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Antigen-specific impairment of adoptive cell therapy against cancer: players, mechanisms, solutions and a hypothesis

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Summary

Adoptive cell therapy (ACT) destroys tumors with infused cytotoxic T lymphocytes (CTL). Though successful in some settings, ACT is compromised due to impaired survival or functional inactivation of the CTL. To better understand the mechanisms involved, we have exploited a mouse model of leukemia expressing ovalbumin as a tumor neo-antigen to address these questions: (i) Is CTL impairment during ACT antigen-specific?; (ii) If yes, which are the antigen presenting cells responsible?; (iii) Can this information assist the development of complementary therapies to improve ACT? Our results indicate that the target (tumor) cells, not cross-presenting cells, are the main culprits of antigen-specific CTL inactivation. We find the affinity/avidity of the CTL-tumour cell interaction has little influence on ACT outcomes, while tumour density is a major determinant. Reduction of tumor burden with mild non-lymphoablative and non-inflammatory chemotherapy can dramatically improve the efficacy of ACT and may minimize side-effects. We propose a general mechanism for the inactivation of anti-self CTL in the same tissues where the activity of anti-foreign CTL is preserved, based on the density of target cells. This mechanism, which we tentatively call Stunning, may have evolved to protect infected sites from self-destruction and is exploited by tumors to inactivate CTL.

Keywords: Antigen Presentation, Dendritic Cells, Myeloid-Derived Suppressor Cells, Cytotoxic T Cells, Cancer, Immunotherapies

Immunotherapy against cancer is quickly being adopted in the clinic. Adoptive cell therapy (ACT) is an exciting strategy where tumour cells are destroyed following the infusion of anti-tumour cytotoxic T Lymphocytes (CTL) (1-4). Until recently, the applicability of ACT appeared limited because it was thought that T cells were inherently incapable of responding effectively against most cancers, and because the number of CTL required for ACT was considered too large for routine production and treatment. These challenges have proven to be superable and ACT is now receiving strong backing from researchers, clinicians and pharmaceutical companies (5). Over 100 clinical ACT trials have been completed or are ongoing (6). The results have been spectacular in some trials, especially against leukemia, but improvements are required for ACT to become standard therapy (1-6).

What are the ingredients of anti-tumour immunotherapy?

The basic goal of tumour immunotherapy is to kill tumour cells with cytotoxic cells while sparing normal cells. NK cells and other types of “innate lymphocytes” are being investigated as the effector cell of choice (7, 8). However the most common subject of research are CD8⁺ T cells, owing to the specificity conferred by their T cell receptor (TCR) and their inherent function as seekers and destroyers of cells presenting non-self or altered-self antigens (9). Normally these antigens consist of proteins synthesized by the tumour cells, or more precisely, peptides produced by the cytosolic degradation of such protein, or their precursor polypeptides, by the proteasome (10). The peptides are transported into the lumen of the endoplasmic reticulum, bound by Major Histocompatibility Complex (MHC) class I molecules and presented on the surface of the cell as MHC class I-peptide complexes (11). CD8⁺ T cells expressing a TCR that can interact strongly with (“recognize”) this complex are candidate killers of the cell presenting the antigen. A tumour that does not display appropriate MHC I-peptide complexes to label it as such, for instance because it has down-regulated MHC I expression, simply cannot be treated with ACT using killer CD8 T cells (12).

It follows that for CD8 T cells to recognize and kill tumour cells, the latter have to present peptides derived from so-called Tumour-Associated Antigens (TAA). These are mainly proteins that either: (i) have mutated so they yield peptides not found in normal cells; (ii) are not expressed by normal cells after the immune system has developed but are expressed by transformed cells; (iii) are expressed by healthy cells at such low levels that they do not yield enough peptides to allow detection (they are ignored) but are overexpressed in tumour cells (13). The changes that convert a normal protein into a TAA are chiefly genetic in origin, but they can also be a consequence of post-translational alterations that may even include the production of whole new amino-acid sequences (14). Regardless, a concept that is important to bear in mind is that the TAA-derived peptides that are presented by MHC class I and ultimately recognized by anti-tumour CD8⁺ T cells do not contain information about their “canceriness”, they are just like any other of the tens of thousands of different peptides (the “peptidome”) presented at any time on the plasma membrane of any healthy or transformed cell (15). Moreover, because MHC class I molecules are constitutively loaded with peptides and these are continually produced from all cytosolically-degraded proteins, TAA presentation never occurs in isolation, but along with presentation of the rest of the peptidome. What makes an MHC class I molecule loaded with a

TAA peptide a target of an anti-tumour reaction against the cell displaying this complex is not a property of the complex itself, but the ability of some CD8 T cell(s) to recognize the complex and become activated by the immune system, turning into a Cytotoxic T Lymphocyte (CTL) (9). This is an important concept to bear in mind when assessing the conclusions of studies employing different types of “natural” or “artificial” TAAs.

The search for the right tumour associated antigen

Decades of research have been dedicated to identify TAAs (13). An ideal TAA would be expressed and presented by all tumour cells in any individual harboring that type of tumour, but not by normal cells. It would also have to play some important function in the tumour cells, otherwise any cell that randomly lost its expression would be selected in the face of CTL pressure, causing growth of an immunoedited tumour that is no longer recognized by the CTL (12). Ideal TAAs are extremely rare. Even TAAs commonly found in a particular type of cancer may be presented by individuals expressing the “right” HLA alleles but not in individuals expressing “wrong” alleles. Cancer cells are resilient and can survive without, and hence immunoedit, many TAAs. The TAAs are often also expressed, even if at low levels, in some normal tissue and induction of a response against the TAA may cause autoimmunity (16, 17). The way around these limitations is to accept and control some damage of normal tissues and its concomitant side-effects when using CTL therapy against cancer, to target several TAAs simultaneously to reduce the chances of tumour escape by immunoediting, and to tailor the CTL therapy against TAAs expressed by each particular tumour and/or individual. The first solution is just an extension of virtually every cancer therapy already in use, often characterized by severe side-effects on healthy tissues. The last two solutions were considered impossible, or at least impractical, until relatively recently due to the difficulties inherent to TAA identification, but this has started to change with the advent of cheap, deep DNA sequencing technology, which is making possible to identify many unique TAAs in any tumour (13). This is supplemented with highly sensitive quantitative proteomic analysis (15). It can be stated that identification of potential TAA targets for CTL-based immunotherapy will become a routine practice, opening the range of TAAs that onco-immunotherapists will be able to target with CTL.

Towards clinical-grade manufacture of anti-TAA CTL

Identification of suitable TAAs solves the first variable of the ACT equation. The second variable is the CTL. How can these be selected and produced? A recent and highly successful approach does neither. It consists of boosting or restoring the activity of endogenous CTL that are already trying to kill the tumor cells in patients but are held at bay by immunosuppressive mechanisms. Administration of check-point blocking Abs does just that (18). This approach is proving its value spectacularly and is still under development, so it is conceivable it will render every other anti-cancer therapy superfluous, though it is more likely the therapy will not work in every individual and/or type of cancer. It is in these conditions where ACT may become the therapy of choice or a complement to other immunotherapies (19). ACT requires to obtain and expand *in vitro* anti-TAA CD8⁺ T cells or CTL to inject them into the patient. These can be derived from tumour infiltrating lymphocytes (20) or manufactured *in vitro* by transfecting T cells with pre-defined anti-TAA TCRs (21). If the CD8 T cells are not yet activated, they have to be primed *in vivo*. Such approach is often impractical and difficult to control, so the most favored method for clinical-grade preparation of CTL involves an *in vitro* activation step (1-6). If large-scale manufacture of CTL of any specificity becomes generally accessible and affordable, this strategy may become standard treatment. It is also possible that a series of “off-the-shelf” CTL will be made available for treatment of large cohorts of cancer patients with shared characteristics, for instance expressing a common HLA-I allele and having a type cancer with well-defined TAAs (22). A variation of this theme is CTL expressing Chimeric Antigen Receptors (CAR) (23-25). The CARs consist of an Ab-derived region that recognizes a native epitope exposed on a plasma membrane protein (rather than an MHC I-peptide complex) expressed by tumour cells, fused to TCR-derived components required to trigger the signaling events that lead the CTL to kill the TAA-bearing cell. Remarkably, the downstream events elicited by newest generation CARs appear to be similar to those triggered by normal TCRs (23-25).

Limitations of ACT: why does it not always work?

Given recent advances in this area, it can be stated that the major remaining obstacle to successful ACT is CTL inactivation (1-6). Failure is associated with impaired expansion, homing, survival or maintenance of cytotoxic activity of the CTL, but the mechanisms remain obscure. Administration of immunostimulatory drugs or pre-conditioning of the patients can increase CTL activity in ACT patients, but also creates adverse effects, namely autoimmunity. Detailed characterization of the mechanisms of CTL inactivation is required to maximize the anti-tumor

activity of ACT while preventing the deleterious effects of reactivity against self tissues. Our own studies in this area are directed to answer these specific questions: (i) Is CTL impairment during ACT antigen-specific?; (ii) If yes, which are the antigen presenting cells responsible?; (iii) Can this information assist the development of complementary therapies to improve ACT? We believe these questions have so far been insufficiently addressed, and that this is an important deficiency in an area where success or failure depend precisely on the outcome of the interaction between MHC I-peptide complexes on cancer cells, and the TCRs (or CARs) expressed by the immunotherapeutic CTL.

In the spirit of Immunological Reviews, what follows does not pretend to be a comprehensive review of the vast literature relevant to this topic that has been published in recent years. Rather, we provide a summary of our own studies and interpret them in the context of commonly-held concepts, some of which are controversial and, in our opinion, in need of re-assessment. We finish with a proposal for a hypothetical mechanism for the inactivation of anti-self CTL in the same tissues where anti-foreign CTL kill infected cells, which can explain our observations and perhaps those of others.

E μ -myc lymphoma as a model for the study of anti-cancer ACT

Our studies aim at eliminating a transplanted E μ -myc lymphoma, a well-studied model of non-Hodgkin lymphoma (26), expressing chicken ovalbumin (OVA) as a neo-TAA. Here we summarize what are in our view the main advantages and disadvantages of this system as a model to gain insights into the parameters that determine the outcome of ACT against cancer.

Advantages of the E μ -myc tumour model

E μ -myc lymphomas (usually pre-B cell lymphomas) emerge spontaneously in transgenic mice expressing the oncogene myc under the control of the promoter region of the Ig heavy chain gene (26). The tumors can be transplanted to syngeneic C57Bl/6 mice simply by injecting a small number of lymphoid organ cells. We generated variants of a spontaneous E μ -myc lymphoma which express Green Fluorescence Protein alone (control E μ -myc-GFP tumor) or with OVA (E μ -myc-GFP-OVA tumor) (27). GFP and OVA were not expressed as a fusion construct but as two separate proteins encoded by a bicistronic RNA with an internal ribosomal entry site. The level of expression of GFP in these cells is a surrogate marker of OVA expression and presentation. We expanded both tumour variants *in vivo* and froze large stocks of lymphoid organ single-cell

suspensions from the tumour-bearing mice. Inoculation of just 5×10^6 of these cells produce lymphomas in naive recipient mice. Of note, the tumors are not repeatedly passaged through mice nor cultured, so they remain essentially as the original tumor (except for the expression of the transfected genes), providing an excellent model of a spontaneous, yet transferable lymphoma. As other transplantable tumour models, the E μ -myc lymphoma provides two advantages over spontaneous tumors: firstly, its genetic make up and phenotype at the time of inoculation is identical in all tumor-bearing mice; secondly, the timing of growth and expansion kinetics can be accurately followed from the time the tumor is injected, and to some extent altered by choosing the size of the initial inoculum. A third benefit of the model is that E μ -myc lymphomas grow not only in lymphoid organs but also in peripheral tissues (e.g. lung) so it is possible to study interactions between the tumor and the immune system at both locations in the same mice (28). Expansion of the tumor can be measured by counting tumour cells in blood, without killing the animals. Yet, the main advantage of our tumor model is that it expresses OVA as a model neo-TAA. This affords exploitation of the sophisticated reagents and tools that have been developed over many years to analyze in fine detail the presentation and recognition of this antigen. Furthermore, comparing immune reactions against E μ -myc-GFP vs E μ -myc-GFP-OVA tumors allows to dissect antigen-specific from non-specific effects, the main driver of our experiments. We believe these benefits override the perceived disadvantages that are sometimes associated with the use of OVA as a model antigen, and that are not always grounded on empiric evidence.

Limitations of the E μ -myc tumor model

Naturally our E μ -myc lymphoma model also has limitations. If left untreated it is fast-growing, causing lethality at approximately 15 days post-injection (27). That is the time window during which experimental therapies can be tested on this model, which may be too short for the tumor to deploy all the anatomical and immunological alterations that characterize the development of spontaneous tumors and that may contribute to immune escape (12). Secondly, the model TAA OVA is a neo-antigen, so some immunoregulatory mechanisms that may allow tumors to escape immune reactions against self antigens may not exist in mice bearing E μ -myc-OVA lymphomas. Naturally, this is a limitation that applies to any other mouse tumor model where the TAA is derived from a non-mouse genome, be it viral, human or from any other species. The third limitation is that, being a pre-B cell lymphoma, E μ -myc tumors may possess idiosyncratic immune escape mechanisms that are not found in other types of cancer (29). On the other hand,

blood cancers are overall more sensitive to immunotherapy than solid tumors, probably because they are in general more accessible to anti-tumor effector mechanisms (6). These two factors mean that conclusions of studies based on the E μ -myc lymphoma model may not be applicable to other tumors. This is unsurprising because, after all, different types of tumors already receive different types of treatment in the clinic, and we know that the rules that govern immune responses against different viruses, or autoimmunity against different self tissues, vary. It would be ideal to develop universal immunotherapies, but onco-immunologists will have to accept that any strategy may be very effective against some tumors, yield mixed results against others, and provide no benefit at all against certain tumors.

In summary, like any other mouse tumor model, the E μ -myc-GFP-OVA model possesses features that make it highly advantageous to address some questions, but that must be interpreted with caution before generalizing to other mouse models, let alone the clinic. The following sections provide an overview of the work we have carried out on this tumor model to uncover basic rules of CTL responsiveness against tumors, and their inhibition, which we hope may contribute to advance the development of clinical ACT against lymphoma and perhaps other cancers.

Ignorance of the E μ -myc tumour and its variants

In mice pre-immunized against OVA and then inoculated with E μ -myc-GFP-OVA tumor cells, the endogenous CTL activity causes a delay in tumor growth, accompanied by selection of cells that no longer express GFP and OVA (27). This process of immunoediting is a common outcome of effective anti-tumor immunity (12), and even though it represents a case of “failed treatment”, outgrowth of GFP-OVA⁻ tumor cells is advantageous in our system because it provides unequivocal evidence of persistent, TAA-specific CTL activity. That the process is driven by a OVA-specific response is further demonstrated by the fact that the E μ -myc-GFP control tumor develops in vaccinated mice with the same kinetics as it does in naïve mice (27). The immunoedited OVA⁻ tumor cells were susceptible to anti-OVA CTL *in vivo* when exposed to SIINFEKL peptide to artificially reconstitute MHC I-peptide complexes recognized by anti-OVA CTL (27), confirming the survival of the immunoedited cells was due to lack of presentation of the antigen rather than to resistance to killing. The purpose of these experiments was just to demonstrate, as has been done with many other tumor models, that prophylactic vaccination can generate an effective, endogenous TAA-specific CTL response that protects against TAA⁺ tumors.

It would be expected that if inoculation of the E μ -myc-GFP-OVA tumor in naïve mice were sufficient to generate an endogenous CTL response, the tumor would not grow or would become OVA⁻ by immunoediting. This was not the case; the E μ -myc-GFP-OVA expanded with the same kinetics in naïve mice as the E μ -myc-GFP and even the parental E μ -myc tumor, and without losing GFP-OVA expression (27). We did not observe expansion of OVA-specific CD8 T cells with tetramer staining, indicating the presence of the tumour did not trigger an immune response (27). We tested the alternative possibility that a response against OVA did start, but was blunted by immunosuppressive mechanisms, causing tolerance. We discarded this explanation because if mice bearing E μ -myc-GFP-OVA tumors were vaccinated against OVA, expansion of OVA⁺ CD8 T cells could be detected with tetramers (27). This meant that in spite of the expression of GFP in E μ -myc-GFP and E μ -myc-GFP-OVA tumors, the additional expression of OVA in the latter, and the fact that these tumors grow in the same organs where immune responses develop, the immune system of naïve mice ignored these tumors. This contradicted the extended view that OVA is inherently a highly immunogenic antigen. We believe the reason the immune system of mice bearing E μ -myc-GFP-OVA tumors do not spontaneously respond against OVA is because the expression of the antigen is too low to prime naïve CD8 T cells in the absence of inflammatory signals, even though it is high enough to trigger killing by pre-existing CTL (see above). This illustrates the well-known concept that the threshold for naïve T cell priming is considerably higher than the threshold for effector activity is (9). It is one reason why injecting CTL pre-activated *in vitro* is considered a more promising therapeutic strategy than inoculating, and then priming *in vivo*, naïve CD8 T cells (4). Another reason is that even when naïve CD8 T cells are primed *in vivo*, they may not become full-fledged CTL. We showed this using normal vaccination to induce an endogenous anti-OVA response in E μ -myc-GFP-OVA tumour-bearing animals. Although endogenous CD8 T cells expanded in the vaccinated animals, they did not affect the tumor because the primed T cells did not become effector CTL (27). Importantly, impairment of CTL formation was antigen-specific because the endogenous CD8 T cells did become CTL in mice bearing E μ -myc-GFP tumors (27). We did not investigate the specific mechanisms that blunted the response because our objective was to demonstrate the E μ -myc-GFP-OVA tumor represents an attractive model to study ACT, with common features to many human cancers: it is susceptible to CTL attack and it is possible to induce an immune response against a neo-TAA in individuals harboring the tumor, but that endogenous response becomes

blunted. It is in this situation where administration of ready-made CTL can potentially have major benefits (1-6).

ACT against E μ -myc lymphoma: when it works and when it does not

Effective elimination of small lymphomas by CTL expressing TCR of different affinities

To ascertain the ability of exogenous TAA-specific CTL to recognize and eliminate E μ -myc-GFP-OVA tumour cells, OVA-specific CTL (OT-I CTL) were generated *in vitro* and adoptively transferred into tumour-bearing mice. To generate the CTL, naïve OVA-specific CD8⁺ T cells from OT-I transgenic mice (30) were stimulated for four days *in vitro* in the presence of irradiated peptide-pulsed splenocytes, IL-2 and LPS (31). When tumour-bearing mice were injected intravenously with the CTL 3 days after tumour inoculation, a time at which the tumour was already clearly detectable, exogenous OT-I CTL were able to eliminate E μ -myc-GFP-OVA cells (Fig. 1 and 2) (32). This tumour reduction was antigen-specific, because E μ -myc-GFP cells were not affected by the presence of OT-I CTL. As in the experiments of prophylactic vaccination (see above), mice therapeutically treated with OT-I CTL succumbed to lymphoma, but this was an immunoedited OVA⁻ variant, demonstrating the persistence and efficacy of the ACT (Fig. 1) (32).

Effective killing of lymphomas by CTL of different affinity

An important question in ACT development is: how strong has to be the recognition of the TAA by the CTL in order to cause effective tumor eradication? The answer has practical implications because the strength (the avidity) of CTL-target recognition depends primarily on the affinity of the TCR for the MHC I-TAA(peptide) complex (22). If effective ACT requires CTL that express high-affinity TCR, this will limit the number of useful clonotypes that can be isolated and expanded from tumour infiltrating lymphocytes (20). Similarly, if the immunotherapeutic CTL are manufactured by transfection of engineered TCRs and only high affinity ones are of practical use, the process of TCR production may become very complex, expensive and perhaps impractical (21). The OVA model allowed us to test this question. OT-I CTL recognize H-2K^b molecules bound to the OVA peptide, SIINFEKL. Recently, another CTL derived from transgenic mice that recognize the same epitope but with a much lower affinity has become available, namely the OT-3 CTL (33). The TCR of the OT-3 cells is undetectable with tetramers (28), and OT-3 CTL only killed 20% of target cells *in vitro* at a 2:1 (CTL:Target) ratio, whereas

OT-I CTL killed 100% of targets at 0.2:1 ratio. We assessed in parallel the anti-tumor activity of OT-I and OT-3 CTL *in vivo* and both were capable of eliminating E μ -myc-GFP-OVA lymphomas (28). This was surprising but consistent with a previous study that showed OT-3 cells can kill self tissues and induce diabetes as efficiently as high-affinity T cells (33). An important lesson from this analysis was that measurements of target cell killing *in vitro* can underestimate the true anti-tumour potential of low-affinity CTL. Our result contradicts studies where CTL expressing higher affinity TCRs were found to be more efficacious against cancer (34-36), but ratifies other studies where high TCR affinity was found to matter little (37, 38) or even to impair (39-41) the anti-tumour activity of CTL. The discrepancies among studies may in part be explained because the correlation between affinity and efficacy is not linear (38) so that, for instance in our case, the two CTL we tested express TCRs with affinity above the minimum threshold required for tumor elimination. More studies will be required to settle this question. In any case, we conclude that CTL that appear to be of low affinity should not be readily disregarded as potential ACT agents.

Antigen-specific impairment of CTL when tumour burden is high

Anti-OVA CTL demonstrated effective killing of E μ -myc-GFP-OVA tumors that had been expanding in mice for three days and were relatively small. When injected four days following tumour inoculation, the OT-I CTL were still able to eliminate E μ -myc-GFP-OVA cells, but the result was more variable, suggesting that at this time some mice had started to develop immunoescape mechanisms that enabled the tumour to avoid killing (Fig. 1) (32). Injection of the OT-I CTL five days after tumour inoculation had no effect on tumour burden (Fig. 1 and 2) because even though the lymphoma cells remained susceptible to CTL killing, the injected CTL were inactivated by two fast-acting mechanisms. First, a large proportion of the CTL died within 24 hours after injection (Fig. 2) (32). Second, the CTL that survived expanded, but they did not exhibit effector function either *in vivo* or upon purification and analysis *in vitro* (Fig. 2) (32). In mice bearing similar burdens of E μ -myc-GFP tumors, OT-I CTL remained functional and capable of killing OVA⁺ normal cells injected subsequently (32). Inactivation of anti-OVA CTL in mice bearing large E μ -myc-GFP-OVA lymphomas was therefore antigen-specific. This was more clearly illustrated by assessing the fate of two CTL co-injected into mice with E μ -myc-GFP or E μ -myc-GFP-OVA lymphomas. The first CTL was OT-I, specific for OVA, the second was

gBT-I (42), specific for a Herpes Simplex Virus antigen absent in the mice. Both CTL retained their activity, and were present in comparable numbers, in mice bearing large E μ -myc-GFP lymphomas (Fig. 3), but in mice with large E μ -myc-GFP-OVA lymphomas the number and activity of the gBT-I CTL remained normal while the number and activity of the OT-I CTL decreased dramatically (Fig. 3) (32). These experiments implied that: (i) neither the E μ -myc tumour cells *per se*, nor the cellular or extracellular environment induced by growth of the tumor, caused overt immunosuppression; and (ii) antigen recognition by the OT-I CTL in mice bearing E μ -myc-GFP-OVA tumors did not induce inactivation of bystander CTL (the gBT-I CTL), even when both CTL were contained in the same site. The corollary of these experiments was that inactivation of TAA-specific OT-I in our model required cognate antigen recognition (formation of an immunological synapse) on a cell presenting OVA. Which cell(s) were those? Answering this question was important because if the cell responsible were a non-tumor cell, a strategy that allowed depletion of those cells might be able to preserve the activity of the anti-tumor CTL and enhance ACT outcomes.

The search for the antigen presenting cell responsible for ACT impairment

TAA's can be presented by the tumor cells that produce them or by other cells capable of cross-presentation, defined as the ability to present via MHC I antigens that have not been synthesized by the antigen-presenting cell (43). Dendritic cells (DC) are the most potent antigen cross-presenting cells *in vivo*, and in mice this activity is performed chiefly by a DC subtype often referred to as "CD8 DC" (44, 45). We could readily demonstrate CD8 DC cross-presented OVA in mice bearing E μ -myc-GFP-OVA lymphomas (Fig. 4) (32).

CD8 DC can induce T cell priming or tolerance (46), so it was possible they were the cells causing CTL inactivation, but when we inoculated the tumor in mice deficient in the transcription factor Batf3, which lack CD8 DC (45), CTL inactivation was still evident (32). The lymphoid organs of mice bearing E μ -myc lymphomas also contained so-called Myeloid Derived Suppressor Cells (MDSC) (47, 48), which in our system were mostly monocytes capable of differentiating into DC or macrophages (32). MDSC reportedly cross-present and inhibit anti-tumor immune responses (49, 50). They did not appear the cell responsible for the antigen-

specific inactivation of anti-OVA CTL in our system because we could not obtain evidence they cross-presented OVA, but perhaps they did it at a level we could not detect. Other cells have been shown to be capable of cross-presentation in certain conditions (e.g. CD8⁺ DC, macrophages or B cells), though their role *in vivo* is questionable (43).

To address a potential role for antigen cross-presenting cells in CTL inactivation in our system we generated a new Eμ-myc lymphoma, in this case from OVA⁺ H-2K^{bm1} mice (32). This lymphoma expresses OVA but its MHC I molecules cannot present the OT-I epitope. The tumor was inoculated into C57Bl/6 × H-2K^{bm1} F1 mice, in which neither C57Bl/6- nor H-2K^{bm1}-derived cells are rejected. As all cells in the recipient mice express H-2K^b, any cross-presenting cell would be able to present OVA to OT-I CTL, but the tumor cells themselves could not. Indeed we could show the CD8 DC of the tumor-bearing F1 mice cross-presented OVA (32). However when we injected the OT-I CTL in these mice bearing large H-2K^{bm1}-OVA lymphomas, the CTL were not deleted and maintained effector function. This demonstrated that the tumour cells themselves directly presenting the antigen, rather than any other cell capable of cross-presentation, was driving CTL inactivation (Fig. 4). While other factors might contribute to induce the inactivation program, our results demonstrated that those factors only acted on CTL that encountered large tumors directly presenting their cognate antigen. An important lesson we learned from these studies is that showing that a particular antigen presenting cell (e.g. CD8 DC) cross-presents an antigen does not imply the cell is actually involved in an immunological outcome (CTL inactivation), even if that cell can be shown to perform the activity *in vitro* or upon transfer to new animals. Only if the experimental system allows selective elimination of the antigen presentation function of that cell in the animals harboring the tumors it is possible to reach conclusions about its role.

What makes Eμ-myc-GFP-OVA lymphomas immunosuppressive?

Having identified the tumor cells themselves as the main culprits for the inactivation of anti-lymphoma CTL, we wondered which properties confer this capacity to the tumour cells.

Idiosyncratic immunosuppressive properties of B cells?

One possibility was that the lymphoma cells were inherently immunosuppressive (29). Indeed, B cells possess immunoregulatory functions and can suppress and/or induce apoptosis of CD8 T

cells via CD95 (51). However, we found that CTL death triggered by encounter of large lymphomas was not mediated by CD95 (S. Prato and J. Villadangos, unpublished results). In fact OT-I CTL killed small E μ -myc-GFP-OVA lymphomas (Figs. 1 and 2) (32). All the CTL showed signs of activation triggered by antigen encounter in these conditions; for example, they expressed higher levels of PD1 than cells injected into mice bearing E μ -myc-GFP lymphomas, though this level was not as high as in inactivated CTL (32). This implied the outcome of interactions between CTL and their lymphoma targets was not automatically suppressive when the latter were in low abundance, arguing against an idiosyncratic property of E μ -myc lymphomas as the cause of CTL inactivation in our system.

Time of tumor growth?

We also considered the possibility that the difference in outcome of ACT administered at day <3 vs day >5 was not determined by tumour size but by the time the tumor had been growing in the animals. Perhaps the tumor required at least five days to acquire immunosuppressive properties? We discarded this option by injecting a much smaller number of E μ -myc-GFP-OVA lymphoma cells, so that it took 5 days for the tumor to reach a similar size as our normal inoculum size would attain in 3 days; the five day-old small tumors did not suppress the OT-I CTL (32).

Affinity/avidity of the CTL-tumor cell interaction?

The third variable we tested was the strength of the interaction between the CTL and its targets. Low-affinity anti-OVA CTL OT-3 (see above) underwent deletion and functional inactivation in animals bearing large lymphomas as the high-affinity OT-I did (28), so this parameter did not appear to dictate CTL impairment. We also addressed the influence of the level of TAA expression and presentation per cell, a major contributor to the avidity of the CTL-target cell interaction. To do this we established two new lymphomas derived from the original E μ -myc-GFP-OVA with very different levels of GFP (and OVA) expression. The E μ -myc-GFP-OVA^{high} cells expressed similar levels as the parental lymphoma. The E μ -myc-GFP-OVA^{low} lymphoma presented OVA but barely above the level of detection by CTL, so that even in cytotoxicity assays *in vitro* the OT-I CTL could not kill more than 50% of E μ -myc-GFP-OVA^{low} cells (28). Yet, both E μ -myc-GFP-OVA^{high} and E μ -myc-GFP-OVA^{low} tumors inactivated the OT-I CTL upon reaching a size above a certain threshold (28). This implied that, provided the tumor cells

presented sufficient antigen to enable detection by CTL, a large enough number of cells could effectively inactivate the CTL.

Inactivation of anti-lymphoma CTL when the target cell density reaches a critical threshold

The variable that all the previous analyses pointed at as the main determinant of ACT outcome in our model was the number of tumor cells, low at day <3 and high at day >5 after tumor inoculation. We made the prediction that if we could specifically reduce the tumor burden without significantly affecting the number of other cells in the tumor environment, subsequently injected CTL would not lose activity. We achieved this by using the common anti-lymphoma drug, cyclophosphamide, to reduce tumor burden. Importantly, instead of using it at a dose that would cause lymphodepletion, as normally used in the clinic, we chose a dose that dramatically reduced the number of E μ -myc lymphoma cells but had no noticeable effects on the number of other hematopoietic cells (Fig. 5) (32). With this approach we limited the impact that creation of “space” through elimination of healthy hematopoietic cells might have on ACT. After reducing the E μ -myc-GFP-OVA lymphoma, adoptively transferred OT-I CTL were not deleted, maintained activity and prevented re-expansion of the OVA⁺ tumor (Fig. 5) (32). The treated mice nearly doubled their life span, finally succumbing to immunoedited OVA⁻ lymphoma (Fig. 5) (32). Tumor reduction with cyclophosphamide did not cause overt inflammation because we did not detect DC activation nor increased release of inflammatory cytokines (32). The benefit of the chemotherapy was also observed in mice that lack CD8 DC (32), so it was unlikely the preservation of OT-I CTL activity was due to increased cross-presentation of OVA captured from dying tumor cells. Notably, combining tumor reduction with ACT only worked if the CTL were administered within 24 hrs after the cytotoxic drug. The tumor was not completely eliminated by cyclophosphamide and if allowed to re-expand for just three days before ACT administration, CTL again underwent deletion and functional inactivation (Fig. 5). This also supported our interpretation that the benefit of the cytotoxic drug was not to induce an inflamed state.

CTL stunning: A hypothetical mechanism for inactivation of anti-self CTL but preservation of anti-foreign CTL based on the density of target cell recognition

Our research on the role of antigen presentation and antigen presenting cells in the context of adoptive cell therapy against lymphoma leads us to propose a model for local regulation of CTL

responses based on the density of target cells. We believe this model for immunoregulation may be applicable to other types of cancer and to viral infections.

It is known that anti-self CD8 T cells escape thymic selection (52). Many of these cells are permanently eliminated or neutralized by mechanisms of peripheral tolerance (53). Yet, some CD8 T cells escape this second layer of control. Anti-self CD8 T cells mostly express low-affinity TCRs for self antigens (54-56), but they can become CTL and cause damage of normal tissues and autoimmunity if other factors lead to their activation (33). Indeed, these are the anti-TAA that comprise the tumor infiltrating lymphocytes and that check-point blockade Abs recruit against cancer (18, 20). Self-peptides are constitutively presented via MHC I by almost all cells, and cross-presented by DC in secondary lymphoid organs both in the steady state and during infection (46). It follows that whenever an infection (e.g. viral) that triggers DC activation and T cell immunity starts, there is a risk that bystander anti-self CTL will be induced along with anti-viral CTL (33). Both CTL will be able to enter the infected site, so how can the immune system enable the anti-viral CTL kill infected cells while at the same time suppress the activity of the anti-self CTL? Our experiments injecting anti-OVA and anti-HSV CTL in mice harboring E μ -myc-GFP-OVA lymphoma demonstrate it is possible to inactivate the former but not the latter within the same site. We propose that a mechanism to achieve this during pathogen infection already exists to protect normal tissues, and that this mechanism is exploited by large tumors to escape CTL. What is the mechanism involved?

Suppressive cytokines like IL-10 or TGF β effectively inhibit immune responses (57), but they cannot discriminate between anti-self and anti-foreign CTL, so they are unlikely to produce the outcome we observe, at least on their own. It is possible that they participate, but their inhibitory activity would have to act only on anti-self CTL. Regulatory T cells (Tregs) can also effectively suppress immunity (58), but it is unclear how Treg would be capable of suppressing responses against, for example, skin self-antigens, while sparing CTL against a virus infecting the same skin site. Again, their participation is possible, but subject to some other mechanism of discrimination. Exhaustion is an effective antigen-specific mechanism of CTL impairment (59). We considered this might be the mechanism responsible for our observations because the inactivated OT-I CTL that expand in mice harboring large E μ -myc-GFP-OVA lymphomas express higher levels of PD1 than the OT-I that maintain activity in mice with small tumors (32).

However, there are several reasons why we think this is not the mechanism responsible in our system. First, exhaustion is defined as a mechanism triggered by prolonged encounter of antigen, but we detect the first signs of inactivation (including high PD1 expression) within 24 hours (S. Prato and J. A. Villadangos, unpublished results). Second, OT-I CTL lacking PD1 expression also underwent inactivation (S. Prato and J. A. Villadangos, unpublished results). PD1 is not the only mediator of exhaustion but, in addition, transcriptomics profiling of inactivated CTL in our model does not appear consistent with exhaustion (G. Segal and J. A. Villadangos, unpublished results). Finally, the mechanisms of exhaustion are deployed by cells chronically infected by viruses to tolerate the presence of the virus rather than promote tissue damage by anti-viral CTL, so this mechanism again cannot explain how anti-foreign CTL would retain activity while inhibiting anti-self CTL.

The mechanism we propose to account for our observations is that multipolar formation of immunological synapses by an individual CTL with multiple targets, or high-frequency formation of non-productive synapses, triggers an inactivation program in the CTL that we tentatively call “Stunning” to express it is quickly triggered by high target density (Fig. 6). This program leads to death of a large fraction of the CTL and, in the CTL that survive and expand, irreversible shut-down of activity. Thus, during a viral infection, if both anti-viral and anti-self CTL were recruited to the same site, the anti-viral CTL would encounter a limited number of cells presenting their cognate (viral) antigen while anti-self CTL would, by definition, encounter their cognate antigen on every cell (Fig. 6). It has recently been shown that during viral infections the number of “productive” encounters between CTL and their targets –those that lead to killing of the target- *in vivo* is much smaller than deducted from *in vitro* studies (60). This study also showed that CTL act cooperatively to kill targets, and a single target is often the subject of simultaneous attack by several CTL *in vivo* (60). For anti-self CTL, the situation would be the inverse, where a single CTL might initiate synapses with multiple targets simultaneously, or establish many unproductive contacts with targets within a short period. It is in this situation where we propose the anti-self CTL becomes “stunned” (Fig. 6). In our experiments, small tumors are successfully attacked by OT-I CTL as if they were low-frequency virus-infected cells, but large tumors are treated as self tissue that should be spared by stunning the CTL.

Stunning does not invoke the participation of new surface receptors, cytokines or cells to turn CTL from killers to stunned, but it does not discard an influence or even a requirement for these players. Proposed mechanisms of immunoregulation usually stipulate that tissues can be “immunopermissive” or “immunosuppressive”, but perhaps it is more accurate to describe the site of infection or tumor growth as an environment that is neither absolutely immunoactivatory nor immunosuppressive, as both activating and inhibitory signals are present. Some of these signals may be ignored while others exert their function; we propose the decision to discriminate which signals should be ignored and which followed is dictated to a large extent by the quality, number or frequency of CTL-target cell interactions. Augmenting the strength of a signal (e.g. increasing the concentration of a cytokine in response to pathogen- or inflammation-associated signals) or decreasing it (e.g. with neutralizing Abs or via recruitment of Tregs) may shift the CTL decision from ignoring inhibitory signals to responding to them or vice versa.

Evidence for and against Stunning in other experimental models

Both normal B cells and large lymphomas can be effectively depleted in human patients treated with CAR CTLs, indicating large tumour size does not necessarily cause CTL inactivation (6). On the other hand, elimination of a mouse lymphoma with CTL expressing a CAR against CD20 was more efficient if normal B cells (which also express CD20) were depleted before administration of the CTL (61). These results resemble our own in that depletion of the normal cells effectively reduced the density of target cells in the tumor-bearing animals. In a different study, B cells were also found to inactivate CTL in a setting of autoimmunity (62). The authors attributed this outcome to an intrinsic property of the B cells, which may also be present in lymphoma cells (29). However, as mentioned previously, we do not observe CTL inhibition when lymphomas are small, which suggests the cells are not intrinsically inhibitory. Evidence for stunning in solid tissues infected with viruses has also been reported. Specifically, CTL against hepatitis virus were deleted and inactivated early in the response if the number of hepatocytes presenting antigen was high (63, 64). The E μ -myc lymphoma grows not only in lymphoid tissue but also in peripheral organs such as the lungs. We observed little deletion of OT-I CTL in the lungs of mice bearing large E μ -myc-GFP-OVA lymphomas, but the CTL present were quickly inactivated and expressed high levels of PD1 (28). Whereas it is well known that reducing solid tumor mass enhances the effectiveness of ACT and other immunotherapies (Robinson), to our knowledge there is no evidence that stunning plays a role in inactivation of CTL against solid

tumors. It is relevant to point out that the hypothesized mechanism of Stunning requires CTL to infiltrate and interact dynamically with multiple target cells, and not all tumors or tissues may nurture this type of dynamic interactions.

In conclusion, there is sufficient evidence to suggest that CTL can maintain high killing activity when the density of target cells is low, but undergo quick inactivation by deletion and functional impairment, -the two components of the mechanism we describe here as Stunning- when they encounter high target density. Future studies should clarify whether this is a common mechanism for prevention of immune pathology and autoimmunity, a peculiarity of the immune response against cancer, a specific mechanism only observed in responses against B cell lymphomas, or just an oddity of our experimental model.

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Figure Legends

Fig. 1. A critical tumor mass impairs tumor killing by adoptively transferred tumor-specific CTL.

(A) Mice bearing E μ -myc-GFP (GFP) or E μ -myc-GFP-OVA (GFP-OVA) tumors were inoculated with exogenously generated OT-I CTL 3, 4 or 5 days after tumor inoculation, or left untreated. Two days later, tumor load in spleens was analyzed by FACS. The contour plots show the FSC/B220 expression in whole spleens that did (lower plots) or did not (upper plots) receive CTL at days 3, 4 or 5. The histograms show the expression of GFP (a surrogate of OVA expression) in E μ -myc-GFP-OVA cells (shaded grey) and endogenous B cells (black line) from mice injected with CTL (or not) at day 5. All contour plots are representative of 2 independent experiments.

(B) Tumor reduction was determined by dividing the number of tumor cells in mice injected with CTL by the number in the untreated controls. The results are the mean (n=4-10) $\pm$ SD of 2 independent experiments.

(C) Mice bearing GFP-OVA tumor were inoculated with OT-I CTL 3 days after tumor inoculation. After 16 days spleens were analyzed by FACS. The histogram shows the expression of GFP in tumor cells (black line). As control, mice bearing GFP-OVA tumor for 11 days, with no CTL, were used (shaded grey). The result is representative of 2 independent experiments.

**** $P < 0.005$, n.s. (not significant, $P > 0.05$).**

Reproduced from (32).

Fig. 2. Tumor size dictates the outcome of ACT against lymphoma.

Left: OT-I CTL inoculated to mice bearing small E μ -myc-GFP-OVA lymphomas kill the OVA⁺ lymphoma cells (top and center) and also normal OVA⁺ splenocytes injected 48 hrs after ACT administration (bottom).

Right: If the OT-I CTL are injected when the tumor burden is high (top), a large number of CTL are deleted and functionally inactivated within 24 hrs (center); although the CTL expand, they remain inactivated and fail to kill not only tumor cells but also normal OVA⁺ splenocytes injected 48 hrs after ACT (bottom).

Fig. 3. The deletion, phenotypic changes and reduced killing capacity of adoptively transferred OT-I CTL are antigen-specific.

Left: Analysis of number, phenotype and killing capacity of OT-I (anti-OVA) and gBT-I (anti-HSV) CTL co-injected into mice bearing large lymphomas. Mice bearing E μ -myc-GFP or E μ -myc-GFP-OVA lymphoma cells were co-injected with gBT-I and OT-I CTL 5 days after tumor inoculation. Three days later, the CTL phenotype, recovery and killing activity in the spleen was determined. (B-C) The gBT-I and OT-I from spleens of mice bearing E μ -myc-GFP (B) or E μ -myc-GFP OVA (C) were analysed by FACS for the expression of (TCR) V α 2 and PD-1 (left histograms) and enumerated (centre graphs), and their killing activity was assessed *in vivo* (right graphs). The results are representative of 2 independent experiments with each circle representing one mouse (n=8). *** $P < 0.0001$, ** $P < 0.005$, n.s. (not significant, $P > 0.05$).

Reproduced from (32).

Right: Schematic representation of the events leading to the results on the left.

Fig. 4. Antigen-specific inactivation of CTL is mediated by direct antigen recognition on a large number of target (lymphoma) cells.

Lymphoma growth promotes accumulation of MDSC in lymphoid organs, but they do not mediate anti-tumor CTL inactivation, as they do not cross-present antigen. CD8⁺ DC cross-present lymphoma TAAs, but neither these cells nor any other cross-presenting cells in the mice bearing lymphoma mediate inactivation of the CTL, which was dependent on direct antigen presentation by the lymphoma cells themselves.

Fig. 5. Tumor reduction with chemotherapy prevents CTL inactivation and allows ACT to effectively eliminate lymphomas.

Administration of mild, non-inflammatory and non-lymphoablative chemotherapy (cyclophosphamide, CTX) to mice bearing large lymphomas (top left) does not eliminate the lymphoma, but reduces the tumour burden (middle left) below the threshold that causes inactivation of anti-lymphoma CTL, so if these are inoculated 1 day after CTX, the CTL effectively kill the tumour cells (middle right), forcing immunoediting of the tumor. If ACT is administered after the tumor re-grows, reaching again the threshold that causes CTL inactivation (bottom left), ACT is ineffective (bottom right).

Inset: (A) Tumor-bearing mice were treated with vehicle (H₂O) or 40 mg/kg CTX 5 days after tumor inoculation. One day later, OT-I CTL were adoptively transferred. The graph shows Kaplan-Meier survival curves. The black line with full black dots includes the following groups: GFP or GFP-OVA tumor-bearing mice treated with H₂O (n=4 mice per group); GFP or GFP-OVA tumor-bearing mice treated with H₂O + CTL (n=4 mice per group). The dashed line with white dots includes the following groups: GFP or GFP-OVA tumor-bearing mice treated with CTX (n=8 mice per group); GFP tumor-bearing mice treated with CTX + CTL (n=8 mice per group). The dotted line with white triangles includes the following group: GFP-OVA tumor-bearing mice treated with CTX + CTL (n=7 mice per group). *P* value (Log-rank analysis) for GFP-OVA tumor (+CTX) *versus* GFP-OVA tumor (+CTX +OT-I CTL) is indicated. (B) The histograms shows GFP (a surrogate of OVA) expression by Eμ-myc-GFP-OVA cells (FSC^{high}B220⁺) in tumor-bearing mice that received CTX alone (left histogram) or CTX + OT-I CTL (right histogram). Expression of GFP on endogenous B cells is indicated as background staining (shaded grey). Results are representative of 2 independent experiments. Reproduced from (32).

Fig. 6. Stunning: a hypothetical mechanism for inactivation of anti-self CTL in the same location where anti-foreign CTL are allowed to maintain activity.

Both anti-foreign (e.g.virus, orange round cells) and anti-self (light blue) CTL have been recruited to a site of infection (top). The effector-to-target ratio for anti-viral CTL is high (top left), resulting in effective killing of the infected (red) cells (bottom left). In contrast, the effector-to-target ratio for anti-self CTL is low (top right), triggering CTL stunning (bottom right). In the

experiments described throughout this review, the anti-lymphoma CTL behaved like the orange CTL in mice bearing small lymphomas, but like the blue CTL in mice bearing large lymphomas.

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