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New dimensions in tumor immunology: what does 3D culture reveal?

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Experimental models indicate that tumor cells in suspension, unlike solid tumor fragments, might be unable to produce life-threatening cancer outgrowth when transferred to animal models, irrespective of the number of cells transferred, although they induce specific immune responses. Human tumor cells cultured in three dimensions display increased pro-angiogenic capacities and resistance to interferons, chemotherapeutic agents or irradiation, as compared with cells cultured in two-dimensional (2D) monolayers. Tumor cells cultured in three dimensions were also shown to be characterized by defective immune recognition by cytotoxic T lymphocytes (CTLs) specific for tumor-associated antigens (TAAs) and by a capacity to inhibit CTL proliferation and dendritic cell (DC) functions. Downregulation of human leukocyte antigen (HLA) or TAA expression and high production of lactic acid might play a role in the elicitation of these effects. Here, we propose that growth in 3D architectures might provide new insights into tumor immunology and could represent an integral missing component in pathophysiological tumor immune escape mechanisms.

Active antigen-specific tumor immunotherapy trials: a discrepancy between immunological and clinical responses

Large numbers of tumor-associated antigens (TAAs) have been identified during the past decade [1], providing the rationale for the development of vaccination protocols targeting a variety of cancers and resulting in many clinical trials [2–6].

A common finding during these studies has been that TAA-specific immune responses, which are detectable by diverse phenotypic and/or functional *ex vivo* or *in vitro* assays or by skin tests [e.g. delayed-type hypersensitivity (DTH) reactions (see Glossary)], can be generated relatively easily upon vaccination. Clinical responses, however, were only observed in a fraction of the cases where successful immunization could be documented. Notably, no assay developed so far for the monitoring of clinical trials has allowed prognostic predictions regarding the clinical effectiveness of the immune responses induced by therapeutic anti-TAA immunization [7–9].

Even cytotoxicity assays that, unlike multimer staining, Elispot or cytokine gene expression tests, exquisitely address the ultimately desired functional immune response, fail to unequivocally correlate with a favorable clinical outcome. More frequently, lack of immune responsiveness, as detected by these assays, is found to be correlated with unfavorable clinical course [10,11].

Remarkably, ^{51}Cr release assays, frequently used in cytotoxic T lymphocyte (CTL) screening or in the monitoring of clinical trials, are mostly performed by using target cell lines of disparate origin that express

Glossary

Bioreactors: devices supporting biologically active environments *in vitro*. They are used to culture cells and tissues in static or dynamic conditions.
Brisk/non-brisk tumor infiltration by lymphocytes: tumor infiltration by lymphocytes is characterized as 'brisk' when TILs are diffused within the tumor, whereas the 'non brisk' TIL infiltration is discontinuous and largely limited to the periphery of the tumor.
Chemokines: families of small proteins inducing direct chemotaxis in responsive cells.
Cytokines: signaling proteins of critical relevance for intercellular communication and functions in innate and adaptive immune responses.
Cytotoxic T lymphocytes (CTLs): subgroups of T lymphocytes capable of killing allogenic, virus-infected or tumor cells upon HLA class I restricted recognition of specific antigens.
Delayed-type hypersensitivity (DTH) reactions: skin lesions predominantly mediated by T cells appearing within 24 to 72 h after intradermal injection of a previously experienced antigen.
Immunological pressure: immune responses specific for antigen(s) expressed by given cells leading to the preferential survival of cell subsets failing to express or present target molecules.
Scaffold: a typically porous, 3D cell culture substrate, which provides the template for cell organization into tissue structures.
Spheroid: a micromass cell culture model, typically achieved by preventing cell adhesion to the culture substrate and thereby promoting cell condensation.
Tumor-associated antigens (TAAs): antigens expressed predominantly or exclusively by tumor cells recognized by T cells or antibodies.
Tumor escape: capacity of tumor cells to avoid elimination by the immune system.
Tumor-initiating cells: cancer cell subsets capable of reproducing the heterogeneity of the original tumor specimen upon engraftment in immunodeficient mice.
Warburg phenomenon: capacity of tumor cells to perform aerobic glycolysis leading to high lactate production.

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defined MHC determinants after loading with specific peptides. TAA recognition might eventually be confirmed by the killing of tumor cell lines of a specific histological origin expressing adequate HLA alleles and TAA. In classic experimental settings, effector and target cells are co-cultured in U- or V-bottom well trays and cells are brought into close contact with each other for optimal elicitation of cytotoxic activity. Critical aspects of the lymphocyte–cancer cell interaction obviously fail to be addressed by these techniques.

Notably, well-defined murine models indicate that single-tumor-cell suspensions are highly effective in inducing protective CTL responses that prevent their *in vivo* outgrowth [12,13]. By contrast, solid fragments containing similar numbers of cancer cells are largely unable to stimulate an immune response [12] and display vigorous *in vivo* proliferation. These effects were mainly attributed to immune ignorance due to the fact that tumor cells from tissue fragments might fail to reach secondary lymphoid organs [12]. Nevertheless, these studies suggest that growth of tumor cells in three dimensional (3D) structures rather than in single-cell suspensions favors cancer development when the cells are transferred to *in vivo* models.

Taken together, these data suggest that the development of 3D culture systems has the potential to allow controlled evaluation of the molecular mechanisms underlying these phenomena.

Tumor escape mechanisms

Tumor escape from immune recognition represents the subject of an active research effort from a large community of scientists.

Downregulation or loss of HLA expression limited to single alleles or involving all of them has been demonstrated in different types of tumors [14]. In several cases, underlying molecular defects, including mutations in genes encoding HLA subunits, have been documented. Furthermore, expression of components of the antigen-presentation machinery has also been shown to be affected [15]. By contrast, loss or downregulation of TAA expression, in particular after successful specific immunization, have also been reported [16,17].

These alterations might result from the selection of resistant variants in cancer cell populations after application of immunological pressure. However, the *in vivo* relevance of these mechanisms is debated [18].

Several more recent studies highlight that *ex vivo*-sampled TAA-specific T cells from tumor metastases might be quiescent [19–22] and functionally impaired. In particular, they appear to be unable to produce interferon (IFN)-γ after T-cell-receptor engagement. Remarkably, these impairments are reversible, and short courses of *in vitro* stimulation with cytokines result in the restoration of these functional capacities, even before the induction of proliferation [20,21,23]. It is of note that, in at least one of the studies cited above, Melan-A/MART-1-specific, *ex vivo*-sampled CTLs from the peripheral blood of melanoma patients were shown to be able to respond with IFN-γ production upon peptide-specific stimulation, whereas $CD8^+$ cells with the same specificity from metastatic lymph nodes or nonlymphoid metastases were unresponsive [21]. These studies are reminiscent of previous studies that suggested that, in tumor samples largely infiltrated by T cells displaying an $HLA\text{-}DR^+$ 'activated' phenotype, the expression of genes typically transcribed upon antigen recognition, such as those encoding IFN-γ or tumor necrosis factor (TNF)-α, is infrequently detectable [24,25]. However, functional unresponsiveness of peripheral blood TAA-specific T cells has also been reported in patients bearing tumors and in experimental models [26,27]. The role of the tumor cells in the elicitation of these effects has not been fully clarified. Transforming growth factor (TGF)-β produced by cancer cells or tumor-associated interstitial cells has been suggested to have a role in the induction of TAA immune tolerance [28]. Alternative mechanisms might be related to the interaction of programmed death-1 (PD-1; CD279) expressed on activated lymphocytes with specific ligands expressed on tumor cells [29]. Exhaustion of CTLs might also result from repetitive exposure to high amounts of specific antigens. Tolerance of TAAs might also derive from the expansion of regulatory $CD4^+/CD25^+/FOXP3^+$ T cells or from the conversion of $CD4^+/CD25^+$/FOXP3-activated T cells into regulatory T cells. Either event might be mediated by TGF-β or by indoleamine 2,3 dioxygenase (IDO), a tryptophan-metabolizing enzyme [30]. A role for interleukin (IL)-10 could also be hypothesized [31].

Taken together these data powerfully emphasize the complexity of the interaction between tumor cells and the immune system. Clearly, mechanistic studies cannot be addressed in sufficient detail in humans by using conventional culture methods, thereby urging the development of models possibly linking experimental *in vivo* models, clinical observations and controlled *in vitro* culture technologies.

Three-dimensional cell-culture models

3D culture models have widely been used across the past two decades with the aim of mimicking the *in vivo* behavior of normal or transformed cells in conditions better amenable to experimental investigation [32]. 3D cultures of tumor cells have been obtained by promoting the aggregation of cells in spheroids via several different methods or by using scaffolds [33].

The liquid-overlay technique requires the culture of trypsinized cell suspensions in dishes coated with a thin layer of agarose. Alternatively, bacterial-grade plastic culture dishes or cell-culture-grade plasticware treated with poly-2-hydroxyethyl methacrylate (polyHEMA) can also be used [34]. Single cells placed on these surfaces do not adhere, yet they proliferate, and for many human cell lines, small colonies of aggregated cells can form within 1 to 3 days.

Cells can also be placed in spinner flasks and stirred to inhibit adhesion to the plastic and to maintain them in suspension. In temperature-controlled bioreactors and in the presence of carefully adjusted amounts of media, homotypic aggregation and subsequent formation of spheroids can take place over time. Rotating wall vessel (RWV) bioreactors have also been used to spin cell clusters while preventing them from settling at the bottom of the vessel [35].

Alginic acid is a 1,4-linked (homopolymeric or heteropolymeric) copolymer of β-d-mannuronic acid and α-l-glucuronic acid. This reagent is not toxic, and its gelling process is thermo-independent [36]. Cells are suspended in water-soluble sodium alginate gel at room temperature, and gelification can be provoked by dropping in ions such as Ca^{2+}, Ba^{2+} or Fe^{3+} in aqueous solution. Alternatively, tumor cells have been embedded in gels (e.g. Matrigel) composed of basement membrane or laminin-rich extracellular matrix (ECM) proteins [37,38].

Solid stress can also facilitate spheroid formation, especially for highly metastatic cancer cell lines. Growth-inhibiting stress in the range of 45 to 120 mmHg can increase ECM (hyaluronan) synthesis by tumor cells [39].

More recently, Fischbach *et al*. reported the possibility of engineering 3D tumor structures by using highly porous scaffolds fabricated from poly(lactide-co-glycolide) [40]. This method offers the opportunity to investigate the role of specific environmental cues, which can be engineered into the scaffold design. Clearly, the complexity of the system is increased, and the biological readouts of the cultures might critically depend on a large number of factors, including scaffold composition (e.g. synthetic or naturally occurring polymer), material surface properties (e.g. wettability, rugosity) and pore architecture (e.g. pore size, interconnectivity, surface area).

These reports highlight the ongoing efforts aimed at the generation of improved 3D culture models amenable to detailed cellular and molecular biology studies.

Features of tumor cells cultured in 3D structures

3D cultures have been used for a long time to study morphogenesis and tissue formation *in vitro* in conditions better controlled than *in vivo* models. More recently, these methods have also been applied to the analysis of malignant transformation and tumor progression. In particular, in regard to breast cancer, the invasion of the glandular lumen, a typical feature detectable in the initial phases of the development of these tumors, has been successfully reproduced *in vitro*, and its mechanisms have been explored in detail thanks to the use of 3D cultures [41–44].

Cancer cells cultured in three dimensions are characterized by several peculiar features differentiating them from monolayer cultures (Table 1) and paralleling those of *in vivo* tumors. In particular, early events of tumor growth before effective vascularization appear to be closely reproduced. Indeed, within a short timeframe, 3D cultures of tumor cells develop hollow cores that resemble the necrotic areas of *in vivo* cancers: areas that are usually observed at a distance from nutrient and oxygen supplies. In addition, the proliferation of tumor cells cultured in three dimensions is typically slower than that of monolayer cultures [45]. This dynamic fits the Gompertz equation, an algorithm used to quantitatively evaluate *in vivo* neoplastic growth [46,47].

Most importantly, it has been shown that tumor cells cultured in three dimensions display different metabolic characteristics compared to those of their 2D counterparts [48]. A predominant feature appears to be represented by increased glycolysis, leading to relatively high lactic acid production [49–52].

Table 1. Main differences in biological functions between tumor cells cultured in three dimensions or in monolayers

Biological function	Changes in 3D as compared to 2D cultures	Refs
Metabolic profiles	Modified	[48,50–52]
Production of lactic acid	Increased	[50–52]
Proliferation capacity	Decreased	[46,47,54,74]
Sensitivity to IFN, drugs, irradiation or apoptosis induced by death receptor ligation	Decreased	[45,48,57,59]
Expression of HLA class I	Decreased in defined cell lines	[51,73,74]
Expression of melanoma differentiation TAA	Decreased in cells included in spheroids with high cell numbers	[51,74]
Expression of HSP70	Decreased	[73]
Polarity and shape of cells	Modified	Reviewed in [53]
Gene expression profiles	Modified	[40,54,72,74] Reviewed in [53]

Tumor cells of epithelial origin cultured in three dimensions have also been shown to change shape and lose polarity, a feature typically associated with tumor progression *in vivo* (reviewed in [53]).

Importantly, gene-expression profiles of cells cultured in three dimensions are different from those of the same cells cultured in monolayers, as observed in studies addressing small series of transcripts or based on oligonucleotide chip hybridization [40,54,55]. Genes encoding several chemokines, such as IL-8, or factors playing a role in angiogenesis, including angiopoietin like 4, hypoxia-inducible protein 2 and vascular endothelial growth factor (VEGF) 1 and 2, have been found to be upregulated in tumor cells cultured in 3D architectures, recalling observations made in clinical tumor specimens [40,54,55] (Figure 1).

Compared to tumor cells cultured in two dimensions, cells cultured in three dimensions display a decreased sensitivity to apoptosis induced by radio-chemo treatments or by death receptor ligation [56]. Similarly, sensitivity to the cytostatic or cytotoxic effects of interferons or chemotherapeutic agents is significantly reduced in cells cultured in three dimensions [40,45,57,58]. Interestingly, the Fas ligand (FasL) gene has been found to be expressed in HRT-18 and CX-2 colorectal cancer cell lines cultured in multicellular tumor spheroids (MCTS) but not in the same cell lines cultured in two dimensions [59].

Culture of tumor cells in 3D scaffolds has also been addressed more recently (see 'Three-dimensional cell-culture models' above). Cells cultured in these conditions display features largely overlapping with those of cells cultured in spheroids, including higher production of pro-angiogenic factors and resistance to treatment with chemotherapeutic agents [40].

An important advantage of 3D cultures is that the interaction of different cell types can be explored. For instance, infiltration of tumor spheroids by endothelial cells has been demonstrated; it depends not only on the production of pro-angiogenic factors by tumor cells but also on the expression of cadherins by endothelial cells [55].

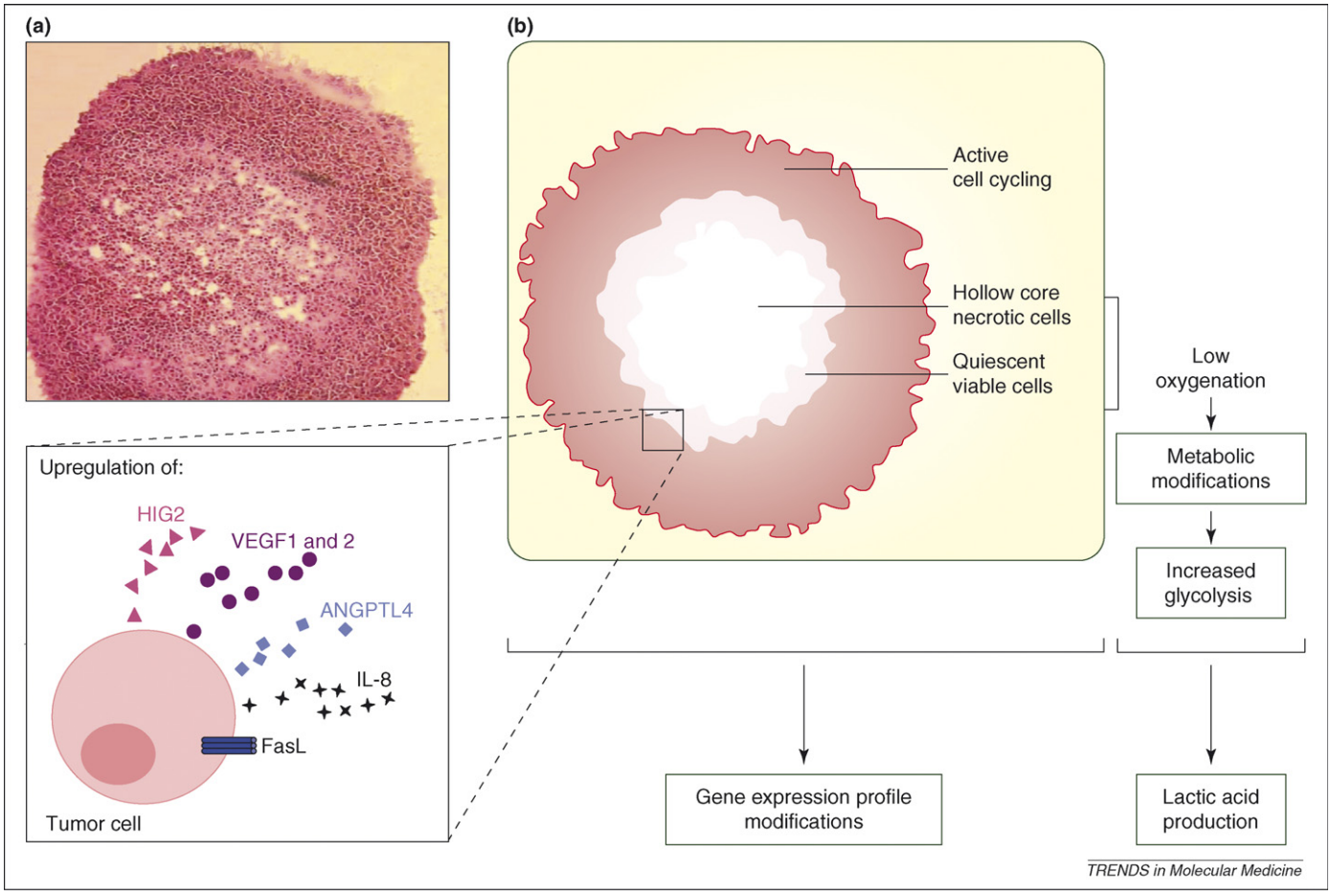

Figure 1. Features of tumor cells cultured in three dimensions. Culture of tumor cells in three dimensions rapidly results in the formation of hollow necrotic cores (**a**). Whereas cells in outer layers do actively proliferate, cells in inner layers are mostly quiescent (**b**). The expression of genes encoding hypoxia-related factors, including VEGF, hypoxia-inducible protein 2 (HIG2), angiopoietin-like 4 (ANGPTL4) and IL-8, is typically enhanced in tumor spheroids, as compared to monolayers. Furthermore, glycolysis with enhanced production of lactic acid is also promoted.

These data show that 3D cultures can mimic defined aspects of *in vivo* tumors and that models of increasing complexity currently under development might help to increase their resemblance to clinical cancers.

Cancer stem cells and growth in 3D structures

Tumor-initiating cells, the so-called 'cancer stem cells' that are capable of transplanting malignancies in immunocompromised hosts, have been characterized in different types of malignancies in the recent past [60]. Their phenotypic profiles have not been fully clarified. For instance, melanoma stem cells were initially described as $CD20^+$ cells [61]. Other studies, however, have emphasized the role of $CD133^+$ cells [62] and, in particular, of their $ABCG2^+$ subset [63]. Cancer stem cells from different tumors appear to share common features, including a state of relative quiescence and a self-renewal capacity in the context of preferentially asymmetric divisions [64]. Furthermore, they are deemed to be highly resistant to chemotherapeutic treatments or irradiation, possibly due to their high DNA repair capacity and to the expression of ATP-binding cassette transporters [65].

Intriguingly, a common tumor-stem-cell characteristic, which has been observed in cells isolated from several human tumors, is represented by their preference to grow in spheroid-like structures *in vitro* [61,66–69]. The molecular background underlying this phenomenon is unclear, but it is possibly related to prevention of adherence caused by loss of polarity or defective interaction with defined ECM components in culture conditions [41,43]. Indeed, culture of embryonic stem cells in two dimensions instead of in embryonic bodies has been reported to favor their differentiation [70]. Notably, different matrices have also been shown to promote maintenance or differentiation of stem cells [53,71]. Future studies are needed to investigate whether culture of heterogeneous populations of tumor cells in 3D structures might favor a relative expansion of cells characterized by stem-cell-like features.

Immune responsiveness to tumor cells growing in 3D structures

There is a curious paucity of studies on immune responsiveness to tumor cells cultured in 3D architectures in humans. However, the few published reports unanimously indicate that, in these conditions, cancer cells are poorly recognized by specific CTLs or that they might even be able to actively inhibit key functions underlying the induction of specific responsiveness. Interestingly, these data were obtained by using tumor cell lines of different histological origin, including melanoma, bladder and lung cancers.

In particular, it has been shown that tumor-infiltrating lymphocytes (TILs) of undefined antigenic specificity, but

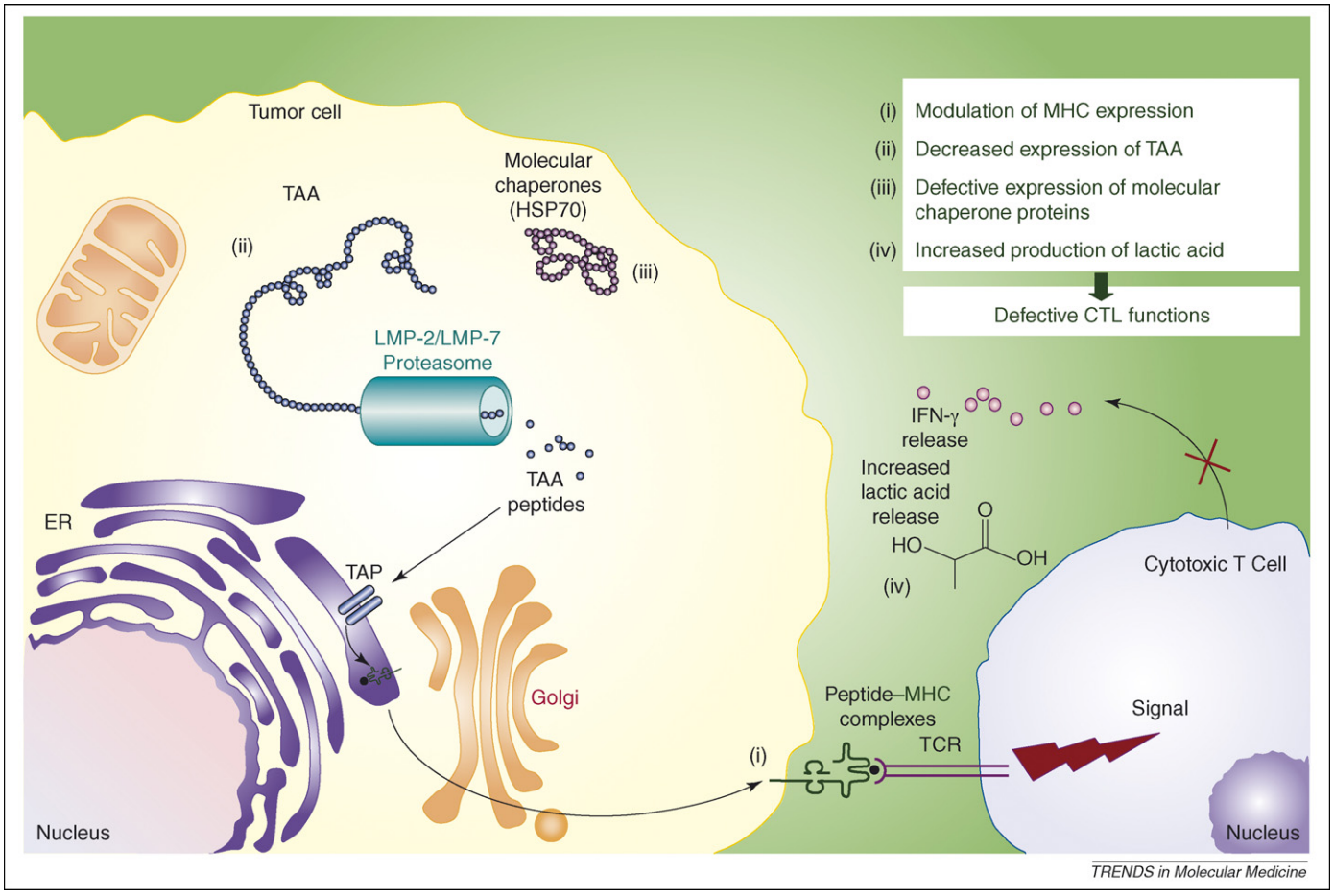

Figure 2. Molecular mechanisms potentially underlying defective responsiveness of tumor-associated antigen (TAA)-specific CTLs to tumor cells cultured in three dimensions. Modulation of the expression of HLA class I molecules has been observed in tumor cells cultured in three dimensions. Furthermore decreased expression of HSP70 molecular chaperone has also been detected. In spheroids including large numbers of tumor cells (>5 000), expression of melanoma-associated differentiation TAA has been found to be significantly decreased. These events might account for an impaired immune recognition of tumor cells by TAA-specific CTLs. Conversely, the increased production of lactic acid typically displayed by tumor cells cultured in three dimensions has been shown to be responsible for defective differentiation of monocytes towards dendritic cells and for an impaired capacity of CTLs to proliferate and produce IFN-γ and cytotoxicity mediators upon T-cell-receptor triggering. A combination of these mechanisms might favor tumor escape from immune response.

able to effectively elicit cytotoxic activity against autologous bladder tumor cells cultured in two dimensions or in suspension, failed to recognize targets cultured in multicellular spheroids [72].

Similarly a CTL clone specific for an HLA-A0201 restricted, mutated α-actinin-4 peptide expressed by autologous lung cancer cells poorly recognized targets growing in three dimensions [73]. The authors suggested a downregulation of heat shock protein 70 (HSP70) expression in spheroids as a possible underlying mechanism.

Our group and others have reported data indicating that melanoma cell lines expressing Melan-A/MART-1 and gp100 are also defectively recognized by CTL clones and lines specific for the HLA-A0201 restricted peptides 26–35 and 280–288, respectively, if cultured in three dimensions, as opposed to monolayers [51,52,74]. Target cells in three dimensions poorly induce IFN-γ secretion and expression of the genes encoding Fas ligand, perforin or granzyme B in TAA-specific effector cells, as compared to targets cultured in monolayers or in suspension.

Interestingly, these TAA-specific CTL clones also fail to infiltrate melanoma spheroids, but rather tend to remain on their surface, resembling the 'non-brisk' T-cell infiltration described in clinical specimens [75].

A multiplicity of mechanisms has been proposed (Figure 2). First, spheroids might provide structural hindrances to CTL attack by limiting the accessible surface of the target cell. However, subtler events could also be involved. Indeed, the expression of melanoma-associated differentiation antigens has been shown to be downregulated in tumor cells cultured in high densities [76], possibly owing to 'antigen silencing' by oncostatin M (OSM) [77]. We have observed that Melan-A/MART-1 and gp100 gene expression is decreased in melanoma cells cultured in three dimensions and, in particular, in spheroids containing high numbers (>5 000) of tumor cells. In these conditions, a downregulation of HLA class I expression, accompanied by decreased expression of at least one gene encoding a specific transcription factor [interferon regulatory factor-1 (IRF-1)], has also been detected in two out of three melanoma cell lines under investigation [51]. Thus, downregulation of the expression of TAA and HLA class I are detectable in tumor cells cultured in three dimensions in the absence of exogenously applied immunological pressure.

Most intriguingly, specific metabolic features of tumor cells cultured in three dimensions might play a peculiar role in the inhibition of TAA-specific CTL response. Lactic

acid, which is known to be produced in greater amounts by tumor cells cultured in spheroids than by those cultured in 2D architectures, has been shown to suppress proliferation and cytokine production capacity of human CTL while markedly inhibiting their cytotoxic activity [52]. Notably, concentrations of lactic acid similar to those produced by melanoma spheroids (10–20 mM) do impair the production of IFN-γ by specific CTLs induced by HLA-A0201^{+} Melan-A/MART-1^{+} cells cultured in two dimensions [50–52]. These effects might be related to the enhanced glucose requirements of activated CTLs, the metabolism of which might be hindered by high extracellular concentrations of lactic acid [78]. The same concentrations have also been shown to affect the differentiation of monocytes towards dendritic cells (DCs) [50]. These findings assume a peculiar relevance because fluorodeoxyglucose (FdG) positron emission tomography (PET) has demonstrated altered glucose metabolism *in vivo* in a large majority of primary and metastatic cancers [79,80].

Taken together, these data suggest that antigen recognition capacity and the resulting functional activities of CTLs might be significantly altered in the presence of tumor cells growing in multilayered architectures.

Concluding remarks

Immunology of human tumors is mostly based on *in vitro* models. Their use has led to substantial advances in our understanding of the molecular nature of TAAs [1]. Furthermore, they have led to the development of a wealth of immunogenic reagents and vaccination procedures and to the establishment of highly sensitive monitoring technologies. However, the discrepancy between T-cell-mediated antitumor immunity and the relatively poor clinical results of TAA-specific vaccination urges an updating of conventional *in vitro* models.

Data obtained by different groups, including ours, suggest that mere culture of cancer cells in three dimensions might result in downregulation of the expression of HLA class I and TAA and in an increased production of lactic acid compared to conventional 2D cultures [50–52,72–74]. Most importantly, combinations of these features have been detected in cancers *in vivo* [15,49]. These events are likely to impact on several essential steps required for the elicitation of effective immune responses, ranging between maturation of antigen-presenting cells (APCs), antigen presentation and recognition and the implementation of effector functions. Thus, results from highly controlled 3D models *in vitro* suggest that structural characteristics of tumor growth and of the resulting microenvironment might play an important role in belittling and defusing natural or vaccine-induced TAA-specific CTL responses, potentially limiting their clinical efficacy.

Indeed, adoptive immunotherapy protocols have provided proof of principle of the possibility of treating solid tumors by administration of high numbers of TAA-specific CTLs [81,82], thereby indicating that they are accessible to effector lymphocytes and potentially sensitive to their activities. However, clinical responses of variable extent are detectable in a fraction of patients treated in different studies [82–85], and they are frequently unrelated to the numbers of TAA-specific CTLs transferred to patients [84,85]. Even upon transfer of lymphocytes genetically engineered to express T-cell receptors specific for melanoma TAAs, resulting in engraftment levels exceeding 10% of peripheral blood lymphocytes, partial clinical responses were detectable in two out of 17 patients treated [86]. Altogether, these data indicate that local conditions might affect the interaction between tumor cells and effector lymphocytes, and such conditions should be investigated in future studies.

Most obviously, 3D cultures fail to thoroughly reproduce the enormous complexity of tumor cell biology *in vivo*. Thus, it is unlikely that they will ever be able to replace experimental *in vivo* models in the analysis of the molecular mechanisms underlying tumor escape from immune response. Conversely, they highlight the relevance of structural features in undermining the effectiveness of TAA-specific immune responses, and they might emerge as an important tool for investigating, in highly controlled conditions, the molecular mechanisms regulating the relationship between cancer and immune effectors.

Several outstanding questions deserve to be addressed (Box 1). Most interestingly, could culture in three dimensions favor the survival or the expansion of cancer stem cells from solid tumors? Could 3D models be of use in the identification of other types of untransformed cells representing necessary components of the stem cell niche?

A common experience in clinical immunotherapy is that patients with relatively high tumor burdens infrequently benefit from TAA-specific immunization. Clearly, several different mechanisms concur in the determination of this outcome. The architecture of tumor tissues might represent one of them. Far from being discouraged, tumor immunologists might rather redirect their efforts to the treatment of patients rendered clinically tumor-free and/or to combination therapies also addressing tumor microenvironment.

Box 1. Outstanding questions

- To what extent is the sensitivity of tumor cells to the cytotoxic effects of defined TAA-specific CTL clones in three dimensions predictive of *in vivo* effectiveness?
- Could 3D culture models be of use in the preclinical evaluation of combination treatments involving the use of TAA-specific T cells and cytokines, monoclonal antibodies or chemotherapeutic agents?
- Is it possible to construct improved 3D culture models that include different cell types other than tumor cells and that take advantage of dynamic perfusion in bioreactors?
- Could 3D culture favor the survival or the expansion of cancer stem cells from solid tumors, and could it help to investigate which other types of untransformed cells represent necessary components of the stem cell niche?

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