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ORIGINAL ARTICLE

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Immunological ignorance of solid tumors

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Abstract Many peripheral solid tumors such as sarcomas and carcinomas express tumor-specific antigens that can serve as targets for immune effector T cells. Nevertheless, the immune surveillance against clinically manifest carcinomas and sarcomas seems relatively inefficient. Naïve cytotoxic T cells are activated exclusively in secondary lymphoid organs including the spleen and lymph nodes. Tumor antigen might be either cross-presented to naïve cytotoxic T cells by professional antigen-presenting cells (pAPC), or presented directly by tumor cells that migrated to secondary lymphoid organs. Direct priming is quite inefficient during early tumor development because metastasis to lymphoid organs is usually limited to advanced stage diseases. Similarly, the process of cross-priming by pAPC seems to depend on relatively large antigen amounts and on maturation stimuli for dendritic cells, and both requirements may be limiting during initial tumorigenesis. Therefore, the immunosurveillance of solid tumors may fail because they are ignored for too long by the immune system. However, these situations may prove promising for the induction of tumor-specific T cell immunity by vaccination, as the T cell repertoire against these antigens has a naïve phenotype and is not yet affected by tolerance mechanisms.

Keywords Immunosurveillance · Tumor · Ignorance · Costimulation · Cross-priming

Introduction

Cancer cells undergo series of genetic modifications during the oncogenic process, leading to multiple cellular proteins (antigens) that are quantitatively or qualitatively different from the parental cell. Different immune effectors are involved in the recognition of tumor antigens, but there is considerable evidence for the central role of cytolytic $CD8^+$ T lymphocytes (CTL) in mediating specific tumor immunity [1, 2]. During the past decades var-

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ious tumor antigens have been characterized [3, 4]. These antigens can be subdivided into four major categories: (1) differentiation antigens, expressed in normal and neoplastic cells of the same lineage; (2) overexpressed antigens with higher expression levels in tumor tissues compared with normal tissues; (3) tumor-specific shared antigens, expressed in different tumors but not in normal tissues, except for the group of cancer testis antigens that is expressed in testis and placenta tissue; and (4) tumor-specific unique antigens, resulting from mutations in a single tumor.

Despite the presentation of antigenic peptides by tumor cells on major histocompatibility complex (MHC) class I, CTL against most of these epitopes are not or not efficiently activated. For differentiation antigens this may be explained by the thymic deletion of high-avidity self-reactive CTL [5, 6]. However, also CTL responses to tumor-specific antigens are often not induced, despite detectable antigen expression by the tumor cells. Why anti-tumor cytotoxic T cells are not induced in this situation may depend on several factors, including the localization of the tumor [7, 8], the activation status of professional antigen-presenting cells (pAPC) [9, 10, 11], inhibitory molecules expressed by the tumor [12] and others that will be discussed in this review.

The endogenous immune response to tumors

The idea that the immune system is involved in the control of tumors dates back to the 1950s when Burnet and Thomson [13] formulated the immune surveillance hypothesis. They suggested that tumors continuously arise in the body, but that these tumors are recognized by a very efficient immune system that eliminates the tumor cells early before they become clinically apparent. Clinically manifest tumors were seen as a rare event that escaped efficient immunosurveillance. In the 70s nude mice became available that lack thymic maturation of CTL. Surprisingly, these mice had a normal incidence of solid tumors [14]. However, due to extrathymic T cell maturation, nude mice possess a small population of functional CTL. In parallel, patients receiving immunosuppressive drugs after organ transplantation had a normal incidence of the most common solid tumors such as breast, lung, or colon cancer [15, 16]. In contrast, the incidence of malignant lymphomas is significantly higher in patients with congenital or acquired immunodeficiencies. In patients with primary congenital immunodeficiency the incidence of associated lymphoproliferative disorders (IALD) ranges from 0.7% for patients with X-linked agammaglobulinemia to 12–15% in patients with ataxia telangiectasia. In patients with post-transplant lymphoproliferative disorders the incidence varies from 0.5% after bone marrow transplantation to 10% after heart and lung transplantation. Although there are some differences between these IALD, they are often associated with Epstein-Barr virus infection [17]. It is clear, therefore, that the immune system is involved in the control of lymphohematopoietic and virus-induced tumors, but due to the normal incidence of spontaneous solid tumors in nude mice and in immunocompromised patients, a central role of the immune surveillance in the control of solid tumor development seemed questionable.

Experimental setups employed to analyze the effector mechanisms necessary for tumor control often include the subcutaneous (s.c.) or intraperitoneal (i.p.) injection of carcinoma or sarcoma cells in recipient mice depleted of or deficient in the effector mechanism of interest. These experiments usually highlighted the importance of $CD8^+$ T cells in a perforin-dependent control of various experimental tumor cell lines [18, 19]. Perforin is exclusively expressed by natural killer (NK) cells and T cells, is essential for inducing tumor

cell apoptosis in vitro, and perforin-deficient mice ($pfp^{-/-}$) show an increase in the growth and metastasis of experimental tumors [18, 20]. In contrast, $pfp^{-/-}$ mice of up to 12 months of age are not abnormally prone to spontaneous malignancy. To accelerate tumor development $pfp^{-/-}$ mice were crossed with p53-deficient mice, that have an increased susceptibility to epithelial, lymphoid and connective tissue tumors. Lack of perforin expression in T and NK cells increased the incidence of disseminated lymphomas but not of solid tumors [21]. These experiments illustrate that the control of tumor development in experimental situations, especially after injection of single-cell suspensions may be overestimated compared to its role in solid tumors that arise spontaneously after clonal expansion of a transformed cell. In addition, even the effector mechanisms necessary to control spontaneously arising tumors in the periphery may be different to the control of single cells experimentally injected i.p. or s.c. in salt solution.

More recent experiments analyzing the spontaneous tumor development in RAG-$2^{-/-}$, in STAT-$1^{-/-}$ and in RAG-$2^{-/-}$×STAT-$1^{-/-}$ mice renewed the interest in the immunosurveillance of solid tumors [22]. In contrast to nude mice that have some limited maturation of functional T cells, RAG-$2^{-/-}$ mice are completely deficient in B and T cells; 50% of RAG-$2^{-/-}$ mice developed benign adenomas and 50% adenocarcinomas of lung and intestine at the age of 15–16 months. The incidence of malign disease was even more pronounced with an incidence of 80% adenocarcinoma at the age of 12–18 months in RAG-$2^{-/-}$xSTAT-$1^{-/-}$ double-knockout mice that in addition lack IFN-γ-meditated effector mechanisms of innate immunity. Tumors derived from methylcholantrene-treated RAG-$2^{-/-}$ mice were more immunogenic in transplantation experiments than tumors induced in control mice, suggesting that the tumors that develop in the presence of a functional immune system are under selective pressure to develop low immunogenic escape mutants, a process that was termed "immunoediting"[23].

In contrast to the extensively studied role of $CD8^+$ T cells in tumor control, the contribution of other effector mechanisms of the acquired immune system is less well defined. The importance of antibodies in the inhibition of solid tumors is not clearly understood and remains controversial. Spontaneous anti-tumor antibodies are frequently detected in the serum of cancer patients, and an increase in the titer seems to be associated with progression of the tumor [24, 25, 26, 27]. For p53-expressing tumors a correlation between detectable anti-p53 antibodies and a poor prognosis has been documented [28]. Over the last few years, the relative efficacy of anti-tumor monoclonal antibodies (mAb) for the treatment of certain breast cancers or B cell lymphomas [29] renewed the interest of immunologists in anti-tumor humoral responses. Antibodies per se are not toxic for a virus-infected cell or a tumor cell, but they act either by blocking vital signaling molecules on the cell surface or by a secondary effector mechanism including antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [30]. Although the relative importance of each effector mechanism in the protection against tumors remains controversial and seems to depend on the experimental system analyzed, ADCC might be the dominant effector mechanism in vivo. The relevance of CDC against tumors in vivo remains to be shown, and is probably limited by the expression of complement regulatory proteins on tumor cells. In mice, anti-melanoma antibodies inhibit tumor growth in a Fcγ receptor (FcγR)-dependent manner [31]. In addition, the importance of the Fc-FcγR interaction for anti-tumor activity was shown for the clinically important antibodies transtuzmab (Herceptin) and rituximab (Mabthera, Rituxan) as well as for intrace-

rebral therapy with an anti-epidermal growth factor receptor antibody in a brain tumor model [32].

The role of innate immune cells such as NK, natural killer T cells (NKT) and $\gamma\delta$TCR$^+$ T cells in immune surveillance remains limited to some experimental systems, although these cells express perforin and many anti-tumor cytokines such as IFN-γ and TNF-α [33, 34]. In contrast to $\alpha\beta$TCR$^+$ T cells that are predominantly localized in secondary lymphoid organs, $\gamma\delta$TCR$^+$ T cells represent the majority of resident T cells in the skin and the gut, termed intraepithelial lymphocytes. These cells may, therefore, represent a frontline defense against tumors arising in the skin or the gut. Indeed, δTCR$^{-/-}$ mice are more susceptible to methylcholantrene-induced skin cancer and to tumor cell lines injected directly intradermally [35]. Immune surveillance by intraepithelial lymphocytes was activated by the murine NKG2d ligands Rae-1 and H60 [35, 36]. However, the tumor incidence was lower than in βTCR$^{-/-}$ mice and tumors developed later, indicating that $\gamma\delta$TCR play a role in the local control of tumors, but there seems to be a predominant role of $\alpha\beta$TCR$^+$ T cells in tumor control. In addition, δTCR$^{-/-}$ have not been reported to be prone to increased risk for spontaneous malignancy.

The activity of NK cells is balanced by opposing activating and inhibitory signals, whereas the down-regulation of MHC class I molecules and the expression of NKG2d have been identified favoring NK activation [37]. NK cells are responsible for tumor rejection and protection from metastasis in MHC class I-negative tumor models. Recent experiments confirmed a role of the innate immune surveillance in the control of spontaneous B cell lymphomas, whereas the incidence of solid tumor was not reported to be increased [38].

Although various effector mechanism of the immune system are involved in tumor immunosurveillance, most experimental data support the notion that eradication of solid tumors rests predominantly on CD8$^+$ T lymphocyte reactivity [1, 2, 23, 39]. It is therefore of importance how other effector mechanisms of the immune system influence the resulting CD8$^+$ T cell response. An interesting possibility is that antibodies influence the induction of cytotoxic T cells. Studies from the 70s showed that sera from rats with tumor regression blocked the cell-mediated destruction of sarcomas [40]. More recent experiments documented an augmented rejection of tumor allografts by mice lacking B cells and an enhanced induction of an anti-tumor response in the absence of B cells [41]. It was speculated that APC, such as B cells, macrophages and dendritic cells (DC) compete for tumor antigen, but an actual role of antibodies in this process could not be documented. In contrast, antibodies have been shown to enhance cross-presentation of tumor antigens. Targeting antigen to FcγR promoted cross-presentation and CTL induction by several orders of magnitude in mouse bone marrow-derived DC [42] and recent in vitro experiments suggested a role for anti-tumor antibodies in the induction of anti-tumor CTL. Coating of myeloma cells with anti-syndecan antibodies increased cross-presentation and cross-priming of the tumor antigens NY-ESO1 and MAGE3 [43]. In a mouse model, CD8$^+$ T cell depletion prevented treatment of established solid tumors with anti-tumor mAb, suggesting a role of antibodies in the induction of CTL in vivo [44]. Similarly, NK cell activation has been shown to influence CTL priming. MHC class I^{low} tumor cells activate NK cells, which in response prime DC to produce IL-12 and to induce anti-tumor CTL [45]. Taken together, various effector mechanisms are involved in the control of solid tumors, and in many tumor situations they may be non-redundant. In addition to their role in direct tumor control in vivo, immunological effector mechanisms may also influence the resulting anti-tumor

CTL response. However, the requirements for either enhancing or inhibiting tumor-specific CTL responses by antibodies or NK cells, and especially their relevance for the protection against tumors in vivo, remains to be analyzed in clearly defined model situations.

Induction of cytotoxic T cells

There is a critical threshold of T cell receptor (TCR) molecules to be engaged with peptide-MHC molecules to trigger a detectable T cell response. In vitro experiments suggested that a small number of peptide-MHC complexes serially triggers up to approximately 200 TCR, leading to T cell activation [46]. The requisite number of occupied TCR can be significantly decreased if costimulatory signals are provided. In situations where TCR triggering becomes limiting, the provision of a costimulatory signal is crucial to reach the threshold for activation and induction of a T cell response [47]. This leads to the concept that a naïve T cell requires two distinct signals for full activation [48, 49]: Signal one is delivered by the interaction between TCR and antigenic peptides presented on MHC molecules. The second signal is provided by at least one of several antigen-nonspecific costimulatory signals, including the interaction of CD28 on T cells with B7 family molecules on pAPC. Additional second signals include members of the TNFR family (CD40-CD40L, CD27-CD70, OX40-OX40L, 4–1BB-4–1BBL) [50, 51], as well as soluble molecules such as IL-2, IL-12 and IL-18 and molecules involved in cell adhesion and T cell stimulation such as LFA-1 and ICAM-1. Although all these costimulatory pathways stimulate different intracellular signaling pathways and some of their roles may be non-redundant, they all result in enhanced activation, proliferation, survival and effector function [50, 51].

In vivo naïve CTL have to interact and collaborate in organized lymphoid tissue with APC to become activated [52, 53]. The term organized lymphoid tissue defines structures such as follicles, marginal zone, germinal center, periarteriolar sheath and red pulp. These anatomical structures determine the localization of antigen, cytokines, interleukins and bystander contacts via costimulatory molecules. Therefore, lymph nodes and the spleen provide the milieu necessary for lymphoid cell interactions and activation. Direct demonstration that secondary lymphoid organs play a pivotal role in adoptive immune responses derived mainly from alymphoblastic aly/aly mice and from lymphotoxin-α-deficient (Lt$\alpha^{-/-}$) and lymphotoxin-β-receptor (LtβR$^{-/-}$)-deficient mice [54, 55, 56]. The interpretation of the immune responses in aly/aly, Lt$\alpha^{-/-}$ and LtβR$^{-/-}$ is complicated by the fact that, apart from the lack of lymph nodes, these animals have other defects in the adaptive immune system. Lt$\alpha^{-/-}$ or LtβR$^{-/-}$ mice have increased numbers of circulating T cells, and spontaneously develop massive infiltrates of activated lymphocytes in their peripheral tissues [55, 56]. Therefore, the necessity of secondary lymphoid organs to induce an immune response may be masked in these mice. Adoptive transfer experiments revealed that T cells from aly/aly mice are functionally normal in a control C57BL/6 host [54, 57]. Aly/aly mice generated detectable, but reduced, CTL responses after infection with vaccinia virus and lymphocytic choriomeningitis virus (LCMV), and the elimination of these viruses was either delayed or virtually impossible; irrespective of the dose or the route of infection, aly/aly mice developed life-long LCMV persistence and splenectomized aly/aly mice did not mount a CTL response at all after infection with LCMV [54]. More recently, it was shown that splenectomized aly/aly mice did not mount a response to alloantigens after heart transplantation [57]. In contrast to naïve T cells that are activated by antigen within

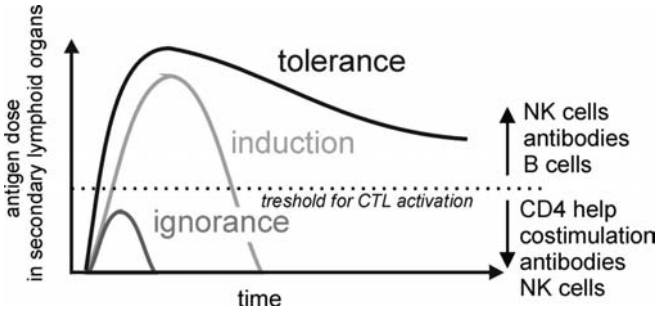

Fig. 1 Antigen dose over time in secondary lymphoid organs determines the resulting CTL response. The *horizontal dotted line* determines the minimal amount of antigen necessary for the induction of a CTL response, "threshold for CTL activation" (*CTL* cytolytic T lymphocyte)

organized structures of secondary lymphoid organs, memory CTL activation and re-expansion seem to be independent of secondary lymphoid structures [58].

How long has antigen to be present in lymphoid organs for optimal expansion of cytotoxic T cells? Several groups have shown that a single brief encounter with antigen results in at least eight to ten "programmed" cell divisions of the responding T cell [59]. The antigen dose and persistence determines the peak size of the $CD8^+$ T cell response by (1) recruitment of antigen-specific precursors from the naïve $CD8^+$ T cell repertoire, and (2) influencing the total number of divisions. This peak expansion is followed by a contraction of T cell numbers to a stable frequency of memory CTL. The contraction phase is initiated by the absence of antigen, but also by the fact that effector CTL are prone to undergo cell death due to the decreased expression of anti-apoptotic protein Bcl-2, c-IAP and tumor necrosis factor receptor-associated factor 2 (TRAF-2), as well as due to direct induction of apoptosis through FAS or TNFR signaling. In addition, cytokine starvation during the peak of CTL expansion, especially of IL-2, may limit further expansion and contribute to the contraction of the $CD8^+$ T cell pool [60]. What happens if the antigen is not cleared and persists at high levels? It is well documented that T cells do not react against antigens that are continuously present in lymphoid organs. Antigen that is continuously present in secondary lymphoid organs will activate naïve T cells to proliferate and expand to a peak similar to that of antigen only transiently present in lymphoid organs. However, despite the persistence of the antigen, T cells do not continue to expand but, in contrast, undergo a contraction phase, resulting in the deletion of all T cells specific for that antigen. The process of activation followed by the physical deletion of T cells is termed exhaustion [61]. Some cytopathic persistent infections that are transmitted from mother to offspring, an overwhelming infectious dose of a replicating virus or advanced stages of leukemia or lymphoma will lead to this form of tolerance [62].

Therefore, the antigen amount, localization and persistence over time are crucial parameters for the induction of naïve T cells (Fig. 1). The threshold for the activation of naïve T cells within lymphoid organs may be influenced by other effector mechanisms of the immune system, as discussed above. For example, the expression of costimulatory molecules on the APC ("cis") or on other non-APC ("trans") lowers the threshold of antigen necessary for the induction of naïve CTL [47]. The presentation of the peptide on the MHC class I, together with costimulatory signals on the same cell, seems more efficient than providing second signals in trans. However, we recently analyzed the contribution of

B7.1 expressed on a fibrosarcoma to the induction of naïve T cells [7]. The same number of B7.1-expressing or -non-expressing fibrosarcoma cells was necessary to induce an anti-tumor CTL response when the tumor cells were injected directly into the spleen. In contrast, CTL priming in lymphatic organs was reduced by a factor of about 100 in CD28-deficient mice, indicating that costimulatory molecules expressed in lymphatic organs may lower the antigen-threshold necessary for CTL activation. The abundance of costimulatory signals including B7.1, B7.2, CD40L, CD70, and soluble factors such as IL-2 are offered most efficiently within the anatomic structure of secondary lymphoid organs in either a bystander or a linked fashion. As outlined above, antibodies may crucially influence the antigen distribution and, therefore, CTL priming (Fig. 1). Natural antibodies are an essential part of the first line of defense against pathogens and provide an important link to the adaptive immune response. One important mechanism by which natural antibodies, but probably also IgM and IgG antibodies of the adaptive immune response, enhance specific CTL and antibody responses is the pooling of antigen to secondary lymphoid organs [63]. Within secondary lymphoid organs antibody-coated antigen may be more efficiently targeted to and processed by FcγR-expressing DC [42, 64]. Furthermore, activation of the complement cascade may enhance $CD8^+$ T cell responses and lower the antigen amount necessary for CTL activation [65].

Cross-priming versus direct priming

How then is the tumor-antigen transported, processed and presented to naïve CTL in secondary lymphoid organs? There are two possibilities how peptides derived from tumor antigens are presented on MHC class I molecules to naïve CTL: (1) direct presentation on tumor cells that migrated to secondary lymphoid organs, or (2) indirect, after uptake and processing by pAPC, a process that is termed cross-presentation (Fig. 2). Cross-priming describes the resulting CTL activation by cross-presented antigen. Similarly, cross-tolerance involves antigen processing and presentation via DC, but the specific T cells are not induced but anergized [66, 67]. There is evidence that both pathways of antigen expression exist in vitro and in vivo. However, there is substantial controversy as to which of the two pathways is of biological relevance [68, 69]. These differences can be partially attributed to the experimental systems analyzed.

The first evidence for the concept of cross-priming was obtained in experiments by Bevan [70], demonstrating that mice immunized with cells expressing foreign minor histocompatibility antigens mounted a self class I-restricted response, even if the cells lacked the restricting class I molecule. Since then, various studies with H-Y and other minor histocompatibility antigens have demonstrated a phenomenon that is compatible with cross-priming or cross-presentation. However, it is interesting to note here that in these early studies no cross-priming could be detected for tumor cells under similar experimental conditions [70, 71]. This was explained by the rapid proliferation of tumor cells favoring one dominant CTL subspecificity by direct induction.

Cross-priming assigns a central role to pAPC in the regulation of the immune response by inducing naïve T cells. Since the early experiments of Bevan, the understanding of the molecular mechanisms allowing the access of exogenous proteins to the MHC class I pathway of antigen presentation of pAPC has drastically increased (reviewed in [66]). Proteins internalized through all pathways of endocytosis, including macropinocytosis, re-

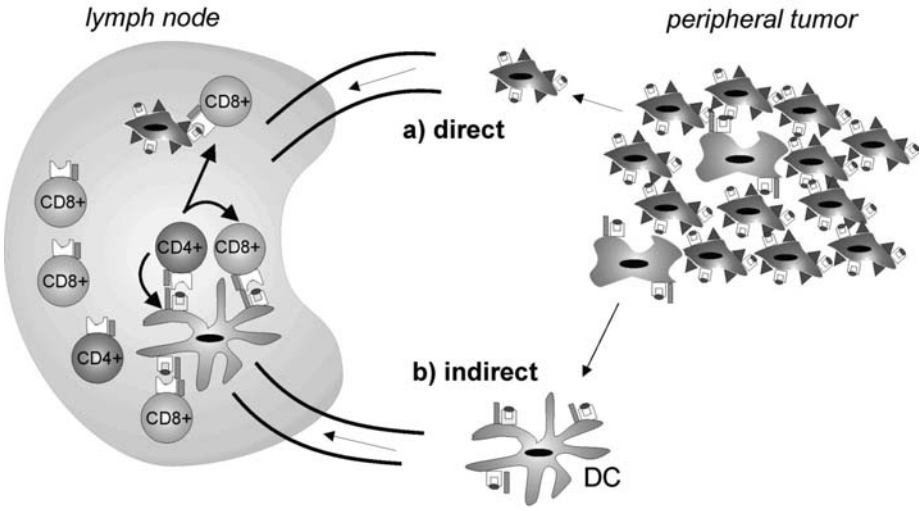

Fig. 2 Induction of an anti-tumor CTL response. Naïve $CD8^+$ T cells are induced in secondary lymphoid organs either direct by tumor cells or indirect via antigen cross-presenting pAPC. Naïve $CD4^+$ T cells are also induced in secondary lymphoid organs, but because most peripheral carcinomas and sarcomas are MHC class II negative, only pAPC are able to activate $CD4^+$ T cells. $CD4^+$ T cell activation provides direct help to $CD8^+$ T cells and, in most tumor models, CTL responses are dependent on the activation of T helper cells. In addition, $CD4^+$ T cells provide signals to pAPC to induce their maturation (*pAPC* professional antigen-presenting cell, *DC* dendritic cell)

ceptor-mediated endocytosis and phagocytosis, may be cross-presented. The process of cross-presentation seems more efficient for apoptotic cells than for necrotic cells [72]. Two models explaining cross-presentation at a molecular level are consistent with the present data. In the first model, the phagosome is self contained and associated with the proteasome and the peptide-loading complex [73]. In the second model, external protein have direct access to the endoplasmic reticulum [74].

Cross-presenting activity has been detected by macrophages, DC populations, B cells, keratinocytes and L cells, but $CD8^+$ DC are probably the important population cross-presenting ovalbumin and herpes simplex virus epitopes [75]. Efficient cross-presentation requires uptake of dead cells by immature DC, followed by the exposure to maturation stimuli. T cell division is induced by both immature and mature DC. However, full activation of effector CTL requires proper activation (maturation) of DC [69]. Activation signals include innate-immunity triggers, such as ligands of Toll-like receptors, or adaptive immunity triggers, such as CD40 ligand (CD40L), provided by activated $CD4^+$ T cells [10, 67]. In addition, opsonized antigen by IgG molecules has not only been documented to facilitate antigen targeting to APC, but may also provide a ligand for DC activation [42, 76]. Usually, high antigen concentrations have been used to document cross-presentation in vitro, but a recent report analyzing the efficiency of cross-presentation of vaccinia virus-derived antigens by human DC revealed that this pathway may be very efficient and comparable to peptide pulsing with 10–100 nM [77]. Of course, one caveat in analyzing cross-priming with potentially replicating agents, such as influenza or vaccinia virus, is the limitation in the detection limit of replicating virus, so that infection of DC can never be excluded absolutely [72, 78, 79].

In contrast to the usually well controllable situations in vitro, the detection of cross-priming in vivo is more controversial and especially dependent on the readout system used. Several methods were employed to analyze CTL activation in vivo as a consequence of cross-presentation by APC: (1) CFSE dilution of TCR transgenic T cells, (2) lysis of peptide-pulsed target cells after in vitro restimulation and expansion, (3) intracellular IFN-γ production after short in vitro restimulation with the respective peptide, (4) analysis of specific $CD8^+$ T cells by MHC class I tetramer staining, and (5) in vivo protection readouts. The adoptive transfer of CFSE-labeled T cells is definitely the most sensitive readout, leading to a precursor frequency of naïve T cells of about 1:10–100. When compared to the estimated physiological LCMV-GP33-specific precursor CTL frequency of 1 in 2×10^5, this is 2,000–20,000 times higher [80]. In different tumor models expressing viral antigens or ovalbumin as model tumor antigens, CFSE dilution as a consequence of antigen cross-presentation by pAPC could be documented [81, 82]. These experiments revealed that the process of cross-presentation in vivo is possible. However, due to the unphysiological high precursor frequency in these TCR transgenic systems, they do not allow conclusions to be drawn regarding the physiological relevance in the induction of an anti-tumor immune response in vivo. In non-transgenic systems, it was shown that minor histocompatibility antigens, together with H-2^b on cells, injected into F1 (bxd) mice prime CTL that are H-2^d restricted [70]. Other experimental systems using defined antigens confirmed that APC and CTL do necessarily have to express the same H-2 restriction for activation of CTL [72, 83]. However, it has to be emphasized that in these experimental systems the antigen-threshold for the induction of a CTL response is often changed (see Fig. 1), e.g., the immunization with an allogenic cell induces T help that lowers the threshold for (direct or indirect) CTL priming. Alternatively, the substitution with a H-2 non-compatible bone marrow prevents the induction of $CD4^+$ helper cells, reducing the threshold for CTL priming. Since anti-tumor CTL responses are usually dependent on the induction of specific $CD4^+$ helper cells and most tumor cells are MHC class II negative, it is obvious that the resulting CTL response is dependent on pAPC, but these experiments neither prove nor exclude a physiological role of cross-presentation of tumor antigens in vivo [7, 84]. Similarly, activation of NK cells by MHC non-matched tumor cells may provide an environment ideal for CTL priming, i.e., lower the threshold of antigen necessary for the detection of a CTL response. Therefore, only analysis of CTL responses to defined peptides will allow conclusions to be drawn regarding cross-priming, whereas protection experiments will not be conclusive. Because of these limitations, experiments in F1 mice from syngeneic parents with different haplotypes may be most instructive since they allow the analysis of direct and indirect CTL priming in the same experimental setting. Experiments by Hang et al. [83] with a colon carcinoma cell line transfected with influenza nucleoprotein (CT26-NP, H-2^b) showed that these cells primed H-2^d-NP336-restricted CTL in F1 (bxd) mice. However, specific lysis of H-2^d NP336-restricted CTL was very low (10–20% at an E:T of 100:1) and a comparison with the efficiency of direct priming by the tumor cells in lymphoid organs was not possible in this experimental system. The role of cross-priming on the peptide level in vivo was assessed in a comparable experimental situation using LCMV-GP and nucleoprotein (NP)-expressing splenocytes and tumor cells (Table 1). (H-2^bxH-2^d) F1 mice were analyzed for the generation of H-2^d- and H-2^b-restricted CTL after immunization with splenocytes from mice that express the LCMV-GP under the H-2 K^b promoter and LCMV-GP or -NP-transfected MC57G (fibrosarcoma, H-2^b), D2 (fibrosarcoma, H-2^d), EL4 (lymphoma, H-2^b) and P815 (mastocytoma, H-2^d) tu-

Table 1 Analysis of cross-priming versus direct priming of LCMV-GP and –NP-expressing tumor cells and splenocytes. Tumor cell lines were transfected with either LCMV-GP or LCMV-NP. LCMV-GP-expressing transgenic C57BL/6 (H-2^b, DEE) were bred with B10.D2 mice to obtain DEE^{bd} F1 and DEE^{dd} offspring. H-2^b-restricted CD8$^+$ CTL responses were analyzed using peptide gp33–41 or np 394–402 pulsed target cells; H-2^d-restricted CTL responses were analyzed against gp283–294 or np118–126 pulsed target cells after 5 days in vitro restimulation. H-2^b restricted CD4$^+$ T cell responses were analyzed by measuring antibody responses after immunization with DNP coupled to the helper epitope gp60–80 (H-2^b). Data are summarized from [7, 8]

Cells	Dose	Recipient mice	CD8$^+$		CD4$^+$
			H-2^b	H-2^d	H-2^b
MC-GP (H-2^b)	5×10^6 i.p.	F1 (H-2^bxH-2^d)	+	–	n.d.
MC-GP (H-2^b) apoptotic	4×10^7 i.p.	H-2^b	–	n.d.	n.d.
MC-GP (H-2^b)	5×10^6 i.p.	H-2^b	+	n.d.	+
MC-GP (H-2^b) anti-CD4 treatment[a]	5×10^6 i.p.	H-2^b	–	n.d.	n.d.
EL-GP (H-2^b)	5×10^6 i.p.	F1 (H-2^bxH-2^d)	+	–	n.d.
D2-GP (H-2^d)	5×10^6 i.p.	F1 (H-2^bxH-2^d)	–	+	n.d.
P815-NP (H-2^d)	5×10^6 i.p.	F1 (H-2^bxH-2^d)	–	+	n.d.
DEE (H-2^{bb})	2×10^6 i.v.	F1 (H-2^bxH-2^d)	+	–	n.d.
DEE (H-2^{bd})	2×10^6 i.v.	F1 (H-2^bxH-2^d)	+	+	n.d.
DEE (H-2^{dd})	2×10^6 i.v.	F1 (H-2^bxH-2^d)	–	+	+
DEE (H-2^{bb}) anti-CD4 treatment	2×10^6 i.v.	H-2^b	–	n.d.	n.d.

[a] M. Matter, unpublished data

mor cells [7, 8]. The resulting CTL response was, in all cases, limited to the haplotype of the immunizing cells, indicating that the efficiency of cross-priming in vivo is low when compared to direct CTL priming. Usually for these kinds of experiments tumor cells are injected as single-cell suspensions i.p. or s.c., an immunization route that is different from a physiologically growing peripheral tumor and which allows the migration of tumor cells to local lymph nodes and to the spleen, and probably favors direct CTL induction [85]. In summary, cross-priming has been very well documented and analyzed in vitro, and the process of cross-priming can also be detected in vivo. However, due to experimental limitations, the conditions when cross-priming is the predominant way, or when direct priming is more efficient in the induction of an anti-tumor immune response, are difficult to assess. In addition, the two processes may be even redundant for the priming of anti-tumor immune responses [86].

Nevertheless, several points can be concluded from the current literature. First, direct priming of tumor cells only occurs in secondary lymphoid organs and the direct injection of antigen-expressing tumor cells into secondary lymphoid organs is about 100–1,000 times more efficient in the induction of a CTL response than s.c. injection [7, 85]. Secondly, DC activation is necessary for cross-priming. Maturation stimuli are very efficiently provided by infectious agents, but may be limiting in a situation of a peripheral self or tumor antigen [69]. Thirdly, relatively large amounts of antigen are necessary to induce cross-priming. Activation of OT-I transgenic T cells is only observed in a transgenic mouse expressing high antigen levels in the pancreatic islets, but not in a strain with low ovalbumin expression [82]. In addition, cross-priming of p14 TCR transgenic T cells was not detectable in a rat insulin promoter (RIP)-GP transgenic mouse model [87]. However, when these mice were crossed to RIP-Tag2 transgenic mice that develop insulinomas, tumor growth enhanced cross-presentation, leading to limited T cell activation [81]. This again may be due to an increased amount of antigen present due to tumor growth and/or

due to APC maturation in the proinflammatory environment associated with the tumor. In line with a relatively high antigen expression necessary for cross-priming of naïve CTL, Spiotto et al. [88] described that solid tumors expressing a model tumor antigen at lower levels grew, whereas the same solid tumors expressing the antigen in a tamoxifen-regulated Cre-loxP system to higher levels were rejected due to cross-priming. Cross-priming does not only depend on a high synthesis rate of the protein, but also on its stability. This may be the reason why cross-priming is less efficiently reached with peptides derived from signal sequences, which are usually rapidly degraded after synthesis [89].

Immunological ignorance

The question then remains as to what happens if the antigen amount in secondary lymphoid organs, over a sufficient period of time, is not sufficient to activate naïve CTL, but the antigen is expressed exclusively on cells outside secondary lymphoid organs. The analysis of this question precludes a strict control of the localization of the antigen-expressing cells. This is difficult to obtain with transplantation experiments, for example using minor histocompatibility differences, since passenger leukocytes expressing the mAg may migrate through lymphatic vessels and reach the draining lymph node. However, the availability of transgenic mice expressing a model antigen under a tissue selective promoter allowed the analysis of immune responses to peripheral self antigens. RIP-GP transgenic mice express the LCMV-GP under the RIP [87]. These RIP-GP mice did not spontaneously develop diabetes. However, LCMV-GP-specific CTL in RIP-GP mice could be activated by immunization with DC expressing LCMV-GP or after infection with replicating LCMV. Interestingly, infection of the same mice with a recombinant vaccinia virus expressing the GP of LCMV resulted in CTL priming, but failed to induce diabetes. These experiments showed that induction of a CTL response alone is not sufficient to cause immunopathological autoimmune disease, but that there is an important quantitative requirement of effector CTL generated, i.e., LCMV infection induces about 100–1,000 times more effector T cells than recombinant vaccinia virus. These experiments illustrate that T cells specific for LCMV-GP are present, but because the antigen is expressed strictly outside secondary lymphoid organs and the antigen does not reach local lymph nodes in sufficient quantity over a sufficient period, no specific CTL are induced, and the peripheral antigen is ignored by the immune system [61]. Immunological ignorance therefore describes a situation in which reactive $CD8^+$ T cells in an individual have not encountered the target antigen and have a naïve phenotype.

Similarly, transgenic mice were produced expressing membrane-bound OVA under the RIP. When RIP-OVA mice were crossed to OT-1 mice, which produce OVA-specific $CD8^+$ T cells, double-transgenic mice showed deletion of OT-1 cells, probably due to thymic expression of OVA. However, adoptively transferred OT-1 cells into RIP-OVA mice expressed activation molecules at the cell surface and entered cell cycle in draining, but not in non-draining lymph nodes due to cross-presentation [90]. In later experiments the same group analyzed RIP-OVA mice with high and with low antigen expression in the pancreatic islets, and showed that the level of antigen expressed in peripheral tissues must be relatively high to be cross-presented by pAPC [82]. In this model, cross-presentation induced an initial activation followed by a deletion of the specific CTL. Below this antigen level, peripheral antigens were ignored by naïve OT-1 cells.

Table 2 Mechanisms limiting antigen-presentation on MHC class I in lymph nodes

Direct	– Late and inefficient migration of tumor cells to lymph nodes
	– MHC-class I down-regulation
	– Low tumor antigen expression
	– Expression of inhibitory molecules
	– Lack of costimulation
	– Walling off
Indirect	– Inefficient cross–presentation of antigen on MHC class I
	– Low level of tumor antigen expression
	– Inefficient maturation of dendritic cells
	– Limited number of apoptotic tumor cells to release antigen

Therefore, immunological ignorance of antigens expressed in peripheral tissues is dependent on several factors (Fig. 1 and Table 2): Firstly, the antigen is exclusively expressed on cells that do not or not efficiently migrate to secondary lymphoid organs. This is probably true for most self and tumor antigens; secondly, cross-presentation of these peripheral antigens is not sufficient for CTL activation due to the level of antigen expression or inadequate maturation of DC, as discussed above.

Ignorance of peripheral antigens must be separated from peripheral tolerance. The term tolerance usually describes situations where CTL have encountered antigen, but they are not properly activated or they are actively suppressed by regulatory mechanisms. Suppression of self-reactive T cells might be important for maintaining immunological unresponsiveness to self constituents (i.e., self tolerance) and preventing autoimmune disease. Recent studies have demonstrated that $CD25^+CD4^+$ regulatory T cells control not only autoimmune reactions but also other immune responses, including tumor immunity (reviewed in [91]).

Ignorance of solid tumors

Tumor antigens are in many respects quite similar to self antigens, and comparable rules may apply for the induction of an autoimmune or anti-tumor CTL response. Tumors develop without the apparent induction of a CTL response and the tumor-specific CTL have a naïve phenotype. $CD8^+$ T cells remain in a naïve phenotype if they have not been activated by tumor cells reaching local lymph nodes or by pAPC cross-presenting tumor antigens. Since peripheral tumors may originate from one malignant cell outside secondary lymphoid organs and metastasis to local lymph nodes occurs at relatively advanced stages of the disease, no or only few tumor cells may reach lymphoid organs early to induce tumor-specific CTL. In addition, tumor cells reaching secondary lymphoid organs have several strategies to avoid efficient CTL induction, including the development of antigen- or MHC class I-loss variants [92]. We recently reported that B16 melanoma cells injected directly into lymphoid organs avoid contact with naïve CTL by encapsulation [7]. Similarly, the requirements to reach the antigen load necessary for cross-priming may not be reached easily in spontaneous tumor development. The initially small tumors do not provide the antigen amount crucial for reaching cross-presentation by pAPC. This may be due to the limited number of tumor cells, but also due to relatively low expression levels of some tumor antigens. It has to be kept in mind that tumor experiments in mice are often done with tumor cell lines overexpressing antigens under the control of very strong viral promoters,

and the transfected tumor cells are selected for maximal antigen expression. This selection of tumor cells expressing high antigen levels is necessary to reliably analyze immune responses. However, there are only few situations with spontaneous tumors in which the tumor antigens are expressed under such strong promoters. Examples are fusion proteins, where some tumor antigens are expressed under very strong promoters such as BCR-ABL [93] or virus-induced tumors where the viral antigen is at the same time a tumor-associated antigen [17]. However, the situation for most solid tumors may be quite different since most tumor antigens are only expressed at low to moderate levels. In addition, tumors resemble normal healthy tissue in many ways, and often cause little or no DC activation [69, 81]. Therefore, both the direct and indirect induction of naïve CTL by tumors is inefficient in vivo, especially during early tumor development, and peripheral tumors are ignored by $CD8^+$ T cells. In more advanced stages of disease, a sufficient number of tumor cells may reach lymphoid organs, and/or, for some tumors, the antigen amount may reach the level necessary for cross-priming, leading to activation of naïve CTL. In these advanced stage diseases the induced CTL response contrasts a very large tumor burden and is, therefore, often not sufficient to eradicate all tumor cells before escape variants occur [23, 92].

In situations where solid tumors are ignored by naïve CTL, vaccination protocols may be most efficient because no tolerance mechanism prevents the induction of the specific $CD8^+$ T cells. We recently showed, for a growing fibrosarcoma expressing the LCMV-GP as model tumor antigen, that detectable CTL responses are relatively easily induced by vaccination. In contrast, rejection of growing tumors is demanding and requires several booster immunizations [8, 94]. Detailed analysis of the CTL frequencies induced by various vaccination protocols with LCMV-gp33-expressing DC revealed that repetitive immunizations during the initial 8–10 days were necessary to reach a high frequency of effector cells, probably due to efficient recruitment of naïve CTL. After the initial expansion, further booster immunizations did not augment the frequency of effector cells, but were necessary to keep the cells activated with lytic function. In this experimental system even the repetitive injection of the tumor cells as single-cell suspensions led to the rejection of established tumors, indicating that ignorance is broken if antigen-expressing cells reach lymphoid organs [95, 96].

Conclusion

Naïve tumor-specific CTL are induced in secondary lymphoid organs, including lymph nodes and the spleen. Solid tumors develop outside organized lymphoid structures and during early oncogenesis they often do not efficiently induce a CTL response. Naïve CTL are only activated if the tumor antigen is presented in secondary lymphoid organs over a sufficiently long period of time. Tumor antigen might be either cross-presented to naïve CTL by pAPC or directly by tumor cells that migrated (metastasized) to secondary lymphoid organs. Both processes are quite inefficient during early tumor development. Metastasis is, for most tumors, associated with advanced disease, and does not occur during early tumor development. The process of cross-presentation in vivo seems to depend on large antigen amounts and on the proper activation (maturation) of DC. Tumors usually start from a single malignant cell and therefore only offer limited inflammatory stimuli for DC maturation and a limited amount of antigen to be cross-presented during early stages of the disease. As a consequence, peripheral tumors may grow because they are ignored by the immune system for too long. In more advanced tumor stages, migrating tumor cells in-

duce an immune response in local lymph nodes or, in some situations, tumor antigen levels might be sufficient to cross-prime naïve CTL. The induced CTL response often contrasts a large tumor burden and is not sufficient to eliminate the tumor completely, but will select for less immunogenic escape mutants. Ignorance to tumors and to self antigens can be broken simply by providing syngeneic antigen-expressing cells to lymphoid organs. Due to the low tumor burden, the naïve phenotype of tumor-specific CTL and the absence of tolerance mechanisms, vaccination protocols against tumors might be very efficient in a situation of peripheral ignorance of solid tumors.

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