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REVIEW

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In situ T cells in melanoma

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Abstract During the past decade new insights have been gained into the role of T lymphocytes in the host's immune response to cancer in general and to melanoma in particular. Several melanoma-associated antigens (MAA) recognized by T cells have been characterized, and a number of HLA class I- and class II-restricted peptides have been identified. These antigens can be divided into three different groups: tumor-associated testis-specific antigens, melanocyte differentiation antigens, and mutated or aberrantly expressed antigens. These proteins give rise to several antigenic peptides. The number of known melanoma-associated peptides that can induce killing by cytotoxic T-lymphocytes (CTL) exceeds 30 and is still increasing. In line with these findings, clinical data indicate that the immune system is essential in the control of tumor growth. A brisk infiltration of lymphocytes is associated with a favorable prognosis, and complete or partial regression of primary melanoma occurs quite frequently. Furthermore, immunomodulatory therapies have accomplished complete or partial tumor regression in a number of patients. However, the immune response is in most cases inadequate to control tumor growth as tumor progression often occurs. Hence, the coexistence of a cellular immune response in melanoma lesions, demonstrated by the presence of clonally expanded T cells, remains a major paradox of tumor immunology. In the present paper we review current knowledge regarding tumor infiltrating lymphocytes (TIL) in melanoma and discuss possible mechanisms of escape from immune surveillance.

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The T cell

T cells are roughly divided into two subgroups, i.e. CD8 and CD4 T cells. CD8 T cells are in general cytotoxic T lymphocytes (CTL) recognizing antigenic peptides in the context of MHC class I molecules whereas CD4 T cells recognize antigenic peptides in the context of class II molecules. Antigen recognition is mediated through the T-cell receptor (TCR), a clonally distributed heterodimer, in most T cells consisting of an α and a β chain, although a small fraction uses a $\gamma\delta$ heterodimer instead [29]. Each T cell expresses a clonotypic TCR on the cell surface together with the non-clonotypic members of the TCR signaling complex: the different chains of the CD3 complex (γ, δ, ε) and the ζ chain homodimer [99]. In the germline, each of the TCR proteins is encoded by variable (V), joining (J) and constant (C) gene segments, and for the β chain, an additional diversity (D) segment [20].

During T-cell maturation in the thymus gene segments are rearranged into a single transcriptional unit for each of the chains, encoding unique $\alpha\beta$ or $\gamma\delta$ TCR genes in each T cell. During the rearrangement process the V, J and D segments recombine to form a single exon adjacent to the C segment. A seemingly limitless repertoire for antigen recognition is provided by the assortment of the various V, J, and D gene segments, as well as the addition or deletion of nucleotides at the junction between the V/J and the J/D gene segments. This so-called "N-region diversity" provides a unique DNA sequence for each individual T cell, forming a clonotype [20].

The naive T cell requires a costimulatory signal in order to reach a state of proliferation and clonal expansion. A well-characterized costimulatory signal is provided by ligation of CD28 and B7-1 (CD80) or B7-2 (CD86) expressed on the antigen-presenting cell (APC). Signaling through CD28 induces an increase in the expression [95] and the stability of the interleukin 2 (IL-2)

mRNA [50] and a reduction in the number of TCR/MHC-peptide interactions necessary for T-cell activation [109]. An early event in CD28-mediated signal transduction is the activation of AP-1 and NFAT thereby integrating the TCR and CD28 activation signals [2]. The CD28/B7 ligation therefore appears to be important, if not mandatory, for T-cell activation. In addition to this costimulatory system, other surface molecules participate in the activation of T cells by directly participating in signaling, by inducing sustained expression of costimulatory molecules [107], or simply by facilitating cell to cell interaction [23]. However, the T cell/APC interaction also modulates the action of the APC. It has recently been shown that the interaction between CD40L on a Th cell and CD40 on dendritic cells (DC) "licenses" the DC to activate CD8 cytotoxic T cells [8, 90].

The activation of a T cell after the encounter with an MHC/peptide complex may take one of several possible routes, meaning that the activation threshold can be gained by interactions between a number of different molecules, possibly differing from cell to cell and from antigen to antigen. The number and character of the different molecular interactions therefore heavily influences the consequences of TCR/MHC-peptide ligation [56, 108]. Furthermore, it is evident that the peptide ligand is of the utmost importance for the outcome of the TCR/MHC-peptide ligation since otherwise agonistic peptides may, if altered at a single position, result in antagonistic TCR signaling and induce unresponsiveness [15].

Evidence for the importance of T cells in the control of melanoma growth

Melanoma lesions are often infiltrated by lymphocytes mostly consisting of T cells. The presence of such tumor infiltrating lymphocytes (TIL) in primary tumors is associated with a better prognosis, suggesting the relevance of lymphocytes in the control of tumor cell growth [16]. This notion is further sustained by the spontaneous regression of primary tumors, partial or complete, associated with an increased number of CD3-positive cells [16, 17, 33]. Although the specific antigens recognized by T cells in situ are not known, a number of melanoma-associated antigens (MAA) recognized by CTL in vitro have been characterized. These antigens can be divided into three different groups: tumor-associated testis-specific antigens, melanocyte differentiation antigens, and mutated or aberrantly expressed antigens. In general, these proteins give rise to more than one antigenic peptide presented in the context of an HLA molecule; the number of known melanoma associated peptides that can induce killing by CTL exceeds 30 and is still increasing [43].

Some of the peptides have only been shown to induce cytotoxicity against peptide-loaded cells, and it is not known whether these peptides are actually processed and presented naturally. Nevertheless, it is evident that melanoma cells do express a number of proteins that give rise to antigenic peptides and that these peptides are recognized by autologous T cells in vitro. Most studies addressing the isolation and characterization of MAA have been carried out using cells expressing the HLA-A1 or the HLA-A2.1 allele. Therefore, even if no additional melanoma-associated proteins are discovered, the number of antigenic peptides derived from the known proteins is likely to increase as more restriction elements are studied.

Several clinical observations provide evidence for the in vivo relevance of T cell-mediated immune responses for the control of metastatic disease. The systemic administration of high doses of IL-2 has been shown to induce tumor regression in metastatic melanoma patients [82, 83], and in recent trials several peptide-based vaccines have been shown to induce tumor regression in some patients [58, 68, 84].

In conclusion, melanoma cells express antigens that are recognized by CTL in vitro, and clinical data suggest that T cells recognize MAA in vivo. However, in most cases the in vivo T-cell response is obviously inadequate to control tumor growth as tumor progression often occurs.

TCR V region repertoire in TIL

Several studies have been conducted with the aim of analyzing the TCR V region repertoire in different pathological T cell infiltrates. In autoimmune diseases, infiltrates of T cells express a restricted repertoire of TCR V regions indicating that T cells carrying TCRs specific for self-antigens are amplified [71]. Based on the assumption that tumor cells may elicit an oligoclonal T-cell response in a similar fashion, analyses of the TCR V region repertoire in TIL have been performed. The method of choice in most laboratories is based on reverse transcription-coupled polymerase chain reaction (RT-PCR) using a set of primers covering the V region families, together with a common C region primer. The majority of the studies have employed RT-PCR in a quantitative setup enabling comparisons of the expression of specific V regions in tumor lesions and peripheral blood lymphocytes (PBL) or in different lesions from the same patient. Nitta et al. have reported a highly restricted TCR Va (AV) region usage in uveal melanoma [69], but in general the V region repertoire in TIL has been found to be heterogeneous and comparable to that in PBL [28, 86, 100, 113]. However, some regions are expressed at high levels compared to the level of expression detected in PBL, which indicates the activation and proliferation of specific T cells.

In general, two to four regions are expressed at elevated levels, although some data show the very high expression of a single region. The presence of highly expressed TCR Vb (BV) regions demonstrated in TIL in situ may indicate the possibility that dominant peptide antigens would select analogue TCR V regions in HLA-matched patients [93]. However, the TCR V region

repertoire appears to be distinct in every patient and even the analyses of several metastases from the same patients have demonstrated substantial differences in V region expression from lesion to lesion [92, 97]. Although the reason for this may be heterogeneity in antigen expression from lesion to lesion, it has become evident that any HLA/peptide complex may be recognized by a number of different TCRs [18, 81].

Another important fact in relation to in situ T cells is that cells specific for MAA may not be present in very high numbers. This was indicated by the study of Salvi et al. in which the expression of BV14 was analyzed due to the demonstrated involvement of this region in the recognition of the MART-$1_{27\text{-}35}$ peptide in the context of HLA-A2 [86]. Interestingly, compared to PBL, the BV14 region was expressed at elevated levels in HLA-$A2^{+}$ tumors, but not in HLA-A^{2-} tumors. Although the expression of BV14 in most cases was intermediate or even low, the data indicate that T cells recognizing melanoma peptides may not expand to high numbers [86].

A striking feature of TIL is the remarkable shift in the V region repertoire that occurs when they are cultured in vitro ([21, 94]; thor Straten et al., submitted for publication). As is discussed below, the biological background for this change in repertoire is unknown. The failure of some in vitro established TIL to recognize fresh autologous melanoma cells, but not autologous melanoma cell lines, could indicate major differences in the expression of antigens between cultured melanoma cells and melanoma in vivo [22]. Nevertheless, the known melanocyte differentiation antigens are expressed by melanoma cells in vivo and in vitro.

T-cell clonotypes in TIL

Although the T-cell infiltrate in melanoma is rather heterogeneous, the presence of clonally expanded T cells has been repeatedly demonstrated [28, 55, 77, 94, 100]. In earlier studies, clonotypic TCR transcripts were detected by means of RT-PCR and subsequent sequencing or cloning of those BV regions expressed at high levels in the tumor [28, 55, 100]. Most studies concerned with this matter have demonstrated the presence of clonal T cells in the TIL population. However, in an intensive study by Ferradini et al. the cloning and sequencing of more than 200 TCR transcripts from a subcutaneous metastasis revealed no evidence of clonotypic T cells [27]. Subcutaneous metastases are frequently infiltrated by a limited number of T cells whereas larger numbers of T cells may be present along the base of the tumor. These T cells are likely to be nonspecific inflammatory T cells that would not be expected to show signs of T-cell clonality. On the other hand, it is possible that clonal T-cell responses are not elicited in all melanoma lesions. Nevertheless, the large number of studies that have demonstrated T-cell clonality in TIL suggest that the that lack of clonality in TIL is the exception rather than the rule.

Mackensen et al. have demonstrated the presence of clonally expanded T cells in a primary melanoma lesion that was characterized by regression, indicating that clonally expanded T cells are involved in the in situ cytotoxicity against tumor cells [54]. The demonstration of clonotypic T cells in both regressive and progressive parts of primary melanoma lesions have indicated, however, that the presence of clonal T cells is not restricted to lesions in regression [101]. It is therefore conceivable that the presence of clonotypic T cells is a general characteristic of melanoma, not dependent on tumor regression. Therefore, the enigma of having regressive areas of tumors in which tumor cell destruction occurs in close vicinity to uncontrolled tumor growth cannot be related to a lack of clonal T-cell responses in the progressive part.

As mentioned, the majority of the studies investigating clonality of TIL have focused on the analyses of TCRs expressed at high levels. Thus, although the general conclusion from these studies is that the presence of clonotypic T cells is a characteristic of TIL, no information has been obtained with regard to the number of clones involved and the diversity of the response. To overcome this limitation Puisieux et al. used the "immunoscope" methodology to analyze the clonality of TIL in more detail [77]. The immunoscope technique amplifies each BV region family and determines the size spectra of the CDR3 regions of the amplified subpopulations. Clonal expansions are detected as a relatively high amount of transcripts having exactly the same length, and further refinement can be achieved by reamplification with TCR BJ-specific primers and repeated size spectra determination [73]. Using the immunoscope technique, Puisieux and colleagues analyzed several subcutaneous metastases from two patients for the presence of clonal T cells [77]. The number of in situ T-cell clones detected in this study ranged from 2 to 15. However, only a limited number of selected BV regions were chosen for refined analysis, so the number and heterogeneity of the in situ T-cell clones may have been underestimated. Interestingly, some of the supposedly clonal transcripts were expressed at low levels ($< 2\%$) confirming an earlier report by Salvi et al. [86].

The complete and detailed analysis of T-cell clonality by the immunoscope technique is rather laborious. It requires a total of 350 PCR reactions and the subsequent separate analysis of these. We recently reported a novel method termed "TCR clonotype mapping", based on RT-PCR and denaturing gradient gel electrophoresis (DGGE) for detection and characterization of clonotypic T cells [103]. DGGE is a powerful method for detection of small deletions, small insertions and point mutations, having a detection efficiency close to 100% [66]. The method relies on the fact that the melting properties of a DNA molecule are highly dependent on the nucleotide composition. During electrophoresis in a denaturing gradient gel, the DNA molecule will partially melt at a sequence-dependent concentration of denaturants, and the resulting partial separation of the DNA

duplex will retard the molecule and thereby reveal a distinct band in the gel. This implies that even single base pair changes in a DNA sequence can be revealed in a denaturing gradient gel by a shift in the position at which the molecule is retarded [66]. In a polyclonal T-cell population, all TCR DNA sequences will, in theory, differ from each other in their melting properties and will therefore be revealed as a smear in the denaturing gradient gel. In contrast, any population of clonally expanded T cells will be revealed as a distinct band that can be recovered for further analysis.

We recently analyzed six subcutaneous metastases from two patients by TCR clonotype mapping for the presence of clonotypic T cells. The results revealed an exceedingly high number of clonotypes ranging from 40 to more than 60 in each lesion. In contrast, the analysis of the corresponding blood samples revealed the presence of only two to four very faint bands in the gel [104]. In each lesion, clonotypes were detected for almost all of the 24 BV families analyzed, in some cases with more than five clonotypes carrying the same BV region. These findings indicate that the in situ T-cell response against melanoma is more heterogeneous and comprise a higher number of T-cell clonotypes than previously appreciated. Furthermore, comparison of the clonotypes in each patient demonstrated that the vast majority of the clonotypes were detected in a single lesion only. Although some clonotypes may be detected in two lesions from the same patient, as reported by others [40], our findings demonstrated that this is a rather rare event. Hence, in general, the T-cell response is confined to strictly localized T cells.

Targets recognized in situ by TIL

The presence of clonally expanded T cells in situ suggests that these cells specifically recognize melanoma cells. However, direct evidence for this is still lacking. Thus, the question as to which antigens are recognized by TIL in situ remains important. One of the major obstacles in defining these antigens is related to the difficulties in supporting the growth of in situ-expanded T-cell clonotypes in vitro [21, 26, 105, 113]. This has been possible only in a few cases and the recognized peptide epitope was not characterized [28].

Experience with the in vitro expansion of polyclonal or clonal T cell lines, has shown that CTL specific for melanocyte differentiation antigens are much easier to establish than CTL specific for cancer/testis-specific antigens, i.e. the MAGE family of proteins [43]. Possibly the reason for this is the presence of structures in cancer/testis-specific proteins that prevent efficient antigen processing [43]. Indications that melanocyte differentiation antigens serve as CTL targets in situ can also be deduced from the depigmentations occasionally observed in patients responding to immunotherapy [42]. This vitiligo-like depigmentation illustrates that T cells recognizing melanoma cells may also destroy normal melanocytes. Recently, we have used TCR clonotype mapping to demonstrate the in situ accumulation of the same T-cell clone in a primary melanoma and a vitiligo-like halo about the tumor (Becker et al., submitted for publication). This study was the first to prove a number of hypothetical considerations drawn from in vitro data. However, as discussed below, the promiscuous nature of peptide recognition by the TCR implies that some of the T cells in situ may have irrelevant peptides, self or viral, as their primary targets.

Immune escape mechanisms in melanoma

The presence of clonotypic T cells in melanoma lesions as well as clinical signs for the immunological control of melanoma growth raises the question as to why this is not sufficient for complete tumor eradication. One possible explanation is that melanoma cells may lose the expression of HLA molecules, and indeed the complete loss of class I expression or selective loss of HLA alleles have been demonstrated in a number of studies [30, 39, 41, 85, 112]. However, only a small percentage of established melanoma cell lines are negative for HLA molecules [102]. Furthermore, clonal T-cell responses occur in progressive as well as in regressive parts of tumors, indicating the expression of HLA molecules in both parts [101].

Immunohistochemistry has demonstrated the heterogeneity of HLA expression in situ [19, 30]. This observation has fostered the hypothesis that the lack of expression in some areas of a lesion could be due to selective downregulation. It has been shown that some breast cancer cell lines do not express HLA molecules when in a quiescent state [49]. Whether this mechanism is relevant for melanoma cells is not known, but it has recently been reported that transcription of LMP2, associated with processing of antigens, is absent in quiescent melanoma cells [13]. It is therefore possible that HLA loss, as demonstrated by immunohistochemistry, does not imply escape from T-cell surveillance but detects cells in a quiescent state. If this is the case, HLA loss is not as widespread a mechanism of immune escape as previously suggested, although it is evident that it does occur. Interestingly, in cell lines established from patients undergoing immunotherapy the loss of HLA expression is more frequent [78], supporting the concept of immune surveillance by T cells.

An alternative immune escape mechanism in melanoma is the induction of T-cell unresponsiveness due to downregulation of the ζ-chain of the TCR/CD3 complex, which has been shown in TIL of several tumors [60]. Only a single study of the ζ-chain expression in TIL and PBL in melanoma patients has been reported, and all the patients included in this study were late-stage melanoma patients [116]. A significant decrease in ζ-chain expression in both TIL and PBL was found. However, even in advanced melanoma patients-MAA specific T cells constitute only a minority of the T cells in

PBL. It is therefore conceivable that downregulation of the ζ-chain may not be directly accomplished by the tumor, but could be a more general property of long-lasting immune responses that are unsuccessful in clearing the antigen from the body, which supposedly induces a state analogous to chronic inflammation [74, 117]. Thus, downregulation of the ζ-chain is secondary to immune escape, merely being the result of having an ongoing unsuccessful immune response.

Melanoma cells are known to secrete a number of cytokines that are able to suppress cellular immune responses, e.g. IL-10 [14, 46] or TGFβ [63, 88]. Normal melanocytes are susceptible to growth inhibition induced by TGFβ, whereas melanomas are not [44]. In other cancers, such an unresponsiveness to TGFβ has been shown to be due to mutations in Smad4/DPC4, which is essential for TGFβ-mediated intracellular signaling [31, 35, 67]. However, in the analysis of more than 40 melanoma cell lines we detected no Smad4/DPC4 mutations (unpublished data). Yet, the unresponsiveness of melanoma to TGFβ may also be acquired by deletions or mutations of p15 which have, to our knowledge, not been extensively tested in melanoma. Whether TGFβ and IL-10 play an essential role in the suppression of T cell antimelanoma responses is not well established.

The cytokine profiles in regressive and progressive melanoma lesions have been analyzed in two recent studies [53, 110]. These failed to prove an important function for TGFβ, and surprisingly, in the study of Wagner et al., elevated amounts of IL-10 were found to be associated with tumor regression [110]. It should be noted that the biological activities of both IL-10 and TGFβ are ambiguous. Besides its immunosuppressive effects, IL-10 has been shown to enhance natural killer (NK) and T-cell cytotoxicity and proliferation [91] and TGFβ may induce the differentiation and proliferation of $CD4^+$ T cells [48]. Nevertheless, it is conceivable that the cytokine balance in the tumor is of importance. For example, a higher concentration of IL-2 has been demonstrated in regressive lesions compared to progressive lesions [53, 110] and tumor regression can be induced by IL-2 [83]. The involvement of melanoma cells in the modulation of this balance is currently under investigation.

It has been demonstrated that melanoma cells express the Fas ligand (FasL) and that such melanoma cells are able to kill T cells expressing the Fas antigen [32]. The first report showed FasL expression in seven out of seven metastatic melanoma lesions selected by the presence of soluble Fas ligand in the serum of the patients. Recently, FasL expression in metastatic lesions has been detected in approximately 50% of nonselected lesions, and in 10% of primary tumors [98]. Although conflicting results have been reported [12], these results strongly indicate that FasL expression by melanoma cells may be involved in immune escape of melanoma. However, it should be kept in mind that T cells are not necessarily susceptible to Fas-mediated apoptosis and that T-cell cytotoxicity may be exerted by mechanisms that do not include the Fas/FasL system [9]. This notion is illustrated in a murine model: the highly aggressive B16 melanoma expresses substantial levels of FasL [11]. Nevertheless, successful T-cell responses are inducible by treatment with IL-2, indicating that T cells are capable of avoiding FasL-induced cytotoxicity [106]. Furthermore, it has recently been shown that in vitro established CTL clones are resistant to FasL-induced apoptosis and that these clones mediate cytotoxicity by mechanisms independent of Fas/FasL ligation [80]. In this regard it has been suggested that the activation of T cells by both antigen and CD28 renders the T cell insensitive to Fas-mediated apoptosis [111].

Killer inhibitory receptors (KIRs) on NK cells are involved in the recognition and subsequent destruction of cells that do not express sufficient levels of HLA molecules [45]. Recently it has become evident that T cells may express KIRs. This finding has a major impact on tumor immunology: a specific T cell may not kill the target cell if the target cell in addition to the specific antigen/HLA complex also expresses the protective HLA molecule [4, 62, 70]. Potentially, the expression of such KIRs could inhibit in situ T-cell responses against melanoma. However, the expression of KIRs in PBL appears to be restricted to a single of a few clonotypic T-cell populations. Preliminary findings have demonstrated the expression of some KIRs, i.e. CD94, on TIL in melanoma (unpublished data), but the functional significance of this remains to be established.

The immune escape mechanisms described previously mainly concern late-stage melanoma patients. Thus, it is likely that these mechanisms are actually acquired by melanoma cells which have been selected by the pressure exerted by the immune system. It is, however, striking that the vast majority of established melanoma cell lines express HLA molecules in addition to several MAAs, and that specific CTL can easily be established in the laboratory [102]. The significant increase in the incidence of HLA and antigen loss variants observed in patients receiving immunotherapy indicates that the immune system needs to be boosted in order to select such antigen-negative variants [47, 57, 78]. This observation stresses the impact of those immune escape mechanisms on immunotherapy of advanced melanoma. On the other hand, they may not be important for early events in tumor immunity, nor for possible immunotherapeutic strategies designed for patients with minimal disease.

However, additional phenomena seem to influence the immune response to melanoma. Even when the tumor is confined to a very limited number of cells it is obvious that the T-cell infiltrate in most cases fails to kill the tumor cells. Immune selection of resistant tumor cells is not very likely because any selection will suffer from a low number of cells. Thus, in a small primary tumor the limited number of cells will substantially reduce the chance of the tumor comprising cells that have the potential to escape the immune system. It is therefore possible that the inadequacy of the immune system to eradicate the tumor is more related to an inefficient T-

cell response than to specific mechanisms of melanoma cells escaping the immune response.

As mentioned, we and others have shown that T-cell clonotypes expanded in situ do not grow using standard in vitro culture conditions [21, 22, 26, 105, 113]. Nevertheless, tumor-specific CTL can be established from TIL, demonstrating that some of the T cells within the TIL are tumor specific, although they are not clonally expanded in situ. This observation suggests the presence of (at least) two different subpopulations of T cells in TIL, one population that consists of in situ-expanded clonotypic T cells not capable of in vitro growth, and another population of T cells that are not clonally expanded in situ, but are capable of in vitro growth. Taking into account that both of these populations contain antigen-specific T cells, their different phenotypic characteristics suggest that the inadequate antitumor response of the two populations is most likely caused by different mechanisms.

Considering the in situ-expanded T-cell clonotypes, these cells are obviously not released from an inhibition of their replicative capacity during in vitro growth. If these cells are in a proliferative state in situ, they obviously stop proliferating during culture. Mammalian cells are limited to a limited number of replications [34]. This replicative senescence is characterized by a finite number of cell divisions, equivalent to the Hayflick limit [34]. There are differences in the Hayflick limit from one cell type to another but in vitro studies indicate that T-cell clones have a lifespan of approximately 33 population doublings [76], although the exact number may vary from T-cell clone to T-cell clone [75].

At the Hayflick limit T cells cease to grow and lose the ability to secrete IL-2. The cells may stay viable for months, but in contrast to anergic T cells, senescent T cells cannot be pushed into a proliferative state by IL-2 [24]. In addition, senescent T cells do not express the CD28 molecule, a feature that appears to be a progressive process, since CD28 expression decreases as the number of population doublings increases [1]. The genotypic feature of senescent cells is a reduction in telomere length. Telomeric DNA, located at the ends of chromosomes, consists of repeated sequences that diminish in normal dividing somatic cells at an approximate rate of 40–100 terminal nucleotides per cell doubling [72].

Several findings have demonstrated that senescence is relevant for in vivo T-cell responses. HIV patients are characterized by chronic antigen stimulation. In these patients the number of $CD8^+CD28^-$ cells is increased and the telomere length of these cells is similar to the length observed in senescent cells [25]. An increase in the number of $CD28^-$ T cells of both CD4 and CD8 subtypes has also been observed in several clinical conditions characterized by chronic antigen stimulation [24], including HIV infection [65, 115], multiple myeloma [64], and rheumatoid arthritis [87]. For these diseases the occurrence of oligoclonal T-cell populations is another characteristic. Furthermore, these oligoclonal T cells have been shown to belong to the $CD28^-$ subset of cells. It therefore appears that clonally expanding T cells gradually reach a phase of senescence during which they lose the expression of CD28. Interestingly, in normal individuals the relative proportion of $CD28^-$ cells increases with age, indicating that the proportion of senescent T cells increases with age [114]. Furthermore, these $CD28^-$ cells are also oligoclonal [89].

Taken together, these findings suggest that in vivo T cells are limited to a finite number of cell divisions after which they enter a state of senescence. It is tempting to speculate that the in situ-expanded clonotypes in TIL have reached a senescent state leaving the cells incapable of proliferation even upon activation with IL-2. However, no evidence is presently available to support this notion and it could be argued that the coupling of T-cell clonality and the $CD28^-$ phenotype has been detected only in diseases characterized by systemic involvement of clonotypic T cells, in contrast to the situation in melanoma in which systemic involvement is limited. Bearing in mind that approximately 33 population doublings are possible for peripheral T cells before they reach senescence, the resulting cell number far surpasses the number of clonotypic T cells in melanoma lesions. However, the majority of melanoma patients investigated have been elderly and the maintenance mechanism for T cells is cell division [61]. T cells in elderly people are therefore likely to have been through several population doublings prior to melanoma development. Furthermore, the clinical course of melanoma, as well as the resulting immune response, is not a linear process; this is important to note because the intermittent nature of immune responses prohibits a simple calculation of maximal attainable cell numbers within the Hayflick limit. Additionally, due to the promiscuous nature of peptide recognition by the TCR [38, 59], it is possible that the vast majority of these T cells have been through a number of population doublings due to the recognition of antigen not originating from the melanoma. Possible targets may be viral [51] or "self" epitopes as has been demonstrated for CTL recognizing the MART-$1_{27\text{-}35}$ peptide [52].

To our knowledge the search for melanoma-associated peptide analogues has only been conducted with the MART-$1_{27\text{-}35}$ peptide, but it is conceivable that the presence of such analogues is not restricted to this protein. The existence of agonistic peptide analogues lacking sequence similarity to the original peptide extends the list of potential analogues [37]. With the high number of T-cell clonotypes involved in the in situ recognition of melanoma it does nevertheless seem unlikely that all of these have reached a senescent stage, i.e. lacking expression of CD28 and having a telomere length corresponding to a state of senescence.

Possibly, T-cell unresponsiveness could be caused by a decrease in the expression of CD28. The expression of CD28 by the T cell has been shown to influence the number and affinity of the peptide epitopes necessary for activation. Thus, CD28 expression lowers the number of

TCRs that need to be triggered in order to reach an activation threshold, and allows activation by ligands that are recognized by the TCR with low affinity [3]. It could therefore be speculated that T cells, before they reach a state of "classical" senescence, i.e. shortened telomeres and loss of CD28, may be growth arrested due to a combination of decreased CD28 expression and the expression of TCRs with low affinity towards the HLA/peptide complex.

There are several reports describing the inability of in situ expanded T-cell clones to be cultured in vitro, and several attempts have been made to explain the supposed discrepancy between in situ and in vitro T cells. It is, however, possible that this discrepancy does not exist. For example, it may be the case that the T-cell clonotypes in situ are growth arrested and continue to be so during in vitro culture. It has recently been shown that transfection of normal retinal pigment epithelial cells and foreskin fibroblasts with a vector encoding the human telomerase catalytic subunit exceeds the lifespan of these cells by at least 20 doublings [10]. If equally efficient in human T cells this could be used to investigate the nature of in situ T-cell clonotypes in melanoma.

The second subpopulation of TIL are expandable under in vitro growth conditions. This is evident in mixed tumor lymphocyte culture (MTLC) in the presence of IL-2. It is therefore obvious that these cells can be stimulated by simple means to proliferate and develop effector functions. It is therefore likely that these cells were rendered anergic in situ [5, 6]. The activation of a naive T cell upon TCR engagement with the HLA/peptide complex requires additional costimulatory signals in order to develop into an effector T cell. The T cell that encounters antigen without a proper costimulatory signal may enter a state of anergy, which is characterized by cell cycle arrest and a failure to develop effector functions. Within the TIL, these cells would be antigen-specific but clonal expansion and secretion of IL-2 would be absent. This basic immunological property of clonal anergy allows tolerance to normal cells to be maintained, and melanoma cells may escape immune surveillance by the induction of anergy. In this respect, it has been demonstrated in a murine model that induction of anergy has an early onset in the course of tumor progression [96]. An important characteristic is that anergy may be overcome by exogenous IL-2. It is therefore quite possible that anergized T cells in situ become responsive after treatment with IL-2, that is they enter the cell cycle and develop effector functions during in vitro culture.

Anergy may also be induced by a low concentration of HLA/peptide complexes or by partially agonistic peptide ligands. Both situations result in insufficient TCR signaling, rendering the T cell anergic [36]. Both situations may be present in the case of melanoma. The presence of partially agonistic and antagonistic peptide analogues to MART-$1_{27\text{-}35}$ has been demonstrated [52]. These analogues were selected among self-proteins for sequence homology to the MART-$1_{27\text{-}35}$ peptide and were shown to elicit cytotoxicity without the secretion of IL-2, or even to inhibit the cytotoxicity of MART-$1_{27\text{-}35}$-specific T-cell clones [52]. It could therefore be speculated that such peptide analogues take part in the preservation of MART-$1_{27\text{-}35}$-specific T-cell clones in vivo and in that way impair the antitumor T-cell response [79]. Interestingly, tumor regressions after immunization with the MART-$1_{27\text{-}35}$ peptide have not been observed despite a marked post-vaccinational increase in the MART-$1_{27\text{-}35}$-specific precursors in the patients' peripheral blood. In contrast, vaccination with the MAGE 3.1 peptide has been shown to induce tumor regression in the absence of detectable systemic involvement. Whether the phenomenon of molecular mimicry of MAA is actually involved in the induction of T-cell unresponsiveness in vivo, however, remains unresolved. The recent finding that peptide agonists can be constituted by peptides with no sequence homology to the antigenic peptide itself indicates that a partial agonistic peptide may be completely different from the original ligand as well [37].

In conclusion, the proposal brought forward in the present review is that the immune response against melanoma for various reasons is rather weak. The escape mechanisms of melanoma cells from immunological surveillance therefore seem to be more related to the properties of the immune system than to the properties of the melanoma cells. Although the presence of clonally expanded T cells in melanoma indicates an ongoing T-cell response, these clonotypic T cells may constitute a population of senescent T cells. On the other hand, some nonexpanded T cells are present in situ which can be expanded in vitro and exert tumor-specific cytotoxicity. Future research will reveal important insights into the nature of the distinct TIL populations present in situ, e.g. whether different targets are recognized or whether they serve specific functions. A better understanding of the biological mechanisms underlying the coexistence of a cellular immune response with a progressing tumor will assist to delineate the optimal strategy for future immunotherapeutic treatments for melanoma patients.

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