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To suppress to rescue? Changing the approach for recalling anticancer immune responses

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1. ABSTRACT

The tumor microenvironment plays a fundamental role in both the organization of and the escape from anticancer immune response. Recent experimental approaches in anticancer therapy show that some anticancer chemotherapeutics – at a different dosage than usually used – can improve tumor control by acting on immunity and can also be used in immunosuppressive treatments. These apparently contrasting effects may provide an explanation by looking at the cancer immunity from a different perspective. Basing our hypothesis on the current inflammatory cancer microenvironment model, we suggest that the tumor escape may derive from a conflict between a deregulated local immune response and the application by the systemic immunity of a homeostatic regulation, physiologically used for terminating inflammatory processes. This regulation applied to the tumor microenvironment can contribute to impede the efficacy of anticancer responses. Therefore, we suggest recovering the anticancer response by paradoxically inducing a temporary immune suppression ("resetting" effect). In this paper, we review present concepts about inflammation and immunity in the cancer microenvironment, as well as experimental data from recent literature supporting this paradoxical intervention hypothesis.

2. INTRODUCTION

When different tasks in a complex system come to overlap and are in conflict, the efficiency of the system is diminished or even halted. Anyone working with a computer has had the experience of its jamming and freezing all functions when overloaded by conflicting activities of too many programs operating simultaneously. To recover, we must shut down and restart the computer. This resets the system and its operating functions. While keeping in mind the necessary differences of this metaphor, we can suppose that a not dissimilar strategy may be useful for rescuing anti-tumor immunity.

Over the years and with updated approaches, immunotherapies have attempted to enhance the anti-tumor immune response by targeting cancer antigens as well as the functions of various immune system cells and their products (1,2,3). The reason why the results still remain interlocutory and have had lower efficacy than expected has been subject of ongoing elucidation over the last ten years. In fact, new insights about immune surveillance, inflammation and the tumor microenvironment have highlighted the reciprocal interplay and the importance of microenvironmental conditions on the efficacy of *in loco* effective immune responses (4). These new insights,

simultaneous with the discovery of new immunomodulatory properties of various therapeutic agents including the anticancer chemotherapeutics, have stimulated an investigation of different possibilities of interventions (5-8). It is useful to summarize some of the main concepts.

3. IMMUNE SURVEILLANCE, TUMOR RECOGNITION, AND INFLAMMATION: SOME CONCEPTS

3.1. Immune surveillance and tumor cell phenotype

Despite the increase of knowledge in the field, the cancer-host interplay at local and systemic level still remains not fully clarified and open to speculation. The understanding of an organism's anticancer response is presently framed inside the still debated *immune surveillance* paradigm, which assumes a continuous activity of the immunity against cells altered by pathogens or mutagens. This activity depends on the capability of the immune system to distinguish the organism's own elements (indicated as "self") from elements normally not present (indicated as "non-self"). The innate immunity plays a critical role in it, both directly and by addressing the adaptive immunity responses (9,10).

Cancer is a multistage process that progressively involves the organism at the systemic level (11). A cancer cell is a self cell that progressively acquires non-self characteristics during the transformation process. These modifications include altered expression of constitutive molecules and the appearance of new molecules on the cell surface. The self phenotype is principally characterized by the expression of major histocompatibility complex (MHC) class I molecules on the surface of all cells. However, under cancer conditions, reduced or altered expression of MHC class I molecules can happen, modifying the self phenotype of the cancer cell (12,13). This modification, as well as the expression level and immunogenicity of newly expressed antigens, elicits responses by the immune system (innate immunity). These immune responses can be modulated by the active microenvironment that the tumor cells induce and in which continue to develop (14,15,10). The unregulated replication of the transformed cells generates stress in the tissue. This produces release of "danger signals" (stress molecules and pro-inflammatory cytokines, as type I interferons – IFN, interleukin [IL]-1beta, tumor necrosis factor [TNF]-alpha, heat shock proteins – HSP, etc.). These molecules together with the changes of the tumor cells from their original self-cell phenotype (reduced expression of MHC class I molecules indicated as "missing self", the expression of neo-antigens, stress proteins like MICA, and changes in the glycosylation) induce the triggering of innate immunity cell responses. Macrophages, granulocytes, mastocytes and dendritic cells (DC) participate in the local immune response. They start of an inflammatory process together with the natural killer (NK) cells that can recognize the missing-self phenotype and carbohydrate changes, becoming actively cytotoxic (16-20).

3.2. Inflammation and tumor inflammatory microenvironment

Inflammation as an important factor during the carcinogenetic process was proposed by Virchow in 1863, and revisited by Balkwill and Mantovani in 2001 (21). An accrual of new evidence in the cellular and molecular immunobiology of cancer sustains this renewed interest.

First, molecules involved in the regulation of immune cells involved in inflammation have been found to be important also for modulation of cancer cell activities and survival. An example is given by the nuclear factor kappa-B (NFkappaB), and the signal transducer and activator of transcription 3 (STAT3). The NFkappaB, an important transcription factor in inflammatory responses, was found to be a key element linking inflammatory cell activity and tumor progression by regulating the expression of genes that encode important proteins for the control of stress response, maintenance of intercellular communications, regulation of cellular replication and apoptosis (22-24). The block of NFkappaB classical activation pathway in a mouse colorectal cancer model (induction by the carcinogen azoxymethane –AOM, and promotion by chronic inflammation agent dextran sulfate sodium salt -DSS) produced effects on cancer incidence. The block was performed by specific deletion of IkappaB kinase-beta (IKKbeta) either in the colonic epithelial cells or in the myeloid cells. In both cases the cancer incidence was reduced, but through a different mechanism. In the KO colon epithelial cells, apoptosis increased under the inflammatory stimulation as a consequence of reduced expression of anti-apoptotic protein Bcl-xL controlled by NFkappaB. On the other hand, the IKKbeta KO myeloid cells were unable to induce proliferation of the colon epithelial cells following inflammation as the consequence of inhibited expression of NFkappaB-controlled proinflammatory genes, the products of which act as paracrine tumor growth factors (25). Another example is seen in models of cancer metastases in mice. After inoculation of CT26 colon cancer cells or by spontaneously metastatic 4T1 breast cancer, an increase of metastases followed the injection of lypopolysaccharide (LPS). This event was inhibited by the block of NFkappaB. The LPS mediator of inflammation is the TNFalpha. Once injected in the tumor, LPS induced TRAIL expression in the tumor surrounding immune cells as well as expression of the TRAIL receptor DR5 on tumor cells. When NFkappaB was present an antiapoptotic effect was induced. To the contrary, the blockage of NFkappaB produced a TRAIL-mediated tumor regression by reducing proliferation and inducing apoptosis. This result confirmed that inflammation produced by LPS stimulates tumor growth by tumor microenvironment production of TNF-alpha and that NFkappaB was linking inflammatory and tumor promoting effects (26). The STAT3 represents another link between cancer proliferation and survival, and cancer activity and immune cell function (inhibition). STAT3 is constitutively activated in cancer cells and this activation can be propagated to immune cells by tumor STAT3-regulated factors, which include IL-6, TGF-beta, Foxp3, VEGF and IL-10. They produce immune suppressive effects and also contribute to further upregulation of STAT3. These factors

can sustain maturation of CD4+ CD25+ Foxp3+ T regulatory (Treg) lymphocytes, and also tumor-promoting inflammation by IL-23/Th17 cells (27,28).

Second, the cells involved in the anticancer immune response (and their products) demonstrated a double-faceted activity as a result of their interplay with the tumour microenvironment. These immune cells can either elicit antitumor responses or help the tumor development and its immune escape in a close cross-talk (for example, through tumor immunoediting, i.e. changes of the tumor cell phenotype induced by the immune response itself, leading to inhibition of the expression of targeted antigens) (29). Examples are furnished by the population of tumor infiltrating macrophages that can shift from an early anticancer activity and immune stimulation (M1 macrophages, producing IL-12, IL-23, TNF-alpha, IL-1, chemokine CXC ligand 10 [CXCL10] and reactive oxygen intermediates [ROI]) toward a tumor microenvironment-driven (M-CSF, IL-4, IL-10) immune suppressive function, neo-angiogenesis and tissue remodelling (M2 macrophages, producing IL-1ra, IL-10, CCL17, CCL22, and CCL18). Similar behaviour is described for the myeloid cells that, in an immature form, can revert from their aggressive function to an immune suppressive one under the effect of environmental prostaglandin E2 (PGE2), IL-1beta and IL-6 (myeloid-derived suppressor cells – MDSC). The MDSCs are characterized by expression of $Gr1^+CD11b^+CD31^+$ in mouse, $CD11b^+CD33^+CD34^+CD14^-$HLA-DR in human, production of nitric oxide (NO), arginase, nitrityrosine, TGFbeta, IL-10, and by down-regulating L-selectin on naive T cells preventing intra-tumor homing (4,15,30-32).

Third, we need not forget that the peculiar microenvironment created by the cancer cells is highly dynamic and self-maintaining by the continuous interaction with the other components of the hosting tissue. It drives changes from an originally acute inflammatory response, activated by release of "danger signals" (33), to a chronically stimulated inflammation influencing the stromal structures of a tissue as well. This suggested the "non-healing wound" hypothesis, originally presented by Dvorak in 1986 (34) and recently reconsidered by Balkwill and Mantovani in 2001(21). The hypothesis suggests that the cancer development is a process mimicking in a deregulated manner the conditions of tissue plasticity (cell proliferation and motility, neo-angiogenesis, stroma reorganization) developing during the healing of a wound (34,35). It is interesting how recently new importance of the stroma components and products was evidenced by various studies that highlighted both the active role of the stroma in collaborating to shape the tumor microenvironment organization, and the role of mesenchimal cells and fibres in establishing favourable niche environments allowing cancer stem cells maintenance and differentiation (36-40).

According to this new picture of the cancer and immunity interplay, an intriguing point is raised: cancer, as a localized process with its own environment, can elicit and sustain a local inflammatory response by the local immunity, but contemporaneously leads to activation of an anti-inflammatory response at the systemic level, independent from the tumor-related local immune impairment. We will try to explain this apparent paradox.

3.3. Three phases of tumor immune microenvironment development

The process of cancer immune microenvironment development can be summarized in three main phases.

During the very early tumor development (first phase), the progressively transformed cell starts unregulated replication. The developing clone induces expression of stress molecules and local delivery of pro-inflammatory cytokines ("danger signals") in the involved tissue (32). Consequently, at the local level, the activity of the innate immunity cells (like macrophages and NK cells) is elicited. If the immune-surveillance response successfully works, by addressing dendritic cell priming and maturation (with presentation of tumor peptides on MHC class I molecules for triggering specific CD8+ cell cytotoxic responses), an effective recognition of and attack against the cancer can follow. Unfortunately, anatomic factors, such as weak antigenicity, can impede the recognition, as well as release of tumor products allowing tumour evolution (39-42).

In a second phase, the progressive cancer pressure on stroma and surrounding cells, together with the release of damaged cell products, sustains and prolongs the local inflammatory reaction. This produces an increase of cytokines, chemokines, growth factors, antigens, chemo-attractants, and NO within the intercellular matrix (36,43-46) continuously attracting macrophages, myeloid cells and primed T cells. Once embedded inside the hyperdynamic tumour microenvironment, these cells find conditions that negatively modulate their maturation and function, allowing the transition from an aggressive acute response to an inadequate and persistent (chronic) inflammation (29-32).

The third phase establishes the paradoxical situation of an anticancer response which is inhibited not only by cancer microenvironment products but also by the same immune system. This inhibitory activity can be considered as the application by the systemic immunity of a homeostatic regulation to terminate the persistent inflammation. Together with the inhibitory molecules released by tumor and stromal cells, the regulatory response increases the inefficiency of the local-regional anticancer response (30,35,47). The consequence is a shift from the initial antitumor cytotoxic response (controlled by CD4+ T helper 1 - Th1 – cells producing IFN-gamma and IL-2, together with NK cells, natural killer T – NKT, and activated CD8+ cytotoxic T lymphocytes - CTL) to a tolerant/suppressive response addressing the naïve CD4+ T helper cells to develop as T helper 2 – Th2- cells, activation of CD4+CD25+Fox3+ cells (activated T regulatory lymphocytes - Treg), maturation of M2 macrophages, and recruitment of immature myeloid cells. These cells release IL-10, IL-6, IL-4, TGF-beta, contributing to the inhibition of the cytotoxic cell activity and DCs maturation. Under

these conditions, the cancer can progress, and the efficacy of immunotherapies is also biased (48-50).

4. THE HYPOTHESIS: A NON-UNITARY IMMUNITY AND A NORMAL HOMEOSTATIC MECHANISM JAM THE ANTICANCER IMMUNE RESPONSE AND NEED TEMPORARY IMMUNE SUPPRESSION TO RESET

4.1. Tumor escape as a consequence of a homeostatic regulation

The shift from a Th1 to a Th2 type of response is a critical point in the failure of the anticancer immune response. According to the described evolution of the tumor immunological network, we can suggest the existence of three levels in the immunity: a first level, the local immune response in the cancer-originating tissue, initially activated; a second level, regional, represented by immunological interplay in the regional lymph nodes between DCs and adaptive immunity cells (DCs move toward regional lymph nodes to maturate and present antigens to the adaptive immunity lymphocytes, which, primed, move to infiltrate the tumor); and finally, a third level, systemic, represented by the regulatory intervention of the systemic immunity against the focus of self-maintaining inflammation produced by the growing tumor together with the accrual of attacking immune cells. In this way, not the tumor per se, but the tumor microenvironment as a deregulated inflammatory focus stimulates a physiological mechanism to control and reconstitute the homeostasis. Consequently, we can assume the possibility for the immune system (as for the nervous system) to simultaneously work on different and partially autonomic levels in which the response hierarchy is determined by the most immediate challenge to the homeostasis in a tissue or organ (51). Thus, a physiological modulation can produce contrary effects in the tumor peculiar contest, finishing to sustain a vicious circle: cancer growth → infiltrated tissue products and tumor antigens → activated immune cells recruitment → insufficient anticancer response → cancer growth → infiltrated tissue products and tumor antigens → and so on.

These two activities (chronically re-activated response and inhibitory immune responses) conflict with the need to eliminate the cancer cells.

The Th2 and Treg cells (and their products) are the arm of the immune system which avoids an immune response which could become excessive and pathologic. Significantly, they are involved, to a various extent, also in autoimmunity and self-tolerance. Together with macrophages and MDSCs, the regulatory cells establish the vicious circle that causes the cancer to persist (15,48,50). Their importance is demonstrated by studies in which the depletion of these cells helps to restore appropriate anticancer responses (52).

Therefore, we can hypothesize that the interruption of this circle can allow for the rescue of a correct immune response against the cancer (the true target), and not against the chronically sustained microenvironmental inflammation. To break the cycle, we suggest the induction of a controlled immune suppression for "re-setting" the system. This (apparently) paradoxical intervention could inhibit part of the inflammatory-cell activities and, in the meantime, block unwanted regulatory immune reactions (Treg). After the controlled suppression, the reactivation of the CTL functions would be enhanced by their previous priming by antigens of the developing tumor; and, eventually, selective stimulations of NK/NKT cells and tumor-primed CTLs could be applied to enforce their anti-cancer responses. The immune suppression should be performed with drugs that target T cells (especially Treg) and macrophages/monocytes, presently considered, the most critical cell populations. While treatment with non-steroid anti-inflammatory drugs (NSAID) appears to have a defined rationale in very early stages of carcinogenesis and transformation (53-55), the kind of intervention suggested here should be applied to developed solid tumors, because we suppose the presence of cancer primed CTLs cells is necessary (56).

4.2. T regulatory cells, CD28, and "anticancer immunosuppressors"

The review of over ten-years of literature, to find indications about possible unusual effects of immune suppressors in cancer conditions, surprisingly reveals an indirect confirmation of this hypothesis of intervention. In fact, various reports indicate the benefit by different drugs with immune suppressive activity (low-dosage cyclophosphamide, rapamycin, sirolimus/everolimus, cyclosporine A) on cancer occurrence, also in association to anticancer treatments, both in experimental models and clinical applications (57-61). Aside from the explanations describing specific effects of the cited drugs on cancer cells, it is possible also to suppose that this treatments can allow the hypothesized Th1-response rescue as a further mechanism by which they improved the rate of cancer control/regression (62,63). Cyclophosphamide treatments at metronomic dosages were linked to the inhibition of activated Treg cells (62,64,65).

Mature Treg (CD4+ CD25+) express Foxp3. Its transcription is mediated by CD28 co-receptor pathway. CD28 plays an important role in the T lymphocyte recognition-activation function, but also on their apoptosis control mechanisms. Particularly, the CD28 triggering appears more critical for the function of CD4+ (Treg maturation) than of CD8+ lymphocytes (66,67). Experiments demonstrated that when activated NK cells interfere with CD28 pathway, they can inhibit the maturation of naïve Treg cells, helping the Th1 response (expression of IFN-gamma) (68). Interestingly, some immune suppressive drugs (rapamycin, cyclosporin A, azathioprine) have been described to target CD28 pathway and Treg cells (69,70).

Low-dosage cyclophosphamide was experimentally found to reverse immunity from a Th2 to a Th1 response and to increase Th1 cytokines (especially IFN-gamma) (62). These effects were proved also in tumor patients in association with different types of anticancer treatment, including immunostimulation (63). The

mechanism producing these effects is not yet fully understood, but of importance are the observations that 1) Treg cells are diminished by the treatment, while CD8+ cells are not especially affected; 2) the presence of pre-existing tumor-sensitized T cells was necessary for the efficacy of this treatment; 3) this intervention increased the efficacy of concomitantly administrated anticancer cell vaccines (56,62,64).

Altogether this data suggest a possibly common mechanism involving the CD28 receptor and its pathway, efficaciously targeting Treg cells and permitting the reactivation of Th1 cells and CD8+ cytotoxic lymphocyte functions.

With this view in mind, azathioprine (AZ) appears to be a perspective drug. Originally used as an anticancer drug, AZ immune suppressive activity has made it more relevant in the treatment of inflammatory bowel diseases (IBD), autoimmune diseases, and anti-rejection treatment after organ transplantation (71-73). The AZ action mechanism was only recently better understood and implies a block of the signalling pathway triggered by interaction of B7 proteins with CD28 receptor particularly on CD4+ T cells. This block can lead both to apoptosis of the cell and inhibition of IL-2 production, a mechanism theoretically useful in the immune microenvironment of established cancers to disable Treg and Th2 cells (70,71).

Interventions also on macrophages appear to be possible by using biphosphonates. Largely used in the treatment of bone metabolism disorders, they are now described to modulate inflammatory processes. They were found to selectively target macrophages, inducing depletion of these cells in cancer microenvironments (74-76). A re-modulation of macrophage activity was also described in mouse experimental melanoma using cyclooxygenase-2 (COX-2) inhibitors, improving anticancer activity and sensitivity to IFN-gamma treatment (77).

5. TESTING THE HYPOTHESIS

Some preliminary tests to verify our original hypothesis were performed on mouse B16F10 melanoma by *in vivo* and *ex vivo* experiments using either an immunosuppressant (azathioprine - AZ) or an anti-inflammatory drug (nimesulide – MES, a COX-2 inhibitor), alone and in association with dendrimers coated with molecules of N-acetyl-glucosamine (glycodendrimers) as immune stimulator (78). These multivalent molecules have resulted as effective in stimulating immune responses mediated by NKR-P1 lectin-like activation receptor isotypes (NKR-P1 C in mice C57/Bl6, also indicated as NK1.1) expressed by NK/NKT cells and activated CTLs in experimental treatment of syngeneic B16F10 melanoma in the C57/Bl6 mice (79). The aims of the tests were: 1) to evaluate if drugs that have different target (chronic and autoimmunity sustained inflammation for AZ; acute inflammation for MES) could down regulate the peculiar inflammatory network of the cancer microenvironment according the period of intervention (early or late stages of the tumor development); 2) if the following re-stimulation of the natural immunity by stimulation through a different pathway than the TCR-CD28 receptors was helping to recover the anticancer response; 3) the possibility to better understand the type of inflammatory network activated in each period of treatment by the effects obtained with the used drugs for addressing more focused treatments. We found that AZ, but not MES, helped to enhance the anticancer immune response both in initial and established tumors, but MES was very efficacious in controlling cancer growth in early treatments. This last observation was in accordance with the reports in literature about the effects of NSAIDs on tumors (54,55,77). While the association of glycodendrimers to AZ was enhancing the therapeutic effect, negative results were obtained with the association to MES. The reduction of cancer growth correlated with an increased Th1 anti-melanoma immune response (enhanced production of IFN-gamma in front of the specific target) and cytotoxicity. These paradoxical results obtained by the use of AZ, especially the quick and intense ex-vivo stimulation to IFN-gamma production after challenge of singular mouse splenocytes with its own tumor homogenate, suggested the triggering of primed CTLs and not only of NK/NKT cells. Further evaluations are ongoing to clarify the levels and targets (Treg, Th2 cells, others?) of the inhibition produced by AZ but absent with MES. The interesting activity of AZ in the treatment of more advanced tumor stages poses also new questions about the quality and components of chronically inflammatory tumor microenvironment, and possibly common immunological pathways shared by cancer and autoimmunity microenvironments (cancer cell remains a modified self cell).

6. PERSPECTIVES

The important advancements in understanding the immunological network elicited by cancer during its development make it necessary to modify our view of the process. The importance of inflammatory processes in regulating and sustaining cancer development and tumor microenvironment organization is a clear and established concept. However, considering the consequences of the “inflammatory revolution”, we suggest reviewing also our concepts about both general immunity network and what a cancer is. As above discussed, we need to rethink in a more dynamic and stratified way the organization of both immune system and immune responses (we suggest a layered, hierarchic perspective). At the same time we need to better understand the importance of the "self" component persisting in cancer, which we believe central for addressing both the immune responses and the related failure. This understanding can enlighten in a newer perspective possible processes and pathways shared with autoimmune diseases.

Many papers were published in recent years by the most important researchers in the field including the word “paradox” and “paradoxical” to describe the new unconventional scenario of anticancer immune response and cancer immune escape. Shifting the perspective away from the traditional view which is still too linked to an antibacterial immunity model, maybe we will be able to

open new avenues towards a more realistic picture of cancer in its complexity. This could be also helpful to better link the clinical and immunobiological observations for the development of more effective immunotherapeutic approaches.

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Abbreviations: AOM: azoxymethane; AZ: azathioprine; CCL: CC chemokine ligand; CD: cluster of differentiation; COX-2: cyclooxygenase-2; CTL: cytotoxic T lymphocytes; CXC: chemokines with the two cysteine residues separated by another aminoacid; CXCL: CXC chemokine ligand; DC: dendritic cell; DSS: dextran sulfate sodium salt; Foxp3: forkhead winged helix protein 3; Gr1: granulocyte-differentiation antigen-1; HLA: human leukocyte antigen; HPS: heat shock protein; IBD: inflammatory bowel diseases; IFN: interferon; IKKbeta : inhibitor of NFκappaB kinase-beta; IL: interleukin; KO: knock-out; LPS: lypopolysaccharide; M-CSF: macrophage-colony stimulating factor; MDSC: myeloid-derived suppressor cells; MES: nimesulide; MICA: MHC class I chain-related gene A; MHC: major histocompatibility complex; NFkappaB: nuclear factor kappa-B; NK: natural killer; NKR-P1: NK cell receptor-protein 1; NKT: natural killer T; NO: nitric oxide; NSAID: non-steroid anti-inflammatory drugs; ROI: reactive oxygen intermediates; STAT3: signal transducer and activator of transcription 3; T : thymus dependent (lymphocytes); TCR: T-cell receptor; TGF: tumor growth factor; Th: T helper; TNF: tumor necrosis factor; TRAIL: tumor necrosis factor-related apoptosis-inducing ligand; Treg: T regulatory; VEGF: vascular endothelial growth factor

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