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Review

Effective Tumor Immunotherapy: Start the Engine, Release the Brakes, Step on the Gas Pedal, ... and Get Ready to Face Autoimmunity

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Abstract. Cellular immune responses can destroy cancer cells, achieving the cure of experimental malignancies. An expanding wealth of knowledge on the molecular basis of how to prime and amplify a T cell response has fueled a number of strategies successful at treating established tumors (rather than merely preventing tumor grafting). The most efficacious approaches operate at different stages, including: 1) priming the immune response using tumor antigen-expressing dendritic cells or tumor cells transfected with genes that render them immunogenic, 2) sustaining and amplifying immunity using agonistic monoclonal antibodies against costimulatory molecules or immune-potentiating cytokines, and 3) eliminating mechanisms that self-regulate the strength of the immune response, such as inhibitory receptors or regulatory T cells. A rational combination of such approaches holds great hope for cumulative and synergistic effects, but there is also evidence that they can open the flood-gates for unwanted inflammatory reactions. The next decade can be envisioned as the time when the first reproducibly efficacious combination regimes for cancer immunotherapy will become available and widely used in the clinic, as clinicians learn the best strategies and try to harness their potentially damaging effects.

Key words: immunotherapy; dendritic cells; costimulation; CTLA-4; 4-1BB; CD40; cytokines.

Antitumor T Cell Priming (Start the Engine)

Tumor cells are antigenic. Many antigenic determinants are encoded by their genes as a result of mutations or ectopic expression[4, 5, 41]. Many of them have been molecularly defined and the interactions of several determinants with MHC antigen-presenting molecules have been studied. However, tumor cells are very poorly immunogenic in the sense that they do not ignite a T cell mediated-immune response by themselves, or if they do, the response is of rather low intensity[41].

A hot topic these days is whether tumors induce tolerance towards their antigens or if their antigens are simply ignored by the immune system[46]. Experimental evidence delivers examples of both modes of action, although in most instances fully-established tolerance, as such, cannot be demonstrated and ignorance is the most prevalent mechanism[9]. In fact, tumor cells perform very poorly as antigen-presenting cells, even to induce tolerance by clonal anergy (or deletion) of T cells[9]. Response or tolerance against tumor antigens occurs as a result of a complex process called cross-

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-presentation in which tumor antigens are taken up, transported and presented by a cell professional for those tasks[21]. Importantly, cross-presentation, either for cross-priming or for tolerance, needs a certain threshold in the level of antigen expression that, if not reached, results in immune ignorance to the antigen[21].

Therefore, several requirements are to be fulfilled in order to start a T cell response to a tumor antigen: 1) transport of the antigen to lymphoid tissue to meet naive T cells, 2) presentation on a cell with the correct array of costimulatory molecules, antigen-presenting molecules and cytokines, and 3) presence of responsive elements in the T cell repertoire[57]. The first two requirements are satisfied if the antigen is given in a form that finally results in its expression on mature dendritic cells (DC)[1]. The presence of a responsive T cell repertoire depends on the ability of the tumor to tolerize against its antigens.

Two general approaches have been followed: a) transfection of cytokines and costimulatory molecules into tumor cells to make them resemble functional DC, and b) an artificial loading of tumor antigens on selected or cultured DC. Either approach has been successful in priming the response against murine tumors. These strategies dominate, in various forms, the current arena of tumor vaccination strategies[41].

Expression of DC genes in tumor cells can be achieved by *in vitro* transfection or by *in vivo* gene transfer of tumor nodules with a number of viral vectors. The best results with this approach are obtained by transfection of granulocyte-macrophage colony-stimulating factor (GM-CSF)[12, 22], a molecule that largely works by easing cross-priming. This is because it attracts and differentiates DC. Interleukin 12 (IL-12) also works well but, again, the cellular events actually elicited probably rely on cross-presentation mechanisms and the induction of a cascade of cytokines with pleotropic functions on leukocyte biology and angiogenesis[38, 53]. Gene transfer of surface molecules of the B7 family[10], MHC class II[2, 47], 4-1BBL[19, 36] and CD40L[54] have been found to be efficacious, but most often they are unable to tackle well-established or proliferated disease.

A major break-through in the field was the possibility to culture DC from monocytes of bone-marrow precursors in the presence of GM-CSF and IL-4[23, 50]. This made feasible the proof-of-concept type of experimentation, showing that tumor antigens pulsed on DC were extremely potent tumor vaccines. Sources of antigen can contain single or multiple antigenic determinants which are given to DC as peptides or a mixture of tumor antigens[40]. Complex sources of tumor antigens are provided as tumor lysate, tumor apoptotic bodies, cell fusion of DC and tumor cells, transfection of total tumor RNA, etc. Viral and bacterial vectors can be used to lead the antigens into the antigen-presenting machinery of DC[56]. An alternative has been to inject DC into malignant tissue in such a way that the artificially injected DC take up antigens and transport them[25, 37, 45]. Complex sources of antigens are better, since they induce a polyclonal type of response against multiple epitopes at the same time (therefore making antigen-loss variants less likely) [40]. However, they could vaccinate against normal sequences shared by proteins in the tumor and normal tissue, leading to autoimmunity. Many clinical trials are currently testing the best source of DC (monocyte-derived, CD34-derived, or Flt-3L-mobilized)[13, 14, 56], the best source of tumor antigen, and the most convenient route of injection. The winning results so far have been for the tumor-cell/DC hybridomas[17, 18], which show impressive efficacy in human renal cell carcinoma[27], but such data are under investigation due to a well-founded suspicion of misconduct[3].

A very appealing source of antigen is tumor proteins of the heat shock protein family (HSP-70 and gp96) that chaperone peptides in the MHC class I antigen-presenting pathway and have been shown to be very efficiently internalized in DC[49], while they also provide cytokine-like activation signals to these cells. This approach is being currently tested with cancer patients.

Costimulation and Immune-Response Maintainance (Step on the Gas Pedal)

The immune system has some tricks that can be exploited in order to amplify a response. A great number of cytokines and membrane-bound costimulatory ligands are able to upregulate and shape the type of effector response. These mechanisms can be grossly exaggerated by properly engineered therapeutic agents in order to get a better antitumor immune response.

In a way, a very simple means to obtain this amplification is to repeat the immunization procedure sequentially over time. If tumor cells present the antigens in a poorly immunogenic fashion, repeated doses of the antigens under immunogenic conditions is a good idea, since these repetitions will not let the immune response fade away[31].

If a patient is successfully primed by active immunotherapy, this can allow the physician to culture his effector T cells *ex vivo* to be reinfused as adoptive therapy[61]. The artificial culturing of these lymphocytes

benefits greatly from this successful priming to increase T cell precursor frequencies[35]. Therefore, active and passive or adoptive immunotherapy are to be combined in the clinic to sustain the response, as has been shown in animal models.

Administration of cytokines, either as proteins or with gene-transfer approaches, can also help the response. Type I interferons and IL-2 may find a role in these therapy combinations. Particularly interesting is the exploration of the use of IL-15, GM-CSF and Flt--3L, which are very promising agents. IL-15 has unexpected properties not shared with IL-2, since it expands memory cytotoxic T lymphocytes (CTLs) and prevents activation-induced cell death[6, 29, 62]. Flt-3L and GM--CSF, to a lesser extent, promote the differentiation and accumulation of great numbers of DC in the treated subjects[32, 33]. This has an antitumor immune effect *per se*[32] and permits an easier isolation and manipulation of DC[14].

A number of agonistic antibodies against costimulatory molecules seem to greatly increase the antitumor immune response. This is the case of anti-4-1BB monoclonal antibodies (mAb)[39], which recognize a surface glycoprotein expressed only on activated T and natural killer cells, providing a very potent costimulatory signal to activated CTLs throughout the body. These antibodies rely on some level of pre-existing T cell priming to activate the expression of 4-1BB on T cells[26]. Agonistic antibodies against CD40, which activate antigen--presenting cells in all lymphoid tissues, also have great potential, according to data obtained in mouse tumors[16, 58]. Here the anti-CD40 mAb reaches and activates, among many others, the small number of DC cross-presenting tumor antigens and license them to activate CTLs[51]. Its function is reminiscent of a physiological concomitant Th1 response activating DC through CD40L/CD40 interactions.

Other means to accelerate the immune response will be very likely found by selectively inhibiting activation-induced cell death of T cells. Such a mechanism offers a target to be manipulated and exploited in order to strengthen the cellular immune response. In this regard, a number of B7-like molecules are being discovered which may mediate these functions.

Finally, a major hurdle on the pathway of cancer immunotherapy is the low migration of T cells into malignant tissue[20]. Such migration is controlled by chemokines and adhesion molecules on endothelial cells, which are upregulated under inflammatory conditions. Addressing effector T cells to cancer tissue by manipulating the expression of such molecules, making the tumor look like an inflamed tissue, is a very attractive possibility[34, 42].

Fighting the Immune Self-Regulation Mechanisms (Release the Brakes)

The immune system has mechanisms that bring the immune responses to an end and downsize the clonal expansions of lymphocytes. On the other hand, certain physiological systems seem to set thresholds and check-point requirements for cellular immunity to proceed. Examples of these mechanisms that have been exploited in tumor immunotherapy are the cytotoxic T lymphocyte antigen 4 (CTLA-4) receptor of T cells and, more recently, immunoregulatory $CD4^+CD25^+$ T cells.

Anti-CTLA-4 antibodies block negative signals that downregulate T cell expansion[8, 60]. This molecule, expressed selectively on activated T cells, recruits tyrosine phosphatases that inhibit CD28-mediated T cell costimulation. In fact, CTLA-$4^{-/-}$ mice develop, in a matter of weeks, a severe autoimmune disease with lymphocyte infiltration in non-lymphoid tissues. Treatment with CTLA-4 mAb, presumably blocking its function *in vivo*, erradicates some malignancies and synergizes with vaccination with tumor cells expressing GM-CSF[7, 8, 59, 60]. In the latter case, autoimmunity in the form of vitiligo has been found in mice as well as in human melanoma patients.

Recently, $CD4^+CD25^+$ T cells have entered the limelight of immunology as suppressor cells of the immune response[11, 24, 28, 55]. They have been found to be very much involved in maintaining tolerance to self tissues. The mechanisms that they use to execute these actions are dependent on cell contact and involve T-T and T-DC interactions[48]. No information has been published on the molecular players of their effector function. Anti-CD25 antibodies deplete this subpopulation and are known to increase the antitumor immunity, in particular in synergy with other means of treatment (i.e. with vaccination with peptide in adjuvant). This depletion must be performed before the immunization procedure, because the depleting anti-CD25 antibody would otherwise deplete the T cells that are becoming activated, since CD25 is expressed on activated lymphoblasts.

The molecular targets involved in this control against overactivation of the immune response will be a productive area of research. A race in the search for attractive candidates has started. Infiltration of non-lymphatic tissues by activated lymphocytes has also been

reported in PD-1$^{-/-}$ mice[15, 44], albeit less intense than that observed in CTLA-4$^{-/-}$ mice. Neutralization of the immune downregulating effects of TGF-β is another field of interest.

… and Get Ready to Face Autoimmunity

Rolf Zinkernagel and coworkers published a paper in which mice developed autoimmunity after immunization against surrogated tumor antigens with DCs. In their model, tumor cell lines and a target transgenic organ shared artificial expression of a viral antigen[30]. In their experiments, tumor rejection correlated consistently with severe autoimmunity. Although this is an important warning call, data from other experiments and from the battlefield of clinical trials are not so worrisome[13, 43], since DC vaccination is known to present self proteins but do not elicit autoimmunity (at least frequently and seriously enough to be a problem). For instance, in mice transgenic for an antigen of hepatitis B virus expressed in the liver, vaccination with antigen-pulsed DC leads to CTL generation, but without liver autoimmunity[52]. However, if these T cells are expanded *in vitro* and reinfused, acute hepatitis takes place, indicating the existence of some control mechanisms. In fact, loading DC with complex sources of tumor antigens containing plenty of normal sequences has not resulted in serious autoimmune conditions.

We do not know what the case will be if we tamper with the control systems and, at the same time immunize intensively. Probably, serious adverse reactions will be witnessed. The scenario would be reminiscent of acute and chronic graft-versus-host reactions in allogeneic bone-marrow transplantation. But do not forget that if we are to fight cancer with these weapons, we have to take some risks. The spectrum of organ damage that can take place is difficult to predict, as is whether the reactions will be acute and self limited or maintained, reaching chronicity.

Conclusion

Our immunotherapeutic arsenal against cancer has increased incredibly in the last decade. Activity against mouse tumor models has been unprecedented and the results in clinical trials are encouraging. We postulate that the ultimately sucessful regimes will consist in a combination of interventions based on each of the three different elements described: priming, amplification and removal of the inhibitions. The potency of the combination will very likely challenge us with some autoimmune adverse effects and, hopefully, we will learn to tilt the balance to the interest of the patient.

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