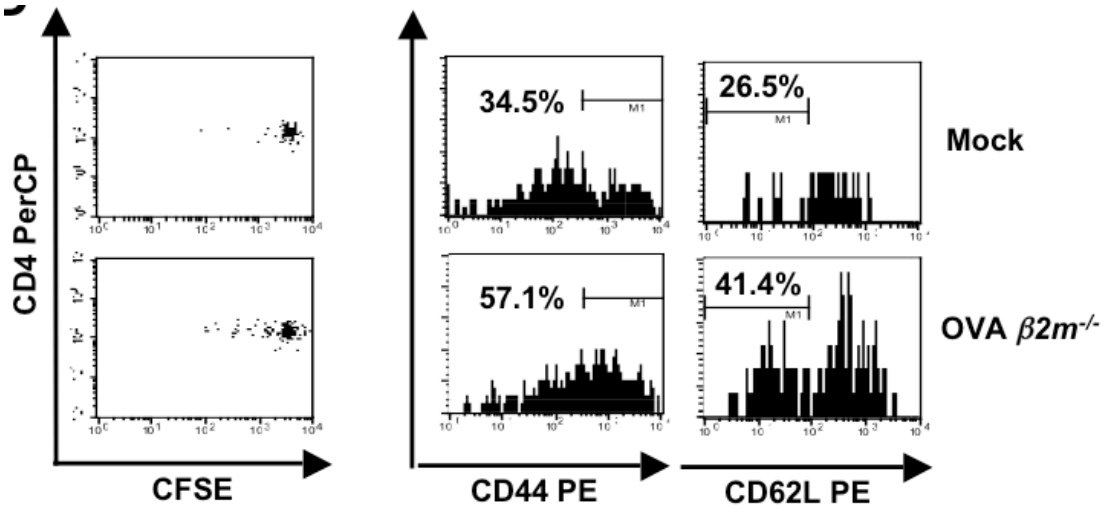

Figure 13 CFSE labeled CD4 OT-II T-cells were transferred into B6 recipients. Twenty-four hours later mice were given mock-transduced (mock) or OVA-transduced GML from $\beta 2m^{-/-}$ (OVA $\beta 2m^{-/-}$) mice. Density dot plots (CFSE, left panels) show OT-II proliferation following the treatment with OVA $\beta 2m^{-/-}$ (left panel). Histograms show the expression of CD44 and down-regulation of CD62L. Data are representative of 2 experiments, performed on splenocytes of two mice/group.

In both the experimental settings, FACS analysis of treated mice showed that OT-II T-cells had undergone cell division, up-regulation of CD44 and down-modulation of CD62L (Figure 13). However, proliferation was less intense than that observed with OT-I $CD8^+$ T-cells. To further confirm that the vaccination with OVA-GML endowed naive $CD8^+$ OT-I T cells with full effector functions capable of controlling tumor growth in vivo, we performed tumor challenge experiments in mice adoptively transferred with OT-I cells and vaccinated with GML. Forty-eight hours after the injection of $5x10^4$ B16-OVA melanoma cells, mice were adoptively transferred with OT-I T-cells and 24 hours later treated with OVA-GML. OT-I T-cells do not spontaneously recognize the tumor; consequently, untreated animals failed to control its growth (data not shown). Only the administration of OVA-GML was able to prime *in vivo* the naive $CD8^+$ OT-I T-cells, enabling mice to successfully control tumor growth (Figure 14).

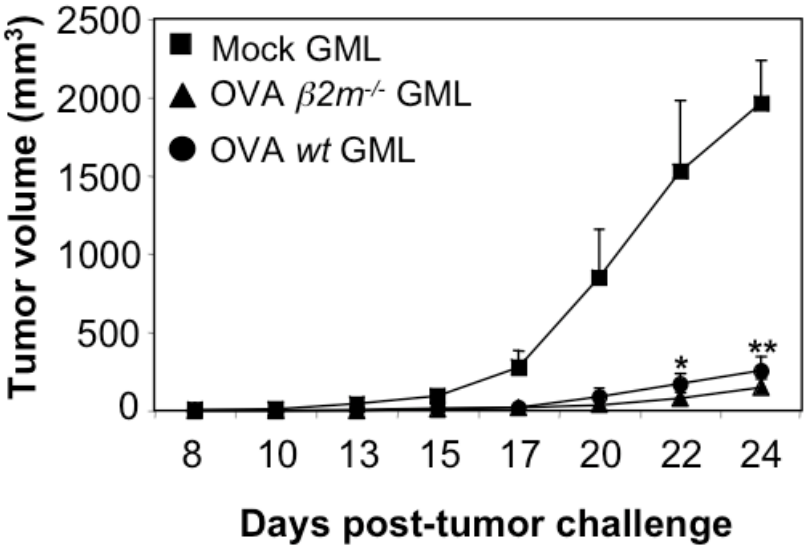

Figure 14 Forty-eight hours after B16-OVA cells injection, mice were transferred i.v. with purified OT-I T-cells. After 24 hours mice were treated with mock-transduced (square) or OVA-transduced GML from wild type (circle) or $\beta 2m^{-/-}$ (triangle) mice. Data are representative of three experiments with five mice/group. Asterisks indicate statistical significant differences (*P <0.05; **P <0.005) using the Student's t test.

The involvement of the above-described cross-presentation mechanism was also confirmed in this experimental setting by the administration of OVA-GML from $\beta 2m^{-/-}$ that resulted in a comparable control of tumor growth (Figure 14).

As expected, mock-transduced GML were ineffective. These results were further confirmed challenging mice 40 days after OVA-GML vaccination (Figure 15).

thus demonstrating that the treatment is able to elicit protective immunity and long term memory in tumor bearing mice.

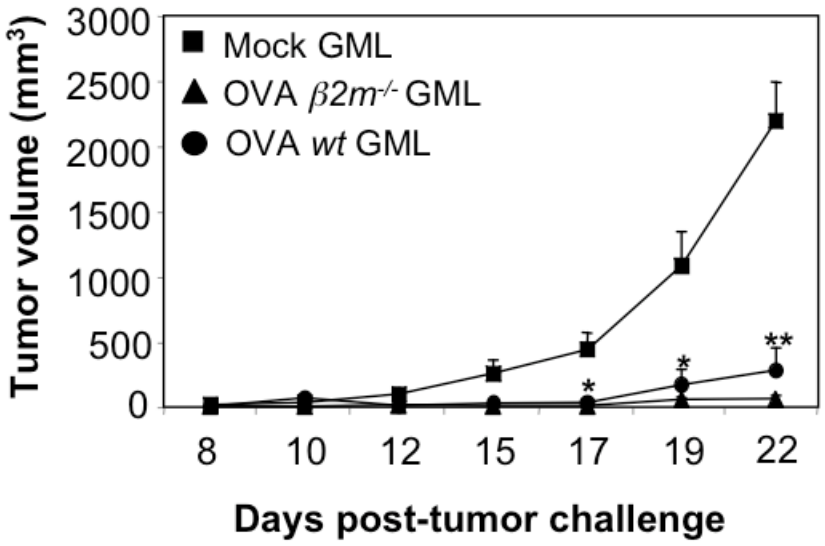

Figure 15 B6 mice were adoptively i.v. transferred with $3x10^6$ purified OT-I T-cells. Twenty-four hours later, mice were treated with $4x10^6$ mock-transduced or OVA-transduced GML from wt and $\beta 2m^{-/-}$ mice. Forty days later $5x10^4$ B16-OVA tumor cells were implanted s.c. and mice were followed up for tumor growth. Data are representative of two independent experiments with five mice/group. Asterisks indicate statistical significant differences (*P <0.005; **P <0.0005) using the Student's t test.

APCs responsible for *in vivo* T-cell priming are bona fide DCs.

To prove that DCs were playing a central role in eliciting T-cell priming, we took advantage of CD11c-DTR transgenic mice. $CD11c^+$ DCs from these mice express the diphtheria toxin receptor and therefore they can be conditionally deleted by administering diphtheria toxin (DT)[281]. CD11c-DTR mice were injected with $1.5x10^6$ CFSE-labeled OT-I $CD8^+$ T-cells. Eight hours later mice were treated intraperitoneally with 4 ng/g body weight DT and the day after they were given $10x10^6$ OVA-GML. Spleen and lymph nodes were harvested 72 hours after GML treatment and analyzed by FACS to evaluate OT-I proliferation and the presence of $CD11c^+$ cells (Figure 16).

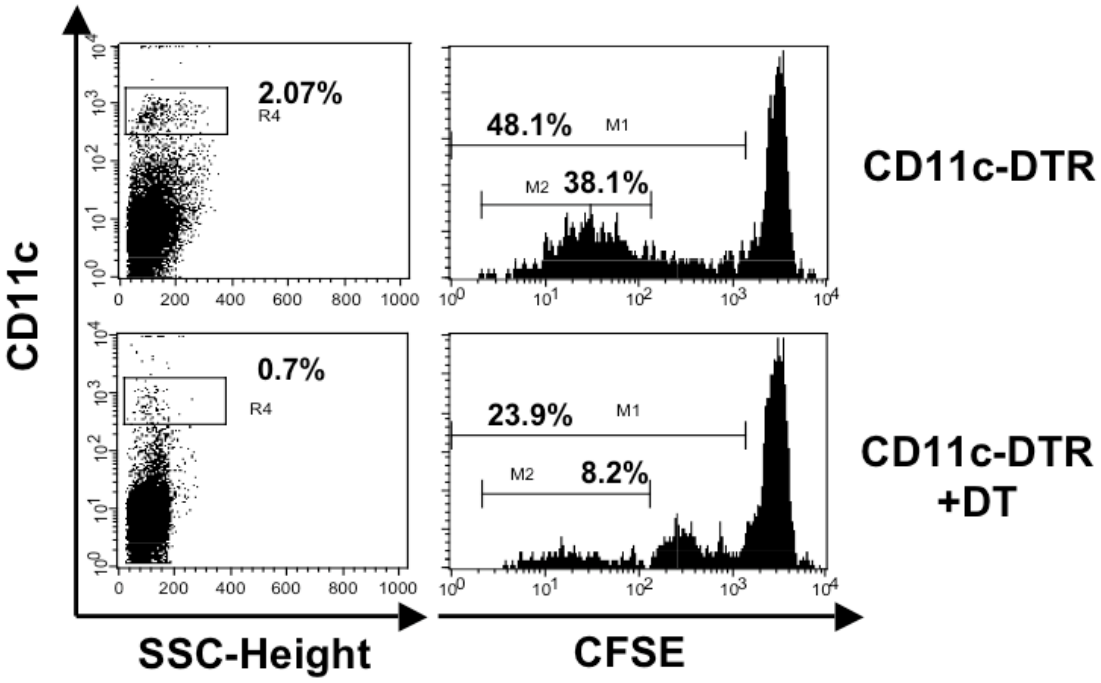

Figure 16 Analysis of OT-I proliferation in DC-depleted mice and characterization of phagocytosing DCs. Purified OT-I T-cells were labeled with CFSE and infused into CD11c-DTR mice left untreated or treated i.p. with 4 ng/g body weight DT. The day after, mice were given i.v. with $10x10^6$ OVA-GML from $\beta 2m^{-/-}$ mice. Seventy-two hours later, spleen and lymph nodes were harvested and analyzed for OT-I proliferation and for the percentage of $CD11c^+$ DCs present in secondary lymphoid organs. Histograms (CFSE dilution on CD45.1 gated OT-I T-cells) show sustained OT-I proliferation in untreated CD11c-DTR transgenic mice (48.1%; upper right panel) compared to that observed in DC-depleted mice (23.9%; lower right panel). The fraction of actively proliferating OT-I cells was remarkably different between the 2 groups of mice (38.1% vs 8.2%). Accordingly, we found 2.07% $CD11c^+$ DCs in untreated CD11c-DTR transgenic mice and only 0.7% in the DC-depleted mice (left panels). Data are representative of 2 experiments, performed on splenocytes of two mice/group.

In untreated CD11c-DTR mice, we observed 38.1% of OT-I cells actively proliferating, whereas only 8.2% of OT-I cells showed some proliferation in CD11c-DTR mice treated with DT. The OT-I proliferation strongly correlated with the percentage of $CD11c^+$ cells present in secondary lymphoid organs of DT-treated (0.7%) and untreated (2.07%) mice (Figure 16, left panels).

These results demonstrate that in our vaccination settings priming of Ag-specific T-cells is mediated by host DCs. We then characterized the subset of SLO-resident APCs responsible for crosspresentation of GML-derived antigens. Twelve hours after the infusion of CFSE-labeled OVA-GML, approximately 20% of the $CD11c^+$ $CD8\alpha^+$ DC subset in the spleen and LNs of treated mice expressed the CFSE dye (Figure 17), with the percentage of $CD11c^+$ $CD8\alpha^+$ $CFSE^+$ cells decreasing after 36 and 60 hours (data not shown).

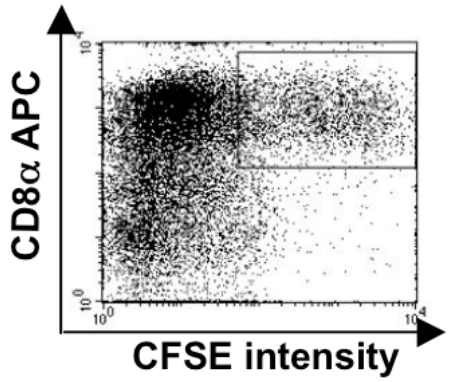

Figure 17 CD11c-purified DCs from mice treated with CFSE-labeled OVA $\beta 2m^{-/-}$ GML, were analyzed by FACS for CFSE uptake and CD8α expression. Data are representative of three independent experiments.

In contrast, only a minority (3-5%) of the $CD11c^+$ $CD8\alpha^-$ population was taking-up CFSE-labeled OVA-GML (Figure 17). The predominant role of the $CD8\alpha^+$ subset of $CD11c^+$ DCs in the phagocytosis of cell-associated antigen[282] was confirmed by confocal microscopy analysis of serial sections of SLO, where we frequently observed the co-localization of CFSE with $CD11c^+$ $CD8\alpha^+$ DCs (Figure 18).

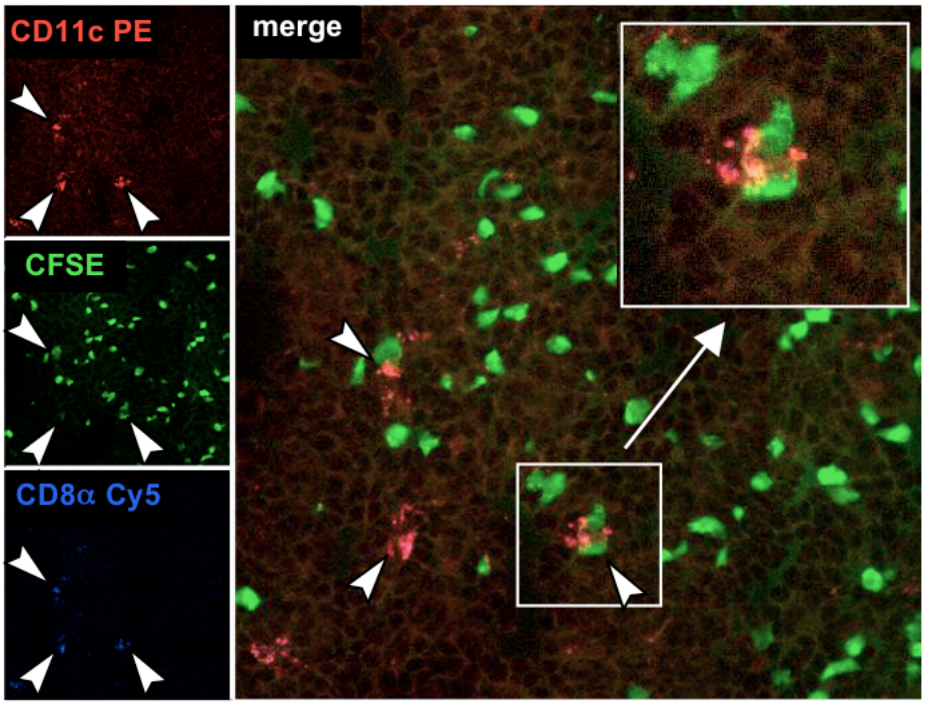

Figure 18 Confocal microscopy analysis of spleen sections. CFSE-labeled GML are displayed as green staining. CD11c and CD8α molecules (DCs) are visualized with CD11c-PE and CD8 α-Cy5 mAbs and displayed as red and blue staining, respectively. Arrows indicate CFSE-labeled GML engulfed by $CD11c^+$ $CD8\alpha^+$ DCs, x40, (shown at higher magnification in the inset).

To investigate whether antigenic material phagocytosed by DCs came from dying GML, we performed TUNEL assay on SLO collected at different time points (6, 12 and 24 hours) upon the infusion of CFSE-labeled GML. A relevant rate of apoptotic GML ($CFSE^+$ $TUNEL^+$) was detected during the entire time of follow up (3.65% at 6 hours, 2.2 and 4.4% at 12 and 24 hours, respectively), whereas only few resident lymphocytes showed picture of apoptosis (1.25% of CFSE-$TUNEL^+$ cells), suggesting that GML, once reached SLO had undergone apoptosis (Figure 19, upper panel).

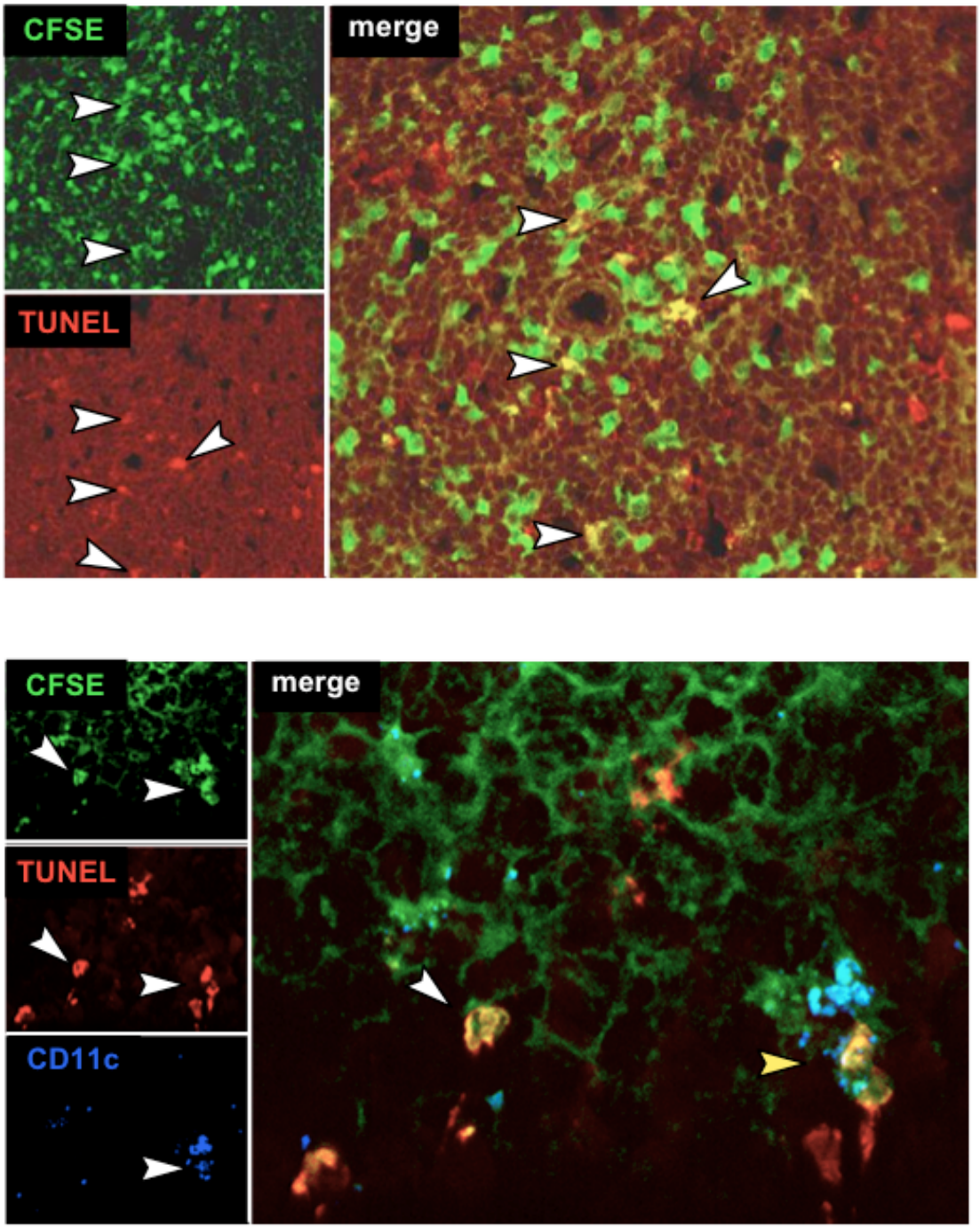

Figure 19 TUNEL$^+$ CFSE$^+$ apoptotic GML (red and green staining, respectively) are detected in SLO of treated mice (upper panel, white head arrows) and displayed as yellow staining in the optically merged confocal image (magnification x40, merge). Lower panels show apoptotic CFSE-labeled GML phagocytosed by SLO-resident CD11c$^+$ DCs (blue staining). The white head arrow indicates TUNEL$^+$ CFSE$^+$ GML, whereas the yellow head arrow shows a dendritic cell phagocytosing apoptotic bodies (triple staining; magnification x63, merge).

Moreover, triple staining for DCs (with anti-CD11c mAb), GML (CFSE) and apoptotic cells (TUNEL) revealed the presence of apoptotic bodies engulfed by DCs (Figure 19, lower panel). Those phagocytosing DCs ($CD11c^+$ $CD8\alpha^+$ $CFSE^+$) belong to a well-characterized subset, which express CD205 (DEC-205), intermediate levels of CD11b, no CD4 (data not shown) and high levels of MHC-II (MFI=8300)[49,283].

Phagocytosing DCs undergo activation *in vivo* and *in vitro* by a cell-to-cell contact mechanism independent of CD40 triggering.

To determine the activation status of SLO-resident DCs, we performed a FACS analysis of phagocytosing ($CFSE^+$) and nonphagocytosing ($CFSE^-$) $CD11c^+$ $CD8\alpha^+$ DCs, collected at different time points. The analysis demonstrated that phagocytosing DCs belonged to the $CD11c^+$ $CD8\alpha^+$ $MHCII^{high}$ subset and showed an activated phenotype, as confirmed by late (72 hours after GML infusion) up-regulation of the costimulatory molecules B7.1 (CD80, 72 hr versus 0 hr; $p<0.05$) and at lesser extent CD40 (Figure 20).

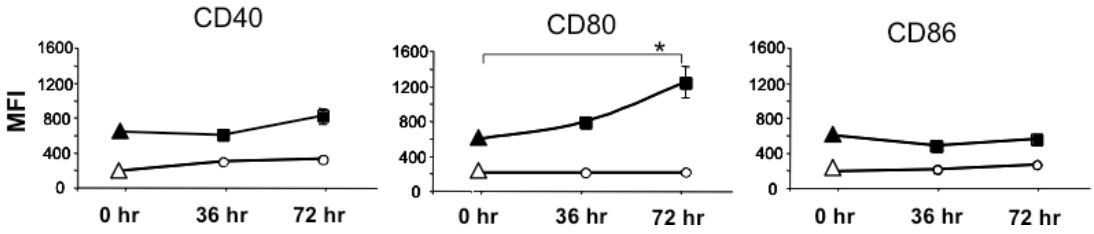

Figure 20 GML induce maturation of phagocytosing DCs *in vivo*. Thirty-six and 72 hours after CFSE-labeled GML infusion, DCs from SLO of treated mice were purified and analyzed for CD80, CD86 and CD40 expression. The analysis was performed on $CD11c^+$ $CD8\alpha^+$ $MHC\text{-}II^{high}$ DCs (full symbols) and $CD11c^+$ $CD8\alpha^+$ $MHC\text{-}II^+$ DCs (empty symbols). Empty circles and full squares identify $CFSE^-$ non-phagocytosing and $CFSE^+$ phagocytosing DCs, respectively. DCs from untreated mice (0 hr) were also analyzed (triangles). Asterisk indicates a significant difference ($P<0.05$) using the Student's t test.

We did not observe such activation in non-phagocytosing ($CFSE^-$) DCs and in $CD11c^+$ $CD8\alpha^+$ $MHC\text{-}II^{high}$ DCs from untreated mice (Figure 20). To further investigate the mechanism responsible for such activation, we carried out in vitro co-culture experiments on bone marrow-derived DCs (Figure 21) and on $CD11c^+$ DCs harvested from SLO of naive mice (Figure 22).

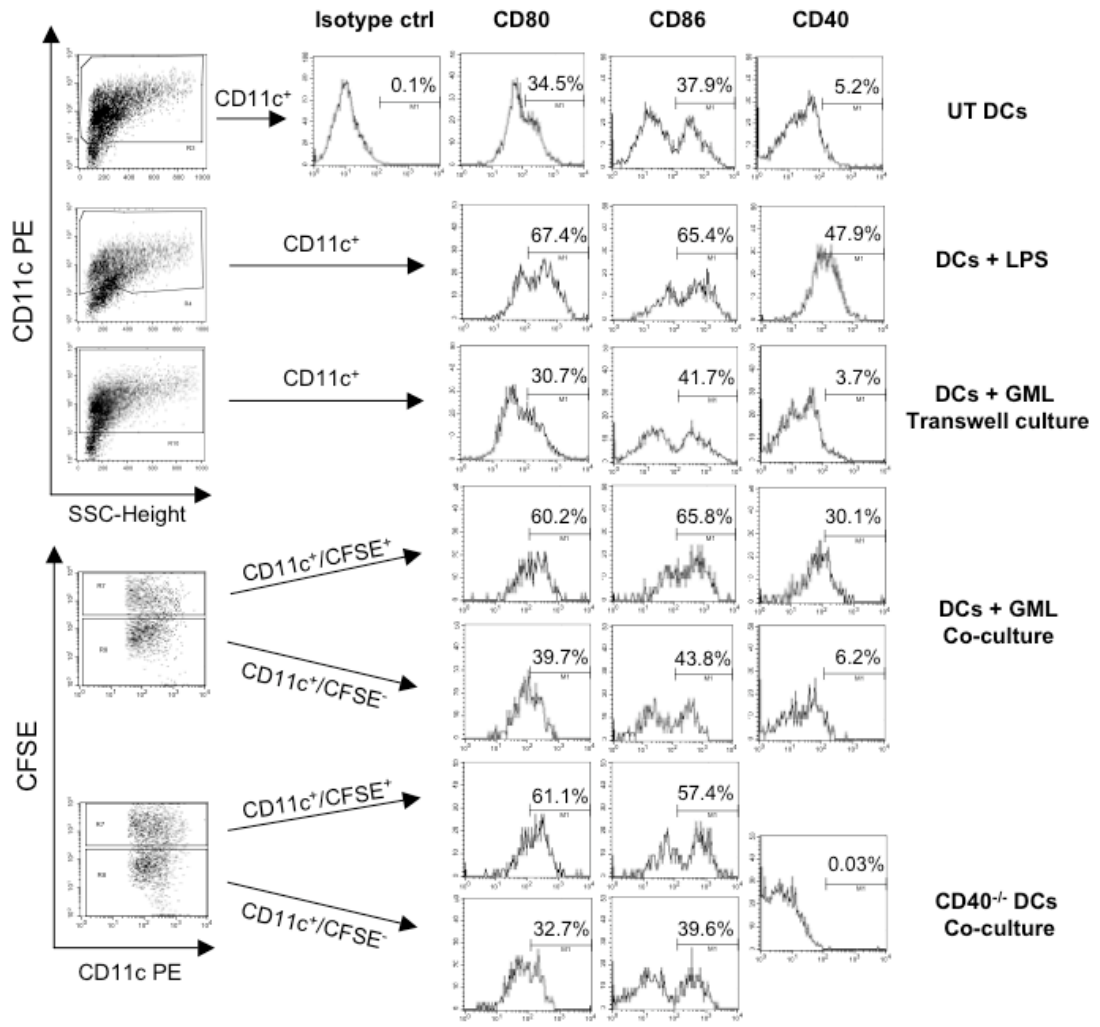

Figure 21 DCs differentiated from bone marrow of B6 mice, were either left untreated, activated with LPS, co-cultured with CFSE-labeled GML or cultured with CFSE-labeled GML in transwell plates. DCs from $CD40^{-/-}$ mice were co-cultured with CFSE-labeled GML. Fourty-eight hours later, cells were analyzed by FACS for CD11c, CD80, CD86 and CD40 expression. In co-culture conditions, the analysis was performed on both $CD11c^{+}$ $CFSE^{+}$ phagocytosing DCs and $CD11c^{+}$ $CFSE^{-}$ non-phagocytosing DCs.

Bone marrow-derived DCs were either left untreated, activated with LPS (1 μg/ml), co-cultured with CFSE-labeled GML or cultured with CFSE-labeled GML in transwell plates. After 24 (data not shown) and 48 hours LPS-activated DCs up regulated CD80, CD86 and CD40, whereas untreated DCs did not (Figure 21). The analysis on DCs co-cultured with CFSE-labeled GML showed that only the phagocytosing DCs (i.e. $CD11c^{+}$ $CFSE^{+}$) had undergone activation, suggesting that DC activation would require cell-to-cell contacts. This hypothesis was then confirmed by experiments performed in the transwell culture system, which did not allow DC maturation by preventing direct DC-GML cell interactions (Figure 21). Interestingly,

DCs differentiated from CD40$^{-/-}$ mice were activated upon co-culture with CFSE-labeled-GML, demonstrating that in our system, DC activation is independent of CD40/CD40L triggering. Similar results were obtained using the CD11c^{+} DCs harvested from SLO of naive mice (Figure 22).

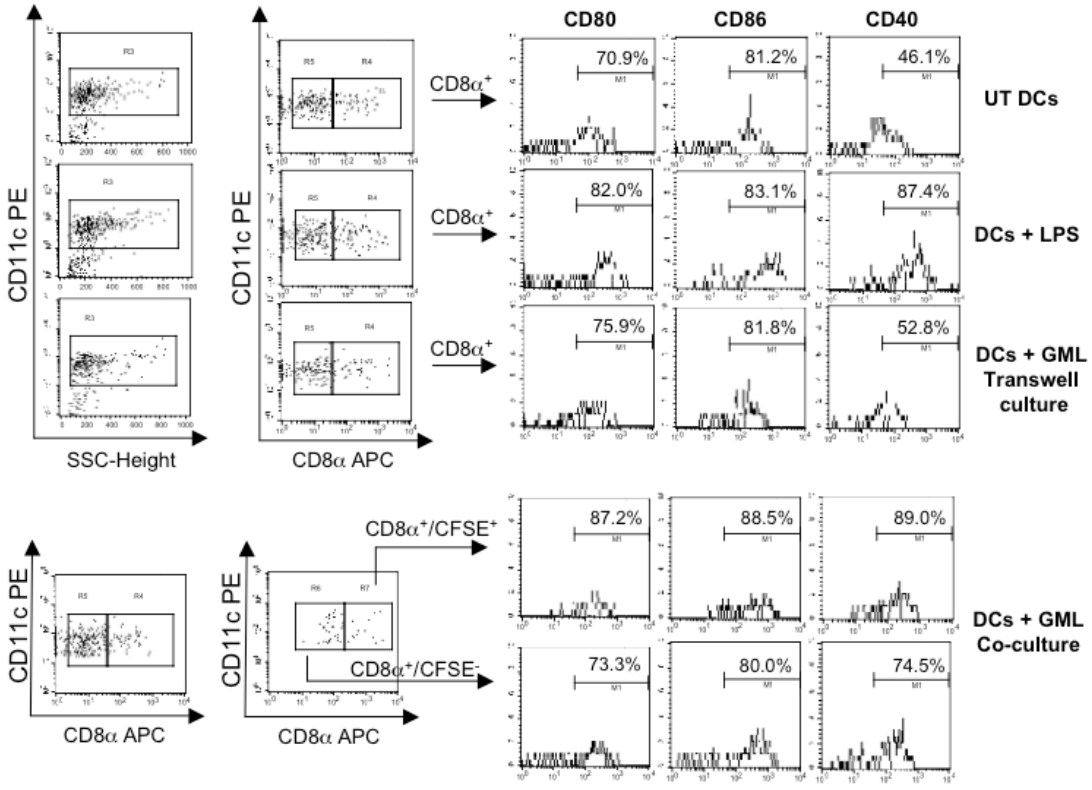

Figure 22 GML induce maturation of phagocytosing CD11c^{+} CD8α^{+} DCs *in vitro*. CD11c^{+} DCs purified from SLO of naive B6 mice, were either left untreated, activated with LPS, co-cultured with CFSE-labeled GML or cultured with CFSE-labeled GML in transwell plate conditions. Twenty-four hours later, CD11c^{+} CD8α^{+} DCs were analyzed for CD80, CD86 and CD40 expression. In co-culture conditions, the analysis was performed on both CD11c^{+} CD8α^{+} CFSE^{+} phagocytosing DCs and CD11c^{+} CD8α^{+} CFSE^{-} non-phagocytosing DCs.

Upon *in vivo* phagocytosis DCs are able to activate naive T-cells.

To determine whether the above-characterized population was, indeed, responsible for antigen presentation, DCs isolated (95-98% $CD11c^+$) from spleen and LN of B6 mice 60 hours after the infusion of OVA-GML from $\beta2m^{-/-}$ mice, were used to stimulate in vitro CFSE-labeled OT-I T-cells. Both OVA-specific IFN-γ release (Figure 23, right panels) and proliferation (Figure 23, left and middle panels) were observed.

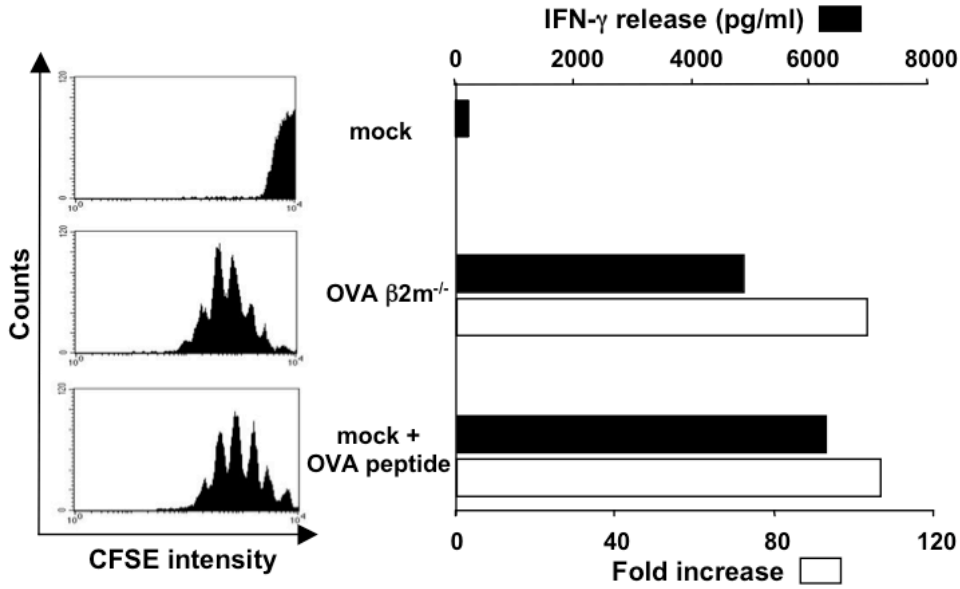

Figure 23 *In vitro* cross-presentation of OVA-GML by $CD11c^+$ DCs. Pure populations (95-98%) of $CD11c^+$ DCs from lymphoid organs of mice treated with mock or OVA-$\beta2m^{-/-}$ GML, were challenged *in vitro* with highly purified CFSE-labeled $CD8^+$ OT-I T-cells. Forty-eight hours later, supernatants were tested for IFN-γ release (black bars, right panels). Cell proliferation, measured as CFSE dilution (CFSE intensity, left panels), was evaluated four days later. Increase of the absolute number of proliferating OT-I T-cells (Fold Increase) is shown in the right panels (white bars). As positive control OT-I T-cells were stimulated with $OVA_{257-264}$-pulsed DCs (mock + OVA peptide).

As expected, there was neither IFN-γ release nor proliferation of $CD8^+$ OT-I cells incubated with DCs purified from mice treated with mock-transduced $\beta2m^{-/-}$ lymphocytes (Figure 23), thus demonstrating that upon *in vivo* phagocytosis of GML, DCs are able to prime OVA-specific T-cells.

Tumor associated antigens delivered by GML are able to persist up to 120 hours into SLO of vaccinated mice.

Finally, we evaluated TAA persistence into secondary lymphoid organs of mice treated with the vaccination protocols used in the prophylactic and therapeutic tumor settings (i.e. i.v infusion of OVA-GML from $\beta 2m^{-/-}$ mice or s.c. injection of peptide-pulsed DCs). $CD11c^{+}$ DCs were harvested from SLO of mice at different times (36, 72 and 120 hours) after the treatments, and were co-cultured with naive OT-I $CD8^{+}$ T-cells. Forty-eight hours later IFN-γ release was measured (Figure 24).

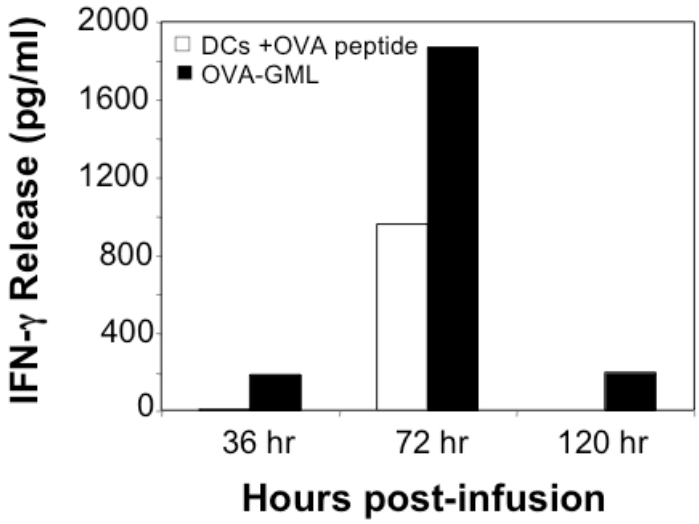

Figure 24 Pure populations (95-98%) of $CD11c^{+}$ DCs recovered from SLO of mice sacrificed 36, 72 and 120 hours after the treatment with either OVA-$\beta 2m^{-/-}$ GML, $OVA_{257-264}$-pulsed DCs or unpulsed DCs were challenged in vitro with $CD8^{+}$ OT-I T-cells as described above. Supernatants collected after 48 hours of co-culture were tested for IFN-γ release.

Seventy-two hours after the treatments, DCs from OVA-GML treated mice induced OT-I T-cells to release approximately 2 fold greater IFN-γ than DC from mice treated with peptide-pulsed DCs (1869.1 pg/ml vs 957.9). Noteworthy, 120 hours later, only DCs from GML treated mice were still able to activate OT-I T-cells (IFN-γ release: 197 pg/ml). These results along with data of GML persistence within SLO (Figure 6), indicate that GML provide SLO resident DCs with a continuous source of antigen for longer time, resulting in a prolonged T-cell activation.

MATERIALS AND METHODS

Mice.

C57BL/6J (B6) mice (8-10 weeks of age) were obtained from Harlan, Italy. C57BL/6J $\beta 2m^{-/-}$ KO mice lack MHC class I expression[276]. OT-I (Ly 5.1) mice[278] are transgenic for a $\alpha\beta$ TCR specific for the H-$2K^b$-restricted $OVA_{257-264}$ peptide. C57BL/6 $CD40^{-/-}$ and CD11c-DTR/GFP (CD11c-DTR) mice were obtained from Orleans, France. Mice were housed under pathogen-free conditions. Animal studies were approved by the Institutional Animal Care and Use Committee of Istituto Scientifico H.S. Raffaele.

Cell lines and reagents.

The murine RMA lymphoma was kindly provided Dr. M. Bellone, (Istituto Scientifico H.S.Raffaele, Milan, Italy). The murine, B16F1 (B16) melanoma cell line was purchased from the American Type Culture Collection (ATCC). Diptheria Toxin (DT) was purchased from Sigma and used as described by Jung et al at 4 ng/g body weight[281].

Flow cytometry, confocal microscopy and TUNEL assay.

MAbs specific for murine markers were all from Becton Dickinson, PharMingen. Isotype controls from the same manufacturers were used. Before staining, cells were incubated with FcR block (purified α-CD16/α-CD32 mAbs, BD PharMingen). Samples were run on a FACS Calibur™ (BD) and analyzed by CELLQuest™ software (BD). $H2K^b$-SIINFEKL OVA pentamer-PE was from Proimmune Limited. TUNEL was performed on SLO from treated mice by using the TUNEL TMR-dUTP assay (Roche) according to the manufacturer's recommendations. For confocal microscopy, B6 mice were injected with CFSE (Molecular Probes) labeled OVA-transduced lymphocytes ($10\text{-}20x10^6$). Six, 12 and 24 hours later, spleen and lymph nodes (cervical, axillary and inguinal) were collected and fixed in 4% paraformaldehyde (Sigma) for 2 hours at R.T. Lymphoid organs were then washed in PBS and incubated with 30% saccharose overnight at R.T., washed, embedded in Tissue-Tek OTC (Sakura Fine Technical Co., Ltd.), frozen in isopentane cooled by liquid nitrogen and stored at -20°C until use. Cryosections (10 μm thick) were

adhered to poly-L-lysine-coated glass coverslips for 1 hour. Before staining, cryosections were treated with PBS 10% FCS and Rat IgG (50 μg/ml) for 1 hour at R.T. and then incubated with rat anti-mouse CD3-PE mAb (BD). To visualize DCs, CD11c PE and CD8α PE-Cy5 mAbs (BD) were used.

Retroviral vectors and transduction procedures.

The OVA-CSM and TRP-2-CSM retroviral vectors were constructed as previously described[284], with OVA or the murine TRP-2 under the control of the viral LTR. Expression of ΔLNGFR was driven by the Sv40 promoter. Splenocytes ($5x10^6$ cells/ml) from B6 or $\beta2m^{-/-}$ mice were stimulated in the presence of 100 U of rhIL-2 (Proleukin, Chiron) and 5 μg/ml of Concanavalin A (Sigma). Forty-eight hours later, splenocytes were transduced with virus-containing supernatant by 2 rounds of centrifugation (120 min., 2400 rpm) a day apart, in the presence of 8 μg/ml of polybrene (Sigma). Three days later, splenocytes were analyzed for ΔLNGFr expression and then expanded for 10-14 days. B16F1 murine melanoma cells were transduced with the OVA-CSM vector and selected for ΔLNGFr expression by magnetic beads (Dynabeads M-450, Dynal) coated with the LNGFr-specific mAb 20.4 (ATCC).

Adoptive transfer, immunization experiments and *ex vivo* recall assay.

OT-I $CD8^+$ T-cells were enriched from spleen and lymph nodes by depletion of B cells with Dynabeads Mouse pan B (B220) (Dynal) and labeled with CFSE. Briefly, cells were incubated at $10x10^6$ cells/ml in CFSE at a final concentration of 4 μM for 8 min at R.T. Labeling reaction was stopped by adding the same volume of FCS. After washing $3x10^6$ CFSE labeled $CD8^+$ T-cells/mouse was injected into the tail vein of sex-matched B6 recipients. After 24 hours recipients were treated i.v. with $4x10^6$ GML. Splenocytes harvested 8 days after vaccination were restimulated *in vitro* (1:1 ratio) with syngeneic irradiated splenocytes, pulsed with 2 μg/ml $OVA_{257-264}$ peptide. Four days later, OT-I $CD8^+$ T-cells were purified by FACSVantage™ sorting using Vα2 Vβ5.1 specific mAbs. OT-I $CD8^+$ effectors ($1x10^4$) were then challenged with $2x10^4$ RMA pulsed with $OVA_{257-264}$. After 24 hours we measured IFN-γ content in the supernatant by ELISA assay (Pharmingen, BD) according to the manufacturer's recommendations. IFN-γ released on unpulsed RMA was always subtracted. OT-II

$CD4^+$ T-cells were purified with the $CD4^+$ T cell isolation kit (Miltenyi Biotec GmbH) and labeled with CFSE as described for OT-I $CD8^+$ T-cells. CFSE labeled $CD4^+$ T-cells ($3x10^6$) was injected into the tail vein of sex-matched B6 recipients. After 24 hours recipients were treated i.v. with 4 or $10x10^6$ GML. For the induction of OVA-specific endogenous repertoire experiments, B6 mice were treated 3 times at 2 weeks interval with OVA-GML ($4x10^6$) and DCs ($3\text{-}5x10^5$) activated with LPS (1 μg/ml) and pulsed with 2 μg/ml $OVA_{257\text{-}264}$ peptide. Forty days later, spleen and LNs were collected, digested, washed and then stained with the $H2K^b$-SIINFEKL OVA pentamer-PE together with mAbs specific for activation and memory murine markers.

***In vitro* cytotoxicity assay.**

Splenocytes from vaccinated mice ($3x10^6$/ml) were cultured with 0.5 μM TRP-2 peptides. After 6 days, activated T-cells were isolated on a lympholyte-M gradient (CEDARLANE, Hornly) cultured one day in the presence of 20 UI/ml hrIL-2. Responder cells were then tested for cytolytic activity in a standard 4-h ^{51}Cr release assay.

***Ex-vivo* cross-presentation assay and kinetic of TAA persistence within SLO.**

$CD11c^+$ DCs were purified from B6 mice after vaccination with OVA-transduced $\beta2m^{-/-}$ lymphocytes ($10\text{-}20x10^6$). Spleen and lymph nodes were digested for 40 min at 37°C with collagenase A,B,D 0,5 mg/ml (Roche Diagnostics, GmbH) and then resuspended in PBS, 0,5% BSA, 2 mM EDTA, to disrupt T cell-DC complexes. Purification of DCs (92-98% purity) was performed with rat anti-mouse CD11c mAb (N418)-coupled magnetic MicroBeads on miniMACS columns (Miltenyi Biotec GmbH). Purified DCs ($1,5x10^5$) were used as stimulators, whereas OT-I $CD8^+$ T-cells ($1,5x10^5$), isolated from lymph nodes of transgenic mice by depletion using B220, CD11c, CD11b, CD4 PE-conjugated mAbs followed by anti-PE MicroBeads incubation, were labeled with 3 μM CFSE and used as responders.

Experiments were performed in triplicates in 96-well U-bottom plates. As positive control DCs from mice receiving mock-transduced $\beta2m^{-/-}$ lymphocytes were pulsed with 2 μg/ml of $OVA_{257\text{-}264}$ peptide. Forty-eight hours later, supernatants were tested for IFN-γ production by ELISA. Two days later, the cells were counted and stained with rat anti-mouse CD3 FITC, CD45.1-PE, and CD8α-Cy-Chrome mAbs and

analyzed by FACS, to evaluate OT-I $CD8^+$ T-cells specific proliferation as a measure of CSFE dilution. For kinetic experiments of TAA persistence into SLO, mice were treated i.v. with OVA-GML ($10\text{-}20x10^6$) from $\beta 2m^{-/-}$ mice or with DCs ($0.5x10^6$) pulsed with 2 μg/ml of $OVA_{257\text{-}264}$ peptide and injected s.c. Thirty-six, 72 and 120 hours after treatments, DCs ($1.5x10^5$) purified as described above were used as stimulators, whereas purified OT-I $CD8^+$ T-cells ($1.5x10^5$), labeled with 3 μM CFSE, were used as responders. Experiments were performed in triplicates in 96-well U-bottom plates. Forty-eight hours later, supernatants were tested for IFN-γ production by ELISA. IFN-γ released by responders and/or stimulators alone was always subtracted.

***In vitro* DCs activation assay.**

$CD11c^+$ DCs were purified from B6 mice with rat anti-mouse CD11c mAb (N418)-coupled magnetic MicroBeads on miniMACS columns (Miltenyi Biotec GmbH) (92-98% purity) or differentiated from bone marrow[284]. Purified DCs ($3x10^5$) were left untreated, activated with LPS (1μg/ml), cultured with CFSE-labeled GML in co-culture or in transwell plates. Twenty-four hours later and 48 hours later, cells were harvested, washed once and then stained with mAbs specific for murine CD3, CD11c, CD8α, CD80, CD86 and CD40.

***In vivo* experiments of DCs depletion.**

CD11c-DTR mice were injected with $1.5\ x10^6$ CFSE-labeled OT-I $CD8^+$ T-cells.

OT-I T-cells were purified and labeled with CFSE as previously described. Eight hours later mice were treated intraperitoneally with 4 ng/g body weight Diptheria Toxin (DT). The day after, adoptively transferred mice were given $10x10^6$ OVA-GML. Spleen and lymph nodes were harvested 72 hours after GML treatment, processed and analyzed by FACS to evaluate OT-I proliferation and the percentage of $CD11c^+$ DCs. Proliferation of OT-I T-cells ($CD45.1^+$ T-cells) was evaluated as CFSE dilution.

***In vivo* antitumor response against B16 or B16-OVA melanomas.**

B6 mice were vaccinated i.v. 3 times at 2 weeks interval with either $4x10^6$ TRP-2-transduced lymphocytes from wt and $\beta2m^{-/-}$ mice; or with LPS-activated DCs (5-$10x10^5$ cells) generated as described by Camporeale et al[284] and pulsed with 2μg/ml of TRP-$2_{181-188}$ (VYDFFVWL)[274], TRP-$2_{180-188}$ (SVYDFFVWL)[96] peptides. Two weeks or 40 days after the last vaccination, mice were inoculated s.c. with $5x10^4$ B16F1 cells. Tumor size was evaluated by measuring perpendicular diameters by a caliper. For curative experiments, mice were inoculated s.c. with $5x10^4$ B16F1 cells. Seventy-two hours later, when the tumor area was approximately 20 mm^3, mice were randomly assigned to one of the following treatments: 3 injections of $4x10^6$ mock-GML, 4 or $10x10^6$ TRP-2-GML from $\beta2m^{-/-}$ mice, 3 injections of $5x10^5$ LPS-activated DCs pulsed with 2 μg/ml of TRP-$2_{181-188}$ and TRP-$2_{180-188}$. Results were evaluated by comparing survival curves by log-rank test. Mice were adoptively transferred with $3x10^6$ OT-I $CD8^+$ T-cells and after 24 hours vaccinated i.v. with $4x10^6$ OVA-transduced lymphocytes from wt, $\beta2m^{-/-}$ mice. B16-OVA cells ($5x10^4$) were injected s.c either 48 hours before OT-I infusion, for therapeutic experiments or 40 days after vaccination for protection experiments. Data are reported as the average tumor volume +/- SD. Statistics. Statistical significance ($p<0.05$) was determined by the two-tailed Student's t test and by the log-rank test.

DISCUSSION

The growth and spread of cancer depends as much on the host response to tumor as on the biological characteristics of the tumor itself. This interaction is at its most intimate and dynamic within the tumor microenvironment. It is here that the battle is fought that leads to mutual evolution of tumor and host cell phenotypes. Contributing to this evolutionary process are physiological changes distinctive for the tumor microenvironment, such as hypoxia, low nutrient levels, low extracellular pH, and high interstitial fluid pressure. These largely result from the chaotic intratumoral vasculature but are impacted by the nature of the tumor and the inflammatory and wound healing responses that are generated. Numerous immune cells, including macrophages, lymphocytes, natural killer cells and dendritic cells infiltrate the tumor, contributing to high levels of growth factors, hormones, and cytokines.

The integrated interplay between host and tumor factors results in distinct phenotypes that determine the response to therapy as well as tumor behavior. Targeting the tumor microenvironment to awaken or reawaken immune cells, or to redirect it from a pro-tumor to an anti-tumor state, requires understanding of this phenotype.

Current conventional therapies target tumors, not tumor cells, and clearly affect the host infiltrate and the physiological characteristics of the tumor microenvironment. This may an advantage that has yet to be effectively exploited due to lack of knowledge of existing phenotypes resulting from the tumor-host interactions. The same lack of knowledge impacts outcomes of clinical immunotherapy trials that have so far not broken through the ceiling of 10% success rate that seems to exist even in melanoma. It seems obvious that more could be achieved by combining therapies that tackle malignancies from multiple angles, with the tumor microenvironment conditioned to support a powerful effector arm generated by immunotherapy. The challenge is how to design combination therapies that modify the tumor microenvironment so as to promote immunity and better combat both local and systemic disease. These improved approaches to induce cytotoxic T-cell responses to tumors are based on a more detailed understanding of the immune system activation and regulation. The higher response rates with modern immunotherapy approaches may allow exploration of the molecular mechanisms that make tumor targets resistant or sensitive to immunotherapy.

In this contest, the identification of genes coding for human tumor-associated antigens (TAA)[253], the characterization of DCs as the most potent APCs[254], as well as the accumulating evidence, refining the concept of tumor immunoediting and immunosurveillance[255], have all renewed interest in the preclinical and clinical aspects of cancer immunotherapy. Indeed, the results of adoptive immunotherapy trials in both solid[256,257] and haematologic[258,259] malignancies confirm that immune effectors represent potent anti-cancer agents and their use may result in clearance of tumor cells.

These results suggest that a suboptimal induction phase could be one of the factors responsible for the limited clinical efficacy of most vaccination trials[260]. On the other hand, a large variety of immune escape mechanisms adopted by tumors (i.e. a large tumor mass secreting immune suppressive factors, generation of TAA and/or HLA loss variants, TAA-specific tolerance induced by the tumor and intrinsic low immunogenicity of several tumor antigens[261,262] as well as heavy pretreatments of the patient could limit the efficacy of anticancer vaccines[263]. Novel strategies of active vaccination should consider all these aspects in order to become effective.

The potency of a vaccination protocol is a function of the magnitude of the immune response induced, type of immunity generated and how long it will persist in the patient. The goal is to channel the tumour antigens into the DC-presentation pathway, to introduce the antigens into the appropriate DC subset and to induce the DC to differentiate into a potent immunostimulatory cell.

There are two general approaches to channel antigens into the DC-presentation pathway, the *in vivo* route, and the *ex vivo* route.

The *in vivo* route is the age-old approach to vaccination, which consists of injecting antigen mixed with adjuvant into a patient, used long before it was known that a primary function of adjuvants was to mobilize the DC system. The *in vivo* approach is arguably the simpler and preferred approach. Several strategies to target antigens to DCs *in vivo* have been described (see introduction); however, their effective value has not been clearly established.

In this study a novel approach of cancer vaccination has been investigated, based on the *in vivo* delivery of tumor-associated antigens directly to secondary lymphoid organs by genetically modified T lymphocytes. This approach could improve the current DC-based protocols[264], e.g. *ex vivo* selection and differentiation of the most

appropriate DC subset[265], the low number of intradermally injected DCs reaching the draining LNs[266], and the limited half-life of MHC-I-peptide complexes on the DC cell surface[267]. In our system, the GML constitutively express the antigen (i.e. TRP-2, OVA), therefore there are no time constraints limiting their migration to SLO, or the transfer of the antigen to DCs. Indeed, up to 60-72 hrs after injection of OVA-GML, DCs harvested from SLO are able to produce maximum stimulation. Noteworthy, DCs harvested 120 hours after OVA-GML infusion, were still able to activate OVA-specific $CD8^+$ T-cells, whereas those from mice treated with peptide-pulsed DCs did not. These results indicate that DC targeting with TAA-GML could overcome some of the above-mentioned limitations of the DC-based vaccines, particularly the low number of DCs reaching SLO and the rapid degradation/loss of MHC-I-peptide complexes. Additionally, the constitutive and strong expression of the transgene products, driven by viral promoters, combined with the SLO-migratory capability of the GML might make them similar to virus-infected cells , which produce enormous amounts of antigen within immunological priming sites[268]. This vaccination approach elicited also antigen-specific $CD4^+$ T-cells. However the proliferation of the $CD4^+$ T-cell compartment was less sustained than that of the $CD8^+$ Tcells. This result is in agreement with recent data[269] showing that MHC-I and -II antigen presentation is achieved by different DC subsets. In particular, the $CD11c^+$ $CD8\alpha^+$ subset is responsible for MHC-I presentation, whereas the $CD11c^+$ $CD8\alpha^-$ subset for MHC-II presentation. In our setting we demonstrated that the majority of the GML-associated antigen is taken up by the $CD11c^+$ $CD8\alpha^+$ subset. Therefore, we hypothesize that the reduced proliferation of $CD4^+$ T-cells would reflect a lower recruitment of naive T-cells due to the limited uptake of GML-associated antigen by the $CD11c^+$ $CD8\alpha^-$ subset (3-5%). However, in our vaccination setting the reduced proliferation of TAA-specific $CD4^+$ T-cells does not dampen the development of an effective antitumor response *in vivo*. Moreover, in a pivotal clinical study using a similar strategy, we have observed the induction of fully active $CD4^+$ effector cells specific for the TAA carried by GML (manuscript in preparation). Mice vaccinated with TRP-2-GML were protected from a subsequent tumor challenge as well as those vaccinated with DCs *ex vivo* pulsed with TRP-2-derived peptides; thus suggesting a comparable efficacy of the two approaches. Noteworthy, also in a more stringent therapeutic tumor model, we observed long term survival benefit of TRP-2-GML vaccination. However, in our

opinion, a more stringent and informative comparison between vaccination strategies based on *ex vivo* and *in vivo* DC loading could be addressed in humans. In this setting, the high degree of MHC-I/II polymorphisms would allow the full exploitation of the immunogenicity of the entire antigenic proteins and the *in vivo* processing and presentation capabilities of SLO-resident DCs. In our murine model, an efficient uptake of antigenic material by LNs/spleen-resident DCs is able to induce the activation of TAA-specific responses. In agreement with other experimental models[270], we show that the activation of naive TAA-specific T-cells following the infusion of TAA-GML, involves the targeting/transfer of the antigen to a defined subset of $CD11c^+$ DCs expressing the CD8α molecule[270,271] and requires the cross-presentation of the TAA, as demonstrated by treating mice with GML from $\beta 2m^{-/-}$ animals. The essential role of DCs was definitely confirmed by the loss of sustained TAA-specific proliferation in DC-depleted mice (i.e. CD11c-DTR mice). Differently from studies carried out by other groups[273], in our model the induction of TAA-specific effector cells was obtained without adding any further DC activating stimulus. However, 72 hours after the treatment we observed an increased expression of activation markers (i.e. B7.1 and CD40) by phagocytosing DCs. These results were further confirmed by in vitro experiments, with bone marrow-derived DCs and $CD11c^+$ DCs harvested from SLO. Only phagocytosing DCs co-cultured with GML were able to undergo maturation, indicating that DC activation would require cell-to-cell contacts. Moreover, the use of DCs from $CD40^{-/-}$ mice allowed to hypothesize that the DC activation could be induced by the GML via a direct interaction with DC activating receptors other than CD40-CD40L[273]. Further genetic modifications of TAA-GML to express SLO-homing molecules, and the combined administration of TAA-GML and ganciclovir to suicide GML, could increase antigen availability and therefore improve the treatment. In conclusion, experimental results clearly demonstrate that transduced T lymphocytes represent an efficient way for *in vivo* loading of tumor-associated antigens on $CD11c^+$ / $CD8a^+$ DCs and for the induction of effective antitumor responses

FUTURE PERSPECTIVES

Cross-priming was first described by Michael Bevan[285] more than 25 years ago. Antigen-presenting cells (APC) have the ability to prime CTL responses against minor histocompatibility antigens captured from forgein donor cells. The term cross-presentation subsequently has been used to describe the uptake and representation of cell-associated antigens primarily in the MHC-class I pathway of professional APC.

However, cross-presentation of diverse antigenic materials has been shown to be effectuated by several types of APCs. Among them, most prominently dendritic cells and macrophages[286-288]

Apoptotic/necrotic cells may represent an antigenic source that both macrophages and dendritic cells have been shown to take up. Dendritic cells, however, due to high-density expression of costimulatory molecules, have the unique capability to strongly induce naive CTL to respond. In certain model sytems apoptotic cell death has been shown to be an attractive immunogenic antigenic source for the crosspriming of CTL.

Our experimental settings offer the possibility to improve the efficiency of *in vivo* loading of DCs by a suicide gene therapy approach. As a matter of fact, T lymphocytes used in our approach, could be transduced with a retroviral vector coding for the tumor antigen and a suicide gene.

The herpes simplex virus thymidine kinase (HSV-*tk*)/ganciclovir (GCV)-based suicide strategy appears to be the most effective and specific and has been widely adopted[289,290]. Cells are engineered to express HSV-*tk*; the addition of ganciclovir leads to cell death through *tk*-catalyzed metabolism of the drug to a lethal product.

Despite this demonstration of efficacy, the study revealed limitations of the HSV-*tk*/GCV approach. As a matter of fact in a clinical trial focused on GVHD prevention by infusion of donor T lymphocytes genetically modified with the suicide gene TK, it has been showed that a specific cytotoxic CD8-mediated immune response developed against genetically engineered T cells and led to the selective elimination of these cells.

Although the cells expressed both HSV-*tk* and the cell surface marker ΔLNGFR (the extracellular and transmembrane domains of the human low-affinity nerve growth factor receptor), the immune response was directed exclusively against HSV-*tk*. This suggests that virally derived proteins were recognized by the immune system and

eliminated, whereas ectopically expressed human products were not targets of immune recognition.

To circumvent these limitations, recently has been set up a novel suicide switch based on the human Fas receptor to trigger cell death in primary human T lymphocytes[293]. Fas (CD95, APO-1) is a member of the tumor necrosis–nerve growth factor receptor superfamily.12 Cross-linking of Fas results in the recruitment of a death-inducing signaling complex, activating a proteolytic cascade of caspases and inducing cell death by apoptosis [291,292].

Experimental data indicate that Fas-engineered T lymphocytes should be as efficacious as HSV-*tk*–engineered cells but able to bypass the intrinsic limitations of the HSV-*tk*/GCV suicide system.

This approach may represent an optimal tool to induce synchronized apoptosis once engineered cells have reached secondary lymphoid organs, thus delivering the antigenic material to DCs in a more immunogenic way. This could improve the DCs loading quantitatively and qualitatively offering stronger maturation stimuli and improving the CTL priming.

As reported in the results, just a small part of engineerd T cells are able to reach secondary lymphoid organs. In order to increase the amount of these cells it could be useful to transduce them with lymph nodes specific homing receptors like CCR7 and CD62L. The CC-chemokine receptor CCR7 has been described to be crucially involved in several fundamental processes shaping the structural and functional organization of the immune system. In particular, the generation of CCR7-deficient mice by targeted gene deletion has contributed substantially to the unraveling of the manifold functions of this homeostatic chemokine recept. Importantly, lymph nodes of CCR7-deficient animals were found to harbor significantly reduced numbers of cells compared with LNs of wild-type animals. This phenotype illustrates the key function of CCR7 and its ligands in the regulation of cell homing to secondary lymphoid organs. CD62L (L-selectin, mel 14) is also involved in the regulation of naive T cell homing into lymph nodes and the migration of leucocytes to sites of inflammation. The possibility to improve the delivery efficiency of engineered T cells to lymph nodes using these molecules will give the opportunity to increase the amount of antigenic material within APCs thus inducing a powerful recruitment and priming of tumor-specific $CD8^+$ / $CD4^+$ T cells.

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