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Local immunostimulation leading to rejection of accepted male skin grafts by female mice as a model for cancer immunotherapy

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ABBREVIATIONS

ACT: Adoptive cell transfer
APC: Antigen-presenting cell
ASCO: American society of clinical oncology
BCG: Bacille de Calmette-Guerin
CARs: Chimeric Antigen receptors
CEA: carcinoembryonic antigen
CpG: short single-stranded synthetic DNA molecules that contain a cytosine triphosphate deoxynucleotide ("C") followed by a guanine triphosphate deoxynucleotide ("G").
CLL : Chronic lymphocytic leukemia
COX: Cyclooxygenase
CR : Complete response
CTL: Cytotoxic T lymphocyte
CTLA-4: Cytotoxic T lymphocyte antigen 4
CV: cancer vaccine
DC: Dendritic cell
DNA: Deoxyribonucleic acid
EBV: Epstein-Barr virus
ELISA : enzyme-linked immunosorbent assay
EMA : European medicines agency
FDA: Food and Drugs Administration
FOXP3: Forkhead box P3
G-CSF : Granulocyte colony-stimulating factor
GM-CSF: Granulocyte macrophage colony-stimulating factor
HBV: Hepatitis B virus
HCC: Hepatocellular carcinoma
HPV: Human Papilloma virus
HLA: Human leukocyte antigen
HSV : Herpes simplex virus
ICAM : InterCellular Adhesion Molecule
IDO: Indoleamine 2,3-dioxygenase
IFN: Interferon
IL: Interleukin
i.v.: intravenous
KIR: Killer-cell immunoglobulin-like receptor
LPS: Lipopolysaccharide
mAb : Monoclonal Antibody

MAGE: Melanoma antigen-encoding gene
MART-1: Melanoma antigen recognized by T cells
MDSC: Myeloid derived suppressor cell
MHC: Major Histocompatibility Complex
mRNA: Messenger ribonucleic acid
MUC-1: Mucin-1
NK: Natural killer
NO: Nitric oxide
NR : No response
NSCLC: Non-small cell lung carcinoma
PAP: prostatic acid phosphatase
PBMC: Peripheral blood mononuclear cell
PD1: Programmed death 1
PD-L1: Programmed death-ligand 1
PGE2: Prostaglandin E2
PFS: Progression-free survival
PR: Partial response
PSA: Prostate-specific antigen
RCC: Renal cell carcinoma
RNA: Ribonucleic acid
mRNA: messenger Ribonucleic acid
RECIST: Response Evaluation Criteria in Solid Tumors
s.c.: subcutaneous
TCR: T cell receptor
TDO: Tryptophan 2,3-dioxygenase
TGF: Transforming-growth factor
TIL: Tumor-infiltrating lymphocyte
TLR : Toll-like Receptor
TNF: Tumor-necrosis factor
TNFR: Tumor-necrosis factor receptor
Treg: Regulatory T cell
VEGF: Vasoendothelial growth factor
WHO : World Health Organization

SUMMARY

The introduction is a review of the literature covering the different immunotherapeutic protocols tested within clinical trial for human practice.

It has been shown that many cancer patients are able to mount a spontaneous T lymphocyte response against their tumor. In metastatic progressing patients, this response has evidently been incomplete. An increasing number of data on human tumors and tumor infiltrating lymphocytes support the scenario of an immunosuppressive environment that appears to be present within the tumor. This immunosuppressive environment seems to be a major factor preventing the success of immunotherapies, especially for anti-cancer vaccination.

Our working hypothesis is that reversing focally the immunosuppressive environment to an immunostimulating environment could potentiate the effect of anti-cancer vaccination and other immunotherapeutic approaches such as immunostimulating antibodies (as anti CTLA-4). Stalled immune responses are occasionally observed in the area of tissue transplantation where a small number of recipients can be withdrawn from treatment with immunosuppressive agents without deleterious effects on the grafted organ. In a mouse strain named CBA, this phenomenon is observed when male skin is grafted onto females. Female of this mice strain do not reject syngeneic male skin grafts even though they mount a T cell response against the male-specific HY antigen. This experimental model was used to test our working hypothesis. In our skin graft model we have tested several agents

that, when applied locally, may relieve the immunosuppression and reactivate the immune response leading to graft rejection.

Some cytokine combinations, locally administered, are effective in promoting the rejection of these skin grafts. Local immunostimulation performed by injecting a combination of cytokines and Toll-like receptor ligands in close vicinity to the graft promoted rejection. This approach was then tested in a few tumor-bearing human patients in combination with anti-tumor vaccination. A clinical phase I/II trial based on the combination discovered was conducted, with encouraging preliminary results.

These data suggest that a therapeutic approach using local injections of the combination described in this work might contribute to boost the efficacy of various cancer immunotherapy modalities. Relief of intratumor immunosuppression may increase considerably the fraction of patients who respond to vaccination directed against tumor antigens recognized by T cells.

RESUME

L'introduction est une revue de la littérature reprenant les différentes méthodes d'immunothérapie du cancer qui ont été utilisées dans le cadre d'essais en clinique.

Il est actuellement bien démontré que la plupart des patients atteints de cancer sont capables de développer spontanément une réponse lymphocytaire cytotoxique dirigée contre leur tumeur. Cette réponse est évidemment incomplète chez les patients en progression métastatique. De plus en plus d'observations basées sur l'analyse des tumeurs humaines et des lymphocytes infiltrant ces tumeurs supportent l'idée qu'un environnement immunosuppresseur est présent au sein même de la tumeur. Cet environnement immunosuppresseur serait un facteur essentiel du manque d'efficacité des vaccinations anti-cancer.

Notre hypothèse de travail est qu'en agissant localement sur l'environnement immunosuppresseur présent dans les tumeurs et en le rendant plutôt stimulant, cela permettrait de réactiver les lymphocytes infiltrant les tumeurs, et de potentialiser l'effet des vaccinations anti cancer.

L'absence de réponse immunitaire efficace est également observée occasionnellement en transplantation d'organe où quelques patients présentent une tolérance opérationnelle à l'organe greffé sans qu'aucune immunosuppression ne soit nécessaire.

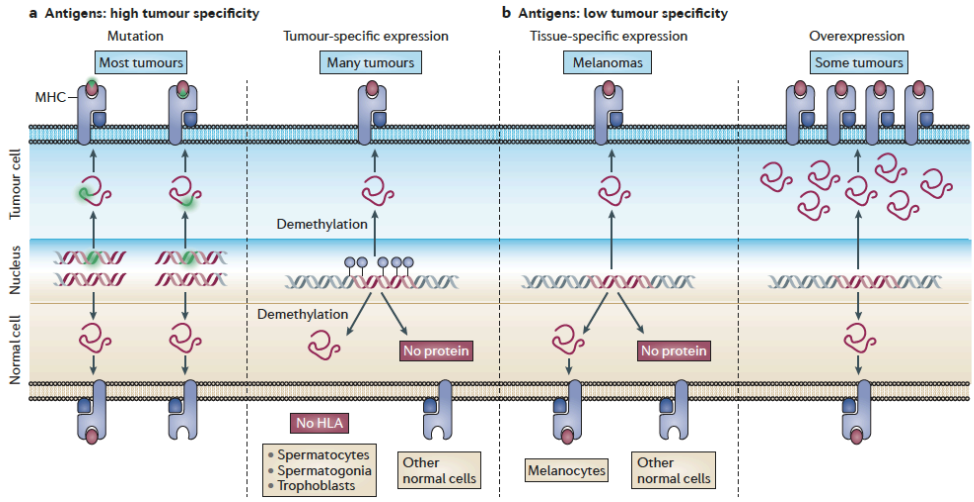
Dans une race de souris appelée CBA, ce phénomène est observé quand on réalise une greffe de peau de mâle sur une femelle. Les femelles de cette race de souris ne rejettent pas les greffons cutanés mâle syngéniques, malgré qu'elles soient capables de monter une réponse immunitaire cytotoxique dirigée contre l'antigène mâle-spécifique (HY). Nous avons utilisé ce

modèle expérimental, proche de la situation observée dans les tumeurs humaines, pour tester nos hypothèses.

Dans ce modèle murin de greffe de peau nous avons testé différents agents qui, injectés localement, pourraient atténuer l'immunosuppression locale et réactiver la réponse immunitaire, ce phénomène menant à un rejet des greffes. Nos expériences nous ont permis d'identifier une combinaison originale de cytokines et de ligands des Toll-Like récepteurs, qui, injectés au contact de la greffe, induisent un rejet du greffon dans 100% des cas. Cette approche a par la suite été testée chez des patients atteints de cancer en combinaison avec une vaccination anti-tumorale. Un essai clinique de Phase I/II a été initié sur base de la combinaison découverte dans notre modèle expérimental. Les résultats préliminaires obtenus sur trois patients sont encourageants.

Une approche thérapeutique par injections locales de la combinaison décrite ici pourrait augmenter l'efficacité de l'immunothérapie du cancer, particulièrement celle des vaccinations anti-tumorales.

INTRODUCTION



From Coulie et al. Nat. rev. cancer 2014, 14 (135-146)

Figure 1 : Tumor antigens
Representation of the different types of tumor antigens classified by considering the genetic mechanisms leading to their expression. Here 4 groups are distributed as antigens of high (a) or low (b) tumoral specificity.

1. Introduction on cancer immunotherapy:

For decades, cancer treatment has relied on surgery, chemotherapy and radiotherapy. Targeted therapy, pioneered by the BCR-ABL inhibitor imatinib in chronic myeloid leukemia, joined the group in the early century, and has since then been extended to many other specific pathway inhibitors. More recently, immunotherapy has emerged as a new treatment modality against cancer.

During the last three decades, therapeutic interventions aimed at activating the immune system against tumors began to attract an increasing interest from both researchers and clinicians.

The idea of cancer immunotherapy was particularly developed after the observation in the 1980s that human T-lymphocytes isolated from the blood or tumors of melanoma patients, and restimulated in vitro with autologous tumor cells, exerted a tumor-specific lytic activity[1]. Tumor-specific cytolytic T-lymphocyte (CTL) clones were derived from these cultures and were used to identify their target antigens[2].

Several classes of tumor antigens have been described[3]. A convenient way to classify tumor antigens is to consider the genetic mechanisms leading to their expression (Figure 1). We will consider 5 groups distributed as antigens of high or low tumoral specificity. An updated list of tumor antigens recognized by T cells is available[4].

-Antigens with high tumoral specificity:

-*Antigens encoded by mutated genes:*

These antigens were among the first to be identified in human tumors[5,6].

The mutation is usually a point mutation in an ubiquitously expressed gene, changing one amino acid in the encoded protein. The wildtype peptide binds to an HLA molecule but natural tolerance ensures that no T cell response occurs against it, whereas the mutated amino acid creates a new epitope that can be recognized by T cells. Another mechanism of antigenicity is that the wildtype peptide does not bind to HLA, but amino acid change resulting from the mutation allows this binding to occur, thereby creating an antigen[5].
Another type of gene alteration is chromosomal translocation. At the level of the breakpoints, the translocated genes code for chimeric peptides that can be processed in the tumor cells and presented on HLA molecules.
Mutated antigens have important advantages for cancer immunotherapy. First, they are truly tumor-specific: T cells that recognize them do not recognize normal cells, thus avoiding immune tolerance. A second advantage of these antigens is their diversity in each tumor. It is estimated that about one half of the identified tumor antigens recognized by the spontaneous tumor-specific T-cell responses in cancer patients are encoded by mutated genes[4].

-Antigens encoded by cancer-germline genes:

The first human gene coding for a tumor-specific antigen recognized by T cells was discovered in 1991 by the group of Thierry Boon from the Brussels Branch of the Ludwig Institute for Cancer Research[7]. It was named *MAGEA1* (for Melanoma AntiGEn). *MAGEA1* is one of the cancer-germline genes, belong to several families of genes with a similar expression profile. The main cancer-germline gene families are *MAGE-A, MAGE-B, MAGE-C*, *BAGE*, *GAGE*, *LAGE* with *LAGE1*[8] and *NY-ESO-1/LAGE2*[9], and the *SSX*

family[10].

The cancer germline gene are expressed in many human tumors of different histological types[11], but they are not expressed in normal adult cells, except in male germline cells and in trophoblastic cells. Those cells do not produce HLA molecules and therefore cannot present antigens to T cells[12].

As a result of this particular expression profile, the cancer germline genes encode antigens that are strictly tumor-specific. Many antigenic peptides, mostly from MAGE and LAGE/NY-ESO-1 proteins[4], have been identified.

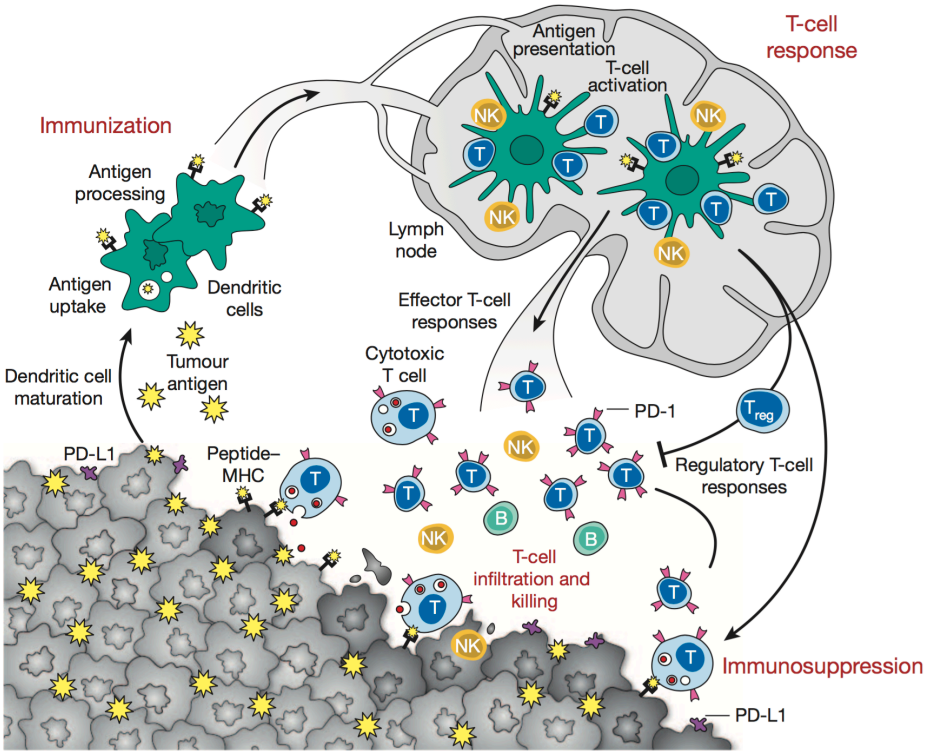
-*Viral antigens:*

Many cancers, approximately 20% of all, are caused by infectious agents[13], such as the oncogenic viruses HPV and EBV, which are sources of tumor-specific antigenic peptides in the associated tumors.

-Antigens with low tumoral specificity:

-*Antigens encoded by tissue differentiation genes:*

Some CTL clones derived from melanoma patients and raised against their tumor cells were also found to recognize normal melanocytes[14]. The target antigens were shown to be derived from melanocyte-specific proteins. The main antigenic peptides recognized by such CTL are derived from tyrosinase, encoded by gene *TYR*[15], Melan-A/MART-1 encoded by gene *MLANA*[16,17], and Gp100/PMEL17 encoded by *PMEL*[18,19]. These proteins are involved in melanin synthesis. There is no convincing evidence that differentiation antigens elicit T cell responses in patients with cancers other than melanoma. The reason why there is an incomplete tolerance against those antigens is unknown.



From Mellman et al. Nature 2011, 480 (480-488).

Figure 2 : Generation and regulation of antitumor immunity.

-Antigens encoded by genes that are overexpressed in tumors:

These antigens are expressed on normal cells, and are present at higher levels in tumor cells. The expression levels required for T-cell recognition are generally not known.

-Initiation of an anti-tumor immune response:

Anti-tumor T cell responses develop spontaneously in most, if not all, cancer patients. The following scenario, summarized in figure 2, for the development of such responses, has been proposed.

Antitumor immune responses begin with the capture of tumor antigens by dendritic cells (DCs), either delivered exogenously or captured from dying tumor cells. Then the DCs process the captured antigens for presentation and cross-presentation on MHC class II and class I molecules, respectively, and migrate to draining lymph nodes[20]. In the lymph node, antigen presentation to T cells elicits a response that depends on the type of DC maturation stimuli received and on the interaction of T-cell co-stimulatory molecules with their receptors on the DCs. If capture and presentation occur in the presence of an immunogenic maturation stimulus, DCs elicit anticancer effector T-cell responses in the lymph node. If no immunogenic stimulus is received, DCs induce tolerance leading to T-cell deletion, anergy or the production of regulatory T cells (Treg)[21]. For example, interaction of CD28 and OX40 on T cells with CD80/86 and OX40L on DCs, respectively, promotes potentially effective T-cell responses, wheras interaction of Cytotoxic T lymphocyte antigen 4 (CTLA-4) on T cells with CD80/86 on APC decreases T-cell responses and possibly promotes Treg

Introduction

formation. Activated T cells primed against the antigen (along with B and NK cells) exit the lymph node, circulate in the blood and enter into the tumor bed to perform their function. There, several immunosuppressive mechanisms can be produced by tumors that block effector T-cell function. These include the downregulation of MHC class I molecules by tumor cells, the upregulation of PD-L1/ L2 in the tumor[22], the release of PGE2, the expression of arginase and IDO (which are all T-cell suppressors)[23], and the release of VEGF (released by intratumoral hypoxia), which inhibits T-cell diapedesis from the vasculature into the tumor bed[24].

Several immunotherapy approaches were developed, acting at different steps of the immunization process. Here I review the principal approaches that have been developed and used in the clinic to trigger or enhance anti-tumor immunity. I make a distinction between immunotherapy strategies that make use of defined tumor antigens from those that do not.

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2. Immunotherapy without the use of tumor antigens:

The first attempt to stimulate the immune system against cancer dates from 1891, when William Coley, a young New-York surgeon, observed tumor regressions following local injection of live or inactivated *Streptococcus pyogenes* and *Serratia marcescens*. Coley tried to reproduce the regressions that were rarely observed in sarcoma patients who had developed erysipelas (*Streptococcus pyogenes* infections). Coley became convinced that he could effectively use bacteria to treat cancer and proposed a mixture of heat-killed bacterial cultures known as Coley's toxin. They were injected at increasing doses until fever was induced[25]. Many patients were then treated with Coley's toxin and some tumor regressions were recorded. But the successes were sporadic, difficult to reproduce and not obtained in a scientifically rigorous fashion[26]. It is now widely accepted that the proinflammatory activity of Coley's toxin, which contains many bacterial components including lipopolysaccharide (LPS), is mediated, at least in part, by the engagement of Toll-like-receptors (TLRs) on immune cells[27].

Bacterial products are still used today to treat cancer. Superficial bladder cancer patients who receive intravesical instillation of live BCG (Bacillus Calmette-Guérin) after surgical resection have less or later relapses and have a prolonged survival compared with patients treated by surgery alone[28].

2.1 Cytokines.

Several immunostimulatory cytokines have been administered to cancer patients, either systemically or locally into metastatic tumors.

2.1.1 Systemic administration.

IFN-α

IFN-α is produced by virtually all cell types, often in response to viral infection. The major producers of IFN-α are plasmacytoid dendritic cells but also lymphoid and myeloid cells. The IFN-α receptor is a heterodimer that is present on most cell types. The main function of IFN-α is to induce viral resistance mechanisms in surrounding cells. It also enhances antigen presentation by upregulating the expression of MHC class I and II molecules[29], and activates NK cells and macrophages.

Many clinical trials have established the ability of IFN-α to induce hematological responses and prolong the survival of patients with chronic myelogenous leukemia, a malignancy of pluripotent hematopoietic stem cells. In those clinical trials, IFN-α was administered at doses from 2 to 9 million units (MU)/m^2/day[30].

In patients with high-risk malignant melanoma, IFN-α has been used in the adjuvant setting. Randomized clinical trials established that systemic administration of IFN-α reduces the risk of recurrence following surgical excision of localized lymphnode metastases. Here 20 MU/m^2/day of IFN-α were administered 5 days/week during 4 weeks[31,32].

Significant toxicities are associated with systemic IFN-α treatment, including fatigue, cytopenia, elevation of liver enzymes, and neurologic symptoms (grade 3 and 4 adverse events following the World Health Organization criteria).

IL-2

IL-2 is a T-cell derived cytokine which was first described as a T-cell growth factor[33]. It is a glycoprotein produced by T lymphocytes. The IL-2 receptor comprises three non-covalently linked chains, termed alpha (CD25), beta (CD122), and gamma (CD132). IL-2 stimulates the proliferation and survival of cells that express the IL-2 receptor, mostly T cells and NK cells[34]. The effects of IL-2 on its target cells include increased cellular proliferation, stimulation of the production of other lymphokines, increased IL-2 receptor expression and enhanced phagocytosis, cell killing or antibody production[29].

Systemic administration of high-dose IL-2 was first tested by the group of Rosenberg for the treatment of patients with metastatic melanoma and renal-cell carcinoma (RCC)[35]. It induced tumor regression in a minority of patients[36,37]. In 1998, the Food and Drug Administration (FDA) approved the use of IL-2 to treat patients with metastatic melanoma. The same team reviewed the outcome of 684 patients with metastatic melanoma treated with high-dose IL-2 (720.000 International Units/kg 3 times a day for 12 to 15 administrations) either alone or in association with melanoma vaccines. IL-2 alone produced an overall objective response rate of 13%, which increased to 16-22% when IL-2 was combined with vaccination[38]. The precise mechanisms underlying this therapeutic activity remain to be defined, but increased cytotoxicity and alterations in tumor blood flow are probably important.

The toxicities associated with IL-2 cover a large spectrum of symptoms and clinical signs and can be severe. Chills, rigors, malaise, nausea, vomiting, diarrhea, erythroderma, fluid retention, hypotension, and

mental status changes are the most common side effects associated with IL-2 therapy. The toxicities are due to a combination of a capillary leak syndrome and lymphocyte infiltration of tissues. These common side effects can be treated with appropriate monitoring, prophylactic antibiotic coverage, analgesics, antiemetic drugs and fluid resuscitation[39]. Over time, experience has resulted in a significant decrease in the number and intensity of toxicities associated with treatment of high-dose IL-2.

Despite its limited efficacy and high toxicity, high-dose IL-2 therapy is still in use. A recent phase II clinical trial has tested subjects for maintenance IL-2 and GM-CSF therapy after high-dose IL-2 therapy. This maintenance treatment appears to prolong partial response, and increases the rate of complete responses (Bernabe M, abstract presented at ASCO meeting 2013). Another phase II trial based on this IL-2 maintenance therapy schedule is currently recruiting melanoma patients (NCT01702896 see clinicaltrials.gov). Studies have been initiated that combine IL-2 with immunostimulatory antibodies, antiangiogenic therapy induced by tyrosine kinase inhibitors, or radiation therapies. A Phase II clinical trial in patients with advanced RCC exploring the efficacy and toxicity of sequential treatment with IL-2 and IFN-α followed by sorafenib showed clinical efficacy and manageable toxicity[40]. A clinical trial is now recruiting patients (NCT01884961) to test radiotherapy as an immunological booster in metastatic melanoma or RCC patients previously treated with high-dose IL2. In view of the recent superior success of immune checkpoint inhibitors, it seems unlikely that IL-2 will continue to be used in the clinic.

Introduction

TNF-α

TNF-α is secreted by activated monocytes and macrophages, and many other cells including B cells, T cells and fibroblasts[41]. There are two receptors for TNF-α, the type I and the type II receptor, both of which are members of the TNFR superfamily. TNF receptors are present on nearly all cell types with few exceptions such as erythrocytes and resting T cells[29]. TNF-α is a potent paracrine and endocrine mediator of inflammatory and immune functions. It is also known to regulate growth and differentiation of a wide variety of cell types.

Systemic administration of TNF-α causes severe side effects precluding its use. However, perfusion into limbs that harbor malignant melanoma or sarcoma, and that are isolated from the circulation during the treatment, confers substantial therapeutic benefit[42].

IL-12

IL-12 was identified as a factor secreted by a human EBV-transformed B-cell line. IL-12 is a heterodimer formed by two covalently linked glycosylated chains, p40 and p35. It is mainly produced by activated antigen-presenting cells[43]. The IL-12 receptor was detected on activated T or NK cells, but neither on B cells nor on resting T or NK cells[44]. *In vivo,* all T-cell subsets respond to IL-12 by IFN-γ production[45]. IL-12 enhances rapidly the cytotoxic ability of resting or activated NK cells[46] due to increased surface expression of adhesion molecules and increased number of granules containing molecules involved in the cytolytic process[47]. IL-12 also enhances the generation of CTL and their cytolytic activity[48]. It has a minimal effect on the proliferation of resting T cells, but enhances the

proliferation of T-cells activated by various stimuli, such as IL-2. However, IL-12 cannot induce T-cell proliferation at the same level as that induced by IL-2, and lymphocytes cannot be maintained in long-term culture with IL-12 alone[49]. IL-12 also exerts anti-angiogenic activity by increasing the production of IFN-γ, which in turn increases the production of CXCL10, which directly exerts the anti-angiogenic effect. These effects of IL-12 suggest a potent activity in anti-tumor immunity.

Systemic IL-12 administration elicits striking antitumor effects in mice, but clinical testing was interupted because severe toxicities were observed during a phase I dose escalation trial. The most common toxicities were flu-like symptoms and reversible hepatic toxicity for the low doses administered in bolus i.v. (3 ng/kg/day) but at higher doses (500 ng/kg/day), oral stomatitis and hepatotoxicity were observed and one death due to septicemia was reported[50].

GM-CSF

T cells, fibroblasts, endothelial cells and, to a lesser extent, eosinophils, neutrophils, and macrophages, secrete GM-CSF after activation by immune or inflammatory stimuli[51]. The GM-CSF receptor is a member of a family that includes the receptors for IL-3, G-CSF and EPO[52]. It is expressed on granulocytes and monocytes and their precursors, as well as on endothelial cells, fibroblasts and Langerhans cells. GM-CSF acts as a hematopoietic growth factor stimulating the proliferation and maturation of myeloid committed progenitor cells, thereby increasing the numbers of blood neutrophils, eosinophils and monocytes[53]. Apart from the stimulation

of proliferation, several functional activities of mature myeloid cells are enhanced[54]. Expression of MHC class II molecules is also increased[55].

GM-CSF was among the first colony-stimulating factors to enter clinical trials. GM-CSF and G-CSF are approved drugs that shorten the chemotherapy-related neutropenia, leading to reduction of infectious morbidity[29].

Systemic administration of GM-CSF was reported to confer some clinical advantages in the adjuvant setting. In melanoma, GM-CSF was administered subcutaneously in 28-day cycles, at a dose of 125 $\mu g/m^2$ delivered daily for 14 days followed by 14 days of rest. This treatment was well tolerated; only one subject discontinued the drug due to an adverse event (grade 2 : injection site reaction). In this study GM-CSF prolonged overall and disease-free survival in patients with stage III and IV melanoma who were clinically disease-free after surgical resection[56]. Prostate cancer patients with serologic progression after complete surgical resection received 250 $\mu g/m^2$/day of subcutaneous GM-CSF on days 1 through 14 of a 28-day cycle. GM-CSF showed an anti tumoral effect, as measured by PSA declines in the serum and modulation of PSA kinetics[57].

GM-CSF is now used in combination with cancer vaccines and with the anti CTLA-4 monoclonal antibody (mAb) ipilimumab (see section 2.4.1.1 below). A recent multicenter randomized phase II trial of GM-CSF plus ipilimumab versus ipilimumab alone in metastatic melanoma patients showed that more than two-thirds of patients who received the combination were still alive one year after the treatment compared with slightly more than half of patients with ipilimumab alone. Additionally, some serious side effects associated with ipilimumab were reduced in patients who received

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the combination[58]. These data argue in favor of combining cytokine therapy with immunostimulatory antibodies in future trials.

Overall, the systemic administration of pro-inflammatory cytokines is associated with specific and significant side effects that are generally consistent with their mode of action. Moreover, systemic cytokine administration has achieved only modest therapeutic benefits, possibly because this approach fails to reproduce the paracrine functions of cytokines produced in tissues, and because the narrow therapeutic margin of these drugs has prevented to use them at higher, more effective doses.

2.1.2 Local administration.

Peritumoral or intratumoral injection of pro-inflammatory cytokines, particularly IL-2, might enhance tumor rejection through a coordinated local host reaction composed of neutrophils, eosinophils, macrophages, NK cells, and lymphocytes[59].

Except for IL-2[60], peritumoral cytokine injection was not tested in patients. However, many pre-clinical studies using gene-transfer techniques to modify tumor cells have been tested to screen a large number of cytokines for their abilities to stimulate tumor rejection. Most of the animal studies involved transduction of tumor cell lines with vectors encoding cytokines, followed by implantation of the genetically modified tumor cells into syngeneic mice. Animals that rejected the cytokine-secreting tumor cells were evaluated for their ability to generate a protection against subsequent challenges with wild-type tumor cells. GM-CSF and IL-12 proved to be the

most potent in many tumor models[61,62]. IL-12 augmented tumor rejection by promoting T-helper 1 responses, associated with high IFN-γ production, increased lymphocyte cytotoxicity, and angiogenesis inhibition[63]. GM-CSF increased the activation of DCs, macrophages, granulocytes and NKT cells, resulting among other in improved tumor antigen presentation[64].
T-helper 2 cytokines, such as IL-4, IL-5, IL-6, IL-10, and IL-13, also contributed to tumor rejection by boosting eosinophil function and increasing antibody concentrations[65].

A combination of several cytokines injected peritumorally or perilymphatically has been used in a phase II study in patients with head and neck cancer. The interleukin mixture, branded as Multikine®, was prepared from selected human blood mononuclear cells stimulated by a mitogen. Subsequently, supernatant was aseptically harvested and the final injectable agent was tested (by ELISA) for the presence of five cytokines: IL-2, IL-1α, GM-CSF, IFN-α, and TNF-α[66]. Patients were injected with Multikine® as local neoadjuvant immunotherapy before surgery or radiation therapy. Multikine® induced T-cell migration into cancer cell nests, as shown by a markedly altered composition of tumor-infiltrating mononuclear cells, and increased the $CD4^+/CD8^+$ ratio, as compared with control patients[67]. In addition, Multikine® injections increased the number of tumor cells undergoing cell cycle entry, an effect that may modulate the susceptibility of cancer cells to radiation therapy and chemotherapy. Multikine® is currently used in a phase III clinical trial (the IT-MATTERS trial, NCT01265849). This clinical study is the first that uses a "neoimmunoadjuvant" treatment, i.e. applied before conventional therapy.

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2.2 Toll-like-receptor (TLR) agonists:

TLRs play an important role in activating immune responses. They recognize conserved pathogen-associated molecular patterns expressed on a wide array of microbes, as well as disease-associated molecular patterns, derived from endogenous products released from stressed or dying cells[68]. TLR1, -2, -4, -5, -6, and -10 are expressed on the cell surface, whereas TLR3, -7, -8, and -9 are associated with endosomes[27].

In humans, TLRs are expressed by many different cell types. Most TLRs are expressed primarily on monocytes, mature macrophages and DCs[69]. TLR stimulation on these cells induces the surface expression of costimulatory molecules including B7.1, B7.2 and CD40, and the secretion of IL-12[70], which are all involved in the induction of primary innate immune responses.

Various TLRs have been reported to be expressed by human tumor cells. The engagement of TLRs on tumors cells can impact tumor growth by various mechanisms, including apoptosis and a potentiation of the effects of chemotherapy[71]. The activation of TLR3 and TLR5 on tumor cells appears to have mostly an antitumoral effect, whereas that of TLR4, -7, -8, and -9 has primarily a protumoral activity.

The engagement of a given TLR on a particular tumor type induces cell death, whereas it promotes cell survival, induces proliferation or leads to chemoresistance in another type of tumor. Therefore, the identification of optimal TLR agonists for cancer immunotherapy requires a careful documentation of the TLR expression profile and of all the functional consequences of TLR signaling in a specific type of cancer[72].

The TLR activating agents that are approved by the FDA for use in

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cancer patients are BCG (which stimulates TLR2, TLR3, TLR4 and possibly TLR9), MPL (a TLR4 agonist), and imiquimod (a TLR7 agonist).

Table 1 shows an overview of TLRs.

TLR	Adaptor molecule	Natural ligand/agonist	Source of ligand	Ligand used in the clinic
TLR1-TLR2	MyD88	Peptidoglycans, Zymosan, lipopotein	Bacteria, Fungi, synthetic	BCG
TLR2-TLR6	MyD88	Diacylate lipopeptides, uric acid, fibronectin, HSPs	Bacteria, synthetic	BCG
TLR3	TRIF	dsRNA, Poly I:C	virus, synthetic	Poly A:U
TLR4	MyD88 or TRIF	LPS, lipoteichoic acid	Bacteria	BCG, MPL
TLR5	MyD88	Flagellin	Bacteria	
TLR7-TLR8	MyD88	ssRNA, CpG	Virus, synthetic	Imiquimod (Aldara), 852A, PF-3512676
TLR9	MyD88	Unmethylated CpG DNA	Bacteria, virus, synthetic	BCG, CpG
TLR10	MyD88	Unknown natural ligand	Synthetic	/

The most impressive antitumor responses obtained with TLR ligands are

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observed in patients with superficial bladder cancer treated with intravesical instillations of BCG (Bacille Calmettes-Guérin). BCG is an attenuated strain of *Mycobacterium bovis,* which contains TLR agonists, and is routinely used as a vaccine against tuberculosis. Treatment with BCG results in the expression on urothelial carcinoma cells of MHC class II and costimulatory molecules including CD86 and ICAM-1[73]. The infection of these cells with BCG also induces cell death and reduced proliferation and motility. BCG has also been administered as an adjuvant with autologous whole cell tumor lysate vaccines in patients with colorectal cancer[74], melanoma[75] and renal cell cancer[76].

The TLR7 agonist imiquimod (Aldara®) used as a topical treatment proved to be effective against genital warts and primary skin tumors such as basal cell carcinomas[77]. Recent results from a Phase II study in breast cancer patients with chest-wall recurrences or skin metastases revealed modest antitumor effects with 2 of 10 patients achieving a partial response[78]. Imiquimod treatment is generally well tolerated, even thought it causes local inflammation.

Another TLR7 agonist (852A) has also been tested for its ability to treat metastatic breast, ovarian, endometrial, or cervical cancer in patients not responding to standard treatment. In this study, most patients exhibited increased cytokine levels in response to 825A treatment (NCT00319748).

In a randomized Phase II trial, the TLR9 agonist PF-3512676 (or CPG7909) was administered to patients receiving standard first-line chemotherapy for non-small cell lung cancer (NSCLC). The objective response rate in patients who received the complete treatment was 30% as compared with 19% in those who received chemotherapy alone[79]. The Phase III clinical trial testing

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the same TLR9 agonist was stopped following the drug's inability to demonstrate efficacy in combination with standard chemotherapy for NSCLC.

In conclusion, TLR agonists can be used as single agent immunomodulators, as vaccine adjuvants and in combination with conventional cancer therapies.

2.3 Oncolytic virotherapy.

Oncolytic virotherapy has recently emerged as a new immunotherapeutic modality. The term "oncolytic viruses" is generally employed to indicate non-pathogenic viral particles that specifically infect cancer cells and cause their lysis, while leaving non-malignant tissues unaffected [80]. Oncolytic viruses can be genetically engineered to produce various immunostimulatory factors, thereby mediating more robust therapeutic effects than their non-engineered counterparts[81].

Intratumoral or intravenous injections of oncolytic viruses such as herpes simplex-, pox-, parvo-, or adenoviruses may reactivate a quiescent antitumor immune response. By generating tumor cell debris *in situ*, oncolytic viruses may overcome tumor resistance mechanisms and release tumor antigens that can be processed by antigen presenting cells and activate anti-tumor T cells. Oncolytic virues are able to kill human tumor cells and stimulate an anti-tumor immune response through increased presentation of tumor-associated antigens, maturation of DCs, and release of pro-inflammatory cytokines.

The safety and preliminary antineoplastic potential of naturally occurring

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and genetically modified oncolytic viruses, alone or combined with immunotherapeutic agents or conventional treatments, have been tested in multiple clinical trials, in patients affected by a wide variety of (mostly solid) tumors[82]. The majority of these studies were phase I/II trials, most often reporting reassuring safety data and sporadic antineoplastic activity, even when oncolytic viruses were employed as standalone therapeutic interventions.

The most encouraging results have been recorded with talimogene laherparepvec (also known as T-VEC), an oncolytic γ34.5-deficient variant of HSV engineered to drive the expression of GM-CSF by infected cells[83]. As a single agent administered intratumorally, T-VEC induced the complete regression of both injected and distant lesions in 8 out of 50 metastatic melanoma patients with superficial lesions[84]. A similar efficacy has been observed with JX954, an oncolytic vaccinia virus engineered to express GM-CSF that was shown to induce objective responses in a significant proportion of hepatocellular carcinoma patients[85].

Many ongoing clinical trials are assessing the safety and antineoplastic potential of oncolytic viruses, including a wild-type reovirus (serotype 3, Reolysin®)[86] administered intravenously (i.v.) or intraperitoneally, often in combination with conventional therapeutic regimens, to patients affected by various types of solid tumors.

In conclusion, accumulating clinical evidence indicates that oncolytic virotherapy is generally well tolerated and, at least under specific circumstances, exerts some antineoplastic effects.

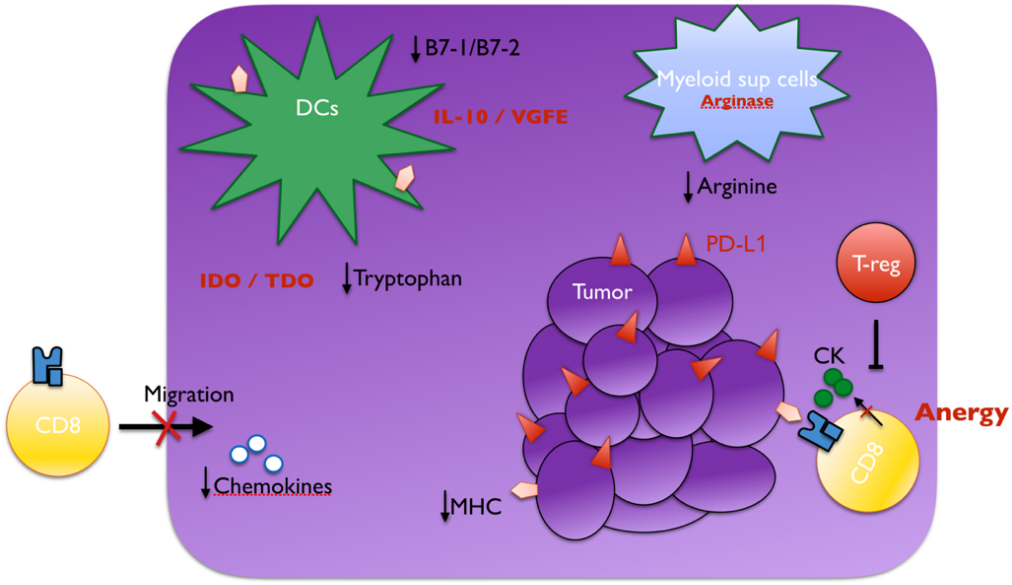
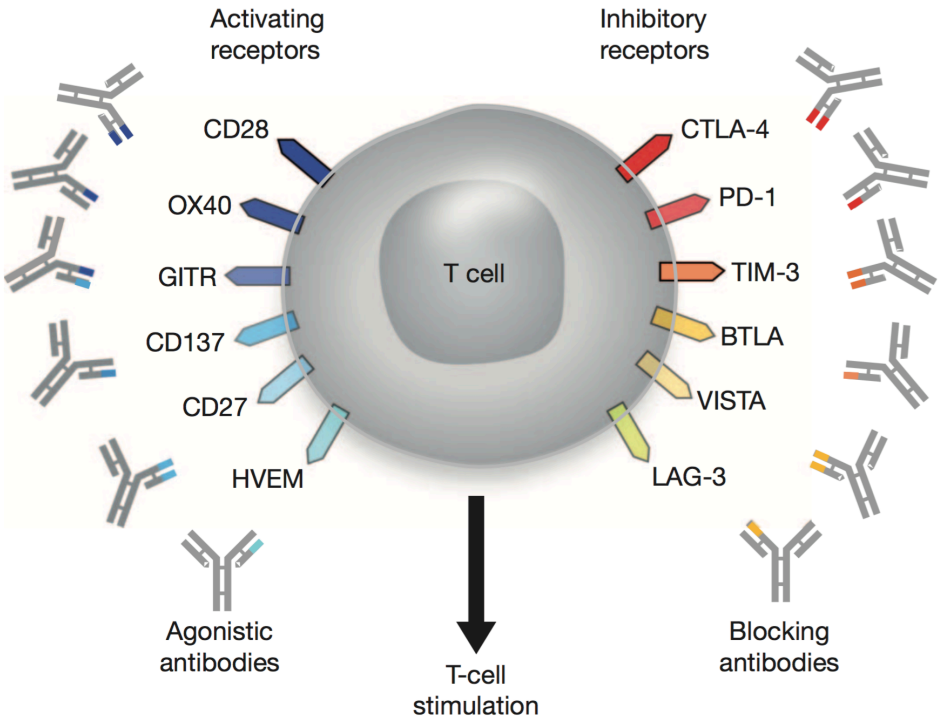


Figure 3 : Tumor bed is an immunosuppressive environment.
Tumors have developed a number of different strategies to escape immune surveillance, many of which operate in parallel.

2.4 Stimulation of spontaneous antitumor T cell responses.

Human tumors express tumor antigens and elicit spontaneous immune responses. Many tumors continue to progress despite these responses. Indeed, tumors have developed a number of different strategies to escape immune surveillance, many of which operate in parallel (Figure 3).

Among all those strategies, tumor cells can directly escape T-cell recognition by downregulating MHC class I or by disabling other components of the antigen processing machinery[87]. Tumor cells may also upregulate surface ligands, which mediate T-cell anergy, including PD-L1 and other ligands to inhibitory T-cell receptors. A variety of cells infiltrating the tumor are also able to suppress T-cell function, including Treg cells, tumor-associated macrophages and myeloid-derived suppressor cells, which have the ability to suppress T and NK-cell activation through several mechanisms including secretion of paracrine mediators like adenosine, NO, arginase, IL-10, TGF-β and VEGF[88]. Such mediators exert multiple immunosuppressive activities, such as suppression of stimulatory DCs, inhibition of T-cell migration into the tumor bed, suppression of effector T-cell activation, and enhancement of the function of Treg cells[89]. Tumor cells can also express enzymes, such as indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO), which degrade tryptophan and limit T-cell function[23,90]. All those mechanisms of immune suppression in tumors may represent potential therapeutic targets. Here we will focus on strategies used in the clinic to inhibit the immunosupressive mechanisms developed by the tumors.



From Mellman et al. Nature 2011, 480 (480-488)

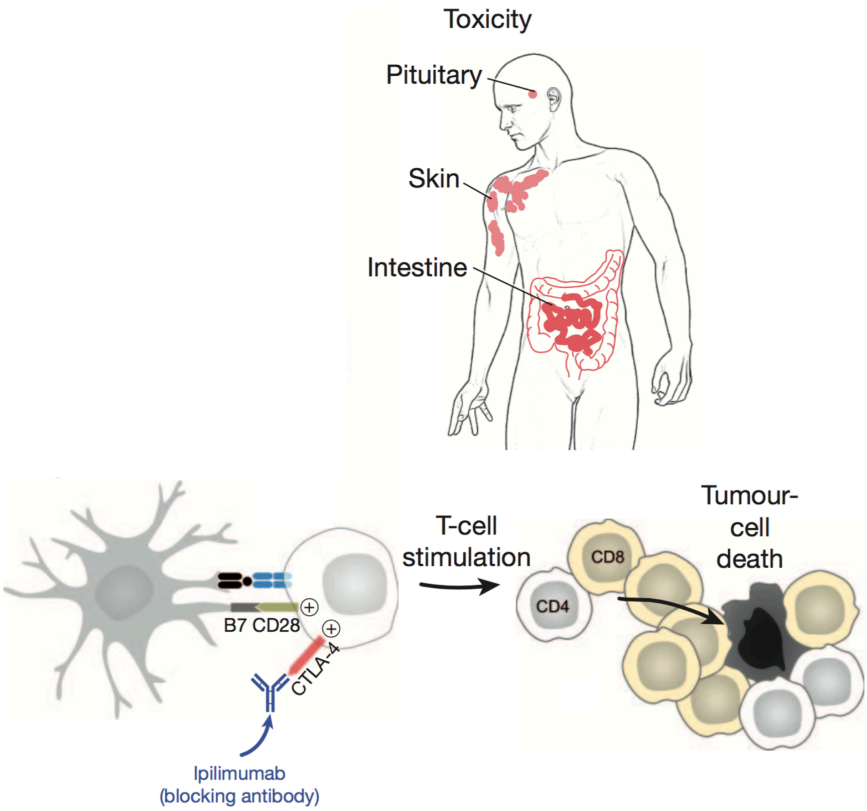
Figure 4 : Activating or inhibitory receptors as potential targets for antibody-mediated immunotherapy
Several molecules expressed by T lymphocytes can either increase or decrease the T cell activation that follows antigen recognition by the T cell receptor. A set of such costimulatory or inhibitory molecules is shown here.

2.4.1 (Re)activation of anti-tumoral T cell responses by either activating or inhibitory receptors:

Several molecules expressed by T lymphocytes can either increase or decrease the T cell activation that follows antigen recognition by the T cell receptor. A set of such costimulatory or inhibitory molecules is shown in Figure 4. Several monoclonal antibodies (mAb) have been developed that engage these surface molecules (Table 2).

Table 2: Monoclonal Ab that increase or decrease T cell activation in patients.

Targets	mAb	Isotype	Source	Activity
CD27	CDX-1127	n.a.	Human	Agonist
CD40	Chi Lob 7/4	IgG1	Chimeric	Agonist
	CP-870,893	IgG2	Human	Agonist
	Dacetuzumab	IgG1	Humanized	Agonist
	Lucatumumab	IgG1	Human	Agonist
PD-L1 / B7-H1	BMS-936559	IgG4	Human	Antagonist
	MEDI4736	IgG1	Human	Antagonist
	MPDL3280A	IgG1	Human	Antagonist
	MSB0010718C	n.a.	Human	Antagonist
CTLA4	Ipilimumab	IgG1k	Human	Antagonist
	Tremelimumab	IgG2	Human	Antagonist
PD-1	AMP-224	IgG1	Human	Antagonist
	Pembrolizumab	IgG4	Humanized	Antagonist
	Nivolumab	IgG4	Human	Antagonist
	Pidilizumab	IgG1k	Humanized	Antagonist
PD-L2	rHIgM12B7	IgM	Human	Agonist
OX40	9B12	IgG1	Murine	Agonist
	MEDI6469	n.a.	Murine	Agonist
GITR	TRX518	IgG1	Humanized	Agonist



Adapted from Mellman et al. Nature 2011, 480 (480-488)

Figure 5: Biological activities of anti CTLA-4 mAb
Ipilimumab blocks CTLA-4 function, thereby allowing enhanced T-cell stimulation and a more potent antitumour reaction. CTLA-4 antibody blockade compromises tolerance to some normal tissue antigens, provoking inflammatory toxicities. Up to 23% of patients treated with ipilimumab develop serious adverse events including colitis, hypophysitis, hepatitis and skin inflammation.

2.4.1.1 Blockade of inhibitory T-cell receptors.

-Anti cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) mAb :

CTLA-4 is a key negative regulator that is expressed on activated T cells where it competes with CD28 for binding to members of the B7 family of molecules expressed by antigen-presenting cells (including DCs). The ligation of CTLA-4 to B7 prevents the formation of the immunological synapse and decreases T cell activation and expansion, controlling the progress of an immune response and attenuating the chances for chronic autoimmunity[91] (Figure 5). The fundamental importance of CTLA-4 in controlling T-cell function is illustrated by the phenotype of *Ctla4-/-* mice, which die of an aggressive lymphoproliferative disorder at a young age[92]. CTLA-4 ligation is also important for the immunosuppressive function of Treg cells.

The rationale for using anti-CTLA-4 antibodies in cancer immunotherapy is the presence in cancer patients of antitumor T cells that appear to be dysfunctional. Indeed it is well-known that cancer (and especially melanoma) patients have TILs with specificity for tumor antigens. Such TILs were shown to express CTLA-4, as shown in Hodgkin disease, melanoma and ovarian carcinoma[93,94].

An anti-CTLA-4-blocking mAb was tested clinically for the first time in 2003. In this pioneering study, 14 patients with metastatic melanoma were treated with serial i.v. administrations of a fully human anti-CTLA-4 mAb in conjunction with s.c. vaccination with two antigenic peptides derived from the gp100 melanoma-associated antigen. The blockade of CTLA-4 induced autoimmune manifestations involving multiple organs in six

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patients (43%), and mediated objective cancer regression in three patients (two complete and one partial responses)[95]. Since then, two different anti-CTLA-4-blocking mAbs have been used in the clinic.

Ipilimumab was first used in a phase II trial which failed to show tumor regression, but a large randomized phase III trial was later initiated in late stage metastatic melanoma[96]. In this trial, 676 patients with unresectable stage III or IV melanoma received either a vaccine containing two gp100 peptides, or ipilimumab alone, or ipilimumab plus the gp100 vaccine. A twofold survival benefit was detected at 12 months in both arms involving ipilimumab. This benefit was maintained after 2.5 years and included several complete responses. In a second phase III trial involving 502 patients with previously untreated metastatic melanoma, the addition of ipilimumab to standard dacarbazine therapy was shown to improve overall survival compared with dacarbazine alone[97].

Recent data support the durability of long-term survival in ipilimumab-treated patients with advanced melanoma. Multiple studies of long term overall survival (>10 years) data for ipilimumab-treated patients with advanced melanoma were pooled. A plateau in the survival curve, beginning at approximately 3 years, was observed, which was independent of prior therapy or ipilimumab dose[98].

Those results led to the approval of ipilimumab by the FDA in March 2011 for the treatment of metastatic melanoma patients (now commercialized by Bristol–Myers Squibb under the trade mark Yervoy®), this drug has now also an EMA approval.

Tremelimumab, the other anti-CTLA-4 mAb, used alone or combined with a TLR9 agonist, has recently been tested in patients with

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metastatic melanoma or other advanced neoplasms[99]. It was well tolerated, but measurable clinical responses were observed in only 2 out of 21 patients. Similarly, in a large randomized phase III study in metastatic melanoma patients, no statistically significant survival advantage could be documented for tremelimumab vs. standard-of-care chemotherapy[100]. Alongside, the safety and efficacy of tremelimumab are being assessed in cohorts of patients with malignant mesothelioma, liver cancer and various other solid neoplasms.

Ipilimumab and tremelimumab have also been tested in many clinical trials for patients with other malignancies than melanoma: together with standard chemotherapies or as first-line therapy in patients with advanced small-cell lung cancer[101]; alone or in combination with a GM-CSF-secreting allogeneic vaccine (GVAX) in pancreatic ductal adenocarcinoma patients[102]; in a phase I/II trial testing ipilimumab a similar GVAX vaccine in prostate carcinoma patients[103]. In these trials the administration of either of the two anti-CTLA-4 mAbs was well tolerated and associated with promising clinical responses.

Since 2013 official sources listed more than 60 clinical trials launched to evaluate the therapeutic profile of immunostimulatory mAbs in patients affected by melanoma, various hematological malignancies and various advanced/metastatic solid tumors (source http://www.clinicaltrials.gov). Due to its approved status, more than half of these studies are investigating the clinical profile of ipilimumab, either alone or combined with radiotherapy, conventional chemotherapies, targeted anticancer drugs or other immunostimulatory agents.

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Even if the recent results are highly encouraging, using anti CTLA-4 mAbs presents clinical challenges. First, the true clinical potential of these mAbs employed as single agents is limited. So far, ipilimumab has been shown to exert robust therapeutic effects only in a minority of patients affected by melanoma[96], a tumor type that is highly immunogenic and responds to other unspecific immunostimulants such as high-dose IL-2. Clinical efficacy has only been observed with ipilimumab and not with the other CTLA4-targeting mAb, tremelimumab[100].

A second concern are the frequent and severe toxicities[104]. Up to 23% of patients treated with ipilimumab develop serious adverse events including colitis, hypophysitis[105], hepatitis and skin inflammation. These adverse events have a strong inflammatory component, and probably include autoimmune reactions. The spectrum of toxicities associated with ipilimumab is different from that of standard cancer treatments and closer to what is observed in inflammatory disorders (Figure 5).

A third clinical challenge with ipilimumab relates to the kinetics of the antitumor response. As with other immunotherapies, the stimulation of T-cell responses and the resulting clinical benefits may take several months, as compared to a few days or weeks for standard cytotoxic therapies. Moreover, tumors may increase in size during this period, and that may be a result of the evolving inflammatory reaction. Such evolutions have led most clinical investigators to conclude that the standard criteria for tumor size (such as RECIST or the modified WHO criteria) could not appropriately document the clinical responses to immunotherapy. This unusual pattern of respons led to the proposal of new clinical endpoints for cancer immunotherapy trials[106].

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Finally, increasing clinical results indicate that anti-PD1 mAbs are clearly superior to ipilimumab, both in terms of anti-tumoral efficacy and clinical tolerance[107].

There is no doubt that the introduction of ipilimumab into clinical practice has been the most important development for cancer immunotherapy to date. First because it offers to some patients at a terminal stage of these disease clinical benefits and longer survival, and second because it validates the concept that activating the T-cell compartment can provide a significant therapeutic effect. In the future, additional benefits may come from using those immunostimulatory mAbs to potentiate antitumor T-cell responses elicited by more specific immunotherapeutic interventions.

-Next generation of T-cells mAb modulator:

In parallel with ipilimumab, mAb that block other T cell inhibitory pathways were developed. A number of T cell other receptors are candidate targets for such antibodies (Figure 4). Like CTLA-4, programmed cell death 1 (PD-1) is expressed by activated T-cells[108]. It binds to its ligands PD-L1/L2 that can be expressed on target cells, thereby inhibiting T cell functions. A variety of tumors including melanoma, glioblastoma, ovarian, renal and hepatocellular carcinomas have been found to express PD-L1 or PD-L2. These expressions correlated with a poor prognosis[109,110].

In the last few years, promising results have been obtained in clinical trials investigating the safety and therapeutic profiles of the anti-PD-1 mAbs nivolumab, a human IgG4[111], and pembrolizumab (formerly lambrolizumab), a humanized IgG4[112], and of the anti-PD-L1 mAb

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MPDL3280A, a human IgG1[113] engineered to neutralize antibody- and complement-dependent cytotoxicity.

Nivolumab was tested in a phase I study involving 39 patients with advanced melanoma, colorectal cancer, castration-resistant prostate cancer, non–small-cell lung cancer or renal cell carcinoma. One durable complete response and two partial responses were seen. Two additional patients had significant tumor regressions. The treatment was well tolerated, with only one serious adverse event being documented[114].

The safety and efficacy of PD-1-targeting mAbs have also recently been investigated in 2 distinct phase I clinical trials in melanoma. Patients received either pembrolizumab as a single agent[115], or nivolumab coupled to ipilimumab. The combined treatment was associated with an objective response in 53% of patients, and with a reduction in tumor burden of 80%[116]. The administration of PD-1-targeting mAbs alone was associated with relatively mild side effects[115], conversely, the co-administration of nivolumab and ipilimumab provoked more severe adverse reactions in a consistent proportion of melanoma patients[116]. Such side effects were qualitatively similar to those previously associated with either nivolumab or ipilimumab monotherapy and were generally manageable.

The FDA has recently approved pembrolizumab (Keytruda®) as a treatment for patients with advanced melanoma following progression on prior therapies. This approval was based on response rates observed in a clinical trial including 173 patients with ipilimumab-refractory advanced melanoma. At the recommended dose of 2 mg/kg, the overall response rate was 24%, with a response duration lasting from 1.4 to 8.5 months. The

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treatment was well tolerated, with no drug-related deaths. The most common drug-related adverse events of any grade were fatigue, pruritus, and rash[117]. Some immune-mediated adverse reactions were observed including pneumonitis, colitis, hepatitis, hypophysitis, nephritis, hyperthyroidism and hypothyroidism. However they occured in less than 2% of the patients treated. This reduced toxicity observed with PD-1 blockade as compared with CTLA-4 blockade is consistent with the milder autoimmune phenotype seen in *PD-1*$^{-/-}$ vs *Ctla4*$^{-/-}$ mice[118].
Over 90 clinical trials are currently under way exploring pembrolizumab across a variety of tumor types (source http://www.clinicaltrials.gov).

The anti PD-L1 monoclonal antibody MPDL3280A was tested in 207 patients with selected advanced cancers (NSCLC, melanoma, colorectal cancer, renal-cell cancer, ovarian cancer, pancreatic cancer, gastric cancer, and breast cancer). Among evaluable patients, an objective response (a complete or partial response) was observed in 12.6% of patients. Responses lasted for
1 year or more in 8 of 16 patients with at least 1 year of follow-up. Toxic effects related to treatment occurred in 9% of patients[119].

LAG-3 is another inhibitory receptor present on T cells, its effect being apparently similar to CTLA-4. It limits the activity of CD4+ and CD8+ T cells, and increases the activity of Treg cells[120]. Blockade of the LAG-3/MHC Class II interaction using a LAG-3 Ig fusion protein (IMP321) is being evaluated in phase I clinical trials, in combination with standard chemotherapy for metastatic breast carcinoma[121] and advanced pancreatic

adenocarcinoma[122] patients, or alone in patients with advanced renal cell carcinoma[123]. No severe adverse events have been attributed to IMP321 until now. The absence of toxicity and the demonstration of activity warrant further studies of IMP321.

The clinical potential of mAbs targeting natural killer (NK) cells rather than T lymphocytes has recently been assessed. Killer inhibitory receptors (KIRs) are inhibitory receptors that prevent NK cell activation upon binding their ligands, most of which being HLA-C molecules. IPH2101 is an anti-killer inhibitory receptor (anti-KIR) mAb that blocks KIR-mediated inhibition of NK cells and enhances their cytotoxicity against tumor cells. This mAb was tested as a single agent in patients with acute myeloid leukemia[124] or refractory multiple myeloma[125] in phase I sequential-cohort dose-escalation studies. It provoked limited side effects at doses that fully inhibited KIRs.

2.4.1.2 Stimulation of T-cell costimulatory receptors:

Agonist Abs can be used to increase T cell activation upon binding their ligands such as CD28 and the tumor necrosis factor receptor superfamily members OX40 (TNFRSF4)[126], GITR (TNFRSF18)[127] and CD40 (Figure 3).

The first-in-man study of the agonistic anti-CD28 Ab TGN1412 showed massive and immediate toxicities, including one death, consistent with a cytokine release syndrome[128]. This Ab has been withdrawn from development.

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The engagement of OX40 by ligands present on dendritic cells increases the proliferation, effector function and survival of T cells. A murine IgG1 that triggers OX40 signaling has been tested in patients with advanced cancers. A single course of the anti-OX40 mAb showed an acceptable toxicity profile and resulted in regression of at least one metastatic lesion in 12 of 30 patients treated[129], validating OX40 as a potential target in cancer patients.

Anti-CD40 (dacetuzumab, lucatumumab and CP-870,893) mAbs have been tested in several phase I trials, either in patients with chronic lymphocytic leukemia (CLL) or refractory multiple myeloma, with lucatumumab as a single agent[130]; in patients with advanced pancreatic ductal adenocarcinoma, with CP-870,893 in combination with gemcitabine-based chemotherapy[131] ; in patients with advanced solid tumors, with CP-870,893 associated with chemotherapy[132] and in patients with refractory diffuse large B-cell lymphoma, with dacetuzumab in combination with rituximab and gemcitabine[133]. Overall, these immunostimulatory mAbs were well tolerated but exerted limited clinical efficacy.

In conclusion, immunomodulatory mAbs have shown impressive results and hold great promise, either as stand-alone drugs or to potentiate anti-tumor immune responses elicited by antigen-specific immunotherapies such as vaccines, dendritic cell-based approaches and adoptive T-cell transfer. An important question remains whether the effector function of TILs co-expressing multiple inhibitory/activating receptors can be fully recovered by targeting a single receptor, or whether combinations of checkpoint blockade/stimulation will be required for optimal immune activation.

2.4.2 Inhibiting immunosuppressive mechanisms within tumors.

Other approaches to increase T-cell activity consist in the inactivation or depletion of immunosuppressive cells. Several cell types have immunosuppressive capacties, such as regulatory T cells (Treg), myeloid-derived suppressor cells, mesenchymal stem cells, tumor-associated macrophages and tolerogenic DCs.

Tregs have the ability to suppress both the proliferation and effector functions of T cells by different mechanisms[134]. Whereas no specific surface marker of human Treg cells has yet been identified, $CD4^+$ T cells with a $CD25^+FOXP3^+$ phenotype are generally considered as Treg[135]. In humans, this definition includes regulatory T cells and activated $CD4^+$ T cells. The most specific marker of human Treg up to now is an epigenetic marker: the demethylation of the first intron of the *FOXP3* gene in $CD4^+$ cells[136].

In many tumor beds, infiltrating Treg cells may counteract effector T-cell functions.

Different treatments aimed at reducing the number of Treg have been administered to cancer patients: cyclophosphamide, the anti-CD25 mAb daclizumab, and denileukin diftitox (Ontak®).

Cyclophosphamide injected at low doses might preferentially target Treg and attenuate their activity in the context of immunization protocols[137]. High doses of cyclophosphamide have been tested in melanoma patients in combination with total body irradiation as a depletion protocol before adoptive T cell transfer and IL-2 therapy. Eighteen of the 25 patients who have received this combined treatment experienced tumor rejection but also severe side effects[138]. Whether some of those cyclophosphamide-mediated effects are due to Treg depletion is unknown.

The anti-CD25 antibody daclizumab depletes Treg and activated T cells, which also express CD25[139]. The administration of daclizumab had no significant effect on the progression-free survival of melanoma patients vaccinated with mature dendritic cells pulsed with tumor peptide[140]. Furthermore, it appeared to blunt the anti-vaccine T cell response, as no anti-vaccine $CD8^+$ T cells were detected in any of the daclizumab-treated patients. This effect is probably a consequence of the transient expression of CD25 on the surface of activated T cells.

Denileukin diftitox (Ontak®) is an engineered protein combining IL-2 and diphtheria toxin. This protein binds to CD25 and introduces the diphtheria toxin into cells that express this receptor, thereby killing the cells. It is approved for the treatment of cutaneous T-cell lymphoma. It was shown to deplete Tregs and to increase endogenous anti-tumor immunity and the efficacy of active immunotherapy in experimental tumor models[141]. It was only used in one pilot clinical study, showing a reduction in circulating Treg cells of cancer patients[142].

Soluble inhibitory molecules:

The tumor microenvironment can produce or deplete soluble mediators that can influence T-cell function. Macrophages and DC present in the tumor bed can express amino-acid depleting enzymes such as IDO, TDO, arginase and NO synthase[143]. Activated macrophages can secrete TGF-β, and activate latent TGF-β into active TGF-β[144]. Macrophages and cancer cells can express cyclooxygenase 2 (COX2), which metabolizes arachidonic acid in prostaglandin E2 (PGE2)[145], an immunosuppressive molecule.

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-Enzymes that degrade aminoacids

IDO catalyzes the first step in tryptophan catabolism along the kynurenine pathway. It is constitutively expressed in a variety of human tumors and is upregulated in many cells in response to IFN-γ. Breakdown of tryptophan inside IDO-expressing cells results in the consumption of available tryptophan in the local tumor environment and thus in a deprivation of this essential aminoacid for the T cells[146]. Moreover, the metabolites generated by IDO, such as kynurenine, can induce T cell hyporesponsiveness and death, and render dendritic cells immunosuppressive[147]. Several IDO inhibitors have been identified, including some that are in clinical development. TDO is another tryptophan-degrading enzyme that is normally expressed in the liver, and has also been found in many types of human malignancies[148].

Two other enzymes that degrade amino acids are expressed by myeloid-derived suppressor cells and could play an immunosuppressive role in cancer: arginase and NO-synthase. Both enzymes metabolize arginine, an essential aminoacid for T-cells. Drugs commonly used in the clinic such as sildenafil, a phosphodiesterase-5 inhibitor used as a vasodilator, were shown to down-regulate arginase and NO synthase and to restore in vitro the proliferation of T cells isolated from patients with multiple myeloma or head-and-neck cancer[149].

-TGFβ

TGF-β is classically described as immunosuppressive[144], but it has pleiotropic effects whose impact on cancer are multiple[150]. TGF-β is considered as an important therapeutic target. Three phase I clinical trials

with the anti-TGF-β antibody GC1008 (fresolimumab) were recently conducted in patients with melanoma and renal cell carcinoma[151], and with relapsing malignant mesothelioma[152]. In patients with advanced malignant melanoma and renal cell carcinoma, multiple doses of GC1008 demonstrated acceptable safety and preliminary evidence of antitumor activity, warranting further studies either as a single agent or in combination treatments.

-PGE2

PGE2 was shown to inhibit *in vitro* human T cell proliferation and production of IFN-γ and IL-2, while it spared the production of the Th2 cytokine IL-4[153]. These observations assimilated PGE2 to an immunosuppressive molecule against Th1 responses. High expression of COX2 in cancers was reported to be associated with a shorter survival[154]. Non-steroidal anti-inflammatory drugs, which are COX inhibitors, have been developed to inhibit prostaglandin production. They could reduce PGE2 at the tumor site, with benefit on tumor fate[145,155].

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3. Immunotherapy with the use of tumor antigens

3.1 Cancer vaccines

Cancer vaccines can be preventive or therapeutic.

3.1.1 Prophylactic vaccines:

Prophylactic vaccines aim at preventing the appearance of a new cancer. Available prophylactic cancer vaccines target infectious agents that cause cancer. Like traditional vaccines, they contain antigens of the infectious agent that are recognized as foreign by the immune system. Prophylactic cancer vaccines have been used with success against cancers caused by viruses such as the hepatitis B virus (HBV) and the human papilloma virus (HPV).

Chronic HBV infection is the most common viral cause of liver disease worldwide, and hepatocellular carcinoma (HCC) arising in the setting of cirrhosis is the main consequence of chronic HBV infection. The most effective measure of prevention against HBV-related HCC is preventive vaccination against HBV. The FDA approved the first HBV vaccine in 1981, making it the first preventive cancer vaccine to be successfully developed and marketed. Through the reduction of HBV seroprevalence in the general population, the incidence of HCC declines significantly, especially in areas where HBV is endemic[156].

In 2006, the FDA approved the vaccine Gardasil®, which protects against infection by 4 serotypes of pathogenic human papillomaviruses (HPV), including the oncogenic types 16 and 18, which are responsible for approximately 70% of all cases of cervical cancer worldwide[157] and for

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HPV-associated vulvar and vaginal cancers. The vaccine is made of virus-like particles. Unlike traditional vaccines, which are often composed of attenuated whole microbes, these virus-like particles are not infectious. They stimulate the production of antibodies against HPV types 6, 11, 16 and 18. A second anti-HPV vaccine, named Cervarix®, has also been developed. It contains HPV-16/18 antigens. The public health benefits of anti-HPV vaccines may extend beyond the reduction of the risks of cervical, vaginal and vulvar cancers. Indeed, chronic infection by HPV is also associated with cancers of the anus, penis, and oropharynx[158]. Prospective studies will tell us whether these vaccines also protect against these other HPV- associated tumors.

3.1.2 Therapeutic vaccines:

Therapeutic cancer vaccines aim at treating existing cancer, by enhancing pre-existing anti-tumor immune responses or by priming new responses. Vaccines remain an elegant approach to cancer immunotherapy, as they are easy to administer, cheap and often devoid of side effects[159].

Distinct vaccine platforms incorporate tumor antigens in different ways to activate tumor immunity. Vaccines can contain defined tumor antigens, such as the peptide-based vaccines, or contain undefined antigens, such as the vaccines containing whole tumor cells or tumor cell lysates. The first cancer vaccines were composed of whole autologous or allogeneic tumor cells that were irradiated or inactivated[160], but their clinical efficacy was modest. The molecular identification of human tumor antigens has provided a major stimulus for the development of various vaccines that could elicit tumor specific immunity without autoimmunity. Many vaccine approaches have

been tested in small numbers of patients, based mainly on synthetic peptides or recombinant proteins, 'naked' DNA, recombinant viruses, and dendritic cells.

3.1.2.1 Protein or peptide-based vaccines:

We will consider the 5 groups of tumor antigens described in the introduction.

-Antigens of high tumoral specificity:

-Antigens encoded by mutated genes:

Because most mutated antigens are caused by passenger mutations, they are unique to an individual tumor. Thus, using them in vaccines requires their identification for every new patient. Therefore, despite their immunogenicity, these antigens have not been used in therapeutic cancer vaccines until now.

One approach that may overcome these difficulties involves the infusion of autologous DC transfected with total mRNA from their patient's tumor. This was tested in a phase I/II trial in which 22 patients with advanced malignant melanoma were included, each receiving four weekly DC vaccinations. No serious adverse events were observed, and it was shown that the vaccine elicited T-cell responses against antigens encoded by the transfected tumor mRNA[161]. Regarding the tumor responses in this study, none of the patients showed regression of disease. All showed progression, except two with stable disease. Only one clinical trial is currently recruiting patients into this type of vaccination (NCT01456104 see clinical trial.gov).

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-Antigens encoded by cancer-germline genes:

Many antigenic peptides have been defined, mostly from MAGE and LAGE/NY-ESO-1 proteins[4].

These antigens have been used in many small phase I/II trials, often in metastatic melanoma patients.

These vaccines contained peptides[162], DCs incubated with peptides[163], recombinant proteins without[164] or with adjuvant[165], or recombinant poxviruses[166,167]. As expected from the tumoral specificity of the antigens, no toxicity related to the activity of anti-vaccine T cells, i.e. T cells directed at tumor antigens present in the vaccine, has been observed. Tumor regressions were observed only in a minority of the vaccinated patients. Table 3 shows all patients evaluable for tumor response, and the status after vaccination encoded as NR (no response), PR (partial response) or CR (complete response). If we consider all studies, 16% of the patients treated showed a partial or complete tumor response (PR or CR) considering the RECIST criteria.

The table is not exhaustive and only the studies with enough data to analyse the tumor response were included.

Table 3:

First author in publication	Tumor	Vaccine	Nr of patients treated	NR	PR	CR
Thurner et al.[163]	Metastatic Melanoma	Mage-3A1 peptide on pulsed mature DCs	11	5	6	0
Kruit et al.[164]	Metastatic Melanoma	recombinant MAGE-3 protein s.c.	26	25	1	0
Van Baren et al.	Metastatic Melanoma / NSCLC/ Head and neck carcinoma		40	37	3	0
Davis et al. [165]	Resected Melanoma (Study unplanned for tumor response analysis)	Recombinant NY-ESO-1 protein i.m.				
Marchand et al.[162]	Metastatic Melanoma	MAGE-3A1 peptide s.c.	25	18	4	3
Jäger et al.[167]	Various solid tumor	Recombinant vaccinia/fowlpox NY-ESO-1	16	13	2	1
TOTAL			118	98	16	4

Table 3 shows selected clinical reports in which patients were vaccinated with germline antigens, with the patients' status after vaccination encoded as NR (no response), PR (partial response) or CR (complete response). If we consider all these studies, 16% of the treated patients showed a clinical response.

A large phase III clinical trial (NCT00480025) has been carried out with a recombinant MAGE-A3 protein in combination with immunologic adjuvant AS15. The trial enrolled phase IB, II, or IIIA resectable non-small cell lung cancer (NSCLC) patients, whose tumor expressed *MAGE-A3*[168]. It was a randomized, double blind study designed to assess the impact of the vaccine on disease-free survival. Patients were separated in 2 groups, according to whether they received additional surgery or not. In each group, the patients received either the vaccine or a placebo on a 2 to 1 ratio[169]. The most common side effects were mild local (pain, redness, swelling) or systemic (fever, fatigue, muscle pain) reactions, observed within 24 hours from injection.

The trial was stopped recently, as the disease-free survival was not significantly extended in the vaccine groups as compared with the respective placebo groups.

Another similar phase III clinical trial (DERMA) was conducted with the same MAGEA3 protein vaccine in melanoma patients. Here also the trial was interrupted for the same reason.

-*Viral tumor antigens:*

The most studied oncoviral antigens are those encoded by the E6 and E7 oncoproteins of HPV-16. Clinical responses have been observed in patients with early stage vulvar intraepithelial neoplasia vaccinated with long peptides in incomplete Freund's adjuvant[170]. In this study, 20 women with HPV-16–positive, grade 3 vulvar intraepithelial neoplasia were vaccinated three or four times with a mix of long peptides from the HPV-16 viral oncoproteins E6 and E7. At 24 months of follow-up, 15 of 19 patients had

clinical responses, with a complete response in 9 of 19 patients. All patients had vaccine-induced T-cell responses, and post hoc analyses suggested that patients with a complete tumor response at 3 months had a significantly stronger proliferative $CD4^+$ T-cell response and a broader response of $CD8^+$ T cells than did patients without a complete response.

A vaccine developed by MGI Pharma Biologics (Eisai), ZYC101a (amolimogene), is currently under phase III clinical trial investigation for the treatment of HPV-associated cervical-dysplasia, which is a pre-cancerous stage. ZYC101a is a polymer-encapsulated DNA vaccine consisting of a plasmid expressing a chimeric peptide comprising immunogenic hybrid epitopes from E6 and E7[171]. ZYC101a has been used in a phase IIb trial (multicenter, placebo-controlled study) conducted in 161 patients with high-grade cervical dysplasia. Subjects were randomized into three groups, receiving three intramuscular doses of either placebo or 100 or 200 μg of ZYC101a. High-grade cervical dysplasia disappeared in 43% of patients treated with ZYC101a versus 27% of patients treated with placebo, but this difference was not statistically significant. This potential benefit was higher when evaluated in women younger than 25 years (70% and 23%, respectively; p=0.007). Adverse events were moderate and generally limited to the injection site[172]. A multicenter phase II/III clinical trial (NCT00264732) is ongoing in 13 to 25 year-old patients with high-grade intraepithelial lesions of the uterine cervix. The same vaccine is inactive in patients with metastatic cervical cancer[173].

Whether HPV vaccines are active against the 20% of head and neck tumors

that are associated with HPV is not known[174]. Vaccines containing antigens derived from the Epstein-Barr virus proteins LMP1 and LMP2 are actively explored against nasopharyngeal carcinoma[175].

-Antigens of low tumoral specificity:

-Antigens encoded by tissue differentiation genes:

Numerous clinical trials have used these antigens for vaccination, either with peptides alone or with immunological adjuvants[176-179], or with peptide-pulsed dendritic cells[180], recombinant proteins, recombinant viruses (fowlpox, vaccinia or adenovirus) [181,182] or naked DNA encoding such antigens[183]. Rosenberg and colleagues reviewed the different cancer vaccine trials conducted at the surgery branch of the National Cancer Institute between February 1995 and April 2004[184]. The analysis included 440 patients with metastatic cancer. Of these, 422 had metastatic melanoma and 18 had other types of cancer. In this meta-analysis, the authors used conventional oncologic criteria to evaluate clinical responses to the vaccines. The overall objective response rate for all vaccines was 2.6%. The same analysis was performed on 35 selected reports of vaccine trials conducted by other groups representative of the majority of published trials. This analysis included patients with multiple cancer types treated with the most common types of cancer vaccines, using mostly differentiation antigens but also some antigens encoded by cancer germline genes. Twenty-nine objective responses were reported for an objective response rate of 3.8%. Altogether, a 3.3% overall objective response rate was reported.

A phase III trial involving 185 patients with advanced melanoma was conducted in 21 centers. Patients were randomly assigned to receive either

IL-2 alone or IL-2 plus a vaccine comprising a gp100 peptide in incomplete Freund's adjuvant (Montanide ISA-51) followed by IL-2 alone. Patients treated with the gp100 peptide had a higher tumor response rate and longer progression-free survival than those receiving IL-2 alone[185].

As expected with these antigens of low tumoral specificity, strong T-cell responses to melanocyte differentiation antigens has led in rare cases to severe adverse events such as ocular and cutaneous autoimmunity[186].

Melanocyte differentiation antigens have also been used in adoptive transfer of T cells in melanoma patients[187], as mentioned below.

Non-melanoma cancer patients do not appear to have spontaneous T-cell responses against differentiation antigens expressed by their tumor, suggesting a unique immunogenicity profile for melanomas. Nevertheless, prostate-specific antigens have been used in vaccines against prostate cancer cells after prostatectomy. Indeed, prostatic tissue contains proteins that are absent from other tissues, such as prostate-specific membrane antigen (PSA) and prostatic acid phosphatase (PAP). Antigenic peptides derived from PSA or PAP have been described, which may be used for therapeutic immunization of patients with prostate cancer [188,189].

A clinical trial was conducted in 28 patients with locally advanced or metastatic prostate cancer, with an HLA-A2 peptide derived from PSA (146-154). Specific T-cell immunity to the peptide was observed in one-half of the vaccinated patients. T cells from vaccinated patients showed a PSA-peptide-specific cytolytic activity and predominantly expressed a T helper type-1 cytokine profile[190].

In 2010, the FDA approved sipuleucel-T, a customized cellular vaccine

composed of autologous monocytes pulsed with a fusion protein linking PAP and GM-CSF[191]. More details on this study are provided in the DC-based vaccine chapter.

-Antigens encoded by genes that are overexpressed in tumors:
These antigens are present on normal cells, and at a much higher level in tumor cells. The expression levels required for T-cell recognition are generally not known. The use of such antigens requires caution, as shown by the severe toxicity reported after adoptive transfer of T cells expressing a chimeric anti HER-2/neu receptor[192].

Gene *ERBB2* (HER-2/neu) is overexpressed in many epithelial tumors including ovarian and mammary carcinomas, due to increased transcription and also to gene amplification. Several antigenic peptides such as GP2 and E75 have been identified[4] and are used in vaccines in ongoing adjuvant or therapeutic clinical trials, without harmful side effects up to now[193,194].

Mucin-1 (MUC1) is another antigen that is overexpressed in many glandular epithelial tissues, especially in colorectal cancer, yet is minimally expressed in normal cells[195]. MUC1 is associated with cellular transformation and can confer resistance to genotoxic agents. High levels of MUC1 cell surface expression were reported to have immunosuppressive and anti-adhesive properties, which contribute to the MUC1 ability to promote tumor cell growth and survival. Moreover, MUC1 overexpression in colorectal cancer correlates with worse prognosis. MUC1 is therefore an attractive target for cancer immunotherapy. Vaccines incorporating MUC1 have induced

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immune responses against MUC1-expressing tumors[196]. Several MUC1-based therapeutic cancer vaccines are currently under investigation in colorectal, breast, renal and prostate cancer, and in NSCLC.
A large phase III clinical trial (NCT01462513) targeting MUC-1 is currently conducted with the BLP25 liposomal vaccine Stimuvax®, consisting of a MUC-1 peptide encapsulated in a liposomal delivery system. The trial is designed for patients with metastatic colorectal carcinoma who have undergone a complete resection of their primary tumor and a recent resection of their liver metastases with curative intent. The study endpoint is the comparative evaluation of recurrence-free survival time in vaccinated and control patients. Based on these results, BLP25 might be approved as an adjuvant therapy after curative resection of hepatic metastases in colorectal cancer patients.

Telomerase is a ribonucleoprotein complex highly expressed in almost all cancer cells. It contributes to their unlimited dividing capacity[197]. GV1001 is a vaccine based on immunogenic telomerase peptides. It is expected to prime patient's T cells to destroy cancerous cells while leaving normal cells unharmed. This vaccine is undergoing clinical development for hepatocellular carcinoma, NSCLC and pancreatic cancer. An ongoing phase III controlled trial (NCT00425360) will include 1110 patients with inoperable pancreatic cancer. Patients are randomized between either standard chemotherapy with concomitant GV1001 or chemotherapy followed or not by GV1001. The primary endpoints are survival and safety (Available from http://www.pharmexa.com Press Release number 14/2008). No toxicity or clinically severe adverse events related to the treatment have

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been observed so far[198], nevertheless telomerase is expressed in normal stem/progenitor cells that represent a concern for severe auto-immune toxicity.

The GV1001 vaccine was previously tested in advanced hepatocellular and breast cancers in two phase II trials that were stopped, whereas a phase II study in advanced NSCLC showed the induction of GV1001-specific immune responses associated with objective tumor responses[199].

There are several other promising « overexpressed » antigen candidates, such as WT-1 for hematologic malignancies and some solid tumors such as ovarian cancer[200], PRAME[201] and MELOE-1[202].

-Conclusion

Twenty years after the discovery of the first human tumor-specific antigen recognized by T cells, encoded by the *MAGE A1* gene, many other antigens have been identified, and numerous vaccine clinical trials have been conducted in patients with metastatic cancer. Peptide-based vaccine strategies are based on the administration of antigenic peptides, which are thought to bind to empty HLA molecules on antigen-presenting cells of the patient. It requires a prior knowledge of which tumor antigens are expressed by the tumor cells and of the HLA class I typing of the patient. An important factor for the choice of a vaccine antigen is its tumor specific expression. Another is the amount of antigen present at the surface of the tumor cells, which depends on the level of expression of the gene encoding the antigenic peptide and HLA molecule, the mRNA stability and translation, and the efficiency of antigen processing. So many factors are

involved that predictions are only indicative, and it is still important to check *in vitro* the proper recognition of tumor cells by T cells specific for a candidate antigenic peptide. This evaluation is not easy to carry out, as it ideally requires a T-cell clone and a tumor cell line. One way to bypass the peptide selection problem is to use proteins or protein-encoding nucleic acids; the processing machinery of the antigen-presenting cells will select the best presented peptides. Other advantages are the simultaneous presentations of peptides to CD8+ and CD4+ T cells and the absence of HLA selection requirement for the patients.

It should be noted that peptides or proteins have poor pharmacokinetic properties and may be rapidly cleared before being loaded onto antigen presenting cells. To overcome this limitation, antigenic vaccines are usually co-administered with substances that enhance their immunogenicity: immunological adjuvants. Facing antigens in the absence of a danger signal provided by adjuvants, the DC might remain inactive and promote tolerance instead of immunity. Adjuvants are broadly defined as pharmacological or immunological agents able to modify the effect of other agents (e.g. vaccines), having no direct effect when given by themselves. In the field of vaccines, adjuvants are mixed with antigens to increase their immunogenicity via a variety of mechanisms involving innate immunity[203]. Local and temporal coadministration with antigens distinguishes adjuvants from immunomodulators, such as cytokines or anti-CTLA-4 mAb. A limited number of adjuvants have been used in clinical trials of therapeutic cancer vaccines, mostly in melanoma patients at an advanced stage of the disease. Thus far, no adjuvant has shown superior clinical effects as compared to others, but there are several difficulties in appreciating their effects. The

impact on clinical outcome has been either absent or not evaluated in studies focused on immune responses. When immune responses were analyzed, comparisons between studies were problematic because of differences in antigens, vaccination schedules, and methods of immune monitoring. The importance of using an adequate adjuvant to stimulate the maturation process of DC was highlighted in a study in which vaccination of cancer patients with the MAGEA3 protein revealed long-term memory if priming occurred with adjuvant and tolerance upon priming with antigen alone[204].

3.1.2.2 Cancer cell-based vaccines:

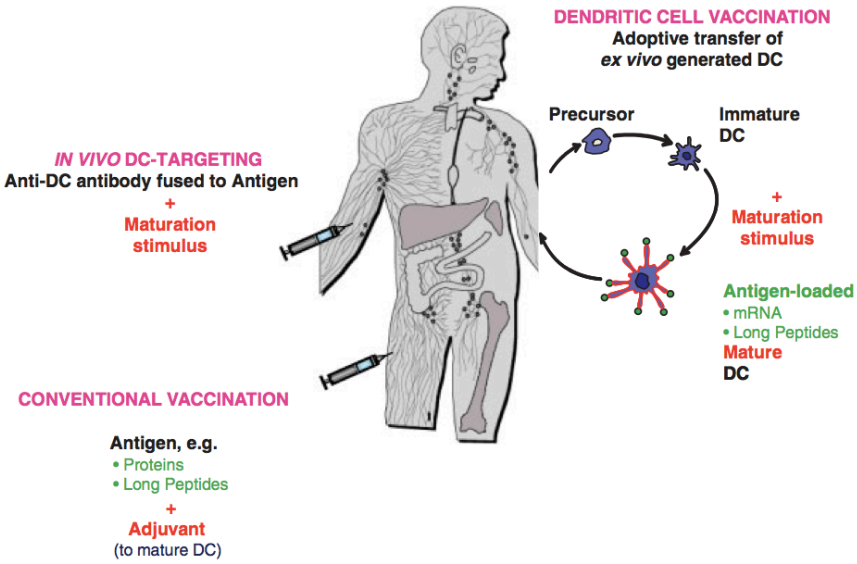
Another strategy for cancer vaccines involves the use of autologous or allogeneic tumor cells. The idea underlying this strategy is that cancer cells carry several tumor-associated antigens including antigens that have not been characterized, and that using them in a vaccine eliminates the problem of antigen identification and selection. A meta-analysis of published immunotherapy trials in various solid tumor types revealed that patients immunized with whole tumor cells had a significantly higher rate of clinical response, 8.1%, as compared to 3.6% when molecularly defined antigens were used[205]. For a given patient, the best choice in terms of tumor antigens is of course his own tumor cells[206]. However, the complexity of manufacturing an autologous tumor vaccine for every new patient has led many investigators to using allogeneic tumor cell lines.

Among these approches, the most advanced clinically is GVAX for prostate cancer. This vaccine product consists of two prostate cancer cell lines engineered to secrete GM-CSF. The phase III trial of GVAX failed due

to a lack of clinical efficacy[207]. This failure might reflect an insufficient immunogenicity due to alterations of the vaccine product during its preparation, or the inability of allogeneic tumor cells lines to recapitulate the full spectrum of antigens present on individual prostate carcinomas. For example, a given patient's tumor will harbor mutated antigens that are absent from the vaccine product. Similar phase I/II clinical studies were conducted for melanoma and renal cell carcinoma[208,209]. GVAX followed with an immune stimulator (CRS-207) also showed extended survivals for patients with metastatic pancreatic adenocarcinoma[210].

Belagenpumatucel-L (Lucanix®) is another allogeneic tumor cell vaccine, composed of 4 lung cancer cell lines modified genetically to block the production of TGF-β, one of the mechanisms whereby tumors counteract the immune system[211]. This vaccine has been used in a phase III multicentric clinical trial (NCT00676507) recruiting stage III-IV NSCLC patients who did not progress following frontline chemotherapy. Patients were randomised 1:1 to receive maintenance belagenpumatucel-L or placebo. The primary endpoint, improved overall survival, was not met, there were also no differences in progression-free survival, primarily because of the late inclusion of patients, more than 12 weeks following the completion of frontline chemotherapy. Improved survival for belagenpumatucel-L is suggested in patients who were randomised within 12 weeks of completion of chemotherapy and in those who had received prior radiation[212].

Belagenpumatucel-L was well tolerated with no serious safety concerns. Pain, fatigue, respiratory problems and cough were the most frequent adverse events observed in the vaccinated patients.



From Schuler et al. Eur. J. Immunology 2010, 40 (2123-2130)

Figure 6: principle of DCs based vaccination
All vaccines depend on DC, and there are three basic approaches to exploit them as vectors for vaccination.

Belagenpumatucel-L has also been tested against advanced brain cancers in a phase I study reporting promising results[213], and a phase II/III trial is about to be activated for glioma patients.

3.1.2.3 Dendritic-cell-based vaccines:

The presentation of antigens by matured DC expressing costimulatory molecules results in robust T-cell proliferation and differentiation, and thus effective T-cell immunity. The optimal design of a vaccine using DC requires antigen uptake and presentation by the DCs and their adequate maturation by adjuvants. These crucial aspects of DC biology have been largely underestimated during a long time, while the choice of antigen and vaccination schedule was prioritized. But since a decade there has been a considerable interest in developing optimized DC-based vaccines. Usually, autologous DC are isolated from a cancer patient, loaded with antigens (peptides, tumor cell lysates, RNA,…) *ex vivo*, activated and reinfused back into the patient[214] (Figure 6).

The first DC vaccination study was published in 1996 and used rare DC isolated directly from the blood to immunize B-cell lymphoma patients against their own tumor-specific idiotype protein[215]. By March 2010, almost 300 papers have reported on 4422 vaccinated patients, mostly with melanoma[216] and prostate cancer[217] (1301 and 510 patients, respectively). Most trials employed monocyte-derived DC, which were often generated from blood monocytes by culture with GM-CSF+IL-4 over approximately 6 days to obtain immature DC, followed by exposure to monocyte-conditioned medium or its mimic (a cocktail composed of TNF-α, IL-1β,

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IL-6 and PGE2) for 1–2 days, to yield matured DC. As yet, no study has demonstrated a significant effect on tumor progression, and the approach has not gained support from the biotechnology industry, given the complexities of cell isolation, ex vivo manipulation and re-infusion, with one exception, Sipuleucel-T (APC8015-PROVENGE). It is a monocyte-based vaccine developed by Dendreon Corporation for the treatment of both androgen-independent and androgen-dependent prostate cancers. It was the first cancer vaccine with proven clinical benefit. This vaccine contains mature autologous monocytes obtained *via* a standard leukapheresis procedure, approximately two days before each infusion. These monocytes are cultured in the presence of a recombinant fusion protein consisting of prostatic acid phosphate (PAP) linked to GM-CSF. PAP is a prostate differentiation antigen expressed in the majority of prostate cancers but absent in non-prostatic tissues, allowing Sipuleucel-T to stimulate tumor-specific immune responses, which should spare normal cells[218]. Dendreon has confirmed in its pivotal phase III study (IMPACT trial) that its cellular vaccine modestly but significantly improved overall survival (by a median of 4.1 months, reducing the risk of death by 22.5% compared to the control group) in asymptomatic or minimally symptomatic metastatic, hormone-refractory prostate cancer, even though classical regressions did not occur and time to progression was not prolonged[191]. The result of the IMPACT trial led to the approval by the FDA of the first therapeutic cancer vaccine ever on April 2010.

In another phase III DC trial in melanoma, a vaccine consisting of matured DC loaded with selected HLA class I and class II-restricted tumor peptides was compared with standard dacarbazine chemotherapy as first-

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line treatment in stage IV metastatic melanoma. The trial was stopped following an interim analysis as there was no significant difference between the two arms[219]. The authors reported that the mean numbers of DC injected into the skin were low and highly variable, and that in addition, the DC were of inferior quality. This result highlights the importance of standardized vaccine production.

A critical factor determining the effectiveness of current DC-based vaccines is the DC activation or maturation status. It was shown that the T-cell stimulatory capacity of DCs pulsed with tumoral antigenic peptides can be considerably increased by activating the DCs through electroporation with mRNA encoding CD40 ligand, CD70, and a constitutively active Toll-like receptor 4 (caTLR4), together called TriMix-DCs[220]. A pilot clinical trial was conducted with a therapeutic vaccination containing autologous TriMix-DC co-electroporated with mRNA encoding a HLA class II-targeting signal linked to each of 4 melanoma antigens (MAGE-A3, MAGE-C2, tyrosinase and gp100) in patients with advanced melanoma. Thirty-five stage III/IV melanoma patients received autologous TriMix-DC (4 i.d. administrations 2 weeks apart). This study showed that therapeutic vaccination with autologous TriMix-DC is feasible, safe, and immunogenic, even though no objective responses to TriMix-DC alone were seen[221]. A case report indicates that administration of autologous TriMix-DC by a combined i.d. and i.v. route mediated a durable objective tumor response accompanied by a broad T-cell response in a chemorefractory multi metastatic melanoma patient[222].

In general, DC vaccines containing peptides, tumor lysate, tumor RNA or DNA have been very well tolerated[223].

3.2 Adoptive cell therapy (ACT):

Adoptive transfer of effector T cells directed at tumor antigens might bypass an established tolerance by the tumor. In this form of immunotherapy, antitumor T cells are manipulated *ex vivo* and then infused back into the patient.

The first adoptive cell transfer used in the clinic was bone marrow transplantation for hematologic malignancies. The first observation that adoptive transfer of lymphocytes can inhibit tumor growth was made in the 1960s by Southam and colleagues who demonstrated that the subcutaneous growth of human tumor autografts to patients bearing advanced cancers was inhibited in half of the patients by the cotransfer of autologous leukocytes[224]. In solid tumors, the first adoptive transfer of *ex vivo*-amplified TILs was reported in metastatic melanoma patients by Rosenberg in 1988[225]. In early trials, *ex vivo* expanded and activated autologous TILs were re-administered to untreated patients, resulting in transitory tumor regressions but no durable responses[225]. Presumably, inoculated cells are subjected to immunosuppression by endogenous Tregs and fail to persist over long periods *in vivo*. Moreover, endogenous B, T and NK cells compete with the re-infused TILs for a limited amount of critical cytokines such as IL-7 and IL-15[226]. To circumvent these critical issues, pre-conditioning regimens based on cyclophosphamide (an agent that at high doses exerts immunosuppressive effects), fludarabine (a nucleoside analog commonly used for the therapy of hematological malignancies) and total body irradiation have been used, resulting in different levels of lymphodepletion. The intensity of lymphodepletion has been shown to

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directly correlate with ACT antitumor efficacy[227], leading to the wide use of preconditioning lymphodepletion in the clinical practice of ACT.

Two types of ACT cells can be considered: autologous T cells derived from the blood or the tumor, or allogeneic anti-tumor lymphocytes. The adoptive transfer of activated allogeneic T cells is hampered by high risks of comorbidity, risks of destruction of allogeneic ACT cells by the patient's immune system and logistic issues related to the identification of suitable allogeneic donors; accordingly autologous approaches are preferred. In humans, adoptive T cell therapy approaches to date have used as the anatomic sources of T cells: blood, tumors, malignant effusions, and draining lymph nodes.

A central issue for the development of clinical adoptive T cell therapy strategies has been the development of culture systems that may produce the very large numbers of effectors T cells required for autologous transfer. Two approaches are used for clinical adoptive T cell therapy [228] (Figure 7).

The first consist in isolating T cells from blood or tumor specimens, and then to use repetitive stimulation with appropriate antigens and cytokines to clonally expand and enrich the antigen-specific T cells. In the second approach, T cells are activated non-specifically *ex vivo* by various means and are then reinfused into the patient with the expectation that they will respond directly to the tumor or to tumor antigens presented by APCs. The first approach guarantees antigen specificity but is expensive and labor intensive; the second is technically simpler and therefore has the potential for large scale clinical trials, but is less specific[229].

ACT involving transfer of TILs has emerged as an effective form of immunotherapy with rates of success as high as 40%, and complete durable responses in a sizeable proportion of melanoma patients[138,230].

Melanoma and renal cell carcinoma are preferred settings for this immunotherapy because of the high numbers of TILs spontaneously present in those tumors and because immunostimulatory interventions such as IL-2 are efficient against these tumors[38]. Adoptive cell transfer of TILs for other malignancies has been hampered by difficulties in isolating sufficient numbers of these cells. To extend the use and benefits of ACT to such types of cancer, genetic engineering can be employed, converting circulating lymphocytes into cells that exhibit antitumor activity. In this setting, normal blood lymphocytes are isolated and genetically engineered to express TCRs that recognize tumor antigens with high affinity. Here rearranged TCRA and TCRB genes cloned from T-cells with high antitumor activity can be incorporated into highly efficient retroviral or lentiviral vectors[231]. Genetic engineering before ACT can also be employed to confer additional features to lymphocytes such as increased proliferative potential (by overexpression of growth factors like IL-2, or of IL-2 receptors)[232]. Genetic engineering might also provide an alternative to TCRs with the use of chimeric antigen receptors (CARs). These receptors bind to tumor antigens via antibody-derived complementarity-determining regions, which are coupled to the intracellular machinery for TCR signaling, *de facto* mediating the functional activation of T cells even when antigens are not displayed in the context of HLA molecules[233]. In the context of genetically engineered T cells, the choice of the targeted tumor antigen is critical, as it has implication in both the efficacy and the safety of ACT. Several tumor antigens such as the

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carcinoembryonic antigen (CEA)[234] have already been tested with encouraging results.

Most of the ACT clinical trials have involved the transfer of $CD8^+$ effectors T cells. The primary advantage of using $CD8^+$ T cells is their ability to directly recognize and kill tumor cells through the recognition of differentially expressed tumor antigens presented on the tumor cell surface. Using T cells for adoptive therapy is attractive due to their long clonal life span[235], which allows therapeutic and immunoprophylactic effects. However, recent studies have reported that adoptive transfer of other immune cell subsets including non-conventional T cells and innate immune cells can also induce effective anti-tumor responses. $CD4^+$ T cell help is required for the generation or maintenance of $CD8^+$ T cell memory[236]. A patient with refractory metastatic melanoma was shown to display a long-term complete remission after adoptive transfer of anti-LAGE/NY-ESO-1 CD4+ T cells[237].

NK cells constitute a potent innate immune cell type, which can quickly recognize 'stressed' cells and kill them using perforin and granzymes. One of the first transfers of NK cells was performed in the mid 1980s, with autologous cytokine-activated killer cells (that comprise NK cells) coadministered with IL-2. It resulted in 11 out of 25 metastatic cancer patients experiencing partial or objective responses[238]. Even if NK cells possess strong anti-tumor activity, the difficulty in generating sufficient numbers of NK cells has created difficulties for a widespread use of these cells. However the feasibility to manipulate and redirect NK killing towards a specific antigen highlights the potential of gene-modified NK cells in the

clinic. A recent study demonstrated that genetic modification of NK cells can confer them higher survival rates and more potent cytolytic activity[239].

Clinical trials with ACT:

Despite more than 60 years of research with ACT for cancer immunotherapy, no FDA-approved T-cell therapy for cancer is available yet. Nevertheless, the efficacy and safety of ACT as immunotherapy has been evaluated in multiple oncological settings. Results of these early (Phase I/II) clinical studies have been reviewed recently[240]. Half of the ACT studies were performed in small cohorts of metastatic melanoma patients[138,187,241-251] while the other half were conducted in diverse malignancies such as hematological malignancies[252,253], renal cell carcinoma[254,255], hepatocellular carcinoma[256], ovarian cancer[257], neuroblastoma[258], metastatic colorectal carcinoma[234], and head and neck squamous cell carcinoma[259].

In about one third of the published studies, patients were re-infused with autologous T cells that had been non-specifically activated *in vitro* with IL-2 or anti-CD3 antibodies, alone or in combination with anti-CD28 antibodies[138,241,245,250,255,260-263]. Alternatively, patients received T lymphocytes that had been restimulated with tumor specific antigens [246,249,251,254,256,264]. In the remaining studies, the re-infused material consisted in genetically modified cells, including blood T cells engineered to express tumor antigen-specific TCRs[187,234,242,244,250,257], CARs[252,253,258,265] or IL-2[247]. In most cases, patients received classical lymphodepleting regimens based on cyclophosphamide + fludarabine, alone or in combination with total body irradiation[138,241,242,244,245,250,252,253,260,266], and were given together with high-

Introduction

dose IL-2[187,234,241,242,265]. As an alternative, cells were co-infused with low-dose IL-2 over 6 days[246] or low-dose IFNγ[251]. The results of the ACT clinical trials are very encouraging, reporting response rates as high as 70%. ACT was associated with limited therapeutic responses in only two trials[255,263], and in two other studies no antitumor effect was observed [256,257].

The most prominent side effects observed with ACT relate to: (1) the specificity of the re-infused cells, which might destroy normal tissues expressing the antigen recognized by the genetically engineered TCRs (or CARs)[244]; (2) the secretion of large amounts of cytokines by the adoptively transferred cells[192,267]; (3) transduced TCR chains that might recombine with endogenous ones, resulting in the acquisition of a new antigen specificity and graft-versus-host disease[268].

However, until now, only a few cases of severe/lethal adverse reactions have been reported[192,234,267,269], suggesting that, in the vast majority of settings, ACT constitutes a safe clinical procedure.

Most of the ACT trials were performed in small cohorts of patients and the therapeutic protocols often differed from one trial to another, yet these phase I/II studies demonstrate that ACT is efficient and safe for the treatment of some types of cancer, in particular melanoma. Nevertheless, ACT is far from becoming a routine clinical practice, as its implementation is laborious and associated with elevated costs. Development of simplified techniques for the *ex vivo* expansion, activation and genetic engineering of lymphocytes might increase the use of ACT as a therapeutic option.

4. Concluding remarks

Cancer immunotherapy has already a long history, and the results obtained recently with immunostimulatory antibodies open a new era in the field. Indeed anti-CTLA-4 and anti-PD-1 mAbs have demonstrated clear evidence of objective responses including improved overall survival and tumor shrinkage and, most importantly, persistent responses driving renewed enthusiasm for cancer immunotherapy in multiple cancer types. Adoptive cell transfer (ACT) was shown in clinical phase I/II trials to be efficient and safe to treat some types of cancer, in particular melanoma. Nevertheless, ACT is far from becoming a routine clinical practice, as its implementation is laborious and associated with elevated costs. Cancer vaccines, though promising and with a remarkably low toxicity profile, have not yet reached the stage of demonstrated clinical efficacy.

There is now an increasing number of data on human tumors and TIL to support the scenario of an immunosuppressive microenvironment present within the tumor being a major factor preventing the success of immunotherapies, especially anti-cancer vaccination. Individual differences observed in clinical responses to immunotherapy might be related to the fact that patients vary in the degree of immunosuppression present in their tumor. The responder patients would be those who have a lower degree of immunosuppression in their tumor. For the vaccinated patients who show tumor regression, it was proposed that a few antivaccine T cells penetrate the tumor and attack some tumor cells. As a result, these CTL are restimulated and produce cytokines, which focally reverse the local immunosuppression, creating instead an immunostimulatory environment. By reversing focally the immunosuppression, it triggers a broad activation

of other anti-tumor T cells that proceed to eliminate the tumor cells. In other words, the anti-vaccine T cells serve only as a "spark" that activates the regression of the tumor.

If the degree of intratumor immunosuppression is the limiting factor for the success of vaccination against tumors, manipulating the tumor micorenvironment locally to reverse the balance in favor of tumor regression could potentiate cancer vaccines.

In the next part of this manuscript we will show results obtained with some local “non-specific” immunotherapy interventions that seem capable to potentiate T cell responses.

RESULTS

Results

Local immunostimulation leading to rejection of accepted male skin grafts by female mice as a model for cancer immunotherapy

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Results

Abstract

Female CBA mice do not reject syngeneic male skin grafts even though they mount a T cell response against the male-specific HY antigen. We show that local immunostimulation performed by injecting cytokines and Toll-like receptor ligands in close vicinity to the graft causes rejection. We feel that this approach should be tested in tumor-bearing human patients in combination with anti-tumor vaccination. Relief of intratumor immunosuppression may increase considerably the fraction of patients who respond to vaccination directed against tumor antigens recognized by T cells.

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The identification of the antigens recognized on human tumors by autologous T lymphocytes demonstrated that a majority of these antigens are highly tumor-specific[2,7]. Moreover, many of these antigens were found to be encoded by "cancer-germline" genes such as the *MAGE* family of genes, which are expressed by a large fraction of tumors of various histological types. This finding opened the possibility of vaccinating large groups of cancer patients with the same antigen in order to elicit T cell responses able to destroy the cancer cells without damaging normal cells. Human tumors were also found to bear strictly tumor-specific antigens resulting from point-mutations affecting ubiquitously expressed genes. These antigens are rarely shared by different tumors. Finally, melanoma patients also have T cells directed against melanocytic differentiation antigens, contrary to the expectation that natural tolerance ought to prevent such responses[2,15].

The initial clinical trials with cancer vaccines were performed on melanoma patients with detectable metastatic disease. Small trials involving vaccination with MAGE antigens confirmed the expectation that complete or partial tumor responses could be obtained in the absence of autoimmunity [162,270]. However, these clinical responses were observed in only 5 - 10% of the patients, with no sign of tumor regression observed in the vast majority. Because these first vaccination trials were performed with antigenic peptides in the absence of adjuvant, it was widely expected that the use of more potent vaccines, such as viral recombinant vaccines or peptide-pulsed

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dendritic cells would produce improved results. However, more than a decade of such vaccination trials has failed to provide significant improvement of clinical efficacy[271].

The initial view of the process elicited by vaccination in responding patients was that anti-vaccine cytolytic T lymphocytes (CTL) would migrate in large numbers to tumor sites and directly destroy the tumor cells. We believe that this view is erroneous. First, even in patients who experience a clinical response, there are insufficient numbers of anti-vaccine T cells. The analysis of T cells present in peripheral blood indicates that most of these patients have at best a barely detectable anti-vaccine T cell response[272]. In some clinical responders we evaluated the number of anti-vaccine T cells present in tumor deposits and we concluded that they are present at a ratio of less than one in a million relative to tumor cells. Moreover, much larger numbers of T cells directed against other tumor-specific antigens are already present within the tumor before vaccination, and some of these T cell populations expand considerably when rejection occurs.

The presence of tumor-infiltrating lymphocytes (TILs) with potential anti-tumor activity was observed as early as 1988 by Rosenberg and his associates, who cultured single-cell suspensions of metastases in the presence of interleukin 2 (IL-2), and obtained large numbers of lymphocytes that exerted cytolytic activity on fresh tumor cell preparations, albeit under conditions where conclusive specificity controls could not be carried out[225,273]. The detailed identification of the set of tumor-specific antigens expressed by some tumors enabled us to examine TILs under conditions

Results

where the antigenic target and T cell receptor sequence were identified. This led to the demonstration that before vaccination, TILs directed against several of these tumor-specific antigens are indeed present in the tumor. After vaccination, in patients who make clinical responses, many TIL clonotypes analysed within the tumor were found to be considerably amplified, with frequencies about 1,000-fold higher than that of the anti-vaccine TILs. New dominant clonotypes, absent before vaccination, were also observed. Collectively, this diverse set of expanding anti-tumoral TILs reached a frequency of 1% relative to the tumor cells[274].

On the basis of these and other findings, it is now clear that most melanoma patients produce a spontaneous T cell response against their tumor[272]. In many patients this attempt at eliminating the tumor evidently fails and a large number of anti-tumor T cells remain in an inactive state in circulation and within tumor deposits. Inside the tumors, this anergic state appears to be reinforced by an immunosuppressive environment. For the vaccinated patients who show tumor regression, we propose the following sequence of events: a very small number of active anti-vaccine T cells penetrates the tumor and attacks some tumor cells. As a result, these CTL are restimulated and produce cytokines which focally reverse the local immunosuppression, creating instead an immunostimulatory environment. This reawakens many of the inactive anti-tumor T cells that are already present in significant numbers *in situ*. Naïve anti-tumor T cells may also be activated. The mobilization of all these T cells, most of which are directed against tumor antigens other than the vaccine antigen, provides the large numbers of effector cells required to reject the tumor.

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Why is it that following vaccination tumors regress in some patients whereas no sign of regression is observed in most patients? A first possibility is that this may be due to differences in the quantity or quality of T cells responding to the vaccine. The lack of improvement of clinical outcome following highly immunogenic vaccination suggests that the strength of the response to the vaccine is not the limiting factor. A second possible difference between regressing and non-regressing patients could be the degree of expression of the relevant antigens on the surface of tumor cells. It is well established that tumors can lose the expression of some or all of their HLA molecules [2 275 276]. However, we derived melanoma cell lines from a large number of non-responder patients, and almost all of them proved to be sensitive to anti-vaccine CTL *in vitro*. We therefore suspect that loss of antigen expression accounts for only a small fraction of the treatment failures. A third possible explanation for the individual differences in clinical response is that patients vary in the degree of immunosuppression present in their tumor. A highly immunosuppressive tumor environment could render the anti-vaccine T cells inactive soon after penetrating the tumor, preventing them from initiating the cascade of events that leads to tumor rejection. The responder patients would be those who have a lower degree of immunosuppression in their tumor.

The anergic state of tumor-infiltrating lymphocytes and the immunosuppressive tumor environment are often considered to result from an active immunosuppressive mechanism of tumor cells acquired in the course of the selection for resistance to the immune response. Agents such

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as IDO, TDO and TGF-b are produced in many tumors and unquestionably contribute to inducing anergy of infiltrating lymphocytes[148,277,278]. But it is also possible that where the immune system makes a rather weak attempt to eliminate a tissue, as expected in the absence of pathogen-associated molecular patterns binding to Toll-like, Nod and RIG receptors, after a length of time it enters an inactive state. Examples of such "immune fatigue" are observed in human transplantation: a small number of recipients can be withdrawn from treatment with immunosuppressive agents without deleterious effects on the grafted organ [279,280]. This process, whereby an incomplete response evolves into an anergic state could be common to all progressive metastatic tumors, whereas the presence and the nature of immunosuppressive factors secreted by tumor cells are likely to vary from one tumor to another. The common anergic state could involve a degree of immunosuppression within the tumor resulting in potential paralysis of incoming T lymphocytes.

If the degree of intratumor immunosuppression is the limiting factor for the success of vaccination against tumors, the use of cytokines to alleviate this immunosuppression could improve outcome. A considerable amount of effort has been devoted to testing systemic cytokine treatment in cancer patients. Impressive results have been obtained on a number of patients treated with IL-2 and interferon-α (IFN-α), but at the dosage required for efficacy significant toxicity has been observed [225 31]. We feel that in contrast, local cytokine treatment of metastases should be tested as an adjunct to vaccination. This local treatment could enable the anti-vaccine T cells to initiate the rejection process. Moreover, the amount of agents used

for local treatment would be orders of magnitude below those used for systemic treatments, thereby reducing the occurrence of adverse side-effects. We realize that this approach will lead to therapeutic progress only if the immune rejection response of the treated metastasis leads to the production of anti-tumor T cells of such quantity and quality that they successfully attack untreated metastases disseminated elsewhere in the body.

We present here a set of observations related to local immunostimulation with cytokines and Toll-like receptor (TLR) ligands. We wished to address first the common component of anergy referred to above as "immune fatigue". To this end we carried out preclinical studies using the mouse HY system, which involves T cell responses to antigens expressed in male and not in female tissues. This model is attractive as it has several features shared with the immune response to human tumors. Whilst in inbred mouse strain C57BL/6 ($H2^b$) females reject syngeneic male skin grafts, in CBA ($H2^k$) they do not, even though we have observed that a CTL response directed against HY antigens does occur following grafting. Thus the process observed in CBA is similar to that observed in progressing human tumors: a failed attempt at rejection followed by the persistence of ineffective memory T cells. Moreover, in contrast with most mouse tumors, skin grafts can be followed and manipulated over a period of several months, a situation similar to that encountered with most human tumors. We report below that some locally administered cytokine combinations are effective in promoting the rejection of these skin grafts.

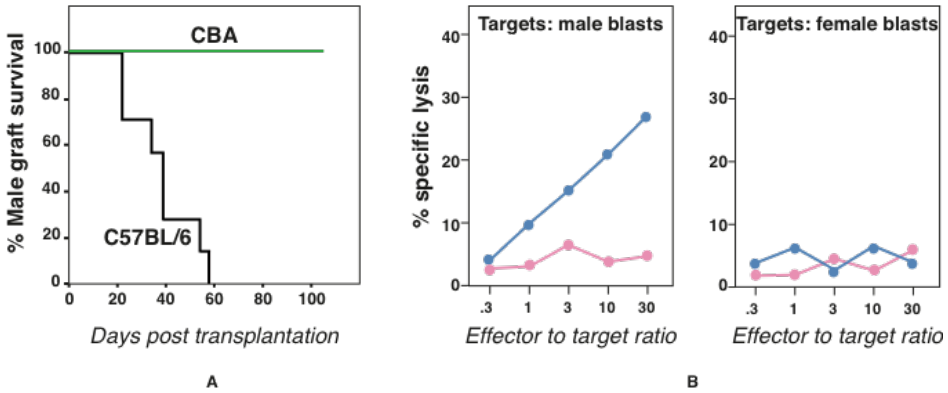


Figure 8

A Seven CBA or C57BL/6 female mice were grafted on the thorax with a 1 cm^2 male skin patch of the same strain. The graph indicates the time of complete rejection.

B Female CBA mice were grafted with either male *(blue)* or female *(red)* skin. After 22 days, 25.105 of their spleen cells were stimulated in vitro for 6 days with 25.105 irradiated male blast cells obtained by incubation of lymphocytes with LPS. The lytic activity was then tested on male and female blasts in a 4-hour chromium release assay.

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As shown in ***Fig. 8***, CBA/Ca ($H2^k$) females do not reject male skin grafts even though they mount a CTL response against HY antigens of the graft.

Elizabeth Simpson and her associates have analysed $H2^k$ restricted HY-specific CTL responses. They have identified HY antigenic peptides encoded by the *Smcy* gene, including dominant peptide RRLRKTLL presented by the H2-D^k class I molecule and peptide TENSGKDI presented by H2-$K^{k\ 281,282}$. We observed that female CBA mice produce CTL against male target cells expressing both peptides after grafting with syngeneic male but not female skin.

Local treatment with IL-12 and interferon α

We tested various immunostimulatory agents to identify combinations which are able to promote graft rejection following local treatment, in the absence of noticeable toxicity.

A number of CBA females grafted with male skin were treated with interleukin 12 (IL-12) and with IFN-α ***(Fig. 9)***. Small volumes were injected subcutaneously under the graft. This local treatment was administered for 3 consecutive days every week either until complete graft rejection or until the end of the fifth week. IFN-α at a dosage of 10^5 units had no effect. Interleukin 12 administered alone was effective at 1,000 units. Clear synergy was observed between the two cytokines, resulting in complete

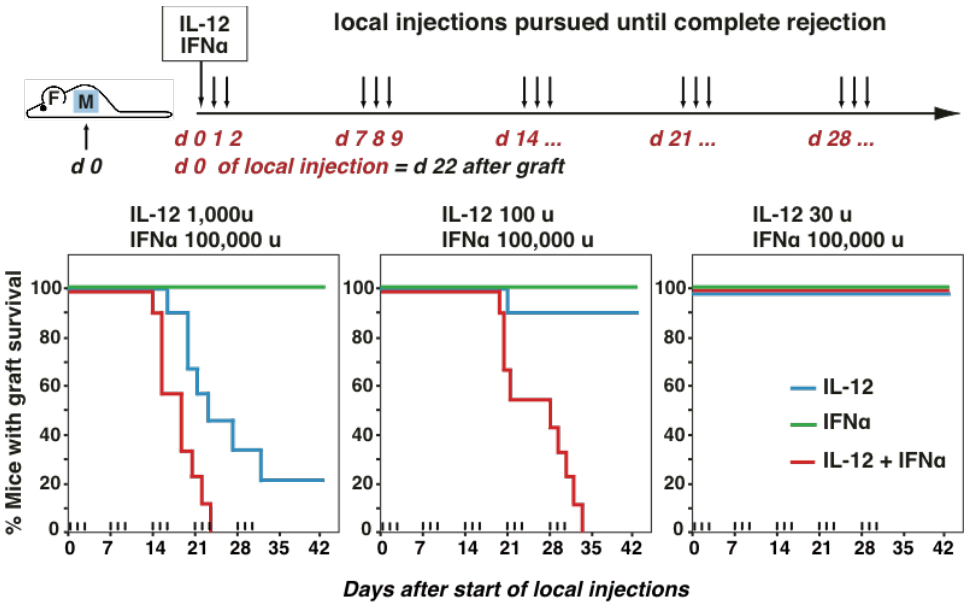


Figure 9
Groups of 9 CBA female mice were grafted with male skin of the same strain. Twenty-two days later, a local treatment involving IL-12, IFN-α or both cytokines was applied with the schedule and the dosage shown on the figure. Injections were carried out on weekly schedules involving injections on three consecutive days. A solution of about 100 µl containing both interleukins was injected subcutaneously under the graft. The graphs indicate the time of complete rejection.

rejection of all the grafts following local treatment with 100 units of IL-12 combined with 10^5 units of IFN-α. No symptoms of toxicity were observed.

The observed rejections do not appear to be due to a non-specific local toxicity of the cytokines as female skin grafts were not rejected ***(Fig. 10)***.

Moreover they seem to result principally from a local as opposed to a systemic effect of the cytokines, as intraperitoneal injections of the same dose of cytokines were clearly much less effective ***(Fig. 11)***.

Whereas some rejections could be observed after treating the graft for only one week, five weeks of treatment were necessary to elicit virtually 100% rejection ***(Fig. 12)***. The treatment involving IL-12 and IFN-α was less effective when given 100 days after grafting rather than after 20 days: 60% versus 100% of the mice showing rejection. This may be due to a decrease in the level of immune memory, likely to diminish between 3 and 12 weeks after grafting. Alternatively, this effect may be due to a gradual decrease of the local inflammation resulting from the surgical grafting process.

IFN-α was not the only cytokine capable of causing graft rejection in combination with IL-12. IL-1α, IL-2, IL-18 also showed definite synergies. So did gardiquimod, a ligand of Toll-like receptor 7 (TLR7) ***(Table 4)***.

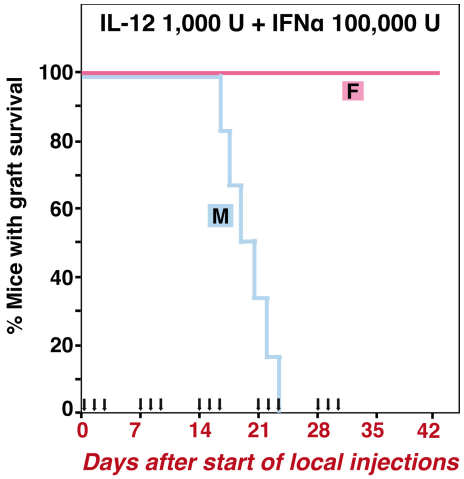


Figure 10
Groups of 6 female CBA mice were grafted with either male *(blue)* or female *(red)* skin of the same mouse strain and treated locally with IL-12 and IFN-α at the dosage indicated. The graph indicates the time of complete rejection. Treatment schedule as in ***Figure 9*** *(arrows)*.

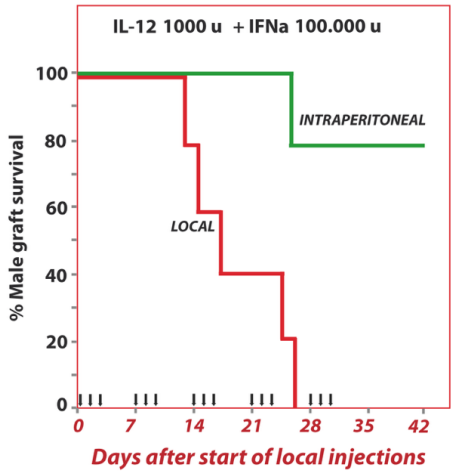


Figure 11
Groups of 5 or 6 female CBA mice grafted with male skin were treated either locally or intraperitoneally with IL-12 and IFN-α at the dosage indicated. Treatment schedule as in ***Figure 9*** *(arrows)*.

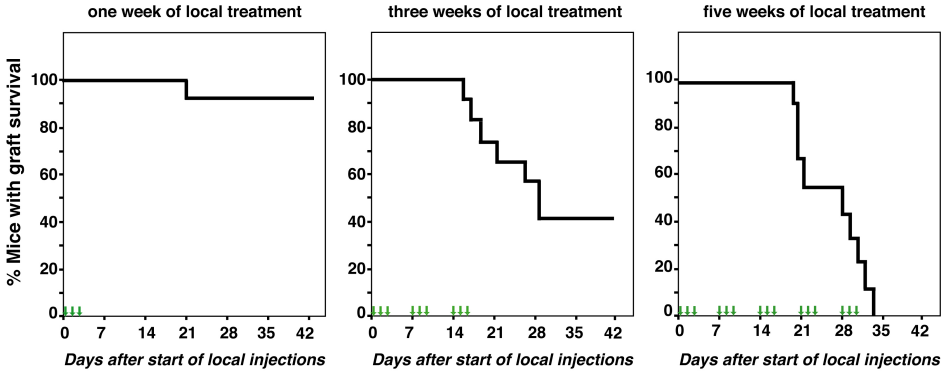


Figure 12
Groups of 9-10 female CBA mice were grafted with male skin and treated locally with 100 u of IL-12 and 10^5 u IFN-α for three consecutive days during either one, three or five weeks.

Results

Table 4: Effect of IL-12 combined with other cytokines

Treatment		Number of mice with surviving graft
IL-12 100 u	alone	13 /14
	+ IFN-α 10^5 u	0 / 9
	+ IL-1α 300 ng	0 / 5
	+ IL-2 300 ng	0 / 5
	+ IL-18 300 ng	0 / 5
	+ gardiquimod 20 μg	0 / 5
IL-12 30 u	alone	5 / 5
	+ IFN-α 10^5 u	5 / 5
	+ IL-2 300 ng	0 / 5
	+ IL-18 300 ng	0 / 5
	+ gardiquimod 20 μg	0 / 5
—	IFN-α 10^5 u	9 / 9
	IL-1α 300 ng	5 / 5
	IL-2 300 ng	5 / 5
	IL-18 300 ng	5 / 5
	gardiquimod 20 μg	5 / 5

The graft was treated locally with the cytokines for three consecutive days every week. Gardiquimod was administered locally once a week. The treatment lasted five weeks.

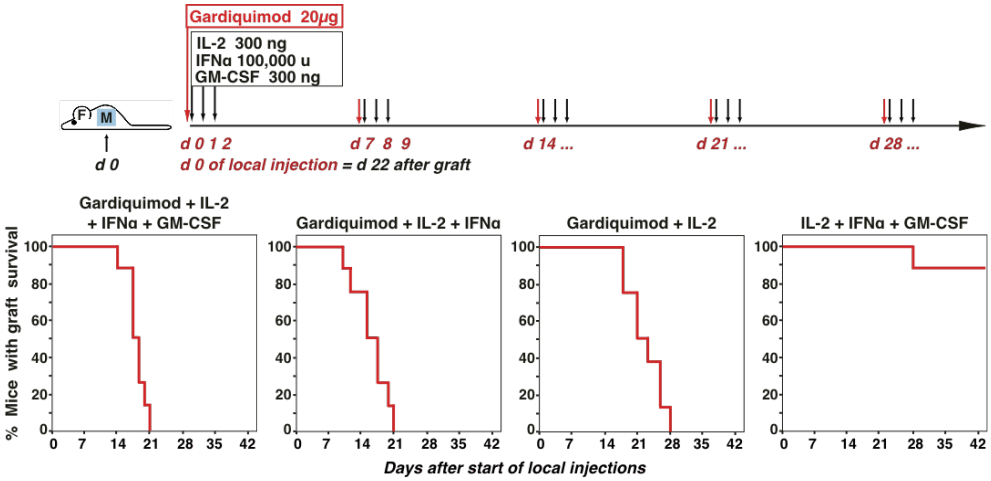


Figure 13
Groups of 8 female CBA mice were grafted with a patch of male skin. Local treatment was initiated 22 days later with a mixture containing a combination of 20 µg gardiquimod, 300 ng IL-2, 10^5 u IFN-α and 300 ng GM-CSF as indicated in the figure. The mixture was injected subcutaneously under the graft. Gardiquimod was injected once weekly and the cytokines were injected on three consecutive days as shown on the figure. The treatment was stopped after either 5 weeks or earlier if complete rejection was observed.

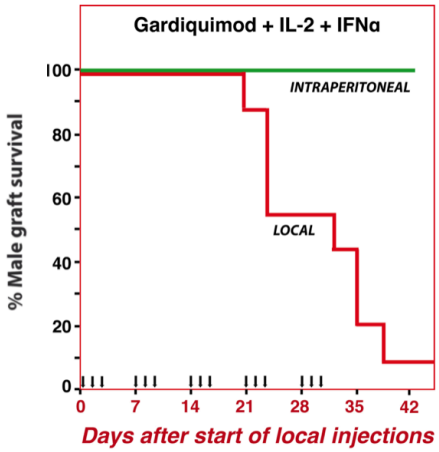


Figure 14
Groups of 9 female CBA mice were grafted with male skin. Fourty days later, they were treated either locally or intraperitoneally with a mixture containing 300 ng of IL-2, 10^5 u of IFN-α and 20 µg of gardiquimod. Treament schedule as in ***Figure 13*** *(arrows)*.

Interleukin 2, Interferon α, GM-CSF and gardiquimod

Considering the difficulty and delays encountered in performing clinical trials with drugs that have not yet been approved for human use, we resorted to our pre-clinical model to test a combination of IL-2, IFN-α and GM-CSF, which are drugs registered for clinical use. Because repeated local injections of 300 ng of IL-2 mixed with 10^5 u of IFN-α and 300 ng of GM-CSF had little effect, we added to the mixture 20 µg of gardiquimod, as it is similar to TLR7 ligand imiquimod which is approved for human use. This resulted in the rejection of the male skin grafts in the absence of noticeable toxicity ***(Fig. 13)***.

The effect was due to a local and not to a systemic action of the agents, as intraperitoneal injection had no effect ***(Fig. 14)***.

Female skin grafts were not rejected. The full effect was maintained when GM-CSF was omitted. The combination of gardiquimod and IL-2 was almost as effective as the complete combination. Increasing the dosage of IL-2 to 3 µg increased the speed of rejection and caused no obvious toxicity. Local injections of 6 µg of IL-2 alone or 20 µg of gardiquimod alone were ineffective.

In contrast to the combination of IL-12 and IFN-α, a Gardiquimod + IL-2 + IFN-α + GM-CSF treatment initiated 100 days after the grafting of the male skin proved as effective as a treatment initiated at 22 days ***(Fig. 15)***.

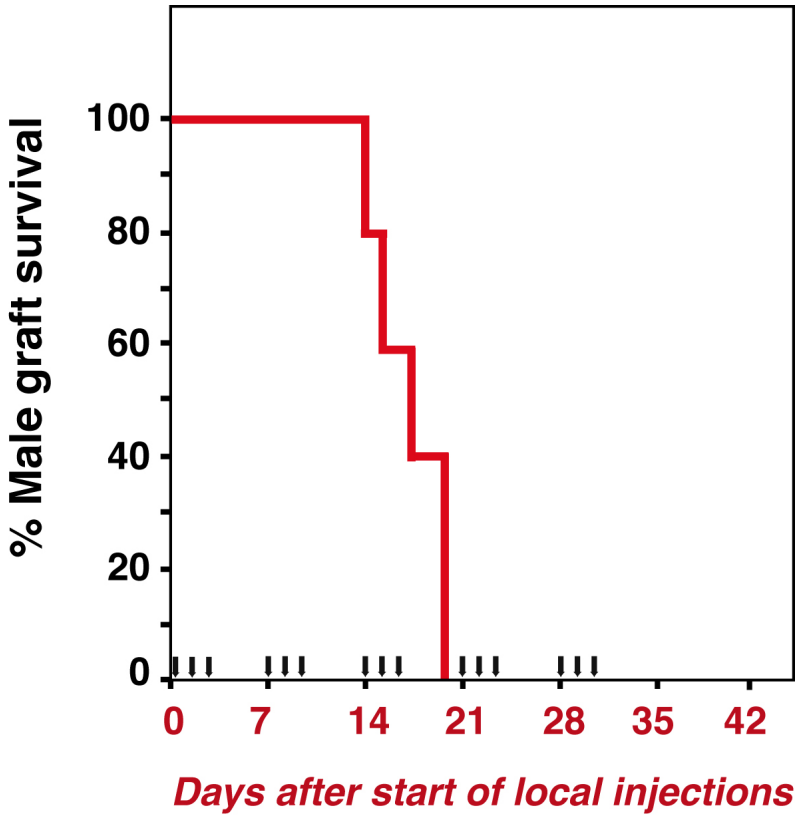


Figure 15
Five female CBA mice were grafted with male skin. After 100 days the grafts were treated locally with gardiquimod, IL-2, IFN-α and GM-CSF as described in ***Figure 13***.

With the combination of gardiquimod, IL-2, IFN-α and GM-CSF, we re-examined the need for repeating local injections for several weeks. As shown in ***Table 5***, in a group of 30 mice, ongoing rejection was observed in 18 mice after 2 weeks. For 9 of those, the treatment was halted and 6 mice proceeded to complete rejection. For the other 9, the treatment was pursued, leading to 8 complete rejections. We conclude that among the mice which show an early onset of rejection, most can complete the rejection process without further local stimulation. The picture was quite different for the 12 mice which displayed a later onset of rejection *i.e.* after 3 to 5 weeks: none of those whose treatment was arrested then rejected their grafts completely, whereas most mice with maintained treatment eventually rejected their grafts.

Table 5: Fate of grafts upon treatment arrest

Nb. mice showing onset of rejection after weeks of treatment	Nb. mice showing complete rejection	
	Treatment halted	Treatment pursued
18 after two weeks	6 / 9	8 / 9
12 after three to five weeks	0 / 6	5 / 6

Thirty female CBA mice received a male skin graft. Fourty days after grafting, we initiated local treatment with gardiquimod, IL-2, IFN-□ and GM-CSF as described in ***Fig. 11***. The 18 mice showing onset of rejection after 2 weeks were divided at random into two equal groups. For the first group the treatment was halted, for the second group the treatment was pursued either until complete rejection or the end of the sixth week. The 12 mice showing onset of rejection after 3-5 weeks were also divided in a treatment and a non-treatment group.

We examined whether other agents could complement the local action of the IL-2 and IFN-α combination. A combination of anti-TGFß1 and anti-IL-10 showed efficacy. So did a combination of anti-CTLA-4, anti-PDL1 and GM-CSF. But neither combination was as effective as gardiquimod ***(Table 6)***.

Table 6: Effect of anti-TGFß1, anti-IL-10, anti-CTLA-4, anti-PDL1

Treatment	Number of mice with surviving graft
IL-2 + IFN-α	10 / 10
IL-2 + IFN-α + anti-TGFβ1 + anti-IL-10	2 / 9
IL-2 + IFN-α + GM-CSF	6 / 6 (including 2 partial rejections)
IL-2 + IFN-α + GM-CSF + anti-PDL1 + anti-CTLA4	5 / 10 (including 2 partial rejections)

The dosages used for the local treatments were 300 ng of IL-2, 10^5 u IFN-α and 150 μg of anti-TGFβ1, 150 μg of anti-IL-10 antibodies or 300 ng of GM-CSF, 200 μg of anti-PDL1 antibodies, 200 μg of anti-CTLA-4 antibodies. The cytokines were administered for three consecutive days every week, whereas the antibodies were administered once a week. The treatments lasted six weeks.

Effect of various cytokines on graft invasion by T cells

To begin evaluating the action of the various immunostimulatory agents that proved capable of promoting graft rejection, we examined their ability to promote the presence of anti-HY CD8 T lymphocytes in the graft. We used CD8 T cells from spleens of T cell receptor transgenic mice expressing a receptor specific for the K^k HY peptide described above. After intraperitoneal adoptive transfer, the number of transgenic T cells present in any given location could be estimated by a quantitative PCR specific for this receptor sequence. No increase in the number of anti-HY transgenic T cells present in the graft was observed following treatment with either IFN-α or GM-CSF. In contrast Gardiquimod, IL-12 and IL-2 produced a very significant increase ***(Table 7)***. We are unable to evaluate whether this increase is due mainly to increased influx, increased proliferation within the graft or decreased efflux. The agents that cause a large increase in the number of anti-HY T cells found in the graft also cause a moderate increase of these cells in the blood and in the spleen. This could be due to some systemic action of these agents or it could be a consequence of T cell circulation between these compartments and the graft. We verified that the local cytokine treatment does not elicit a non-specific influx of lymphocytes: no increase in lymphocytes was observed within female grafts.

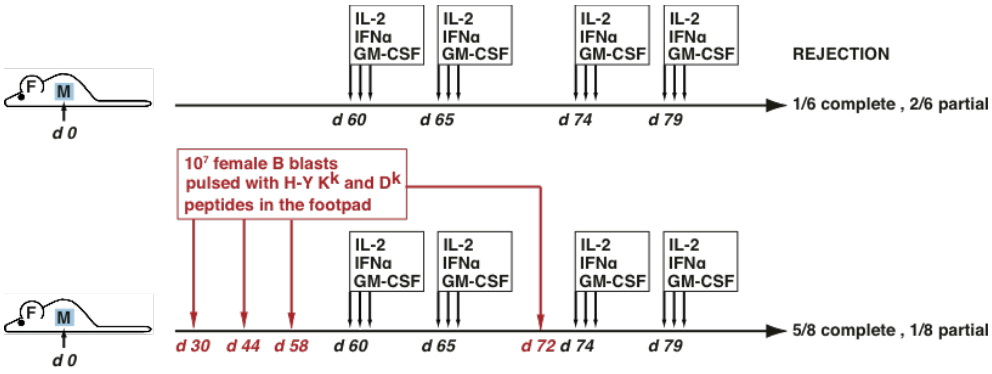


Figure 16
Female mice grafted with male skin received local treatments with 300 ng of IL-2, 300 ng of GM-CSF and 10^5 u of IFN-α. Mice of one group were vaccinated repeatedly with female lymphoblastic cells pulsed with the HY D^k and K^k peptides described above.

Table 7: Anti-HY T cell presence in graft caused by immunostimulatory agents

Local treatment	Total number of anti-HY transgenic cells in:		
	Skin graft	Blood	Spleen
PBS	4,000	8,000	28,000
IL-12 300 u	114,000	16,000	42,000
IFN-α 10^5 u	800	4,000	21,000
Gardiquimod 20 μg	98,000	24,000	49,000
IL-2 300 ng	47,000	12,000	35,000
GM-CSF 300 ng	5,000	6,000	21,000

Female CBA mice received an intraperitoneal injection of 15.10^5 spleen CD8 T cells transgenic for the T cell receptor directed against the K^k HY peptide. One day later these mice received a male skin graft. Three weeks later the graft was treated locally with the agents indicated for two consecutive weeks. Gardiquimod was injected once a week. The other reagents were injected on the first, second and fourth day of each week. The graft tissue was collected one week after the end of treatment. The DNA was extracted and the number of specific T cell receptor coding DNA sequences was evaluated by PCR. The results represent the average of four mice. The total number of transgenic T cells is presented based on estimates of a total number of nucleated cells of 2.10^6 in the graft, 2.10^7 in the blood and $7. 10^7$ in the spleen. We observed large differences between individual mice.

Local immunostimulation combined with vaccination

We next examined whether the local effect of cytokines could be boosted by vaccinating the mice with male antigen after graft healing. This is a situation similar to that encountered in cancer patients who receive therapeutic anti-tumor vaccination. For this purpose we reduced the efficacy of local treatment by using IL-2, IFN-α and GM-CSF and omitting gardiquimod. As shown in ***Fig. 16***, under these circumstances vaccination with peptide-pulsed female lymphoblasts appeared to boost the effect of local treament. In another similar experiment, the combined treatment

Results

resulted in 7/10 of the mice showing complete or partial rejection, whereas vaccination alone and local treatment alone produced respectively 3/10 and 1/5 complete or partial rejections. Vaccination with the two peptides alone was ineffective**.**

Results

Systemic effects in mice and humans

For the combination of therapeutic vaccination and local immunostimulation to be effective in the treatment of human metastatic cancer, it is not sufficient to obtain rejection of the metastases which are treated locally. The rejection must result in such an activation of the immune effectors that they acquire the ability to eliminate the other metastases which have not been treated.

A majority of the mice which received a local treatment with either IL-12 and IFN-α or with IL-2, IFN-α and gardiquimod rejected subsequent grafts in the absence of further local treatment, indicating a degree of systemic increase of anti-HY memory T cells in these mice ***(Table 8)***.

Table 8: Fate of an untreated second male graft

First graft graft	Time of second graft	Rejection of second
untreated	25 days after first	0 / 8
untreated	55 days after first	0 / 8
untreated	117 days after first	0 / 4
treated with IL-12 and IFN-α	105 days after first, *i.e.* 60 days after median rejection time	5/5 complete
treated with gardiquimod, IL-2 and IFN-α	113 days after first, *i.e.* 65 days after median rejection time	10/19 complete, 2/19 partial

The first graft was treated locally either with 100 u of IL-12 and 10^5 u of IFN-α for three consecutive days during five weeks or with gardiquimod, IL-2 and IFN-α as described in ***Figure 13***.

Results

However, when female mice grafted with male skin on both flanks received treatment only on the right graft, only modest effects were observed on the left graft. Even in those mice where the right graft was completely rejected, most left grafts remained intact ***(Table 9)***. Moreover, it is possible that rejection of some of the untreated left grafts was due to a systemic effect of the cytokines, rather than being due to the activation of anti-HY lymphocytes resulting from the rejection of the treated grafts.

Table 9: Fate of an untreated concomitant male graft

Treatment of right graft		Rejection of right graft	Rejection of left
IL-12 and IFN-α	exp. 1	5/5 complete	2/5 complete, 3/5 partial
	exp. 2	5/9 complete, 2/9 partial	1/9 complete
gardiquimod, IL-2, IFN-α and GM-CSF	exp. 1	6/7 complete, 1/7 partial	0/7
	exp. 2	11/12 complete, 1/12 partial	1/12 complete, 5/12 partial
	exp. 3	10/10 complete	1/10 complete, 4/10 partial
	exp. 4	7/9 complete, 2/9 partial	0/9
	exp. 5	5/5 complete	3/5 complete, 1/5 partial

The right grafts were treated as in ***Table 8***. The left grafts were not treated

For local immunostimulation to be effective in the treatment of human metastatic cancer, it is not sufficient to obtain rejection of the treated

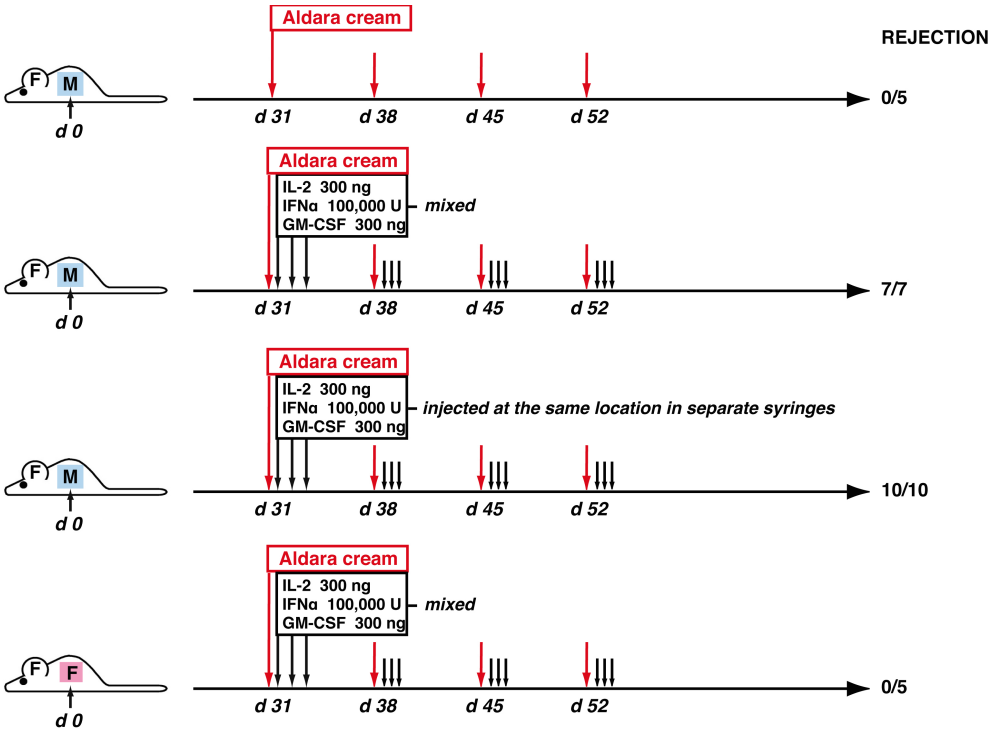


Figure 17
Female CBA mice were grafted with male (M) or female (F) skin. After 31 days, the grafts were treated locally with Aldara (imiquimod), IL-2, IFN-α and GM-CSF as described in the figure. Aldara was applied as a cream on the graft and a bandage was placed on the graft to prevent mice from removing the cream. The rejections reported were complete.

metastases. The rejection process should be systemic and lead to the regression of non-injected tumors. Unfortunately we were not able to observe this systemic effect in our model. It is obvious that our combination will be clinically usefull only if distant metastases regress after the treatment of one superficial metastasis.
Nevertheless, we felt that the situation encountered with cancer patients might differ in this respect from that encountered with male skin grafts on female mice, because the immune system of cancer patients appears to mount a considerable T cell response against a multiplicity of tumor-specific antigens before becoming anergic. We therefore initiated a small clinical trial involving patients with metastatic melanoma, with the objective to analyze the anti-tumoral effect of our association of cytokines and to confirm that such combination is well tolerated. Before initiating the trial, we performed an experiment on mice with the combination of TLR-7 ligand imiquimod (Aldara cream), which replaced gardiquimod, and IL-2, IFN-α and GM-CSF injected at the same location, but in separate syringes as requested for the patients. The results were identical to those obtained with the combination involving gardiquimod ***(Fig. 17)***.

Vaccination with the MAGE-3.A1 or NA17.A2 antigenic peptide is expected to cause regression of melanoma metastases by induction of a cytolytic T lymphocyte response. In previous clinical trials, objective tumor responses following vaccination with each of these two peptides were observed in 10 to 15% of melanoma patients[162]. In our trial, our hope was that co-treatment with immunomodulatory agents would improve the tumor response rate by enhancing CTL responses. An observed tumor response

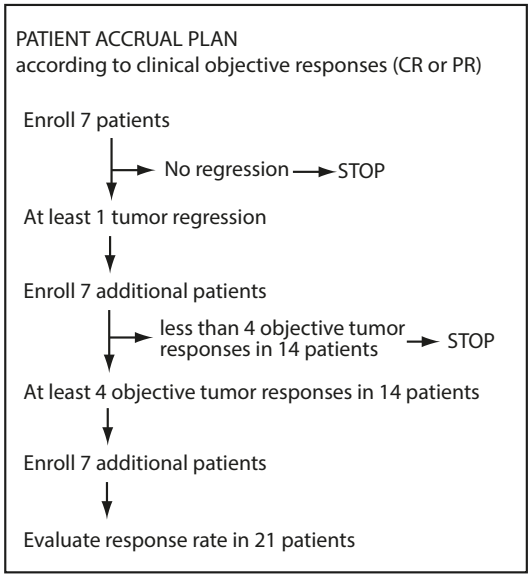


Figure 18
Patient accrual plan

PATIENTS :

- 7 to 21 patients with measurable metastatic melanoma
- Disease stages : regional disease (any T; N2c or N3; M0) or distant metastatic disease (any T; any N; M1a, M1b or M1c) except uncontrolled brain or CNS metastases, and elevated LDH (superior to 1.5 times the normal value).

St. I and II	Stage III					Stage IV			
Primary tumor only	1 regional lymph node met	2 or 3 regional lymph node micromets	2 or 3 regional lymph node macromets	regional skin mets	>3 regional lymph node mets or regional skin & LN mets or matted nodes	distant skin and/or lymph node mets	lung mets	any other visceral met	except uncontrolled brain mets, elevated LDH (> 1.5 x normal limit)
T1-4	N1	N2a	N2b	N2c	N3	M1a	M1b	M1c	
NO				YES					NO

- at least two cutaneous metastases at study entry (equal to or greater than 5 mm)
- Expected survival of at least 6 months
- Tumor must express the MAGE-3.A1 and/or the NA17.A2 antigen (requires HLA locus A typing and RT-PCR on pre-treatment frozen tumor for assessment of MAGE-3 and NA17 expression)

Figure 19
Patient inclusion criteria

rate lower than 10% would be considered insufficient for further development of the treatment combination. To avoid treating too many patients, our aim was to proceed in three steps. The clinical trial was designed to enroll the patients in 3 phases as shown in ***(Fig. 18)***.

The tumor response was assessed in accordance with the Modified RECIST version 1.1.
The main inclusion criteria in the trial was metastatic melanoma patients with regional disease (any T; N2c or N3; M0) or with distant metastatic disease (any T; any N; M1a, M1b or M1c), except uncontrolled brain or CNS metastases; at least one of the two following conditions: 1. HLA-A1 type and MAGE-3 expressed by the tumor; 2. HLA-A2 type and NA17 expressed by the tumor; at least 2 cutaneous metastases (at least 5 mm in largest diameter); an expected survival ≥ 6 months; standard inclusion criteria for phase I/II clinical trials ***(Fig. 19).***

The study was restricted to St-Luc university hospital. After 40 months, only 3 patients could be included in the trial. It was difficult to recruit patients for the trial, probably due to the many studies recruiting the same category of patients, especially trials involving immunostimulatory agents (such as anti CTLA-4). It was decided to stop the trial, even though some interesting responses were observed.

Three HLA-A2 patients whose tumor expressed the NA17 melanoma-specific transcript [283] were treated. They received 4 rounds of vaccination with the NA17.A2 peptide at two-week intervals. After each of the third and fourth rounds, one or two superficial metastases received the following local

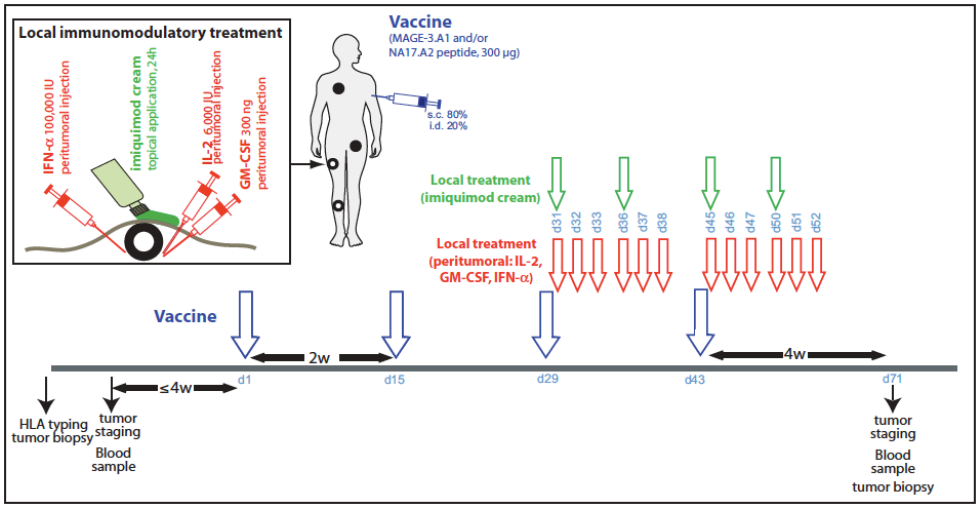


Figure 20
Treatment plan.

treatment peritumoral injection of a low dosage of IL-2, IFN-α and GM-CSF and topical applications of imiquimod (Aldara cream) ***(Fig. 20)***.

One patient showed no response (patient 3, fig. 21) to the treatment but interesting observations were made on the two other patients (patient 1 & 2, fig. 21). The first of these patients had previously been treated with a Tyrosinase-A2 vaccine without response. This patient had about 40 cutaneous leg metastases. He was vaccinated with the NA17.A2 peptide and two cutaneous metastases received the local treatment. Two months after the end of the treatment the majority of the cutaneous metastases had completely regressed and two months later all metastases had either completely or partially regressed.

The second patient had been treated in the preceding months with a MAGE-A3 protein vaccine without observable effects. He had 8 cutaneous leg metastases. He was vaccinated with the NA17.A2 peptide and the local treatment was applied to two metastases. No immediate effect was observed and one month later the patient received four courses of dacarbazine, again without clinical response. Four months later, *i.e.* seven months after the end of our treatment, a routine examination revealed that all the cutaneous metastases had undergone very significant regression. A metastasis in a mediastinal node had also regressed. However, a few months later this patient relapsed in several locations, including one of the treated metastases.

These observations are only suggestive, but they do open the possibility that the rejection process occurring in locally treated metastases can spread to untreated ones.

FIGURE 21: Patient 1

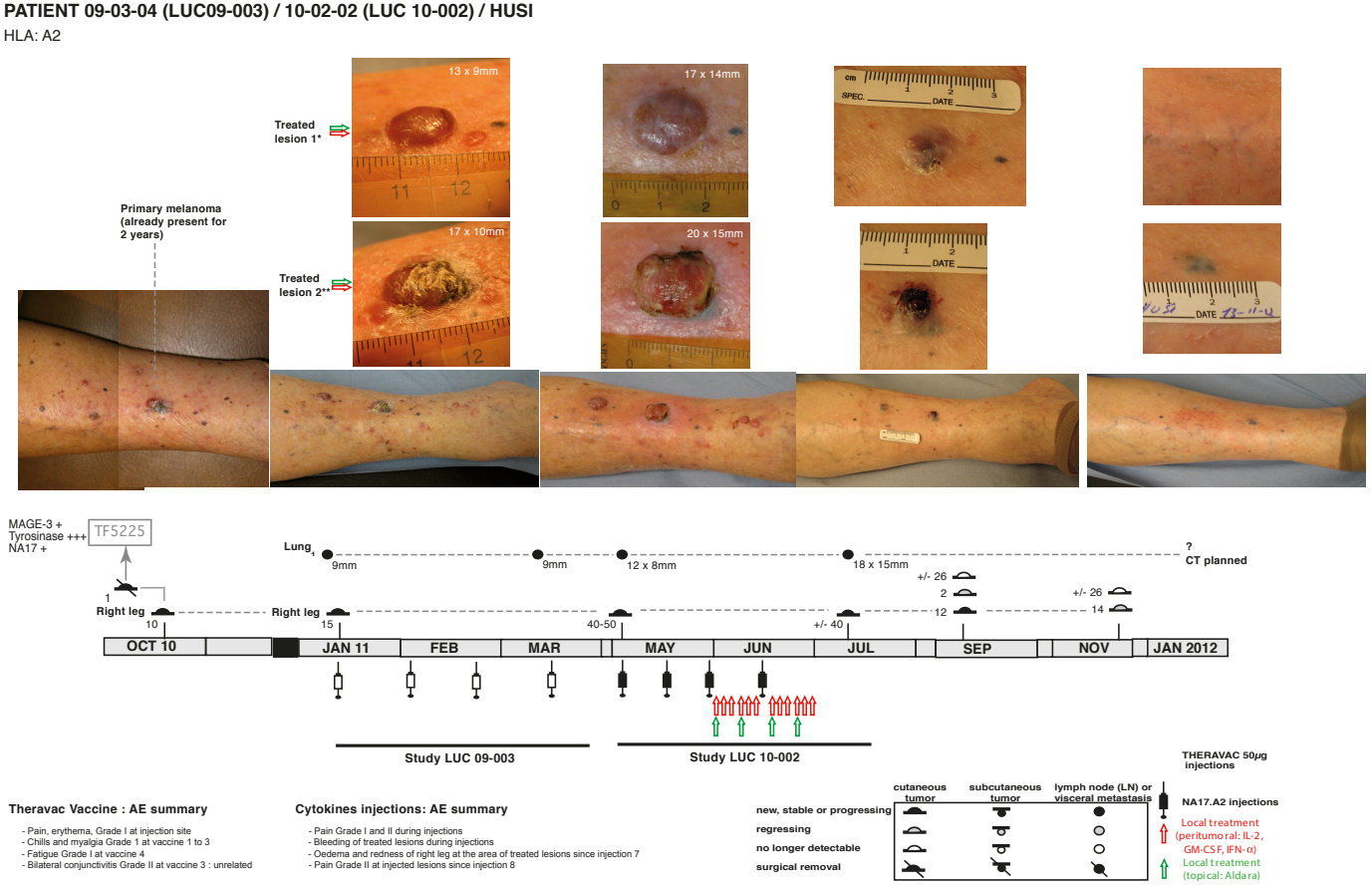


FIGURE 21: Patient 2

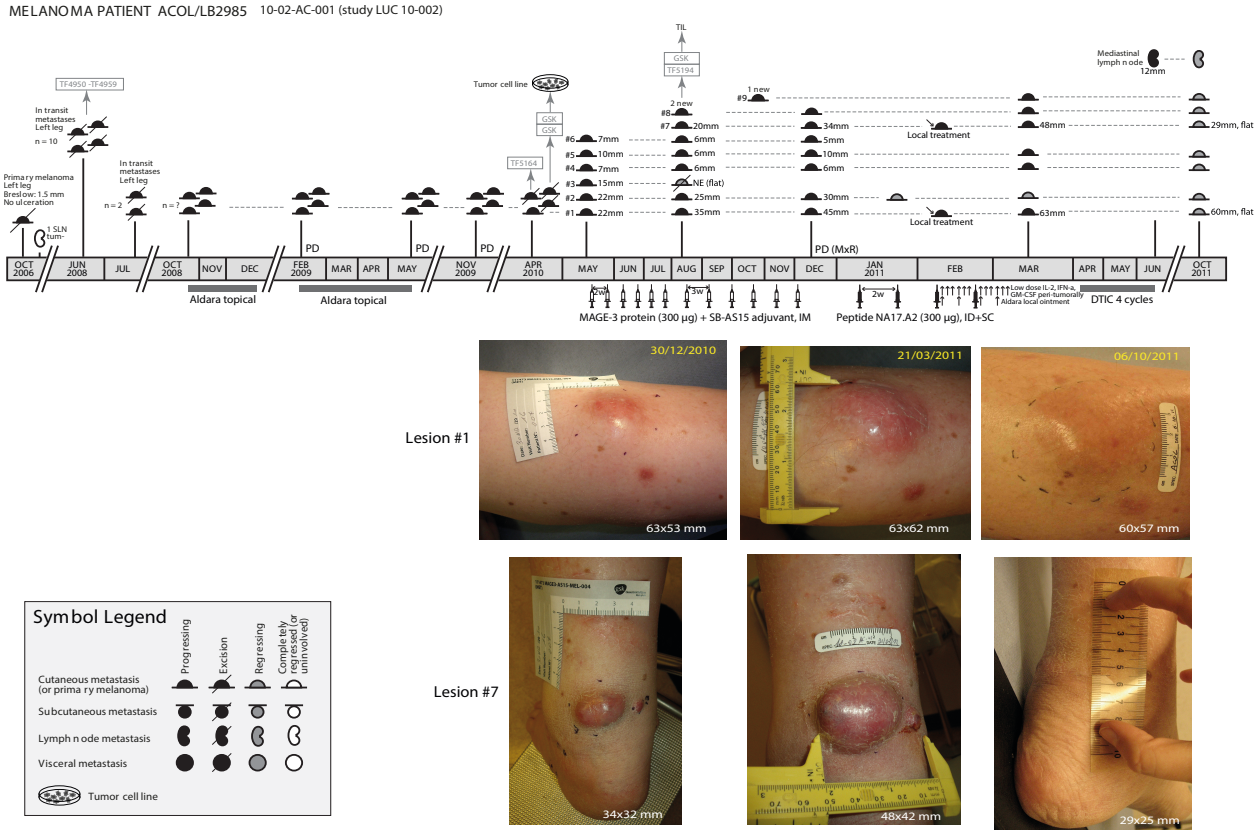
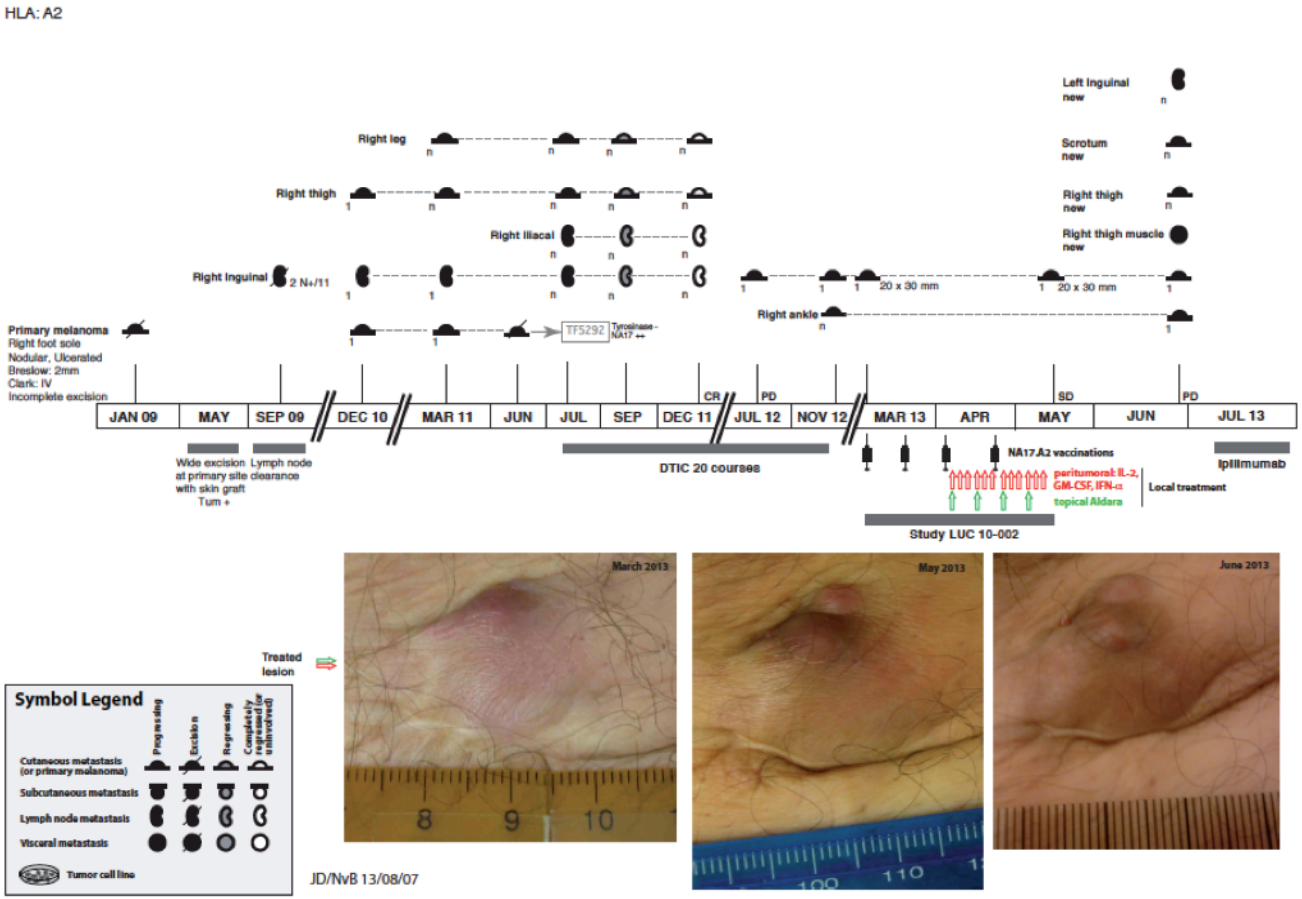


FIGURE 21: Patient 3



Materials and methods

Mice. CBA/Ca (CBA) and C57BL/6 mice were purchased from Harlan (Horst, The Netherlands) or produced under SPF conditions in the animal facility of the Ludwig Institute, Brussels Branch. C6ab TCR transgenic mice expressing a TCR specific for the Y-chromosome–derived male antigen HY K^k Smcy were bred in our facility [284,285]. The strain is on a CBA/Ca background. Mice were used at age of 6–10 weeks. All animal procedures have been approved by the Institutional Review Committee.

Skin grafts. Skin grafts were performed essentially according to the method of Billingham and Medawar [286] using tail skin grafted onto the lateral thorax. Ten days later the plaster cast was removed. A minimum of 20 days elapsed before any local treatment was performed on the graft to ensure perfect healing of the graft with the surrounding skin, and the onset of hair re-growth.

Skin graft rejection monitoring. We designated grafts as completely rejected when at least 90% of the surface of the graft showed scabbing, crust formation and hair loss. At that point it was difficult to ascertain whether any part of the graft had remained intact. The designation "mice with graft survival" corresponds to those with grafts that were not completely rejected. In most instances these grafts were intact by the end of the observation period. We designated grafts as partially rejected when 20%-90% of the graft showed scabbing, crust formation and hair loss.

Results

Cytokines. Recombinant murine IL-1α and GM-CSF were purchased from PeproTech (Paris, France), IL-2 was purchased as recombinant human IL-2 from Novartis Pharma (Vilvoorde, Belgium), IL-18 was purchased from MBL (Nagoya, Japan), IFN-α was purchased as Intron A from Schering-Plough (Uccle, Belgium), toll-like receptor ligand gardiquimod were purchased from Invitrogen (San Diego, USA). Recombinant murine IL-12 was produced as described [287].

Assay of cytolytic T lymphocytes

Spleen cells from grafted mice were stimulated in vitro for 7 days with irradiated male blasts (30 gray) in 24-well microplate containing 2 ml per well with $2.5x10^6$ responder and stimulator cells. The lytic activity of CTL was tested in a chromium-release assay. Briefly, CTLs were incubated with 5,000 ^{51}Cr-labeled target cells at various effector: target ratios in 96-well conical microplates in a equal volume of 200 µl. The supernatants were collected after a 4-h incubation at 37°C for the measurement of ^{51}Cr released from lysed cells. The percentage of ^{51}Cr-specific release was then calculated. Target cells were generated by activating spleen cells of male or female CBA mice (10^6/ml) with 25 µg/ml LPS for 2 days.

Local treatment of the grafts. The cytokines were diluted and mixed with PBS containing 1% of CBA mouse serum. A volume of 100 microliters was injected subcutaneously under the graft using a 30 Gauge needle. The point of penetration was located about 1 mm outside the boundary of the graft and varied between injections. Aldara was used as a cream, applied on the graft and protected for 24h by a bandage.

Results

Vaccination. For immunization of female mice, female spleen cell blasts were obtained by activating female spleen cells (10^6/ml) with 25 µg/ml LPS for 48h in cell medium. These female blasts were pulsed with the male specific peptides HY D^k Smcy, RRLRKTLL and HY K^k Smcy, TENSGKDI at a concentration of 10 µm for 1h at 37°. They were then washed 3 times with PBS. The pulsed blasts were resuspended in 50 µl PBS and implanted via a 30-gauge needle into the hind footpads.

Adoptive transfer. $CD8^+$ T cells were collected from the spleen of C6ab TCR transgenic mice and isolated using MACS separation columns and CD8a (Ly-2) microbeads according to the protocol of the manufacturer (Miltenyi Biotec). The resulting population comprised more than 90% of $CD8^+$ T cells bearing the anti-HY Smcy K^k receptor, as ascertained with PerCP rat anti-mouse CD8a antibody (BD Pharmingen) and PE H-2 K^k Smcy tetramer (Dan L. Duncan Center, Baylor College of medicine, Houston) using FacsCalibur (Becton Dickinson). Using an 18-gauge needle, 2.10^6 cells were injected in the peritoneal cavity.

PCR. To evaluate the presence of transgenic anti-HY T cells in the graft we used a quantitative PCR directed at the DNA sequences of the alpha chain of the receptor and its CD2 promoter. The sense primer was 5'-TGTGGACTCCACCAGTCTCAC and the anti-sense primer was 5'-GGAGCACAAGAACTGAGCAGGTGCC. The probe was FAM-5'-CATGAAGCGGAATTCGGCCTTGTCTG-TAMRA. This PCR provides a product of 183 bp with an annealing extension step of 1 min at 64°C.

Results

Quantitative PCR performed on purified transgenic cells provided an estimation of 25 copies of the transgene per cell.

Treatment of melanoma patients. Patients enrolled on the clinical trial LUC10-002 were required to have measurable metastatic melanoma with regional involvement (any T; N2c or N3; M0) or distant metastatic disease (any T; any N; M1a, M1b or M1c), including at least one cutaneous metastasis with size ≥5 mm. The patient's tumor had to express either the MAGE-3.A1 or NA17.A2 antigen, as determined by HLA-A typing and RT-PCR on a tumor fragment. Standard inclusion criteria for phase 1/2 clinical trials in oncology had to be met, including written informed consent before inclusion. The study objectives were to evaluate the anti-tumor effects, the toxicity and the immunogenicity of a peptide vaccine associated with the local administration of a cocktail of immunostimulatory drugs. This phase 1/2 study addresses a first series of 7 patients, which can be extended to up to 21 patients in case of evidence of efficacy in the absence of severe toxicity. The study protocol was approved by the Institutional Review Board, and was registered as 2010-020435-40 in EudraCT, and NCT01191034 in the ClinicalTrials.gov database. The three patients included up to now received sequential vaccinations with the NA17.A2 peptide (amino acid sequence VLPDVFIRC), a melanoma-specific antigen [283]. The peptide was administered at a dose of 300 µg in 1 ml of NaCl 0.9%, every 2 weeks on 4 occasions, with 20% of the dose administered intradermally and 80% of the dose subcutaneously, in arm or thigh. In addition, one or two superficial metastases received peritumor injections of IL-2 (Proleukin), IFN-α (IntronA) and GM-CSF (Leukine) (respectively

6,000 IU, 100,000 IU and 300 ng per tumor) on days +2,+3,+4,+7,+8 and +9 following vaccines 3 and 4, as well as topical applications of imiquimod (Aldara® cream) during 24h on days +2 and +7 following vaccines 3 and 4.

Acknowledgements

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DISCUSSION

1. Limited clinical success of cancer vaccines:

Cancer immunotherapy has made significant progresses in the past few years, especially since the development of the immunomostimulatory antibodies (such as anti-CTLA-4 or anti -PD-1). On the other hand cancer vaccines have failed to meet the high expectations that they had raised, being associated with rare clinical benefits[184].

Several reasons may explain this limited clinical success:

-The antigens used were not always tumor-specific or their presence at the surface of tumor cells was not ascertained. The side effects that have been observed following some adoptive T-cell therapies[244] suggest that tumor-specific antigens (such as MAGE-type or mutated antigens) [288,289] should be preferred whenever possible. In addition antigen expression within a tumor can be heterogeneous. Vaccines containing allogeneic tumor cell lines are limited by the fact that a given patient's tumor will probably harbor mutations that are not found in the vaccine product. The discovery and application of predictive biomarkers, which could identify those patients most likely to benefit from a given vaccine, is an important challenge for future development.

-Current vaccine modalities that can be used in man are poorly immunogenic to CTLs, and new modalities have to be developed. Viral vectors are promising, as viruses stimulate strong CTL responses.

-Immunosuppressive mechanisms active inside tumors have thus far been neglected in the cancer vaccine trials. It appears mandatory to block some of the immunosuppressive circuits and to enhance migration of T cells into tumor sites in order to increase the clinical efficacy of cancer

vaccines[290], as indicated by the clinical success of immunostimulatory agents.

-The clinical efficacy of cancer vaccines can partly be attributed to the fact that most cancer vaccine trials have been performed in patients with advanced disease, after surgery and administration of multiple chemotherapy regimens. It is likely that cancer vaccines will be more effective in a minimal disease setting before the administration of chemotherapies or other immunosuppressive treatment regimens.

- Investigators have initially been enthusiastic about the results of active immunization for patients with solid tumors because of an over-reliance on surrogate and subjective endpoints on trial design. Several cancer vaccine trials did not specify the precise criteria to determine clinical responses; some trials use 'soft' criteria such as histologic evidence of tumor necrosis or lymphocyte infiltration, rather than objective measurements of tumor sizes. The analysis of the results obtained in cancer vaccine trials should be base on objective clinical responses, such as the RECIST criteria (a 30% reduction in the sum of the maximum diameters of lesions to indicate a response, along with no appearance of new or progressive lesions)[291]. Adherence to these guidelines in cancer vaccine trials is essential to compare the results of different treatment protocols. Nevertheless, In some clinical studies, biopsies of metastases from vaccinated patients revealed the presence of immune infiltrates that may mediate tumor destruction in association with extensive edema, which could be followed by fibrosis[292]. This finding suggests that monitoring tumor size alone may be inadequate for assessing the overall therapeutic effects of vaccination, which highlights the need to modify tumor response criteria

according to new insights into the biology of immunotherapy[293].

The clinical success of cancer vaccine trials might be enhanced if each of the limitation described here could be solved.

2. The model used:

We have shown here that local immunostimulatory treatments involving certain combinations of cytokines and TLR ligands can reactivate stalled T cell responses against male mouse skin grafts. As it is highly unlikely that skin grafts develop the same mechanisms as tumor do to counteract immune rejection responses, we expect that the local immunostimulatory approaches described here would contribute to the reactivation of all stalled T cell rejection responses, observed in tumors as well as in grafts.

Two types of tumor models are classically used to analyze antitumor immunity in mice. First, the injection of tumor cell lines into mice. These models are convenient but the very fast tumor progression (weeks) makes them poor models of human tumors, which develop over several years. Second, inducible tumors which constitute a much improved model of human tumors but are difficult to handle. Moreover, no mouse tumor model has been so far a good predictor of a successful human response to immunotherapy. For example, treatment with anti-CTLA-4 antibodies induces antitumor responses in mice and is well tolerated[294] whereas in humans the antitumor responses can be accompanied by life-threatening colitis or hypophysitis[295,296].

To study the ability of pro-inflammatory cytokines to induce a rejection response, we used the model of rejection of male skin, which

carries HY antigens, by female mice. This model has several advantages. It is easy to handle, allowing experiments on a long time scale to study the immune responses to the grafted tissue. The HY model allows to explore graft rejection responses to a genetically isolated minor histocompatibility antigen encoded by a gene on the Y chromosome, a situation that is similar to the response to a MHC class I-restricted tumor peptide. In this model, most of the genes that have been shown to be involved in the response to HY, and the MHC class I and II-restricted peptides that they encode, have been identified[281,282,297,298]. In addition, the identification of MHC class I-restricted HY peptides has permitted the use of tetramers to enumerate ex vivo the effector $CD8^+$ T cells that are responsible for the skin graft rejection[299].

As demonstrated by Eichwald and Silmser (1955)[300], C57BL/6 ($H2^b$) female mice consistently reject grafts of syngeneic male skin; in contrast, females of other strains, such as CBA ($H2^k$), usually accept such grafts, even though we have observed that a CTL response directed against HY antigens does occur following grafting. Thus the process observed in CBA is similar to that observed in progressing human tumors: a failed attempt at rejection followed by the persistence of ineffective memory T cells, as observed previously by the group of E. Simpson[281,282,298].

We do not understand why the T cell responses against male skin grafts become stalled in CBA female mice. But relevant observations have been made in CBA females that were injected with male spleen cells [301]. Here, most mice did not reject the male spleen cells but some did. When tetramer-selected anti-HY CD8 T cells were transferred into responder mice, they expanded, whereas they did not in non-responder mice. In line

with this, significantly higher numbers of $CD4^+CD25^+$ putative regulatory T cells were observed in non-responder mice. The type of tolerance encountered in our system has been named "in vivo anergy" or "adaptative tolerance"[302]. Recent work indicates that a multiplicity of factors may contribute to the complex process leading to the establishment of T cell anergy. The inhibition of IL-2 production and signalling pathways and that of the PI3 kinase-AKT-mTOR pathways appear to be particularly crucial elements in this process [303]. Regarding the role of Tregs, it is noteworthy that even though IL-2 is a crucial factor for their activity, in our experiments it plays an essential positive role in promoting rejection when combined with gardiquimod but shows no efficacy when administred alone. In this respect, it is interesting to note the essential role of IL-2 observed here in the combination with gardiquimod but also the lack of efficacy of IL-2 alone.

3. Intralesional treatment:

Several reports have described the use of cytokines, TLR ligands or activating antibodies such as anti-CD40, for intralesional treament of mouse or human tumors[304,305]. Impressive results have been obtained on cutaneous metastases of melanoma patients[60,306]. Our approach focuses on the search for synergic combinations of various cytokines and TLR ligands with the aim of obtaining immune rejection with much lower dosages (50-500x) than those reported previously. This ought to minimize harmful side effects. We have tested, many pro-inflammatory cytokines and TLR ligands that are registered for human use, with the aim to find a combination that could also be tested on patients. We found an interesting combination based on GM-

CSF/IL-2/IFNα and Aldara® (imiquimod), which was very effective at inducing a graft rejection following a local administration in the graft.

Such combination was tested in 3 patients and it is interesting to note that no immediate effect was observed but responses occurred from 2 to 7 months after the end of the treatment. Such delayed response suggests that tumor regression only occurred after the immune activation was strong enough, an interval that can be long. For the third patient who did not respond, the superficial lesion was located on a scar of a previous surgical resection. It is possible that this location, possibly with fibrotic and less vascularized tissue, reduced the local effect of our injections.

Considering the absence of toxicity observed with anti-tumor vaccination and the extremely low dose of immunostimulatory agents applied locally, no significant toxicity was observed during the treatment, the only adverse event being a local inflammation with tenderness at the site of injection. Indeed the treatment involves repeated injections of inflammatory agents in a territory which is highly inflamed by the previous injections. Moreover our local immunostimulating treatment is presently difficult to apply to metastases other than those that are cutaneous or subcutaneous. But future technical advances could overcome both difficulties. In particular, it should be possible to trap the agents into microbeads that will ensure slow, long term delivery[307,308]. This could reduce the frequency of injections to once a week.

4. Conclusions:

The purpose of this report is to stimulate clinical cancer immunotherapy research combining vaccination with local and systemic

immunostimulatory agents. We are convinced that local immunostimulation should be supplemented with vaccination against specific tumor antigens. The influx into the tumor of anti-tumor T cells, which have been freshly produced by the vaccination and therefore have not yet been rendered anergic, may prove to be a key condition for success. Considering the absence of toxicity observed with anti-tumor vaccination and the extremely low dose of immunostimulatory agents applied locally, we expect this combination treatment to produce very little or no toxicity. The combinations of immunostimulatory agents described here could be used in a first step. Additional agents could be put into play later according to the observations that will be made. Agents that counteract immunosuppressive factors produced or elicited by cancer cells may be particularly useful. A low systemic dosage of anti-PD1, anti-PDL1 or anti-CTLA-4 might provide added efficacy without provoking autoimmune side effects.

Local immunostimulation as described here might also prove useful in combination with anti-PD1, anti-PDL1, and anti-CTLA-4, in the absence of vaccination since these agents clearly can have an impressive efficacy on their own. This again might provide the possibility of reducing their dosage resulting in lower toxicity.

It is interesting to make a parallel with the clinical experience with the T-VEC oncolytic virus. Its ability to induce regression of the treated tumor, but also of distant metastases is thought to reflect, apart from direct viral-induced tumor damage, also an anti-tumoral immune reaction boosted by the viral infection. Indeed, viruses are potent stimuli for innate and adaptive immune reactions. Their use as therapeutic agents faces the risks associated with infectious agents and genetically modified organisms.

Replacing the viral particles by mixes of immunostimulatory cytokines and ligands of immune cell receptors, aimed at mimicking these immune reactions, appears as a useful and realistic goal. Our approach can be regarded as a first step towards this goal.

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ANNEX

Local immunostimulation leading to rejection of accepted male skin grafts by female mice as a model for cancer immunotherapy

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Female mice of inbred strain CBA do not reject syngeneic male skin grafts even though they mount a T-cell response against the male-specific HY antigen. We show that local immunostimulation performed by injecting cytokines and Toll-like receptor ligands in close vicinity to the graft causes rejection. We feel that this approach should be tested in tumor-bearing human patients in combination with antitumor vaccination. Relief of intratumor immunosuppression may increase considerably the fraction of patients who respond to vaccination directed against tumor antigens recognized by T cells.

immunology | transplantation

The identification of the antigens recognized on human tumors by autologous T lymphocytes demonstrated that a majority of these antigens are highly tumor-specific (1, 2). Moreover, many of these antigens were found to be encoded by "cancer-germline" genes such as the *MAGE* family of genes, which are expressed by a large fraction of tumors of various histological types. This finding opened the possibility of vaccinating large groups of cancer patients with the same antigen to elicit T-cell responses able to destroy the cancer cells without damaging normal cells. Human tumors were also found to bear strictly tumor-specific antigens resulting from point mutations affecting ubiquitously expressed genes. These antigens are rarely shared by different tumors. Finally, melanoma patients also have T cells directed against melanocytic differentiation antigens, contrary to the expectation that natural tolerance ought to prevent such responses (1, 3).

The initial clinical trials with cancer vaccines were performed on melanoma patients with detectable metastatic disease. Small trials involving vaccination with MAGE antigens confirmed the expectation that complete or partial tumor responses could be obtained in the absence of autoimmunity (4, 5). However, these clinical responses were observed in only 5–10% of the patients, with no sign of tumor regression observed in the vast majority. Because these first vaccination trials were performed with antigenic peptides in the absence of adjuvant, it was widely expected that the use of more potent vaccines, such as viral recombinant vaccines or peptide-pulsed dendritic cells, would produce improved results. However, more than a decade of such vaccination trials has failed to provide significant improvement of clinical efficacy (6).

The initial view of the process elicited by vaccination in responding patients was that antivaccine cytolytic T lymphocytes (CTL) would migrate in large numbers to tumor sites and directly destroy the tumor cells. We believe that this view is erroneous. First, even in patients who experience a clinical response, there are insufficient numbers of antivaccine T cells. The analysis of T cells present in peripheral blood indicates that most of these patients have at best a barely detectable antivaccine T-cell response (7). In some clinical responders, we evaluated the number of antivaccine T cells present in tumor deposits, and we concluded that they are present at a ratio of less than one in a million relative to tumor cells. Moreover, much larger numbers of T cells directed against other tumor-specific antigens are already present within the tumor before vaccination, and some of these T-cell populations expand considerably when rejection occurs.

The presence of tumor-infiltrating lymphocytes (TILs) with potential antitumor activity was observed as early as 1988 by Rosenberg and his associates (8), who cultured single-cell suspensions of metastases in the presence of interleukin-2 (IL-2) and obtained large numbers of lymphocytes that exerted cytolytic activity on fresh tumor cell preparations, albeit under conditions where conclusive specificity controls could not be carried out (8, 9). The detailed identification of the set of tumor-specific antigens expressed by some tumors enabled us to examine TILs under conditions where the antigenic target and T-cell receptor sequence were identified. This led to the demonstration that before vaccination TILs directed against several of these tumor-specific antigens are indeed present in the tumor. After vaccination, in patients who make clinical responses, many TIL clonotypes analyzed within the tumor were found to be considerably amplified, with frequencies about 1,000-fold higher than that of the antivaccine TILs. Dominant clonotypes that were absent before vaccination, were also observed. Collectively, this diverse set of expanding antitumoral TILs reached a frequency of 1% relative to the tumor cells (10).

On the basis of these and other findings, it is now clear that most melanoma patients produce a spontaneous T-cell response

Significance

Many cancer patients mount a spontaneous T-lymphocyte response against their tumor. In metastatic progressing patients, this response has evidently been incomplete. Moreover, it has stalled, and an immunosuppressive environment appears to be present within the tumor. This may constitute a major factor preventing success of immunotherapies such as antitumoral vaccination. Similar stalled responses are occasionnally observed in the area of tissue transplantation. In a mouse strain named CBA, this phenomenon is observed when male skin is grafted onto females. We used this system to find combinations of cytokines and Toll receptor ligands that, when applied locally, relieve the immunosuppression and reactivate the response. The combinations reported here may prove generally useful to boost the efficacy of cancer immunotherapy.

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against their tumor (7). In many patients, this attempt at eliminating the tumor evidently fails and a large number of antitumor T cells remain in an inactive state in circulation and within tumor deposits. Inside the tumors, this anergic state appears to be reinforced by an immunosuppressive environment. For the vaccinated patients who show tumor regression, we propose the following sequence of events: a very small number of active antivaccine T cells penetrates the tumor and attacks some tumor cells. As a result, these CTL are restimulated and produce cytokines that focally reverse the local immunosuppression, creating instead an immunostimulatory environment. This reawakens many of the inactive antitumor T cells that are already present in significant numbers in situ. Naive antitumor T cells may also be activated. The mobilization of all these T cells, most of which are directed against tumor antigens other than the vaccine antigen, provides the large numbers of effector cells required to reject the tumor.

Why is it that, following vaccination, tumors regress in some patients whereas no sign of regression is observed in most patients? A first possibility is that this may be due to differences in the quantity or quality of T cells responding to the vaccine. The lack of improvement of clinical outcome following highly immunogenic vaccination suggests that the strength of the response to the vaccine is not the limiting factor. A second possible difference between regressing and nonregressing patients could be the degree of expression of the relevant antigens on the surface of tumor cells. It is well established that tumors can lose the expression of some or all of their HLA molecules (1, 11, 12). However, we derived melanoma cell lines from a large number of nonresponder patients, and almost all of them proved to be sensitive to antivaccine CTL in vitro. We therefore suspect that loss of antigen expression accounts for only a small fraction of the treatment failures. A third possible explanation for the individual differences in clinical response is that patients vary in the degree of immunosuppression present in their tumor. A highly immunosuppressive tumor environment could render the antivaccine T cells inactive soon after penetrating the tumor, preventing them from initiating the cascade of events that leads to tumor rejection. The responder patients would be those who have a lower degree of immunosuppression in their tumor.

The anergic state of tumor-infiltrating lymphocytes and the immunosuppressive tumor environment are often considered to result from an active immunosuppressive mechanism of tumor cells acquired in the course of the selection for resistance to the immune response. Agents such as indoleamine 2,3-dioxygenase, tryptophan 2,3-dioxygenase, and TGF-β are produced in many tumors and unquestionably contribute to inducing the anergy of infiltrating lymphocytes (13–15). However, it is also possible that where the immune system makes a rather weak attempt to eliminate a tissue, as expected in the absence of pathogen-associated molecular patterns binding to Toll-like, Nod, and RIG receptors, after a length of time the immune system enters an inactive state. Examples of such "immune fatigue" are observed in human transplantation: a small number of recipients can be withdrawn from treatment with immunosuppressive agents without deleterious effects in the grafted organ (16, 17). This process, whereby an incomplete response evolves into an anergic state, could be common to all progressive metastatic tumors, whereas the presence and the nature of immunosuppressive factors secreted by tumor cells are likely to vary from one tumor to another. The common anergic state could involve a degree of immunosuppression within the tumor, resulting in potential paralysis of incoming T lymphocytes.

If the degree of intratumor immunosuppression is the limiting factor for the success of vaccination against tumors, the use of cytokines to alleviate this immunosuppression could improve the outcome. A considerable amount of effort has been devoted to testing systemic cytokine treatment in cancer patients. Impressive results have been obtained in a number of patients treated with IL-2 and IFN-α, but at the dosage required for efficacy significant toxicity has been observed (8, 18). We feel that, in contrast, local cytokine treatment of metastases should be tested as an adjunct to vaccination. This local treatment could enable the antivaccine T cells to initiate the rejection process. Moreover, the amount of agents used for local treatment would be orders of magnitude below those used for systemic treatments, thereby reducing the occurrence of adverse side effects. We realize that this approach will lead to therapeutic progress only if the immune rejection response of the treated metastasis leads to the production of antitumor T cells of such quantity and quality that they successfully attack untreated metastases disseminated elsewhere in the body.

We present here a set of observations related to local immunostimulation with cytokines and Toll-like receptor (TLR) ligands. We wanted to address first the common component of anergy, referred to above as "immune fatigue." To this end, we carried out preclinical studies using the mouse HY system, which involves T-cell responses to antigens expressed in male and not in female tissues. This model is attractive as it has several features shared with the immune response to human tumors. Whereas inbred mouse strain C57BL/6 ($H2^b$) females reject syngeneic male skin grafts, CBA ($H2^k$) females do not, even though we have observed that a CTL response directed against HY antigens does occur following grafting. Thus, the process observed in CBA is similar to that observed in progressing human tumors: a failed attempt at rejection followed by the persistence of ineffective memory T cells. Moreover, in contrast with most mouse tumors, skin grafts can be followed and manipulated over a period of several months, a situation similar to that encountered with most human tumors. We report below that some locally administered cytokine combinations are effective in promoting the rejection of these skin grafts.

Results

As shown in Fig. 1, CBA/Ca ($H2^k$) females do not reject male skin grafts even though they mount a CTL response against HY antigens of the graft.

Elizabeth Simpson and her associates have analyzed $H2^k$-restricted HY-specific CTL responses. They have identified HY antigenic peptides encoded by the *Smcy* gene, including the dominant peptide RRLRKTLL presented by the H2-D^k class I molecule and the peptide TENSGKDI presented by H2-K^k (19, 20). We observed that female CBA mice produce CTL against male target cells expressing both peptides after grafting with syngeneic male, but not female, skin.

Local Treatment with IL-12 and IFN-α. We tested various immunostimulatory agents to identify combinations that are able to promote graft rejection following local treatment in the absence of notable toxicity.

A number of CBA females grafted with male skin were treated with IL-12 and with IFN-α (Fig. 2). Small volumes were injected

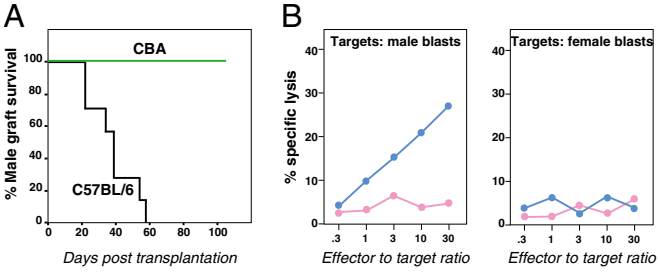


Fig. 1. (*A*) Seven CBA or C57BL/6 female mice were grafted on the thorax with a 1-cm^2 male skin patch of the same strain. The graph indicates the time of complete rejection. (*B*) Female CBA mice were grafted with either male (blue) or female (red) skin. After 22 d, 25.10^5 of their spleen cells were stimulated in vitro for 6 d with 25.10^5 irradiated male blast cells obtained by incubation of lymphocytes with LPS. The lytic activity was then tested on male and female blasts in a 4-h chromium release assay.

s.c. under the graft. This local treatment was administered for 3 consecutive days every week either until complete graft rejection or until the end of the fifth week. IFN-α at a dosage of 10^5 units had no effect. IL-12 administered alone was effective at 1,000 units. Clear synergy was observed between the two cytokines, resulting in complete rejection of all of the grafts following local treatment with 100 units of IL-12 combined with 10^5 units of IFN-α. No symptoms of toxicity were observed.

The observed rejections do not appear to be due to a nonspecific local toxicity of the cytokines as female skin grafts were not rejected (Fig. S1). Moreover, they seem to result principally from a local as opposed to a systemic effect of the cytokines, as i.p. injections of the same dose of cytokines were clearly much less effective (Fig. S2*A*). Whereas some rejections could be observed after treating the graft for only 1 wk, 5 wk of treatment were necessary to elicit virtually 100% rejection (Fig. S3). The treatment involving IL-12 and IFN-α was less effective when given 100 d after grafting rather than after 20 d: 60% versus 100% of the mice showed rejection. This may be due to a decrease in the level of immune memory, likely to diminish between 3 and 12 wk after grafting. Alternatively, this effect may be due to a gradual decrease of the local inflammation resulting from the surgical grafting process.

IFN-α was not the only cytokine capable of causing graft rejection in combination with IL-12. IL-1α, IL-2, and IL-18 also showed definite synergies. So did gardiquimod, a ligand of TLR7 (Table S1).

Interleukin 2, IFN-α, Granulocyte-Macrophage Colony-Stimulating Factor, and Gardiquimod. Considering the difficulty and delays encountered in performing clinical trials with drugs that have not yet been approved for human use, we resorted to our preclinical model to test a combination of IL-2, IFN-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF), which are drugs registered for clinical use. Because repeated local injections of 300 ng of IL-2 mixed with 10^5 units of IFN-α and 300 ng of GM-CSF had little effect, we added to the mixture 20 μg of gardiquimod, as it is similar to the TLR7 ligand imiquimod, which is approved for human use. This resulted in the rejection of the male skin grafts in the absence of notable toxicity (Fig. 3). The effect was due to a local and not to a systemic action of the agents, as i.p. injection had no effect (Fig. S2*B*). Female skin grafts were not rejected. The full effect was maintained when GM-CSF was omitted. The combination of gardiquimod and IL-2 was almost as effective as the complete combination. Increasing the dosage of IL-2 to 3 μg accelerated rejection and caused no obvious toxicity. Local injections of 6 μg of IL-2 alone or 20 μg of gardiquimod alone were ineffective.

In contrast to the combination of IL-12 and IFN-α, a gardiquimod + IL-2 + IFN-α + GM-CSF treatment initiated 100 d after the grafting of the male skin proved as effective as a treatment initiated at 22 d (Fig. S4).

With the combination of gardiquimod, IL-2, IFN-α, and GM-CSF, we re-examined the need for repeating local injections for several weeks. As shown in Table 1, in a group of 30 mice, ongoing rejection was observed in 18 mice after 2 wk. For 9 of those mice, the treatment was halted, and 6 mice proceeded to complete rejection. For the other 9, the treatment was pursued, leading to eight complete rejections. We conclude that, among the mice that show an early onset of rejection, most can complete the rejection process without further local stimulation. The picture was quite different for the 12 mice that displayed a later onset of rejection, i.e., after 3–5 wk: none of those whose treatment was arrested then rejected their grafts completely, whereas most mice with maintained treatment eventually rejected their grafts.

We examined whether other agents could complement the local action of the IL-2 and IFN-α combination. A combination of anti-TGFβ1 and anti–IL-10 showed efficacy. So did a combination of anti–CTLA-4, anti-PDL1, and GM-CSF. However, neither combination was as effective as gardiquimod (Table S2).

Effect of Various Cytokines on Graft Invasion by T Cells. To begin evaluating the action of the various immunostimulatory agents that proved capable of promoting graft rejection, we examined their ability to promote the presence of anti-HY CD8 T lymphocytes in the graft. We used CD8 T cells from spleens of T-cell receptor transgenic mice expressing a receptor specific for the K^k HY peptide described above. After i.p. adoptive transfer, the number of transgenic T cells present in any given location could be estimated by a quantitative PCR specific for this receptor sequence. No increase in the number of anti-HY transgenic T cells present in the graft was observed following treatment with either IFN-α or GM-CSF. In contrast, gardiquimod, IL-12, and IL-2 produced a very significant increase (Table 2). We are unable to evaluate whether this increase is due mainly to increased influx, increased proliferation within the graft, or decreased efflux. The agents that cause a large increase in the number of anti-HY T cells found in the graft also cause a moderate increase of these cells in the blood and in the spleen. This could be due to some systemic action of these agents, or it could be a consequence of T-cell circulation between these compartments and the graft. We verified that the local cytokine treatment does not elicit a nonspecific influx of lymphocytes: no increase in lymphocytes was observed within female grafts.

Local Immunostimulation Combined with Vaccination. We next examined whether the local effect of cytokines could be boosted by vaccinating the mice with male antigen after graft healing. This is a situation similar to that encountered in cancer patients who receive therapeutic antitumor vaccination. For this purpose, we reduced the efficacy of local treatment by using IL-2, IFN-α, and GM-CSF and omitting gardiquimod. As shown in Fig. 4, under these circumstances vaccination with peptide-pulsed female lymphoblasts appeared to boost the effect of local treament. In another similar experiment, the combined treatment resulted in 7/10 of the mice showing complete or partial rejection, whereas vaccination alone and local treatment alone produced, respectively, 3/10 and 1/5 complete or partial rejections. Vaccination with the two peptides alone was ineffective.

Systemic Effects in Mice and Humans. For the combination of therapeutic vaccination and local immunostimulation to be effective in the treatment of human metastatic cancer, it is not sufficient to obtain rejection of the metastases that are treated locally. The rejection must result in such an activation of the immune effectors that they acquire the ability to eliminate the other metastases that have not been treated.

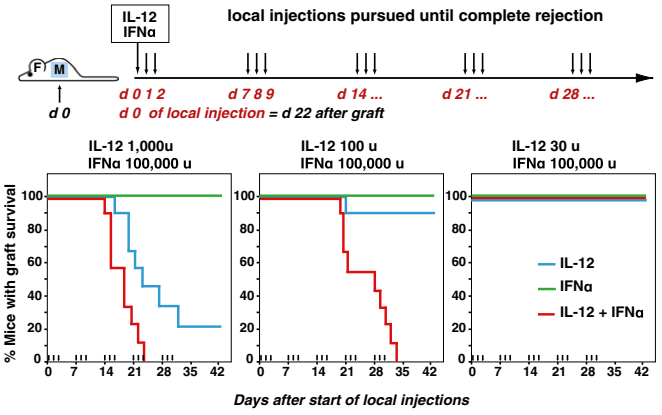


Fig. 2. Groups of nine CBA female mice were grafted with male skin of the same strain. Twenty-two days later, a local treatment involving IL-12, IFN-α, or both cytokines was applied with the schedule and the dosage shown. Injections were carried out on weekly schedules involving injections on 3 consecutive days. A solution of about 100 μL containing both interleukins was injected s.c. under the graft. The graphs indicate the time of complete rejection.

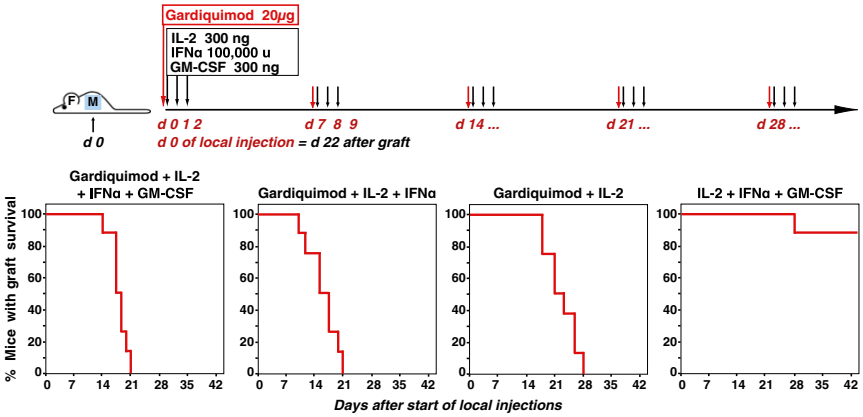


Fig. 3. Groups of eight female CBA mice were grafted with a patch of male skin. Local treatment was initiated 22 d later with a mixture containing a combination of 20 μg gardiquimod, 300 ng IL-2, 10^5 units IFN-α, and 300 ng GM-CSF as indicated in the figure. The mixture was injected s.c. under the graft. Gardiquimod was injected once weekly, and the cytokines were injected on 3 consecutive days as shown. The treatment was stopped after either 5 wk or earlier if complete rejection was observed.

A majority of the mice that received a local treatment either with IL-12 and IFN-α or with IL-2, IFN-α, and gardiquimod rejected subsequent grafts in the absence of further local treatment, indicating a degree of systemic increase of anti-HY memory T cells in these mice (Table S3). However, when female mice grafted with male skin on both flanks received treatment only on the right graft, only modest effects were observed on the left graft. Even in those mice where the right graft was completely rejected, most left grafts remained intact (Table S4). Moreover, it is possible that rejection of some of the untreated left grafts was due to a systemic effect of the cytokines, rather than being due to the activation of anti-HY lymphocytes resulting from the rejection of the treated grafts.

Nevertheless, we felt that the situation encountered with cancer patients might differ in this respect from that encountered with male skin grafts on female mice because the immune system of cancer patients appears to mount a considerable T-cell response against a multiplicity of tumor-specific antigens before becoming anergic. We therefore initiated a small clinical trial involving patients with metastatic melanoma. Before initiating the trial, we performed an experiment on mice with the combination of TLR-7 ligand imiquimod (Aldara cream), which replaced gardiquimod, and IL-2, IFN-α, and GM-CSF injected at the same location, but in separate syringes, as requested for the patients. The results were identical to those obtained with the combination involving gardiquimod (Fig. S5).

Three HLA-A2 patients expressing the NA17 tumor-specific antigen (21) were treated, receiving four rounds of vaccination with the NA17.A2 peptide at 2-wk intervals. After each of the third and fourth rounds, these patients received a local treatment on one or two superficial metastases, namely six peritumoral injections of a low dosage of IL-2, IFN-α, and GM-CSF and two topical applications of imiquimod (Aldara cream) (Fig. S6). One patient showed no response to the treatment, but interesting observations were made of the two other patients.

The first of these patients had previously been treated with a tyrosinase-A2 vaccine without response. This patient had about 40 cutaneous leg metastases. He was vaccinated with the NA17.A2 peptide, and two cutaneous metastases received the local treatment. Two months after the end of the treatment, the majority of the cutaneous metastases had completely regressed and 2 mo later all metastases had completely or partially regressed.

The second patient had been treated in the preceding months with a MAGE-A3 protein vaccine without observable effects. He had eight cutaneous leg metastases. He was vaccinated with the NA17.A2 peptide, and the local treatment was applied to two metastases. No immediate effect was observed, and 1 mo later the patient received four courses of dacarbazine, again without clinical response. Four months later, i.e., 7 mo after the end of our treatment, a routine examination revealed that all of the cutaneous metastases had undergone very significant regression. A metastasis in a mediastinal node had also regressed. However, a few months later this patient relapsed in several locations, including one of the treated metastases.

These observations are only suggestive, but they do open the possibility that the rejection process occurring in locally treated metastases can spread to untreated ones.

Discussion

We show here that local immunostimulatory treatments involving certain combinations of cytokines and TLR ligands can reactivate stalled T-cell responses against male mouse skin grafts. As it is highly unlikely that skin grafts develop particular ways to counteract immune rejection responses, we expect that the local immunostimulatory approaches described here would contribute to the reactivation of all stalled T-cell rejection responses observed in tumors as well as in grafts.

We do not understand why the T-cell responses against male skin grafts become stalled in CBA female mice. However, relevant observations have been made in CBA females that were injected with male spleen cells (22). Here, most mice did not reject the male spleen cells but some did. When tetramer-selected anti-HY CD8 T cells were transferred into responder mice, they expanded, whereas they did not in nonresponder mice. In line with this, significantly higher numbers of $CD4^+CD25^+$ putative regulatory T cells were observed in nonresponder mice.

Table 1. Fate of grafts upon treatment arrest

No. of mice showing onset of rejection after weeks of treatment	No. of mice showing complete rejection	
	Treatment halted	Treatment pursued
18 after 2 wk	6/9	8/9
12 after 3–5 wk	0/6	5/6

Thirty female CBA mice received a male skin graft. Forty days after grafting, we initiated local treatment with gardiquimod, IL-2, IFN-α, and GM-CSF as described in Fig. 3. The 18 mice showing onset of rejection after 2 wk were divided at random into two equal groups. For the first group the treatment was halted, and for the second group the treatment was pursued either until complete rejection or the end of the sixth week. The 12 mice showing onset of rejection after 3–5 wk were also divided into a treatment and a nontreatment group.

Table 2. Anti-HY T-cell presence in graft caused by immunostimulatory agents

Local treatment	Total no. of anti-HY transgenic cells in: Skin graft	Blood	Spleen
PBS	4,000	8,000	28,000
IL-12, 300 units	114,000	16,000	42,000
IFN-α, 10^5 units	800	4,000	21,000
Gardiquimod, 20 μg	98,000	24,000	49,000
IL-2, 300 ng	47,000	12,000	35,000
GM-CSF, 300 ng	5,000	6,000	21,000

Female CBA mice received an i.p. injection of 15.10^5 spleen CD8 T cells transgenic for the T-cell receptor directed against the K^k HY peptide. One day later, these mice received a male skin graft. Three weeks later, the graft was treated locally with the agents indicated for 2 consecutive weeks. Gardiquimod was injected once a week. The other reagents were injected on the first, second, and fourth day of each week. The graft tissue was collected 1 wk after the end of treatment. The DNA was extracted, and the number of specific T-cell-receptor–coding DNA sequences was evaluated by PCR. The results represent the average of four mice. The total number of transgenic T cells is presented based on estimates of a total number of nucleated cells of 2.10^6 in the graft, 2.10^7 in the blood, and 7.10^7 in the spleen. We observed large differences between individual mice.

The type of tolerance encountered in our system has been named "in vivo anergy" or "adaptative tolerance" (23). Recent work indicates that a multiplicity of factors may contribute to the complex process leading to the establishment of T-cell anergy. The inhibition of IL-2 production and signaling pathways and that of the PI3 kinase-AKT-mTOR pathways appears to be a particularly crucial element in this process (24). IL-2 is a crucial factor for Treg activity. In this respect, it is interesting to note the essential role of IL-2 observed here in combination with gardiquimod but also the lack of efficacy of IL-2 alone.

Several reports have described the use of cytokines, TLR ligands, or activating antibodies, such as anti-CD40, for intralesional treament of mouse or human tumors (25, 26). Impressive results have been obtained on cutaneous metastases of melanoma patients (27, 28). Our approach focuses on the search for synergic combinations of various cytokines and TLR ligands with the aim of obtaining immune rejection with much lower dosages (50–500×) than those reported previously. This ought to minimize harmful side effects.

Local immunostimulation is presently difficult to apply to metastases other than those that are cutaneous or subcutaneous. Moreover, the treatment involves injections of inflammatory agents in a territory that is highly inflamed by the previous injections. This can be painful. However, we have little doubt that technical advances will overcome both difficulties. In particular, it should be possible to trap the agents into capsules that will ensure slow, long-term delivery (29, 30). This could reduce the frequency of injections to once a week.

The purpose of this report is to stimulate clinical cancer immunotherapy research combining vaccination with local and systemic immunostimulatory agents. We are convinced that local immunostimulation should be supplemented with vaccination against specific tumor antigens. The influx into the tumor of antitumor T cells, which have been freshly produced by the vaccination and therefore have not yet been rendered anergic, may prove to be a key condition for success. Considering the absence of toxicity observed with antitumor vaccination and the extremely low dose of immunostimulatory agents applied locally, we expect this combination treatment to produce very little or no toxicity. The combinations of immunostimulatory agents described here could be used in a first step. Additional agents could be put into play later. Agents that counteract immunosuppressive factors produced or elicited by cancer cells may be particularly useful. A low systemic dosage of anti-PD1, anti-PDL1, or anti–CTLA-4 might provide added efficacy without provoking autoimmune side effects. Perhaps our cancer immunotherapy strategies should emulate the immune system by administering a multiplicity of agents that complement each other.

Materials and Methods

Mice. CBA/Ca (CBA) and C57BL/6 mice were purchased from Harlan or produced under specific pathogen-free (SPF) conditions in the animal facility of the Ludwig Institute, Brussels Branch. C6ab TCR transgenic mice expressing a TCR specific for the Y-chromosome–derived male antigen HY K^k Smcy were bred in our facility (31, 32). The strain is on a CBA/Ca background. Mice were used at the age of 6–10 wk. All animal procedures were approved by the Institutional Review Board of the Université Catholique de Louvain.

Skin Grafts. Skin grafts were performed essentially according to the method of Billingham and Medawar (33) using tail skin grafted onto the lateral thorax. Ten days later the plaster cast was removed. A minimum of 20 d elapsed before any local treatment was performed on the graft to ensure perfect healing of the graft with the surrounding skin and the onset of hair regrowth.

Skin Graft Rejection Monitoring. We designated grafts as completely rejected when at least 90% of the surface of the graft showed scabbing, crust formation, and hair loss. At that point, it was difficult to ascertain whether any part of the graft had remained intact. The designation "mice with graft survival" corresponds to those with grafts that were not completely rejected. In most instances, these grafts were intact by the end of the observation period. We designated grafts as partially rejected when 20–90% of the graft showed scabbing, crust formation, and hair loss.

Cytokines. IL-1α, GM-CSF, IL-2, IL-18, IFN-α, and gardiquimod were purchased (*SI Materials and Methods*). Recombinant murine IL-12 was produced as described (34).

Local Treatment of the Grafts. The cytokines were diluted and mixed with PBS containing 1% of CBA mouse serum. A volume of 100 μL was injected s.c. under the graft using a 30-gauge needle. The point of penetration was located about 1 mm outside the boundary of the graft and varied between

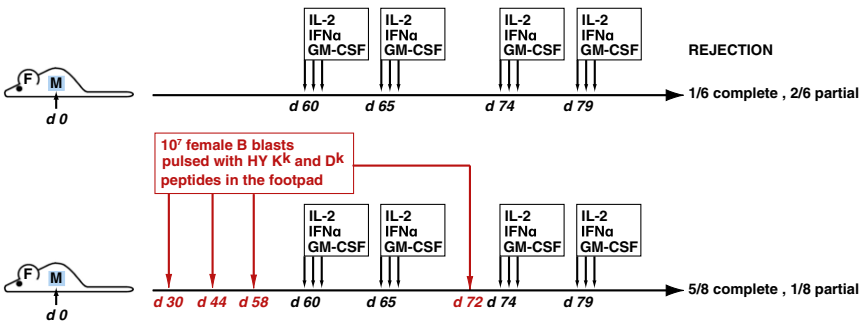


Fig. 4. Female mice grafted with male skin received local treatments with 300 ng of IL-2, 300 ng of GM-CSF, and 10^5 units of IFN-α. Mice of one group were vaccinated repeatedly with female lymphoblastic cells pulsed with the HY D^k and K^k peptides described in the *Results* section.

injections. Aldara was used as a cream, applied on the graft, and protected for 24 h by a bandage.

Vaccination. For immunization of female mice, female spleen cell blasts were obtained by activating female spleen cells (10^6/mL) with 25 μg/mL LPS for 48 h in cell medium. These female blasts were pulsed with the male specific peptides HY D^k Smcy, RRLRKTLL and HY K^k Smcy, TENSGKDI at a concentration of 10 μm for 1 h at 37°. They were then washed 3 times with PBS. The pulsed blasts were resuspended in 50 μL PBS and implanted via a 30-gauge needle into the hind footpads.

Adoptive Transfer. $CD8^+$ T cells were collected from the spleen of C6ab TCR transgenic mice and isolated using MACS separation columns and CD8a (Ly-2) microbeads according to the protocol of the manufacturer (Miltenyi Biotec). The resulting population composed more than 90% of $CD8^+$ T cells bearing the anti-HY Smcy K^k receptor, as ascertained with PerCP rat anti-mouse CD8a antibody (BD Pharmingen) and PE H-2 K^k Smcy tetramer (Dan L. Duncan Center, Baylor College of Medicine, Houston) using Facs-Calibur (Becton Dickinson). Using an 18-gauge needle, 2.10^6 cells were injected into the peritoneal cavity.

PCR. To evaluate the presence of transgenic anti-HY T cells in the graft, we used a quantitative PCR directed at the DNA sequences of the α-chain of the receptor and its CD2 promoter. The sense primer was 5′-TGTGGACTCCAC-CAGTCTCAC and the antisense primer was 5′-GGAGCACAAGAACTGAG-CAGGTGCC. The probe was FAM-5′-CATGAAGCGGAATTCGGCCTTGTCTG-TAMRA. This PCR provides a product of 183 bp with an annealing extension step of 1 min at 64 °C. Quantitative PCR performed on purified transgenic cells provided an estimation of 25 copies of the transgene per cell.

Treatment of Melanoma Patients. Patients enrolled in the clinical trial LUC10-002 were required to have measurable metastatic melanoma with regional involvement (any T; N2c or N3; M0; American Joint Committee on Cancer Staging System) or distant metastatic disease (any T; any N; M1a, M1b, or M1c), including at least one cutaneous metastasis with a size ≥5 mm. The patient's tumor had to express either the MAGE-3.A1 or NA17.A2 antigen, as determined by HLA-A typing and RT-PCR on a tumor fragment. Standard inclusion criteria for phase 1/2 clinical trials in oncology had to be met, including written informed consent before inclusion. The study objectives were to evaluate the antitumor effects, the toxicity, and the immunogenicity of a peptide vaccine associated with the local administration of a mixture of immunostimulatory drugs. This phase 1/2 study addresses a first series of 7 patients, which can be extended to up to 21 patients in case of evidence of efficacy in the absence of severe toxicity. The study protocol was approved by the Institutional Review Board of the Université Catholique de Louvain and was registered as 2010–020435-40 in EudraCT and as NCT01191034 in the ClinicalTrials.gov database. The three patients included up to now received sequential vaccinations with the NA17.A2 peptide (amino acid sequence VLPDVFIRC), a melanoma-specific antigen (21). The peptide was administered at a dose of 300 μg in 1 mL of NaCl 0.9%, every 2 wk on four occasions, with 20% of the dose administered intradermally and 80% of the dose subcutaneously in arm or thigh. In addition, one or two superficial metastases received peritumor injections of IL-2 (Proleukin), IFN-α (Intron A), and GM-CSF (Leukine) (respectively, 6,000 IU, 100,000 IU, and 300 ng per tumor) on days +2, +3, +4, +7, +8, and +9 following vaccines 3 and 4, as well as topical applications of imiquimod (Aldara cream) during 24 h on days +2 and +7 following vaccines 3 and 4.

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