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Mechanisms of specific immune response interactions with tumor cells

Mechanismy interakce specifické imunity s nádorovými buňkami

Bakalářská práce

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Poděkování:

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Prohlášení:

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Podpis

Abstract

Interactions between the immune system and tumors have been among the highlights of present immunological research. An extensive body of new knowledge recently substantiated the long-presumed concept of cancer immunosurveillance. Immune system searches the organism for cells expressing tumor antigens or cellular stress signals and destroys them. T-cells, NK-cells and dendritic cells, as well as cytokine signaling and direct cell cytotoxicity play dominant role in this process. However, a fraction of nascent tumors can evade these mechanisms and create a dynamic equilibrium, gradually sculpting its phenotype by clonal selection. Eventually, tumor cells escape immune control by concealing themselves from recognition or by actively subjugating local immune response. This immunosubversion results in formation of immunosuppressive tumor microenvironment by recruiting protumorigenic cell populations, such as Treg cells, macrophages and myeloid derived suppressor cells. Soluble signaling molecules, as well as surface-expressed immune checkpoint molecules are exploited by tumor cells for inhibition of anti-tumor immunity. Highly effective therapeutic antibodies blocking these checkpoints have been developed for clinical use, with many more in current trials. Several other promising immunotherapeutic approaches (tumor vaccines, adoptive T-cell therapy with chimeric antigenic receptors) have been used or are in clinical trials.

Keywords

cancer immunity, immunosurveillance, cancer immunoediting, tumor-specific antigens, tumor-associated antigens, NKG2D, TGF-β, CTLA-4, PD-1, cancer immunotherapy, immune checkpoint blockade

Abstrakt

Interakce imunitního systému s nádory jsou jedním z nejdůležitějších témat současného imunologického výzkumu. Velké množství nových znalostí recentně prokázalo platnost dávno předpokládaného konceptu imunitního dohledu. Imunitní systém prohledává organismus a deteguje buňky nesoucí nádorové antigeny nebo signály buněčného stresu a ničí je. V tomto procesu hrají dominantí roli T-buňky, NK-buňky a dendritické buňky, cytokinová signalizace a přímá buněčná cytotoxicita. Část vznikajících nádorových buněk ale může těmto mechanismům unikat pomocí klonální selekce. Nakonec tyto nádorové buňky vyvinou i aktivní mechanismy lokálního potlačení imunitní odpovědi. Dochází k infiltraci buněčnými populacemi podporujícími růst nádoru (regulační T-lymfocyty, makrofágy, myeloidní supresivní buňky) a ke vzniku imunosupresivního nádorového mikroprostředí. Nádorové buňky zneužívají k inhibici protinádorové imunity jak rozpustné signální molekuly, tak hlavně povrchové receptory sloužící jako “kontrolní body”. V posledních letech bylo schváleno pro klinické použití několik vysoce efektivních terapeutických monoklonálních protilátek blokujících tyto “kontrolní body” a mnoho dalších je testováno v probíhajících klinických studiích. Několik dalších velmi slibných imunoterapeutických přístupů (protinádorové vakcinace, využití T-lymfocytů geneticky modifikovaných pomocí chimerických antigenních receptorů) bylo použito anebo je klinicky testováno.

Klíčová slova

nádorová imunita, imunitní dohled, imunoeditace, tumor-specifické antigeny, s tumorem asociované antigeny, NKG2D, TGF-β, CTLA-4, PD-1, imunoterapie nádorů, blokáda imunitních “kontolních bodů”

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Aims of the Thesis

The microcosmos of interactions between the immune system and tumor cells has been in the spotlight of immunology, molecular and cell biology and other scientific disciplines for decades. It has, however, not been until recently, that thanks to a number of significant new discoveries in the field and mainly crucial clinical achievements, this topic rapidly became one of the highlights of present biomedical research.

With great quantity of new papers being released annually, it is a rapidly evolving subject and many formerly unclear questions have been elucidated in the past few years. The aim of this thesis is to present a comprehensive review of contemporary understanding of molecular and cellular processes occurring in the interaction of tumor with the immune system. While wide range of general immune-oncological topics is mentioned, a few specific areas of research, alluring most interest in the field are discussed in more depth.

1. Introduction

Among various important functions of the immune system of higher chordates, protection against tumor genesis, survival and growth has always receiving considerable scientific attention. The understanding of the role of immune cells in these processes has been slowly evolving in the past century, with the concepts of cancer immunosurveillance (Burnet, 1957, 1964) and cancer immunoediting (Shankaran et al., 2001, Dunn et al., 2002, Schreiber et al., 2011). It was, however, only in the past few years, that a substantial expansion of our knowledge in the molecular processes occurring in the tumor microenvironment (TME) brought about a revolution in the field of immunooncology.

Mutual interactions between various immune cells, stromal and tumor cells in the TME recently became the major topic of the immunooncological research. It is clear that the tumor cells do not play just a passive escaping role in the fight against the destructive forces of host's immune mechanisms, but rather actively shape the quantitative and qualitative features of the immune response, resulting in a dynamic cancer-promoting environment. This state is achieved via interaction of numerous molecular signaling networks. The increasing understanding of these molecular and cellular mechanisms is currently bringing a number of remarkably efficient novel therapeutic approaches in cancer treatment.

2. History of cancer immunosurveillance concepts

The first prediction of the role of human immune system in protection against cancer is attributed to Paul Ehrlich (Ehrlich et al., 1909), but due to the limitations of the contemporary science, little further development was possible. A major step forward occurred only almost fifty years later, with Frank Macfarlane Burnet's postulation that the immune system continuously monitors the organism for nascent tumors and eliminates them (Burnet et al., 1957, 1964). This concept of tumor immunosurveillance, while theoretically and intuitively attractive, has been accepted with a lot of skepticism when studies on

immunocompromised mice failed to show an increase in tumor incidence in these animals (Stutman, 1974, 1979). While it was hardly apprehensible at the time, in hindsight the design of these studies did not allow to perceive the role of the immune system in cancer prevention (Dunn et al., 2004**b**). The immunosurveillance hypothesis however became again more plausible as a result of later studies employing *e.g.* mice with selectively disturbed function of interferon-γ (IFN-γ) signaling (Dighe et al., 1994, Kaplan et al., 1998, Shankaran et al., 2001) and those elucidating the roles of direct T-cell cytotoxicity (Smyth et al., 2000), mostly via perforin function interference (van den Broek et al., 1996, Street et al., 2001).

The initial understanding of tumor formation in conditions of active immunosurveillance was based on clonal selection of transformed cells resulting in dominance of immunologically inert variants. The host immune system thus acts as an extrinsic shaping factor of tumorigenesis, leaving its marks on the eventual tumor behavior. This important phenomenon was proven experimentally (Svane et al., 1996, Shankaran et al., 2001) and has been termed "immunologic tumor sculpting". This mechanism paradoxically results in creation of more aggressive tumor cell variants.

Moreover, the gradual illumination of circumstances involved in cancer mass formation revealed, that malignant cells are not merely passively escaping the mechanisms of immunosurveillance, but also actively modify the actions of immune cells in the TME (Zitvogel et al., 2006). In addition, it has been becoming increasingly obvious that the immune system may under certain conditions actually stimulate the growth of tumors (Dunn et al., 2004**a**). This progress in understanding of the complex relationship between tumors and the immune system resulted in formulation of the current concept of "cancer immunoediting" (Dunn et al., 2002).

3. Immunoediting in tumor progression and growth

The current perspective on the immune mechanisms and their failure associated with tumor formation was introduced in the early 21st century (Dunn et al., 2002) as a theory explaining the commonly observed process of tumor progression leading to successful cancer mass outgrowth in three phases: elimination, equilibrium and escape.

3.1 Elimination phase

According to our current understanding, transformed cells emerge with considerable frequency, but the immune system of an immunocompetent host promptly eliminates the absolute majority of them. Below the processes and players acting protectively in immunosurveillance are briefly described.

3.1.1 Modes of tumor cell recognition

The first requirement of deploying an effective immune response is the recognition of dangerous malignant and pre-malignant cells and their differentiation from normal healthy cells. Two general basic mechanisms of immunologic determination of present or imminent cell transformation can be distinguished: the antigen-specific (adaptive) and the non-specific (innate) ones.

In order for the **adaptive** immune system cells, *i.e.* T- and B-lymphocytes to recognize a transformed cell, such cell needs to possess antigenic structures dissimilar to normal tissue cells. This fact had been envisioned for decades, ever since the first studies suggesting the existence of such discerning antigens on tumor cells (Foley, 1953, Old, 1982), but only started to be supported by evidence since the discovery of first tumor antigens (van der Bruggen et al., 1991). Multiple studies ensued, identifying ever-increasing number of structures only or dominantly present on malignant cells acting as antigens (reviewed in Coulie et al., 2014). Presently, three groups of these antigens are distinguished: tumor-specific antigens (TSAs); tumor-associated antigens (TAAs) (reviewed in Vigneron et al., 2013) and more recently acknowledged cancer testis antigens (CTAs) (Simpson et al., 2005). It was shown that it is indeed the immune response against these antigens, that is the underlying cause of the bi-directional immunoediting process (DuPage et al., 2012).

It was identified more than two decades ago that some of these TSAs originate in accumulated mutations in nucleotide sequences of protein-coding genes (Monach et al., 1995). The causal mutations can be described as either "driver" mutations in proto-oncogenes or in tumor-suppressor genes, providing malignant cells with capacity of autonomous growth and other hallmark features of cancer, or "passenger" mutations, non-beneficial, acquired due to incompetent mutation-sensing and repair mechanisms (Greenman et al., 2007). These mutations result in altered translated peptide structures and consequently their distinctive antigenic properties, determinable as foreign. TSAs encompass both altered surface-expressed proteins, specifically via differential glycosylation, and more importantly fragments of various modified cell-proprietary peptides, presented to immune cells via major histocompatibility complex (MHC) glycoproteins. It has long been recognized, that nearly ubiquitously expressed MHC type I molecules are crucial for internal-origin antigen presentation to cytotoxic $CD8^+$ αβ T-lymphocytes, whereas MHC type II is limited in expression to professional antigen presenting cells (APCs), which present processed fragments of tumor cell peptides to helper $CD4^+$ T-cells (Dunn et al., 2004**a**). Both of these ways of recognition and immune response initiation were found to play important roles in cancer immunity in a number of studies. Hence, T_H-cells seem too to have their place in elimination phase (Wang et al., 1999**a**, et al., 1999**b**, Saeterdal et al., 2001) even though cytotoxic $CD8^+$ αβ T-cells are likely of a higher significance (Wölfel et al., 1995, Hogan et al., 1998, Echchakir et al., 2001, Clark et al., 2001).

TAAs on the other hand represent unaffected surface-expressed protein structures that exhibit differential expression patterns. They can either be developmental, *i.e.* oncofetal antigens, physiologically solely expressed early in ontogenesis; or normal cell surface proteins usually specific for original tissue from which the tumor arises, but aberrantly produced in great excess (Coulie et al., 2014). TAAs can also provide beneficial effects for tumor cells and directly add to tumorigenesis,, *e.g.* in the case of HER2/neu receptor

(Kawashima et al., 1999). CTAs are actually related to TAAs, as they are also are of undisturbed structure, but aberrantly expressed in cancer cells - they are normally expressed in testis, trophoblast and fetal ovaries, but can also be present on a wide variety of cancer cells (Simpson et al., 2005).

The mechanisms of tumor cell recognition and elimination by **innate** immune response mechanisms was discovered and elucidated more recently, with NK-cells being the most important element. NK-cells possess several inhibitory receptors, including CD94–NKG2A heterodimers and the family of killer cell immunoglobulin-like receptors (KIRs). These receptors bind the MHC class I molecules of targeT-cells. If the contacted cell has a normal level of MHC class I molecules, the inhibitory signals provided by the interactions prevent the activation of cytotoxic mechanisms. Many tumor cells however strongly down-regulate MHC class I expression in order to avoid recognition by adaptive immune system. If such a cell is recognized by a competent NK-cell, the inhibitory signals are too weak and activating signals from stimulatory receptors *(see below)* prevail resulting in activation of cytotoxic mechanisms (Lanier, 2005, reviewed in Tu et al., 2016).

The activating receptors of NK-cells include *e.g.* the molecules NKp30, NKp44, NKp46, 2B4, CD226, and Fc-receptor CD16 (reviewed in Pegram et al., 2011 and Tu et al., 2016). Probably the most important role is, however, played by the stimulatory receptor called NKG2D recognizing numerous stress-induced ligands (Guerra et al., 2008). Upon ligand binding, the signal is transduced through the NKG2D-associated adaptor protein DAP10 and following activation of PI3K and Grb2-Vav1 (Upshaw et al., 2006) pathways ultimately leads to cytolytic function activation (Hayakawa et al., 2002).

NKG2D recognizes several stress-inducible proteins structurally related to MHC class I molecules (López-Larrea et al., 2008), such as MICA, MICB and at least 6 members of UL16 binding protein family (ULBPs). While ULBPs have their respective orthologs in mice, no such ortholog has been identified for MICA and MICB (Bahram et al., 1994, Cosman et al., 2001, Pende et al., 2002). All of these NKG2DLs are upregulated under cellular stress conditions, such as increased temperature (Groh et al., 1999), human cytomegalovirus infection (Groh et al., 2001), binding to E.*coli* surface antigens, (Tieng et al., 2002), genotoxic stress (Gasser et al., 2005), as well as in transformed cells as demonstrated in tumor samples (Groh et al., 1999) and in cancer-derived cell lines (Pende et al., 2002). Hence, it is not surprising, that heat-shock factor 1 (HSF-1) is one of the transcription factors involved in regulation of NKG2D ligands expression (Groh et al., 1996). Their surface expression by tumor cells has been furthermore linked to apoptosis regulation (Nausch et al., 2006), cell cycle control via G_1/S-checkpoint transition associated transcription factor E2F (Jung et al., 2012) but also by housekeeping gene regulator Sp1/Sp3 (López-Soto et al., 2006). This is probably due to epigenetic chromatin-remodeling gene activation or repression (Andersen et al., 2007, López-Soto et al., 2009) and even was connected to epithelial-to-mesenchymal transition (López-Soto et al., 2013).

In addition to NK-cells, NKG2D is also expressed on several other cell types associated with cancer immunoediting, namely $CD8^+$ αβ T-cells and γδ T-cells, (Bauer et al., 1999) but its function in T-cells is less understood. Due to the interconnected signaling patterns with the main T-cell-stimulatory molecule CD28 it

was proposed that NKG2D on T-cells could act as a co-stimulatory signal to classical CD3/TCR activation pathway (Groh et al., 2001, Diefenbach et al., 2004) but it is not clear whether or not NKG2D can activate T-cells as a solitary signal (Meresse et al., 2004, Ehrlich et al., 2005). NKG2D receptor expression is regulated by several cytokines - while interleukin-4 (IL-4) (Brady et al., 2010) and transforming growth factor beta (TGF-β) (Castriconi et al., 2003) cause down-regulation of NKG2D surface expression, several stimulatory interleukins increase NKG2D signaling intensity both on cell surface (Park et al., 2011) and intracellularly (Horng et al., 2007).

3.1.2 Interferons and other damage signals in tumor elimination

The studies demonstrating the cancer-preventive effect of functional IFN-γ signaling in mice were among the first to produce evidence for then disputed immunosurveillance hypothesis at the turn of last century and introduced the immunoediting concept (Shankaran et al., 2001). Its pivotal role in immune protection against cancer was demonstrated both by directly inhibiting IFN-γ receptor function and indirectly via incapacitation of STAT1, the IFN-γ effector transcription factor (Dighe et al., 1994, Kaplan et al., 1998). It was also shown that the effects of IFN-γ were mediated largely by lymphocytes (Shankaran et al., 2001). Further experiments hinted that γδ T-cells might act as important producers of IFN-γ (Gao et al., 2003) but ensuing research discovered a specific $B220^{+}$ $NK1.1^{+}$ subset of dendritic cells, which were identified as an essential cell population responsible for IFN-γ production. They were termed IFN-producing killer dendritic cells (IPKDCs) (Taieb et al., 2006). $CD56^{bright}CD16^{-}$ subset of NK-cells is also recognized as an important cytokine producer, including IFN-γ (reviewed in Carotta, 2016).

Concerning the IFN-γ effector cells, even though much had been known about the mediation of generation and recruitment of tumor-specific $CD4^{+}$ T_H1-cells, $CD8^{+}$ cytotoxic T-cells and macrophage M1-activation (Bach et al., 1997), malignant cells themselves seem to be IFN-γ responsive (Dighe et al., 1994, Kaplan et al., 1998). Upon exposure to IFN-γ, the tumor cells exhibit an increase in immunogenicity, where the up-regulation of MHC class I expression likely carries the host-protective effect (Shankaran et al., 2001).

Type I interferon (IFN-α/β) signaling is generally viewed as a cell distress signal produced to the surrounding environment, thus activating immune responses. As such, it also seems to have certain significant, if not fully understood role in tumor immunity (Gresser and Belardelli, 2002). IFN-α/β expression is regulated *i.a.* by a p53-dependent pathway, suggesting a link to tumor cell-mediated signaling (Takaoka et al., 2003). In contrast to IFN-γ, these type I interferons do not target tumor cells, but rather act via various host immune system cells, namely hematopoietic precursor cells (Dunn et al., 2005b). Their function seems to be specifically irreplaceable early in the cancer response, when it stimulates CD8α/ $CD103^{+}$ dendritic cells and thus augments the presentation of tumor antigens to $CD8^{+}$ T-cells (Diamond et al., 2011, Fuertes et al., 2011).

In addition to the type I IFNs and above described intracellular stress signaling via NKG2D ligand expression up-regulation, there is one more way, by which cells alert the immune system of incipient danger.

Multiple damage-associated molecular pattern molecules (DAMPs) are released from dying tumor cells; these molecules tend to stimulate inflammatory immune functions within the TME (Sims et al., 2010).

3.1.3 Effector mechanisms of tumor cell elimination

While it is possible that a full spectrum of effector killing and inhibitory mechanisms is involved in tumor elimination, only several of them have been shown to be critical. The killing effectors with the most pronounced role in cancer immunity were observed to be cytotoxic granule release upon formation of immunologic synapse and apoptosis induction, mostly via Fas-ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL) signaling upon direct cell contact.

The former mechanism is dependent on exocytosis of cytolytic enzymes termed granzymes, which are in their action dependent on another granule protein, perforin and initiate target cell death. While granzymes seem to be mutually redundant in their cancer-protective function (Cullen et al., 2010), perforin was shown to be an irreplaceable molecule in the granzyme-mediated cytotoxicity (Kägi et al., 1994). Perforin gene knock-out in mice caused significantly higher incidence of both spontaneous and chemically induced malignancies (van den Broek et al., 1996, Street et al., 2001) supporting the essentiality of perforin-granzyme destructive pathway in cancer immunosurveillance.

The TRAIL molecule is constitutively expressed on a subset of NK-cells and is greatly upregulated on NK-cells, monocytes and dendritic cells upon their activation by IFN-γ (Smyth et al., 2003). Its potent tumor-annihilating effects have been recognized for over twenty years (Wiley et al., 1995, Takeda et al., 2002, Cretney et al., 2002). Based on TRAIL receptor p53-dependent up-regulation, it has been postulated, that TRAIL signaling can be acting as an intermediary between intrinsic cellular stress sensing and extrinsic immune regulatory mechanisms (Dunn et al., 2004**a**). A natural presumption, that this ligand can be exploited in cancer therapy caused a wave of optimism, but unfortunately the clinical outcomes have not been satisfactory, at least without further selection of patients with better outcome potential (reviewed in Dimberg et al., 2013).

When it comes to the classical apoptosis-inducing interaction of Fas/FasL, the situation is more complicated. While normally immune cells, mostly NK-cells, express FasL to induce apoptosis of target cells (Carotta, 2016), tumor cells too can express this ligand. On one hand, this results in inflammation reaction in TME potentiating elimination efforts, but on the other hand it may cause apoptosis induction of the infiltrating lymphocytes (Whiteside, 2002, Kim et al., 2004).

3.1.4 Participating immune cells

3.1.4.1 Dominant role of adaptive immunity

Both innate and specific adaptive immunity is shown to be required for undisturbed function of immune surveillance. The principal role of adaptive immune system was demonstrated in experiments with recombinase activating gene 1 (Smyth et al., 2000) and 2 (Shankaran et al., 2001) deficient mice ($RAG1^{-/-}$ and $RAG2^{-/-}$), which were consequently unable to form both αβ and γδ T-cells, B-cells and NKT-cells (Shinkai et al., 1992). These mice developed more frequently and more rapidly malignant tumors both spontaneously and after chemical induction with methylcholanthrene (MCA). Further studies were performed to determine the respective roles played by individual cell populations.

For a time, γδ T-cells were believed by some to be of a higher importance (Girardi et al., 2001, Gao et al., 2003) but αβ $CD8^+$ cytotoxic T-lymphocytes (**CTLs**) are now thought to assume the dominant role (Girardi et al., 2003, reviewed in Mittal et al., 2014). This became particularly apparent by demonstrating that it is mainly CTLs, which are responsible for the clinical efficiency of novel immunotherapeutic approaches (Snyder et al., 2014, reviewed in Hirayama and Nishimura., 2016). $CD4^+$ T-cells are also among tumor-infiltrating lymphocytes (TILs) and can function synergically with CTLs in tumor elimination, possibly via prevention of CTL exhaustion analogically to chronic inflammation (Matloubian et al., 1994, Kim et al., 2010). $CD4^+$ T-cells, specifically their $Foxp3^+$ Treg subset, can however act also as a crucial pro-tumorigenic regulatory cell population *(see below)* (Bos et al., 2013).

Similarly, various **B-cell** populations can play dual roles in cancer immunosurveillance. Anti-TSA immunoglobulin production is generally present in cancer patients, but is thought not to mediate immune protection (Preiss et al., 2005), even though in certain cases IgG-coupled antigen presentation via Fc-receptor on dendritic cells was shown to indeed activate $CD8^+$ T-cell-mediated tumor cell killing (Baker et al., 2013). On the other hand, the $CD19^+$ $CD25^{hi}$ Breg cells (Olkhanud et al., 2011) and immunosuppressive plasmocytes (ISPC) (Shalapour et al., 2015**a**) have been shown to exhibit pro-tumorigenic effects.

3.1.4.2 Dual roles of innate immune cells in immunosurveillance

There has been much discussion about the role of innate immune cells in cancer immunoediting. For efficient adaptive immunity mounting, a population of professional antigen presenting cells (APCs) is required. In tumor immunity, dendritic cells (**DCs**) and specifically their recently described $CD103^+$ subset, are the most important APCs, capable of direct CTL activation (Broz et al., 2014). This happens via CD8α signaling, which is dependent on type I IFN production (Fuertes et al., 2011). Tumor cell lysis releases DAMPs and tumor antigens. DAMPs stimulate DC maturation and the tumor antigen presentation to T-cells (Ma et al., 2013). Due to IFN-γ production and TRAIL-dependent tumor cytolysis, IPKDCs are also thought to be a significant cell population involved in immunosurveillance (Taieb et al., 2006). Another distinct population of plasmacytoid DCs (pDCs) have been shown to suppress anti-tumor immune responses by inhibiting both CTLs and other DC populations (reviewed in Kerkar et al., 2012).

The importance of **NK-cells** and NKT-cells in elimination of transformed cells was described following initial studies with RAG2$^{-/-}$ mice (Smyth et al., 2000, et al., 2001, Hayakawa et al., 2003). Their function had been originally understood to be prevalent in the early phase of tumor rejection, where they mediated direct tumor cell destruction before the activation of adaptive immunity (reviewed in Carotta, 2016). However, recently it was demonstrated, that innate immunity does contribute to immunosurveillance even in the absence of adaptive immune system (O'Sullivan et al., 2014). By comparing RAG2$^{-/-}$ γc$^{-/-}$ mice lacking all lymphocyte populations, including NK-cells, with the original RAG2$^{-/-}$ strain, it was documented, that the mice lacking all lymphocytes were even more susceptible to tumor formation than the original recombinase negative mice. Moreover, the immunogenicity of tumor cells was observed to be also shaped in mice with only NK-cells. NK-cells produced IFN-γ, which was concluded to be responsible for this immunoediting capacity. It was also postulated, that IFN-γ acts via induction of M1 activation state of tumor-associated macrophages (**TAMs**).

Tissue macrophages normally serve both as APCs and as cytokine producers regulating *i.a.* tissue regeneration. These functions are dependent on their respective activation state, which is a result of cytokine induction. It is likely, that the activation states form a continuous spectrum ranging in between two polarized states: the "classical" M1 and the "alternative" M2. Both IFN-γ and DAMPs acting via toll-like receptors (TLRs) lead to M1 activation, which in turn stimulates inflammation through production of pro-inflammatory cytokines such as IL-12 and tumor necrosis factor-α (TNF-α) as well as their antigen-presenting capacity through MHC class II up-regulation (reviewed in Mantovani et al., 2002). Hence, the M1-type TAMs play an important role in cancer immunosurveillance, especially in early stages of tumor development (Wang et al., 2011, O'Sullivan et al., 2014) and also in immunogenic cell death (Kroemer et al., 2013).

Significantly more discussed, however, are the protumorigenic effects mediated by TAMs. These have been shown to be largely dependent on M2 mode of activation by IL-4 and IL-13 (Gocheva et al., 2010). In TME, hypoxia and lactic acid seem to strongly stimulate M1 to M2 trans-activation (Colegio et al., 2014). Moreover, M2-like activation by a not-fully-understood effects of apoptotic tumor cells exert similar pro-tumorigenic activity (Lauber and Herrmann, 2015). In addition to immunosuppressive IL-10 secretion (Mantovani et al., 2002), the M2 TAMs have been shown to increase intratumoral angiogenesis (De Palma et al., 2003) and tumor metastatic potential (DeNardo et al., 2009).

Similarly ambiguous functions in tumor immunity have been attributed to tumor-associated neutrophils (**TANs**). On one hand, these cells directly damage tumor cells by ROS and elastase production, as well as counteract TGF-β effects exerted on T-cells, but mostly they sustain chronic inflammatory state within the TME, leading to the exhaustion of adaptive immune mechanisms (Houghton 2010). They also produce various mediators promoting angiogenesis (Houghton 2010) and tumor invasiveness (Queen et al., 2005). Analogously to TAMs, some authors describe tumor-inhibitory N1 activation mode and protumorigenic N2 mode, differentiated based on TGF-β signaling (Fridlender et al., 2009).

Other cell populations of innate and adaptive immune system play important roles in tumor immunity, but often predominantly favor tumor sustaining and progression. The most important of those are myeloid-derived suppressor cells (MDSCs) and regulatory T-lymphocytes (Treg), both of which are mentioned under the escape phase.

3.2 Equilibrium phase: immune-mediated tumor dormancy

As documented by numerous studies mentioned above, immune system undoubtedly plays an important role in prevention of transformed cells ultimately becoming a relevant threat to the host organism. In current understanding, most of emerging tumors are either completely destroyed or their expansion is permanently blocked via the innate and adaptive immune response mechanisms during the elimination phase of immunoediting (Dunn et al., 2004**a**). However, a small percentage of tumor cells may evade effective immune annihilation in a dynamic process of immunoselection. A balance arises between immune system-mediated destruction of an asymptotically increasing proportion of tumor cells and the multiplication of their constantly developing new antigen variants (Schreiber et al., 2011). This represents cryptic part of the immunoediting process regarded as an immune-mediated component of tumor dormancy (Uhr et al., 1991, reviewed in Aguirre-Ghiso, 2007).

Although in this phase the tumor cannot be externally detected, there have been well-designed studies supporting validity of this concept (Farrar et al., 1999, Saudemont and Quesnel, 2004, Loeser et al., 2007, Koebel et al., 2007, Eyles et al., 2010). It is presumed to be the longest phase of cancer immunoediting, proposed to be lasting throughout the life in most individuals and thus not progressing into the escape phase. Some cancers may disseminate early, yet fail to produce clinically apparent metastases due to the effective control by the immune system (Eyles et al., 2010). This view is supported by clinical observations of new, unexplained tumor outgrowth in transplanted organ recipients, mostly documented as a dormant melanoma transmission (Penn, 1996, Suranyi et al., 1998, McKie et al., 2003).

The quilibrium phase seems to be predominantly maintained by the adaptive immune response mechanisms, specifically IL-12, IFN-γ and T-cells (Koebel et al., 2007). Interestingly, one study found a direct association between the length of the tumor dormancy and its consequent faculty to actively suppress immune processes in the tumor via PD-L1 and CD80 *(see below)* surface expression upon transition to escape phase (Saudemont and Quesnel, 2004).

3.3 Escape phase: loss of immune control of tumor progression

Not all tumors probably follow the three-phase development scheme. However, the ones actually progressing from the equilibrium phase apparently stochastically acquire novel abilities under the selection pressure of the immune-mediated clonal destruction and these new abilities enable such tumor cell variants to escape the control by the immune system. The beginning of the escape phase is characterized by a relatively rapid tumor advancement leading to clinical observable malignancy outgrowth (Screiber et al., 2011).

The following paragraphs describe two general means, by which tumors escape the immunosurveillance: (1) the effects of immunoselection *i.e.* the processes affecting tumor cells and (2) immunosubversion *i.e.* the somewhat paradoxical pro-tumorigenic effects exerted on or by immune system of the host organism (Zitvogel et al., 2006).

3.3.1 Tumor-affecting phenomena permitting immune escape

New clonal variants of transformed cells, harboring novel resources of immune escape emerge with high frequency, since the genetic instability is inherently one of the hallmarks of malignant cells (reviewed in Lengauer et al., 1998). The resulting genetic and epigenetic changes may result in phenotypic changes competitively advantageous in the struggle with the immune system. These can be associated with either one of two escape-promoting effects: inhibition of tumor recognition and/or hindering the immune killing mechanisms.

The former can happen either via selection of clones lacking any strong tumor neoantigens (Jäger et al., 1996) or mutants characterized by defective antigen presentation (Khong et al., 2004). These disturbances can interfere with proper recognition of tumor cells by the immune system. Multiple mutations affecting MHC class I molecules expression (reviewed in Algarra et al., 2000), β2-microglobulin expression (Restifo et al., 1996) as well as other molecular processes essential for antigen presentation have been observed. Among these the constituents of antigen-processing machinery such as transporter associated with antigen processing 1 and 2 (TAP1 and TAP2), (Maeurer et al., 1996, Seliger et al., 1997, White et al., 1998), proteasome subunits low molecular weight protein 2 and 7 (LMP-2 and LMP-7) (Seliger et al., 1997) and tapasin (Schoenhals et al., 1999) are frequently mutated. Equally, several studies demonstrated defective up-regulation of MHC class I-associated antigen presentation after IFN-γ stimulation in tumors (Wong et al., 1997, Kaplan et al., 1998, Dunn et al., 2005**a**), mostly due to post-receptor deregulation of STAT proteins (Wong et al., 1997) and Janus-activated kinase 1 (JAK1) (Dunn et al., 2005**a**). The defects of these mechanisms render tumor cells hypo-immunogenic, thus allowing them to remain concealed to MHC-dependent recognition by T-cells.

Down-regulation of MHC class I expression naturally results in increased destruction of tumor cells by NK-cells, since their recognitory MHC I molecules provides an inhibitory signal. Thus, a close regulation of NK-cell function needs to be maintained in the TME, mostly by their receptor down-regulation (Platonova et al., 2011). Transformed cells also have been shown to avoid direct NK-cell activation by down-regulation of NKG2DL expression, occurring predominantly via a microRNA dependent mechanism (Stern-Ginossar et al., 2008).

Furthermore, some intracellular effector systems of immune cell mediated killing have been demonstrated to be defective in tumor cells, namely the ones involved in apoptosis-activating pathways. Tumor cells often up-regulate anti-apoptotic molecules, specifically FADD-like apoptosis regulator (FLIP) (Kataoka et al.,

1998) and Bcl-2 family member Bcl-XL (Hinz et al., 2000) or express mutated apoptosis-regulating membrane receptors incapable of death signal transmission, such as TRAIL receptors 1 and 2 (Shin et al., 2001) and CD95/Fas (Takahashi et al., 2006).

3.3.2 Immunosubversion and immunosuppressive TME

The tumor-sculpting process of immunoselection undoubtedly plays a considerable, if passive, part in tumor escape. However, the focus of immunooncological research has recently shifted to the mechanisms employed by tumors in order to actively derail the host-protective immune system. This is mainly due to the clinical ramifications of the interference with such mechanisms. The sum of all factors actively dampening and adapting immune functions has been termed immunosubversion (Zitvogel et al., 2006) and was shown to be the result of mostly locally active effects leading to establishment of immunosuppressive TME (Radoja et al., 2000). The ways by which tumor cells achieve this can be divided into three fundamental groups: (1) regulatory immune cell populations present in the TME; (2) alteration of TME composition by production of soluble suppressive signals to immune cells or enzymatic activity eventually favoring tumor escape; and (3) cell surface-bound signaling proteins termed immune checkpoint molecules (Adachi and Tamada, 2015).

3.3.2.1 Immunosuppressive cell populations

Among the cell populations negatively regulating immune responses **Treg** cells are probably the most important ones. Treg cells constitute a heterogenous population of T-lymphocytes defined by their function rather than phenotype. Their unifying trait is the expression of forkhead box P3 transcription factor (FoxP3), a crucial activator of suppressive proteins expression (Fontenot et al., 2003). Although $CD8^+$ $FoxP3^+$ T-cells were described to take part in tumor escape (Kiniwa et al., 2007), the pivotal regulatory T-cell population generally referred to as Treg are $CD4^+$ $CD25^{hi}$ $FoxP3^+$ T-cells (Bos et al., 2013). Treg cells present in tumors can originate from two distinct populations: thymus-derived natural Treg cells (nTreg), normally responsible for maintenance of peripheral self-tolerance; or *de novo* differentiated induced Treg cells (iTreg), generated upon antigen recognition by naïve $CD4^+$ $CD25^-$ T-lymphocytes in the suppressive conditions of TME (reviewed in Jacobs et al., 2012). It is widely accepted that iTreg cells are the cell population most closely associated with immune suppression in cancer immunoediting, but in various tumors different iTreg to nTreg ratios have been observed (Lindau et al., 2013).

In order to maintain Treg cell presence and function in TME, intact semaphorin-4a/neuropilin-1 (Sema4a/ Nrp1) pathway must remain intact, while no such requirement applies for non-tumor-associated immunosuppressive Treg function (Delgoffe et al., 2014). Sema4a is *i.a.* expressed on immune cells, where it acts as a T-cell response activator potentially inhibiting tumor growth and angiogenesis (Toyofuku et al., 2007). Curiously, Nrp-1 receptor was shown to be highly expressed on nTreg cells, but not on iTreg cells (Weiss et al., 2012), suggesting the importance of the natural Treg population in immune escape processes. Chemotaxis plays an important role in nTreg recruitment to the tumor site. Tumor cells were shown to produce CCL2 (Kimpfler et al., 2009) and CCL22 (Curiel et al., 2004) chemokine ligands, which bind to CCR4 receptor on Treg cells.

It was demonstrated, that the Treg function is dependent on T-cell receptor (TCR)-based (auto)antigen recognition but their immunosuppressive effect is exerted antigen-non-specifically (Fourcade et al., 2010). Treg cells employ several suppressive mechanisms, some of which are probably still poorly understood. They include mainly expression of immune checkpoint molecules and production of various soluble immunosuppressive signaling mediators *(see below)*.

Recently, a heavily discussed concept of tumor-associated inflammation emerged. Tumor microenvironment indeed does exhibit certain attributes of chronic inflammation, such as infiltration by myeloid cell populations, namely TAMs, TANs and MDSCs. They produce high levels of IL-1, IL-6, TGF-β and reactive oxygen and nitrogen species (RONS), contributing to tissue damage, extracellular matrix degradation and aggravation of local hypoxia. This state may aid immune system in early phases of elimination, but ultimately creates pro-tumorigenic conditions (reviewed in Shalapour et al., 2015**b**). MicroRNAs seem to act as both regulators and effectors of inflammation-associated changes in the TME (reviewed in Marques-Rocha et al., 2015).

In this concept, **MDSCs** are an indispensable group of cells (Meyer et al., 2011). They encompass a wide spectrum of myeloid precursor cell populations. Based on their surface markers, two main groups were distinguished in mice: granulocytic (G-MDSC) and monocytic (M-MDSC). Classification of human MDSCs is more complicated and ambiguous (reviewed in Lindau et al., 2013). It has not yet been determined, whether MDSCs present in tumors are a distinct terminal cellular population or if they represent immature forms of DCs, TAMs and TANs. MDSC recruitment and expansion in the TME happens via chemotaxis and proliferation stimulation by tumor and stroma cell derived factors, such as vascular endothelial growth factor (VEGF), (Gabrilovich et al., 1998) granulocyte/macrophage colony-stimulating factor (GM-CSF) (Bronte et al., 1999) and IL-1β (Song et al., 2005).

Despite their varied phenotype, all of these MDSC populations are uniform in their immunosuppressive effects and the specific mechanisms employed are not strictly limited to individual populations. Predominantly in G-MDSCs, NADPH-oxidase 2 (NOX2) was shown to be upregulated, producing reactive oxygen species (ROS) (Corzo et al., 2009). Similarly, principally M-MDSCs over-express inducible nitric oxide synthase (iNOS) and arginase-1 (ARG-1) (Corzo et al., 2010). MDSCs also activate Treg cell induction from naïve $CD4^+$ $CD25^-$ T-lymphocytes, further suppressing tumor elimination (Huang et al., 2006).

3.3.2.2 Immunosuppressive composition of the TME

Various substances are being produced into, or depleted from the TME by either transformed cells, stromal cells or infiltrating immune cells, many of which carry strong immunosuppressive and even tumor progression-stimulating effects. Three types of such substances can be recognized: effector agents of immune cells present in the TME, such as ROS and NO; altered concentration of low-molecular metabolites, actively changed by upregulated enzymes such as ARG-1 and indoleamine-2,3-dioxygenase (IDO); and most importantly, soluble intercellular signaling factors, namely IL-10 and TGF-β.

Important dominantly suppressive agents produced mostly by MDSCs and TANs are **RONS**. They were shown to inhibit T-cell-mediated recognition (Kusmartsev et al., 2004) which is particularly pronounced, when superoxide anion, one of ROS, reacts with NO. Resulting production of peroxynitrite nitrates tyrosine residues on TCR/CD8 complex, rendering CTLs anergic (Nagaraj et al., 2007), but also inactivating CCL2 chemokine, thus inhibiting lymphocyte migration to tumor site (Molon et al., 2011).

Mechanisms of **IDO**-mediated immunosuppressive effect are plentiful. In normal conditions, IDO enzymes serve as an evolutionally-conserved pathway of inflammation regulation. Natural IDO-producing cells are recruited to TME (Shields et al., 2010) and the expression of IDO or functionally related tryptophan dioxygenase (TDO), is upregulated in tumors (Munn et al., 2004, Opitz et al., 2011). Their expression has been shown to be dependent on Treg cell function (Fallarino et al., 2003). The enzymatic activity of IDO and TDO catalyzes the degradation of tryptophan to kynurenine. Kynurenine acts as an endogenous ligand for aryl hydrocarbon receptor (AhR), leading to iTreg differentiation (Mezrich 2010). The decreased concentration of tryptophan transduces signal via two important regulators of metabolism: mammalian target of rapamycin (mTOR) signaling inhibition; and GCN2 kinase activation *(see below)*. It was demonstrated, that mTOR inhibition leads to iTreg cell formation and immune suppression (Cobbold et al., 2009).

Increased expression of **ARG-1** by MDSCs and M2-activated TAMs results in consumption of L-arginine from the TME. Lack of L-arginine shortens CD3 ζ-chain mRNA half life, leading to decreased expression of TCR on effector T-cells (Rodriguez et al., 2002). The CD3 ζ-chain was later shown to be downregulated by activated GCN2 also in IDO-dependent manner (Fallarino et al., 2006). Moreover, both arginine (Rodriguez et al., 2007) and tryptophan (Munn et al., 2005) depletion-dependent activation of GCN2 kinase caused inhibition of downstream eukaryotic initiation factor 2 (eIF2α), leading to cell cycle arrest in effector T-cells.

It has been know for a long time, that tumor cells produce a suppressive cytokine, **IL-10** (Pisa et al., 1992). Apart from the autocrine stimulation of tumor cell immune recognition escape (Yue et al., 1997), IL-10 signaling in metastatic tumors also mediates MHC-like CD1 molecule downregulation on infiltrating DCs and thus inhibits tumor antigen presentation (Gerlini et al., 2004). However, it was also shown, that IL-10 can in certain conditions aid anti-tumor immunity by maintaining CTL function (Fujii et al., 2001). Tumor-resident B-cells have been shown to be an important source of IL-10 production (Inoue et al., 2006).

Of much more interest has however been another inflammation-associated cytokine, **TGF-β**. Although TGF-β receptor signaling via SMAD proteins has tumor-suppressing effect, on the other hand, in advanced tumors, TGF-β plays a crucial role as an inducer of immunosuppressive conditions in the TME (reviewed in Yang et al., 2010). Apart from tumor cells themselves, this potent immunosuppressive mediator is produced by Treg cells (Lindau et al., 2013) TAMs, but even more by MDSCs, largely dependent on IL-13 signaling from NKT-cells (Terabe et al., 2003).

It was demonstrated that although almost all anti-tumor immune mechanisms are suppressed by TGF-β, it is the inhibition of CTLs, that allows tumors escape immunosurveillance (Gorelik and Flavell, 2001). T-cell-

mediated cytolytic functions are hindered partly directly via SMAD-dependent downregulation of crucial lytic molecules, such as perforin, granzymes and FasL, but also IFN-γ (Thomas and Massagué, 2005). CTL function is, however, also inhibited indirectly by TGF-β via FoxP3 activation and thus iTreg cell generation (Nakamura et al., 2001) and also via suppressive specific T_H17-cell line differentiation (Mangan et al., 2006). TGF-β inhibits also NK cells.. As discussed above, activating receptors of NK-cells, namely NKG2D and NKp30 (Castriconi et al., 2003) as well as NKG2DLs on tumor cells are directly downregulated by TGF-β signaling (Friese et al., 2004). Anti-tumor actions of NK-cells are further suppressed indirectly via TGF-β-dependent Treg induction (Ghiringhelli et al., 2005). Furthermore, DC-mediated antigen presentation is impaired and M2/N2-activation mode of TAMs/TANs was reported to be induced by TGF-β (reviewed in Yang et al., 2010).

3.3.2.3 Immune checkpoint molecules

Immune cell function inhibition can be mediated by direct intercellular contact. Surface expression of various suppressive molecules triggers decreased proliferation, anergy or apoptosis in target lymphocytes. These membrane-bound proteins and glycoproteins activate inhibitory immune checkpoint pathways, physiologically occurring in peripheral tolerance induction (Fife and Bluestone, 2008). Tumor cells, as well as tumor-recruited immune regulatory cells, exploit these peripheral tolerance mechanism and thus suppress adaptive anti-tumor immunity.

Among these immune checkpoint molecules, the most vigorously studied ones belong to immunoglobulin (Ig) superfamily. A specificity of T-cell antigen recognition is mediated via TCR/MHC binding, however, a co-stimulatory signal is required to determine further fate of the T-cell. A classical activating signal is transduced via additional interaction of CD80 (also known as B7-1) and CD86 (B7-2) ligands expressed on the surface of APCs with T-cell co-stimulatory receptor molecule CD28 (Collins et al., 2002). Another T-cell surface receptor structurally similar to CD28, **CTLA-4** (CD152), has a much higher affinity to the CD80/86 ligands and therefore competitively (Fallarino et al., 1998) or possibly even directly (Masteller et al., 2000) inhibits the CD28-based co-stimulation. While in naïve T-cells, the CTLA-4 protein is sequestered in intracellular vesicles and is driven to the cell surface only upon strong TCR signal activation (Linsley et al., 1996), Treg cells express it constitutively on their surface (Takahashi et al., 2000). In addition to inhibition of T-cell proliferation and IL-2 secretion (Krummel and Allison, 1996), the CD80/86-CTLA-4 binding also downregulates CD80/86 on APCs, rendering them incapable of further T-cell activation (Qureshi et al., 2011).

In addition to the CD28-dependent T-cell co-stimulation, other distinct regulatory immune checkpoint pathways exist. The most prominent of them may be the one based on the **PD-1** (CD279) receptor signaling. PD-1 is present on the surface of T-cells, as well as B-cells and myeloid cells (reviewed in Fife and Bluestone, 2008). Its expression is significantly upregulated on exhausted CTLs, which underwent long-term activation without $CD4^+$ T_H-support (Kim et al., 2010). While CTLA-4 intracellular signal transduction is TCR-independent, PD-1 directly attenuates TCR signal (Parry et al., 2005).

PD-1 ligand 1 and 2 (PD-L1 and PD-L2) molecules are also expressed more ubiquitously than CD80/86. PD-L1 (CD274) is naturally present on lymphocytes, but also on non-hematopoietic cells, can be induced on various other cell types by inflammatory cytokines and has been described on a wide variety of tumor cells (reviewed in Fife and Bluestone, 2008). PD-L2 has more limited expression, constitutively present on DCs and monocytes/macrophages, but inducible on various other cells (reviewed in Rozali et al., 2012). PD-1 activation by its individual ligands seems to exert different, possibly opposing effects (Akbari et al., 2010). Moreover, PD-L1 was shown to also bind CD80 and mediate T-cell inhibition (Butte et al., 2007). Besides, it aids differentiation of iTreg cells, further suppressing CTL function (Wang et al., 2008).

Although these two immune checkpoint pathways have recently been in the spotlight of research due to the immunotherapeutical opportunities their blockade offers *(see below)*, various other checkpoint molecules have been described, most of them belonging to Ig superfamily. Among these, lymphocyte activation gene 3 protein (**LAG-3,** CD223) has been known for over 15 years (Triebel et al., 1990). It is structurally similar to CD4 and also binds MHC class II molecules (Huard et al., 1997). LAG-3 is expressed on most lymphocyte populations, but its function was shown to be also important in pDCs (Workman et al., 2009). Its surface expression is equally upregulated alongside PD-1 on exhausted CTLs (Grosso et al., 2009, Baitsch et al., 2012). LAG-3 suppresses anti-tumor immunity both by directly hindering CTL function (Grosso et al., 2007) and by mediation of Treg cell inhibitory functions (Durham et al., 2014). LAG-3 thus acts synergistically with PD-1 in peripheral tolerance regulation and potentially also in tumors (Okazaki et al., 2011).

A functionally similar **TIM-3** protein, expressed on T-cells, NK-cells and monocytes, was also described to participate in peripheral tolerance induction (Sánchez-Fueyo et al., 2003). Similarly to the LAG-3 signaling (Durham et al., 2014), upon binding its best known ligand galectin-9, TIM-3 specifically inhibits T_H1 $CD4^+$ T-cells (Zhu et al., 2005) likely inducing CTL exhaustion. TIM-3 is also co-expressed with PD-1 on these exhausted T-cells (Sakuishi et al., 2010), completing the vicious circle.

Another immune checkpoint molecule is a TNFR superfamily member herpesvirus entry mediator protein (**HVEM,** CD270). It is expressed on majority of lymphocytes, DCs and tumor cells and exhibits dual function in immune response regulation, dependent on the binding of particular ligands (Duhen et al., 2004). The TNF-family molecules LIGHT and lymphotoxin-α mediate mostly stimulatory signals upon HVEM binding (Duhen et al., 2004), whereas dominantly negative signaling occurs after interaction with Ig superfamily members CD160 and **BTLA** (CD272) (reviewed in Murphy and Murphy, 2010). BTLA is expressed on many lymphoid and myeloid populations. While normally BTLA is downregulated upon T-cell activation, in tumors its high expression seems to be preserved on TSA-specific CTLs and associated with their dysfunction (Derré et al., 2010).

Other molecules exploited by tumor cells in their fight against immune system have been reported, but generally, there is still insufficient amount of knowledge of their physiologic and cancer-related functions. Similarly to CD80/86, poliovirus receptor (PVR, CD155) was shown to bind two functionally opposing ligands: stimulatory CD226 and inhibitory checkpoint molecule **TIGIT**. TIGIT is highly expressed on exhausted CTLs, along with PD-1 and other checkpoint molecules (Johnston et al., 2014). **B7-H3** (CD276), a

B7 family molecule, exhibits both pro- and anti-tumor functions by stimulation (Chapoval et al., 2001) and inhibition (Suh et al., 2003) of T-cell-mediated immunity. Another immune checkpoint molecule, **VISTA** is dominantly expressed on MDSCs, inhibiting T-cell effector functions and inducing Treg cell generation (Lines et al., 2014).

4. Cancer therapy reverting immune escape

For decades, in addition to surgery, chemotherapeutic and radiation therapeutic cytostatic treatments were the only modalities available to cancer patients. These conventional treatments nonspecifically target fast-proliferating cells, but were actually shown to also cooperate with immune system. Chemotherapy and radiation therapy dramatically accelerates tumor cell lysis, releasing DAMPs and tumor antigens. DAMPs stimulate DC maturation via TLRs and thus the tumor antigen presentation to T-cells. This ultimately leads to potentiation of anti-tumor response, often being capable of complete tumor elimination or equilibrium re-instatement. Destruction of tumor cells mediated in this way is called immunogenic cell death (ICD) (reviewed in Ma et al., 2013). Since the beginning of 21st century, new treatments were being developed, which specifically target the immune system-tumor interactions.

4.1 Tumor vaccines

As is demonstrated above, specific T-cells as well as APCs are present in tumors. This fact led to efforts to develop tumor-antigen containing vaccines capable of stimulating anti-tumor immune response. Initially, efficiency of such vaccines was very low (Rosenberg et al., 2004), mainly due to improper tumor antigen selection and immunosuppressive TME, not permitting adequate antigen presentation (Schwartzentruber et al., 2011). A novel approach was developed, capitalizing on selective efficiency of DCs as APCs in non-suppressive environment (reviewed in Palucka et al., 2012). These DC-based vaccines are developed by culturing DCs with tumor antigen *ex vivo* and reinfusing them back to a patient. DC-based vaccines exhibited modest efficiency and one of them (Sipuleucel-T, trade name Provenge, manufactured by Dendreon Corporation) was even approved for clinical use. Further research and clinical trials apparently bring encouraging results (Schwartzentruber et al., 2011), so this approach may in future deliver clinically useful therapies.

4.2 Adoptive cell therapy

Similarly to DC-based vaccines, another approach, in which isolated immune cells are potentiated *ex vivo* and reinfused, was developed. In adoptive cell therapy (ACT), T-cells more or less enriched for the tumor antigen-specific ones are isolated from patient's peripheral blood, resected tumor mass or draining lymph nodes and stimulated to proliferate and activate their anti-tumor functions outside of immunosuppressive TME. Remarkable clinical effects were observed in some studies, especially in those involving lymphodepleting conditioning (reviewed in Hinrichs et al., 2014). Removal of suppressive cell populations,

as well as competitive increase in IL-7 and IL-15 concentrations were proposed as mechanisms responsible for the positive effects of the lymphodepletion (Gattinoni et al., 2005). Following application of radiation-myeloablative regimen, the use of tumor-infiltrating T-lymphocytes showed efficiency, albeit with considerable side-effects (Rosenberg et al., 2011).

A further approach to increase T-cell-mediated anti-tumor activity of ACT therapy was developed by genetically engineering tumor antigen-recognizing proteins and transfecting DNA encoding these proteins into cultured T-cells. Antigen-specific TCR α and β chains provided capability of specific tumor antigen recognition to all cultured T-cells, amplifying their potential of antigen-specific response (Gross et al., 1989). More sophisticated chimeric antigen receptors (CARs) capable of direct T-cell activation independently from TCR/MHC binding were designed. CARs consist from antibody-derived variable domain, separating segment including trans-membrane domain and activating signal-generating domains derived from T-cell co-stimulatory molecules. Specific domain mediates tumor antigen recognition and is usually derived from single-chain variable fragment of a monoclonal antibody. It is connected at the C-terminus with a IgG, IgD, or CD8-derived spacer segment, allowing for effective antigen binding and sufficient CAR surface expression. To circumvent the requirement of co-stimulatory signal upon antigen binding, activating domains of one or more intracellular T-cell signaling proteins are included in CAR constructs, such as those derived from intracellular parts of CD28, 4-1BB, and CD3ζ, alone or combined (reviewed in Dai et al., 2016). The CAR-transfected T-cells are capable of potent direct CTL-mediated tumor cell killing and/or cytokine release (Chmielewski et al., 2011). T-cells expressing such CARs are capable of highly effective malignant cell destruction (Hinrichs et al., 2014).

Similarly, a very efficient T-cell activation and thus potent anti-tumor response can be achieved by a group of specifically designed molecules called bi-specific T-cell engagers (BiTEs). In BiTEs, two single-chain variable fragments are connected by a linker, one of them being targeted against specific tumor antigen and the other one against CD3 component of TCR. Upon binding to the tumor antigen, T-cells can be nonspecifically recruited to form immunological synapse, resulting in direct tumor cell killing (Brischwein et al., 2006). This approach is particularly effective in hematological malignancies. Blinatumomab, a CD19-targeting BiTE has shown extraordinary clinical efficiency against B-cell leukemias (Topp et al., 2011).

4.3 Immune checkpoint blockade

The above described modes of cancer immunotherapy are based on artificially increasing the potency of immunostimulatory mechanism. These can however only fulfill their limited potential, while the tumor-protective immunosuppressive mechanism are in place. A “next generation” immunotherapy concentrates on dealing with the immunosubversion mechanisms exerted by the tumor (Adachi and Tamada, 2015). To date, the only such clinically approved approach is based on blockade of immune checkpoint molecules CTLA-4 and PD-1.

The first immune checkpoint-blocking monoclonal antibody approved for routine human medical use was anti-CTLA4, ipilimumab. Its moderate efficiency was accompanied by immune auto-agressive adverse

effects (Hodi et al., 2010); this is not surprising when considering lethality observed in CTLA-4-deficient mice (Waterhouse et al., 1995). It has, however, paved the road for subsequent more efficient checkpoint-blocking antibodies.

Pembrolizumab and nivolumab, PD-1 blocking monoclonal antibodies, have followed ipilimumab with significantly better clinical efficiencies. They were shown to induce long-term remission in advanced melanoma patients (Topalian et al., 2014, Garon et al., 2015), while causing reasonable level of adverse effects (Topalian et al., 2012). The considerably lower toxicity of anti-PD-1 antibodies when compared to ipilimumab is obviously due to the dominantly pathological activation of PD-1 pathway and is in agreement with the observed milder phenotype in PD-1 knock-out mice (Nishimura et al., 1999). As most of tumor-protective effects of PD-1 signaling are caused by PD-L1, selective blocking of this ligand has also been explored. Indeed, PD-L1 knock-out mice suffer from only very limited autoimmune damage (Dong et al., 2004). Multiple anti-PD-L1 antibodies are currently in clinical trials, *i.a.* BMS-936559 (Brahmer et al., 2012), atezolizumab (Powles et al., 2014) and durvalumab (Planchard et al., 2016, Antonia et al., 2016).

Additional anti-CTLA-4 antibody, tremelimumab, applied mainly synergistically with durvalumab (Antonia et al., 2016) and a novel antibody directed against PD-1 receptor, pidilizumab (Westin et al., 2014), are also undergoing clinical trials. Furthermore, antibodies targeting other immune checkpoint molecules, such as LAG-3, TIM-3, BTLA, TIGIT, but also those against other tumor-promoting proteins such as IDO, KIRs and TGF-β are being developed or are in preclinical trials (Cohn et al., 2014, reviewed in Topalian et al., 2015).

Intriguing results were obtained by combining individual checkpoint-blocking antibodies together or with conventional means of cancer therapy and targeted biological therapy. Evidence of significantly higher clinical efficiency was produced for ipilimumab/nivolumab combination therapy in melanoma patients, admittedly at the cost of more severe immunological adverse effects (Wolchok et al., 2013, Weber er al., 2015). Similarly, combination of checkpoint inhibitors with chemotherapy, radiation therapy and targeted biological therapy has been exhibiting optimistic results (reviewed in Melero et al., 2015). This synergic anti-tumor effect was attributed to immunogenic cell death *(see above)*, but also to depletion of tumor-infiltrating MDSCs (Vincent et al., 2010) and Treg cells (Le and Jaffee, 2012).

A critical problem of all currently used or tested immune checkpoint-blocking antibodies is the difficulty in determining which patients will benefit from which individual immunotherapeutic modality. Therefore, major efforts are concentrated on finding such predictive biomarkers. Patients receiving CTLA-4-blocking antibodies displayed a correlation of the tumor mutational load (and thus quantity of TSAs) with treatment response (Snyder et al., 2014). It was however not sufficiently predictive to be used as a response determinant. More optimistic is the situation in the case of PD-1/PD-L1 blockade. Several studies showed association between PD-L1 expression on tumor-infiltrating immune cells and clinical effectiveness of both PD-1 and PD-L1 targeting antibodies (Herbst et al., 2014, Powles et al., 2014, Garon et al., 2015). These are, however only retrospective analyses, hence there is a certain degree of doubt associated with their clinical implementation and further research is undoubtedly necessary.

5. Conclusions

The immunooncological research of the past three decades brought about a revolution in the way we perceive interactions of immune system with tumor cells. While formerly it was anticipated that tumors can evade immune recognition and destruction, recent findings expose the many ways by which tumors also actively subjugate immune system of the host. Since this immunosubversion is one of critical requirements for tumor to persist and grow, it can be assumed, that if we would understand the entirety of the tumor-immune system interactions, we could specifically target the ones exploited by each particular tumor. Hence, the gradual elucidation of these mechanisms has direct consequences for our capabilities in cancer treatment.

Development of therapeutical methods harnessing the natural power of immune system in fight against cancer has been one of the recent most significant biomedical breakthroughs. Many of currently applied modes of immunotherapy provide clinicians with effective and very promising options for cancer patients, which are beyond capabilities of classical therapy. As evidenced by exciting preclinical results and a number of current clinical trials, the immunotherapy field is likely only at the beginning and further developments will almost certainly bring even much better clinical results in near future. As each patient and each tumor are more or less unique, a high priority must be given to identification of biomarkers predicting the optimal type of immunotherapy for individual patients. Although the novel immunotherapies appear to be relatively safe, the risk of adverse effects cannot be underestimated. An important issue is also the economical burden associated with these novel expensive therapies; this problem may become very serious when large numbers of patients will require them as the only effective treatment option.

In conclusion it can be stated, that the decades of often painstakingly slow and frustrating basic research finally brought fascinating breakthroughs of major clinical relevance. It is no exaggeration to speak about true immunological revolution in cancer treatment and about the coming era of broadly applicable cancer immunotherapy.

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