Cancer-reactive memory T cells from bone marrow: Spontaneous induction and therapeutic potential (Review)

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Abstract. Cognate interactions between naïve tumor antigen (TA)-specific T cells and TA-presenting dendritic cells (DCs) are facilitated by secondary lymphoid organs such as lymph nodes or the spleen. These can result either in TA-specific tolerance or, depending on environmental costimulatory signals, in TA-specific immune responses. In the present review, we describe such events for the bone marrow (BM) when blood-borne TA, released from the primary tumor or expressed by blood circulating tumor cells or DCs enters the BM stroma and parenchyma. We argue that cognate T-DC interactions in the BM result in immune responses and generation of memory T cells (MTCs) rather than tolerance because T cells in the BM show an increased level of pre-activation. The review starts with the spontaneous induction of cancer-reactive MTCs in the BM and the involvement of such MTCs in the control of tumor dormancy. The main part deals with the therapeutic potency of BM MTCs. This is a new area of research in which the authors research group has performed pioneering studies which are summarized. These include studies in animal tumor models, studies with human cells in tumor xenotransplant models and clinical studies. Based on observations of an enormous expansion capacity, longevity and therapeutic capacity of BM MTCs, a hypothesis is presented which suggests the involvement of stem-like MTCs.

Contents

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1. Introduction

Immunology textbooks refer to BM only as a primary lymphatic organ with the function of hemato- and lymphopoiesis. There is no mentioning of secondary immune functions (initiation of antigen-specific immune responses), in particular not about T cell mediated immune responses. Secondary immune functions are described only for secondary lymphoid tissues such as lymph nodes, spleen, mucosa-associated lymphoid tissues (MALT) and skin-associated lymphoid tissues (SALT) (1).

One reason for the relative paucity of data on BM physiology is the fact that normal BM is surrounded by thick cortical bone that impedes direct observation and experimental manipulation. Bones represent the inner skeleton of vertebrates and it is exclusively in vertebrates where the adaptive immunity sytem was invented and further developed about 400 million years ago. When some of the technical hurdles about bones were solved and immune-histological stainings could be performed using frozen tissue sections, we made some surprising observations: there were follicle-like lymphoid structures which had an architecture and a cellular composition similar to that of secondary lymphoid organs. Functional studies corroborated this impression. In 2003, we reported for the first time that BM is a priming site for T cell responses to blood-borne antigen (2). Ten years after this discovery, it was confirmed and extended by 2-photon dynamic imaging. This revealed cross-presentation of blood-borne antigens to naïve $CD8^+$ T cells in the BM (3).

The compact outer matrix of bone surrounds a central cavity. This is filled with a spongy honeycomb structure made up of thin strands of connective tissue called trabeculae. Similar to spleen, BM is vascularized by blood, not by lymphatic vessels

and is part of the lymphocyte recirculation network (4). The total BM cell population consists of ~60-70% myeloid lineage cells and 20-30% erythroid lineage cells. Lymphoid lineage cells represent ~8-20% of BM mononuclear cells. Mouse BM contains 1-2% $CD11c^+$ DCs in different stages of maturation and 1-5% $CD3^+$ T cells. These DCs and T cells are embedded in parenchyma and stroma consisting of a fibrous network of reticular and stromal cells lined by an extracellular matrix and a blood microvasculature. Approximately two thirds of BM T cells express surface markers [$CD44^{hi}$ and CD122 (IL-2Rβ)] relating to antigen experience (2,5,6).

DCs are BM derived and reside in most tissues as sentinels of the immune system. Immature DCs sample extracellular fluid in short periods of time in search of antigenic materials. They constitutively ingest bits of their environment by endocytosis and macropinocytosis. They also internalize cell debris from cells that have undergone cell death by apoptosis or necrosis (7). Furthermore, DCs are equipped with pattern recognition receptors (PRRs) that mediate recognition of 'danger' motifs, be they exogenous from pathogens (PAMPs) or endogenous from tissue damage (DAMPs). DCs use their PRRs and phagocytic power to sample tissue microenvironments for the presence of PAMPs and DAMPs that indicate 'danger' and also the presence of peptide-major histocompatibility complexes (pMHCs) in a way that can trigger the activation of pMHC antigen-specific T cells. $CD11c^+$ DCs from BM are highly efficient in taking up exogenous blood-borne antigen and processing it via MHC class I and class II pathways (8). They, thus, become professional antigen-presenting cells (APCs) for $CD8^+$ and $CD4^+$ T cells. Naïve, antigen-specific T cells home to BM where they can be primed by such antigen-laden DCs.

The present review will not deal with regulatory aspects and T regulatory (Treg) cells and not with B-cell aspects in the BM. Instead, it will focus on cancer-reactive MTCs from BM, their spontaneous induction, their dynamics and their therapeutic potential. The review will include findings from a Graft-versus-leukemia (GvL) animal tumor model, MTC longevity studies in nude *(nu/nu)* mice, pre-clinical data with human cells in immune incompetent NOD/SCID mice and finally summarize results from a phase I clinical study. These results, mainly from own studies, will be combined with general background information and with a discussion of their potential clinical relevance. Special attention is given to the fact that MTCs from BM can rapidly expand after cell transfer, show longevity and strong antitumor protective activity. A hypothesis is presented to suggest that such characteristics are in part due to the presence of stem-like memory T cells.

2. BM and tumor dormancy

A serendipity observation led us to look into the BM of mice: we had intended to establish tumors from a metastatic Eb lymphoma variant (ESb) in the ear pinna of mice in order to establish a model for post-operative metastasis and anti-metastatic immunotherapy. Ear pinna resection would have been easier than tumor operation from an intradermal or subcutaneous inoculation site. Unexpectedly, the highly aggressive tumor cells did not grow from this inoculation site. We then found out that the animals shortly after ear pinna (i.e.) inoculation of the tumor cells became tumor resistant also at other sites of inoculation and this for prolonged periods of time. Thus, the i.e. inoculation of live proliferation-competent murine lymphoma cells in syngeneic DBA/2 mice was a very effective way of antitumor immunization (9). The ear pinna was superior over a subcutaneous inoculation site also for induction of specific cytotoxic T cell (CTL) responses following immunization with naked lacZ DNA vectors (10).

The i.e. inoculation of Eb lymphoma cells genetically marked with the bacterial lacZ gene into syngeneic mice allowed to visualize rapid migration and, surprisingly, long-term persistence of tumor cells at a low level (<100 cells/10^6 BM mononuclear cells) selectively in the BM. The proliferating fraction (% Ki-67^+X-Gal^+) of EblacZ tumor cells from BM of tumor dormancy mice was 21% compared to 87% in cells from tissue culture and to 40% in BM from immune incompetent mice (11). EblacZ tumor dormancy was under active control by $CD8^+$ immune T cells and correlated with long-term (>6 months) immunological protection (11). These findings provided the motivation to investigate the special properties of the BM.

Long-term persistence of tumor cells in a dormant state (up to 20-25 years) is suggested from clinical observations in cancer patients, notably breast cancer (12). Primary operated breast cancer patients (25-43%) exhibit micrometastatic disease in BM (13). More details can be found in a review of T cell memory, anergy and immunotherapy in breast cancer (14).

3. BM as priming site for spontaneous T cell responses to blood-borne tumor antigens

The lacZ product β-galactosidase (Gal) served not only as a marker to visualize individual tumor cells in tissues (15,16) but also as a surrogate TA. The first T cell response observed after i.e. inoculation of live lacZ transfected ESb (ESblacZ) tumor cells was observed in the BM, as revealed by dominant Gal peptide-MHC I (L^d) tetramer analysis (2). This response was stronger than that of spleen and draining lymph nodes and showed a peak after 10 days. This suggests that the tumor cells from the i.e. inoculation site quickly entered the blood stream and from there the BM where they were taken up by DCs which functioned as APCs, cross-presented TAs and Gal and initiated primary $CD8^+$ T cell responses. Primary T cell responses to blood-borne antigen occured in spleenectomized mutant *aly/aly* mice, thus, demonstrating their independency from secondary lymphoid organs (2).

T cell activation in the BM correlated with DC-T cell clustering (2) and immunological synapse formation (17,18). The responses were not tolerogenic and resulted in generation of CTLs, protective antitumor immunity and immunological memory (2). These and other findings (19-28) highlighted the uniqueness of BM as an organ important for primary as well as for secondary immune functions (systemic B- and T-cell mediated immunity). Table I lists some of the important steps in BM relating to T-cell mediated immunity.

Further evidence for primary T-cell responses in BM came from direct intra-BM vaccination. Vaccination with a class I MHC restricted T cell epitope from human papilloma virus HPV-16 E7 induced large numbers of activated, IFN-γ

Table I. Important steps in the generation of antitumor effector and memory T cells in the BM.

Steps	Description
1. Homing:	T cells from the blood enter vascular sinuses in BM and transmigrate via diapedesis through endothelium into BM parenchyma; this involves traffic/adhesion molecular interactions[a], chemokines[b] and cytokines and is true for circulating T cells and tumor cells; the chemokine axis CXCL12/CXCR4 also plays an intriguing role in BM homing (108); the collagen II receptor CD49b on stromal cells is required for migration of memory CD4 T-cell precursors into their survival niches[c] (110).
2. Antigen cross-presentation:	$CD11c^+$ DCs and $F4/80^+$ macrophages but not $B220^+$ B cells were capable to cross-present i.v. injected OVA to the OVA peptide specific hybridoma B3Z (2). These BM resident APCs can also take up tumor cells, process their proteins to peptides and cross-present TAs as peptide-MHC complexes to T cells.
3. APC scanning:	Infiltrating T cells scan these APCs to find out whether they present peptide-MHC fitting to their own antigen-specific receptor.
4. T-APC cluster formation:	Once a cognate interaction event has taken place between a TA-specific T cell and an APC with a corresponding TA epitope, an immunological synapse is formed and the two types of cells stay together and exchange signals (e.g. IFN-α, IL-12 from DC to T, IFN-γ, TNF-α from T to DC (17,18).
5. T cell activation, proliferation and memory formation:	Upon activation[d], T cells around the T-APC clusters transform to lymphoblasts; upon cell division, they are released from the clusters and either recirculate to the blood or remain in the BM compartment as MTCs.

[a]LFA-1a and alpha4 integrins on T cells interacting with VCAM-1, MadCAM-1 and ICAM-1, in BM stroma (2); [b]CXCR3 ligands guide CMTs in recall responses (108); DCs in lymphoid tissues express the chemokine SLC that binds to CCR7 expressed on naïve T cells; [c]memory T cells late of an immune response also dock on IL-7(+)/collagen XI(+) stromal cells (110). [d]Antigen availability determines $CD8^+$ T cell-DC interaction kinetics and memory fate decisions (111). CXCL12, cystein-intervening amino acid-cystein motif ligand 12, produced by BM resident reticular (CAR) cells (109); OVA, ovalbumin.

producing E7-specific lymphocytes in the BM. Adoptive transfer of these to naïve animals conferred complete protection against HPV-16 immortalized and oncogene transformed TC-1 tumor cells (29). The potential of T-cell priming in BM for the induction of long-lasting protective antitumor immunity has been reviewed (30).

4. Evolutionary aspects

The innate immune system has its origin in the bilateria approximately one billion years ago. The cells express innate immune receptors that are encoded in the germline DNA. While the majority of organisms survive utilizing only innate immune mechanisms, vertebrates have developed a second immune defense system. The genes of the antigen-specific T cell receptor (TCR) and of the antigen-specific B cell receptor (BCR; immunoglobulin genes coding for antibodies) first appeared in the cartilaginous fish, such as shark, ~400 million years ago. Each lymphocyte of this system (B or T cells) has learned to generate a unique antigen receptor by somatic rearrangement of multiple gene segments. The beneficial features unique to the adaptive immune response are great diversity for antigen recognition, high specificity and immunological memory. The vertebrate immune system relies on natural positive and negative selection mechanisms within the individual organism between developing cell lineages. This ensures the generation of a broad repertoire of diverse B and T cells that recognize foreign antigens, but not antigens from the organism itself.

The emergence of a thymus and spleen in the teleost (bony fishes) was associated with MHC molecules, cell-mediated immunity, CTLs and allograft rejection. The organization of the peripheral lymphoid tissues and the homing of B and T cells to distinct areas evolved under guidance of TNF-family proteins and chemokines. BM first occured in amphibians. Lymph nodes, MALT and SALT appeared only later, namely in birds. Lymph nodes and spleen are the most highly organized of the secondary lymphoid organs. In addition to lymphoid follicles [which we also observed in BM (2)], they have distinct regions of T- and B-cell activity and they are surrounded by a fibrous capsule.

Thus, the adaptive immune system is found exclusively in vertebrates. It is likely that the formation of an inner skeleton consisting of bones with BM and cartilage was helpful for the evolution of a second immune defense system. BM was found to be capable of performing adaptive T cell mediated immune functions and this in an autonomous way, in the absence of secondary lymphoid organs (2). The function of a central

organ such as the BM was perhaps to protect the blood circulation while that of peripheral lymphoid organs was to protect the lymphatic circulation. Such diversification would have achieved a division of labor between protection from within and protection against the outside world.

5. Memory T cells

A characteristic feature of MTCs is their long-term survival in the absence of re-exposure to the antigen. During a primary immune response, IL-7Rα (CD127) is downregulated on most $CD8^+$ T cells. Only a small subset of cells that are $CD127^{hi}$ contribute to the pool of memory cells. Priming of naïve T cells leads to association of the tyrosine kinase Lck with the $CD8^+$ coreceptor, thereby enhancing TCR signaling (31). The association between Lck, which phosphorylates CD3 immunoreceptor tyrosine-based activation motifs (ITAMs), and CD8 is maintained in MTCs which explains their enhanced sensitivity to antigen re-exposure.

Memory $CD8^+$ T cells (Tc) generated without CD4 (Th) help can be defective in their ability to respond to secondary encounters with antigen (32). An important mechanism by which $CD4^+$ T cells provide help to $CD8^+$ T cells is through CD40-CD40L(CD154) interactions. For naïve $CD4^+$ T cells, the binding of the TCR to antigenic peptide displayed on MHC class II by mature DCs delivers signal 1 to the Th nucleus and also upregulates CD40L on the Th cell surface. The binding of CD40L to CD40 on the DC upregulates on these cells the expression of B7 (CD80 and CD86) molecules. Signal 2 to the Th nucleus is delivered when these B7 molecules interact with costimulatory CD28 molecules on the Th cell surface. Expression of cytokine receptors is then upregulated and the Th cell starts to produce IL-2. Signal 3 is delivered when the Th cell's IL-2 receptors (IL-2R) are engaged by IL-2. The DC which is now considered to be 'licensed' due to its upregulated B7 expression, detaches from the Th cell (33). A naïve Tc receives signal 1 when its TCR binds to peptide presented on MHC class I expressed by a licensed DC. Signal 2 is delivered via B7-CD28 interaction and signal 3 when IL-2 produced by a Th cell engages the Tc cell's IL-2Rs (34). The Tc cell detaches from the licensed DC, proliferates and generates daughter cells that differentiate into effector CTLs and MTCs.

T cell help provided to Tc cells consists among others in the production of IL-2 and possibly other cytokines necessary for Tc proliferation. Naïve Th or Tc cells that have received signals 1 and 2 upregulate the receptors which permit to receive signal 3 in the form of cytokines, type I interferons (35), chemokines and growth factors.

The progeny generated from an activated IL-2 producing Th cell are called Th0 cells. Approximately 48-72 h after the original antigenic stimulation of the naïve Th cell, these Th0 cells terminally differentiate into various subsets of resting effector Th cells. The type of effector Th subset is determined: i) by the cytokines and other factors present in the immediate microenvironment; and ii) by the nature of the DC by which the original naïve Th cell was activated. In the BM it were $CD11c^+$ DCs and $F4/80^+$ macrophages, but not $B220^+$ B cells that cross-presented blood-borne antigen (2). Normally, intracellular pathogens such as viruses and intracellular bacteria trigger macrophages and DCs to produce IFN-γ, IL-12 and IL-27. A Th0 cell that has its TCR engaged by specific pMHC and encounters these cytokines will activate the transcription factor STAT4. This drives a gene expression program that causes the Th0 cell to commit to the Th1 subset. Once in the site of inflammation where their effector function is required, Th1 cells are stimulated by antigen in this site and activate the transcription factor T-bet. This drives IFN-γ production and opposes intracellular signals promoting Th2 differentiation. The duration of both Th1/2 and CTL responses is controlled by T cell exhaustion and activation-induced cell death (AICD) mediated by both extrinsic and intrinsic apoptotic pathways. Only MTCs remain from such an antigen-specific T cell response.

The Th1/Th2 lineage decision, in which DCs play an important role, has important consequences for the primary immune response. Memory $CD4^+$ T cells, upon re-encountering antigen rapidly re-express the cytokines elicited during their primary activation. Epigenetic chromatin remodeling during the primary T-cell response accounts for specific cytokine expression profiles of reactivated MTCs.

Apart from Tc and Th MTCs there are at least two further sublasses, namely effector memory T cells (EMTs) and central memory T cells (CMTs) (36). CMTs express high levels of the lymphoid tissue homing molecules CD62L and CCR7. Accordingly, these cells tend to migrate through lymphoid tissue thereby maintaining a long-term central reservoir of MTCs. EMTs express only low levels of CD62L and CCR7, so that they circulate mainly through non-lymphoid tissues. EMTs constantly patrol the peripheral tissues and are able to quickly migrate into sites of inflammation.

The duration of TCR signaling required for activation is ~20-30 h for naïve T cells but <1 h for effector T cells and MTCs. The life span of naïve T cells is 5-7 weeks, that of effector T cells is 2-3 days, while that of MTCs can last up to 50 years.

A tumor vaccine with a combination of all three T cell activation signals, delivered simultaneously via CD3, CD28 and CD25, triggered the strongest activation of naïve human T cells, thereby inducing CTL and MTCs. A single stimulation caused durable bystander antitumor activity in a tumor monolayer assay *in vitro* with repeated effector cell transfers lasting for >10 days (37).

6. Maintenance of T cell memory by BM microenvironment

BM microenvironment. BM stroma was shown to express constitutively multiple adhesion molecules (ICAM-1, VCAM-1, MadCAM-1 and P-selectin) relevant for the infiltration of BM by blood-derived T cells and also CD80, relevant for T cell costimulation (38). BM resident MTCs can be activated by incoming APCs from the blood (21) or by BM resident APCs. Further aspects relate to stroma derived cytokines and chemokines. For instance, BM contains a high proportion of MTCs in close proximity to IL-7 and/or IL-15 secreting stromal cells which serve to regulate their homeostatic proliferation and survival (39-42).

Long-term presence of T cell memory was found for CD8 T cells (2,3,11,20,23-27,39,41) as well as for CD4 T cells (17,26,39,42). The mechanisms for maintenance of T cell memory in the BM is still a matter of intensive research.

Table II. Important findings relating to bone marrow derived T cells.

Steps	Description
1. Tumor dormancy:	Selectively to BM; balance between proliferating EblacZ or ESblacZ tumor cells and proliferating Gal-specific CD8 MTCs; balance/dormancy persisting for months; importance of antigen availability[a] immunization site: ear pinna; primary Gal-specific CD8 T cell response in BM; BM microenvironment: ICAM-1, VCAM-1, MadCAM-1, P-selectin, CD80, CD57, CD28 (CD8 T cells less differentiated and more proliferative than those in PB).
2. Dynamics:	iBMT to iPEC and back to iBMT (resting vs. active state and back); >80-fold enrichment of Gal-specific EMTs; reversion to BM niches; residual TA-expressing dormant tumor cells boosting specific T cell frequencies.
3. Longevity:	Repeated iPEC transfer in *nu/nu* mice over a period of 8 months; with each transfer a tumor challenge is necessary to preserve longevity; competition for BM MTC niches;
3. Turnover rate:	MTCs: BM (41.6%) > spleen (8.7%) > lymph node (3.4%) [% BrdU uptake (44)] naïve T cells: BM (12.8%) >spleen (0.9%) > lymph node (0.2%).
4. Expansion capacity:	i) GvL late stage model; $2x10^7$ iBMT transfer leads to complete tumor remission (62); ii) clinical study in late stage breast cancer; $2x10^7$ APC-reactivated BMT: Th1 and Tc1 greatly expand *in vivo* within 7 days in responder patients (87).

[a]T cells that experience stable contacts with APCs develop long-lived memory, whereas conditions without stable contacts yielded immunological amnesia (111); BMT, bone marrow T cell; EblacZ, ESblacZ, mouse lymphoma lines transfected with a gene coding for bacterial β-galactosidase, which expresses a dominant CD8 T cell epitope (Gal-L^d); MTC, memory T cell; PB, peripheral blood; iBMT, immune bone marrow T cells (i.e. from mice immunized against ESblacZ by ear pinna inoculation of 10^5 live tumor cells); iPEC, immune peritoneal effector cells (i.e. mostly EMT, from primed mice 3 days after i.p. challenge with irradiated tumor cells); EMT, effector memory T cell; TA, tumor-associated antigen; GvL, graft vs. leukemia (3 week established ESb-MP leukemia from DBA/2 treated with immune cells from B10.D2 mice); APC, antigen-presenting dendritic cell; Th1 and Tc1, IFN-γ producing polarized T helper and T cytotoxic T cells.

Turnover rate of MTCs and competition for niches. Naïve ($CD44^{int/lo}$) and memory ($CD44^{hi}$) T cells from non-immunized DBA/2 mice were analysed for bromodeoxyuridine (BrdU) uptake. MTCs consistently exhibited a higher uptake rate than naïve cells, and BM resident cells had a significantly higher uptake rate than their spleen or lymph node resident counterparts. The highest uptake rate was detected in MTCs from the BM (Table II, turnover rate) (44). These findings were later basically corroborated employing a more sophisticated technology and suggesting that the higher turnover is mostly attributable to a higher proliferation rate (45).

BM contains special cellular niches for lymphopoiesis (22), for naïve lymphocytes (46), for B cells (47) and for MTCs (43). Niches are microenvironmental domains or functional compartments in the BM that sustain and modulate hematopoietic stem cell (HSC) and immune cell behavior. Within the MTC niches IL-7 and IL-15 play an important role for survival of CD4 and CD8 MTCs (38-42). In addition, type I interferons are important as stimulators of DC-mediated cross-priming and have an impact on anti-tumor responses (35). Mesenchymal stem cells induce in $CD4^+CD45RO^+$ MTCs IL-17 production and this promotes neutrophil activation (48). Neutrophils transport antigen from the dermis to the BM, initiating a source of memory $CD8^+$ T cells (49).

MTCs have been described to compete for BM seeding since the number of respective niches is restricted (50). The homing phenotype also plays a role. Human virus-specific $CD8^+$ BM T cells were found to exhibit a unique $CCR5^+CXCR6^+CXCR3^-$ homing phenotype which has not been observed on T cells from classical secondary lymphoid organs or peripheral organs (51). Tissue-selective homing receptor expression on effector and memory T cells was reported to be governed by inductive as well as suppressive signals from both DCs and tissue microenvironments (52).

Role of persisting tumor antigen. Further data suggested that small amounts of persisting TA produced by dormant tumor cells from the BM contributes to the maintenance of TA-specific long-term memory. This mechanism apparently produced a delicate balance between proliferating 'dormant' tumor cells and proliferating TA-specific T cells. Notably, the phenotype of BM resident MTCs differed between tumor-inoculated and tumor-free hosts: while in TA containing hosts the majority of CD8 T cells was $CD45RB^{lo/neg}$ $CD62L^-$ $CD44^-$ (cycling cells), in hosts containing no TA they were mainly $CD45Rb^{hi}$ $CD62L^-$ $CD44^+$ (resting cells) (44).

The importance of persisting antigen for the maintenance of T-cell memory and for the balance between CMT and EMT has been a matter of extensive debate. It is now clear that MTCs do not need persisting antigen for their survival. Yet, persisting antigen keeps the T cells in a more activated state, facilitating maintenance of immediate effector function. Such stimulation might also help to induce the expression of base levels of anti-apoptotic molecules that would permit MTCs to survive. Optimal long-term protective T-cell memory is

induced by viruses that persist at a low level in the host, such as LCMV or CMV. Containment of persisting viruses and other pathogens is probably one of the most important tasks of T-cell memory. This may be true for cancer as well in situations of tumor dormancy or stable disease.

7. Dynamics of tumor-reactive memory from the BM

To engage tumor-reactive MTCs from the BM in immunotherapy of cancer outside the BM requires their proper recruitment and activation. The ins and outs of MTCs from the BM were studied in the above-mentioned model system of tumor dormancy.

DBA/2 mice were primed with live ESblacZ cells in the ear pinna. Following T-cell priming in the BM, Gal-specific MTCs could be recruited from the BM to the peritoneal cavity by intra-peritoneal (i.p.) vaccination with irradiated ESblacZ cells. The secondary anti-Gal response in the peritoneal cavity involved a >80-fold enrichment of epitope specific CD8 T cells and the release of various cytokines (53). Two months later the MTCs returned from an activated state (mostly EMTs) into a resting state (mostly CMTs) and from the peritoneal cavity back to the BM (53).

Similarly, upon *in vitro* activation, human $CCR7^-CD62L^-CD8^+$ and $CCR7^-CD62L^-CD4^+$ EMT cells were shown to exhibit dynamic differentiation, involving transient as well as stable changes to CMT phenotype and function (54).

Within 2 months after their activation in the periphery, most antigen-specific CD4 T lymphocytes re-locate to the BM where they play a key role in immunological memory by modulating the generation of memory B cells and the maintenance of memory CD8 T cells.

8. Longevity of BM MTCs

Gal reactive peritoneal EMT cells were transferred from immunocompetent DBA/2 mice to athymic *nu/nu* mice together with live ESblacZ tumor cells in order to study longevity of tumor-protective T cell memory. The EMT transfer prevented tumor outgrowth and resulted in the long-term persistence of a high frequency of Gal-specific T cells in the BM and spleen. MTCs could be recruited to the peritoneal cavity, like in immunocompetent DBA/2 mice, by tumor vaccination of immunoprotected *nu/nu* mice. These *nu/nu* derived EMTs could again be transferred together with live ESblacZ cells to secondary *nu/nu* hosts and protected against tumor outgrowth. Long-term immune memory and tumor protection could be maintained in this way over four successive transfers starting from DBA/2 derived EMTs. While naïve nude mice died within 10 days following injection of $1x10^5$ tumor cells, memory nude mice were able to reject a tumor dose of $5x10^7$ and survived longer than 8 months (44).

Considering the limitations of numbers of cell division during a cells lifetime by telomere shortening (55,56) and considering the high proliferation rate of MTCs (44,45), the longevity of Gal-specific MTCs over four passages in *nu/nu* mice is remarkable.

In conclusion, longevity of protective antitumor immunity could be established in T-cell deficient nude mice. TA-reactive CD8 T cells survived over a long period of time (>8 months) in antigen-inexperienced hosts. The presence of TA-expressing dormant tumor cells was indispensable to boost specific T cell frequencies to levels detectable by peptide-MHC multimers, giving TA-specific T cells a selective advantage over irrelevant clones (44).

9. Therapeutic potential of BM MTCs in a GvL animal model

Complete remission of cancer is a rare phenomenon, especially when the cancer has already progressed to late-stage disease involving multiple organ metastases and cachexia. Most immunotherapy studies in animal tumor models are performed in early stages of disease. The model system we have established and chosen for testing the therapeutic potential of cancer-reactive MTCs from the BM is therefore unique and a rare exception. It makes use of the knowledge obtained over years of immunogenetic studies of immunoresistance to the DBA/2 lymphoma ESb in allogeneic mouse strains (57). These resulted in selection of an optimal MHC-matched donor strain for immune T cell transfer studies. This strain, B10.D2, contains among others a high frequency of vβ6 T cells recognizing on ESb-MP lymphoma cells the endogenous viral superantigen 7 (vSAG7) from DBA/2 mice leading to an extremely rapid type of tumor cell death (58).

The GvL animal model of advanced metastasized cancer. The GvL system involved metastasizing sublines (ESb and ESb-MP) of the above-mentioned murine lymphoma Eb. Immunoresistant donor mice were immunized on day 0 by i.v. inoculation of 10^5 live ESb-MP lymphoma cells. At the same time, recipient DBA/2 mice were inoculated intradermally (i.d.) with $2x10^5$ syngeneic ESb-MP tumor cells. At day 20, when a tumor had been established in DBA/2 (but not in B10.D2) mice and metastasized to the liver and kidneys, the recipients were whole body X-irradiated (10 animals per group). One day later, they were treated i.v. with donor immune cells.

When tumor-bearing hosts were pre-treated with 4.5 Gy to reduce host-versus-graft (HvG) reactivity, a single adoptive transfer of 10^7 immune spleen cells (iSPL) from the donor mice led to a break of tolerance to vSAG7 (Mls^a) (59), to complete remission of the primary tumor and of metastases within weeks and to significant life prolongation (60,61).

Results obtained with BM MTCs. A further study compared in this model cancer-reactive MTCs from different compartments (spleen, peritoneal cavity, BM) of pre-immunized donor mice. To broaden the therapeutic window, ESb-MP tumor-bearing hosts were irradiated either with 5 or with 7.5 Gy. Results were the following: at the higher dose of host irradiation the transfer of allogeneic iSPL led to GvL but also to graft-versus-host (GvH) reactivity. Immune peritoneal effector cells (iPEC) were even worse: They exerted strong GvH reactivity so that the mice from this group died even faster than those from an untreated tumor-bearing control group. Apparently the higher dose of host irradiation had reduced HvG reactivity so that allogeneic naïve T cells caused the GvH disease. In contrast, immune cells from the BM (iBM) of donor mice conferred GvL without GvH reactivity, no matter whether the hosts were

irradiated by 5 or 7.5 Gy. BM cells from naïve donor mice or T cell depleted iBM cells were completely devoid of GvL reactivity (62).

Antitumor immunization resulted in a 20% increase in BM resident $CD44^{hi}$ CD8 T cells and in an increased frequency of $CD44^{hi}$ $CD62L^{+}$ cells in both the CD4 and CD8 T cell compartment. iBM cells isolated either 2 weeks (short-term memory) or 4 weeks after the last immunization (long-term memory) were equally potent in the transfer of GvL reactivity. The efficiency of long-term memory T cells to mediate the GvL effect observed becomes evident when calculating the numbers of adoptively transferred T cells. Since BM contains only ~2% T cells and since one transfer of $2x10^7$ total BM mononuclear cells was sufficient for complete tumor remission, this GvL effect was dependent on maximally $4x10^5$ allogeneic T cells, with only a fraction of these being tumor specific (62).

Mechanism of function of effective immune rejection. Protective immunity and donor T cell infiltration of liver metastases in this model is dependent on CD4:CD8 immune T cell interactions (60,63) as well as on donor T cell:host macrophage interactions (64). In donor-host cell cluster (64), CD40-CD40L(CD154) interactions led to the induction of inducible nitric oxide synthase (iNOS). Blocking donor-host cell interaction with anti-CD40L monoclonal antibody led to inhibition of the therapeutic effect (65). The donor T cells induced and activated in the liver of the tumor-bearing host sialoadhesin (Sn^{+}) positive macrophages which apparently functioned as scavengers, antigen-presenting cells and as producers of NO (66). These cells localized strikingly around the metastases.

Role of inducible nitric oxide synthase (iNOS) in other tumor models. Targeted activation of the tumor microenvironment may overcome inefficient T cell migration and tumor infiltration. Interestingly, neoadjuvant local low-dose gamma irradiation (LDI) caused normalization of aberrant vasculature and efficient recruitment of tumor-specific T cells in xenotransplanted human pancreatic carcinomas. LDI was shown to program the differentiation of $iNOS^{+}$ M1 macrophages that orchestrate CTL recruitment into and killing within solid tumors through iNOS by inducing endothelial activation and by suppressing the production of angiogenic, immunosuppressive, and tumor growth factors (67).

Downregulation of iNOS activity was found to be associated with breakdown of stable disease (68). Local tumor growth following intradermal inoculation of ESblacZ cells revealed progressive growth during the first 10 days, then a plateau phase from day 10 to 20 and a second phase of progressive growth until day 30. The plateau phase was caused by the effect of a T cell mediated immune response to Gal. A similar plateau phase with stable metastatic disease was observed in the liver: in this phase, in which ~10% of re-isolated liver sinusoidal cells represented tumor cells, there was a distinct regular mosaic type of pattern of metastases, visible only after whole organ staining (16). The breakdown of this transient growth control coincided with a breakdown of iNOS activity in Kupffer cells and liver sinusoidal endothelial cells (69). Transfer of B10.D2 donor iBM cells in this critical time period could prevent the breakdown of endogenous immune control and cause rejection of the tumor in the skin and of the liver metastases.

Clinical relevance. Naïve allogeneic lymphocytes are commonly used in the clinic for donor lymphocyte infusion (DLI) to transfer GvL activity under non-myeloablative conditioning. BM T cells were reported to be superior to splenic T cells to induce chimeric conversion after non-myeloablative BM transplantation (70). Our studies suggest that cancer-reactive MTCs are superior to naïve allogeneic T cells to obtain optimal GvL effects. This was recently corroborated for $CD8^{+}CD44^{hi}$ but not $CD4^{+}CD44^{hi}$ MTCs (71). To generate cancer-reactive MTCs, one strategy would consist of *in vitro* pre-stimulation of donor T cells with host DCs presenting target tumor antigens (72).

10. Cancer-reactive MTCs from the BM of cancer patients

During the last decade, numerous studies clearly demonstrated the spontaneous induction of functional TA-specific T cell responses in cancer patients (73-77).

Enrichment of MTCs in the BM. We (5) and others (20,23-28) observed an enrichment of antigen-specific MTCs in BM, suggesting that this compartment is a preferential site for migration, homing, survival and retainment of MTCs.

Repertoire of specificities. Cancer-reactive MTCs were searched for in the BM and peripheral blood of 84 primary-operated breast cancer patients and 11 healthy donors by HLA-tetramer analyses and by IFN-γ ELISPOT assays (78). Altogether, T cells reactive against the entirety of TAs in autologous tumor cells (as presented by autologous tumor cell lysate-pulsed DCs) could be detected in ~40% of the patients. These T cells existed in frequencies of 1:200 to 1:10.000 of total T cells and included Tc cells and Th cells. Similar frequencies of tumor-reactive T cells were detected in patients with different types of cancer, including colon (79) and pancreatic carcinoma (80), melanoma (81), glioblastoma and haematological malignancies (82). Such spontaneous T cell responses result in the accumulation of cancer-reactive T cells, especially in lymphoid organs such as the BM.

A majority (~70%) of tumor-reactive BM T cells belonged to the population of MTCs, including ~40% CMTs and 60% EMTs (38). The presence of EMTs is interesting since this subset is generated by appropriate re-activation of long-lived BM resident CMTs that subsequently differentiate into the rather short-lived subset of EMTs which mediate protective effector functions. The dominance of EMTs in the BM of cancer patients suggests that presentation of TAs to T cells is a rather frequent event in the BM parenchyma and stroma. The importance of this phenomenon for maintaining high frequencies of TA-specific MTCs was discussed above in chapter 5.

The repertoire of specificities of MTCs from the BM was investigated in more detail for primary operated breast cancer patients. The T cell repertoire was highly polyvalent and exhibited pronounced interindividual differences in the patterns of TAs recognized by each patient. Compared to healthy individuals, the repertoire of tumor-reactive MTCs in

the BM of breast cancer patients was clearly shifted towards tumor recognition (83,84).

Therapeutic potential in xenotransplanted NOD/SCID mice. To evaluate the therapeutic potential of the natural repertoire of tumor-reactive T cells from the BM of cancer patients, we adoptively transferred these cells, after appropriate *ex vivo* reactivation with tumor lysate-pulsed autologous DCs, into NOD/SCID mice previously xenotransplanted with small pieces of breast cancer or normal skin from the same patient (38,78). Separated BM derived $CD45RA^{-}CD45R0^{+}$ memory but not $CD45RA^{+}$ naïve T cells infiltrated autologous tumor but not skin tissues 2-3 weeks after i.p. cell transfer. The tumor-infiltrating lymphocytes had a CMT or EMT phenotype and produced perforin (38).

A single transfer of restimulated BM MTCs was associated with the induction of tumor cell apoptosis and significant tumor reduction. T cells from the peripheral blood showed much lower antitumor reactivity (38,78). Notably, immunohistology revealed the infiltration of tumors not only by human T cells but also by human DCs. Both types of cells expressed the chemokine receptor CCR7. Tumor infiltration included cluster formation in tumor tissue by MTCs with cotransfected DCs. The DCs had been present in the restimulation cultures as APCs. Removal of the DCs from the T cells of the stimulation culture resulted in a reduction of therapeutic potential (38). These results suggest that reactivated MTCs together with APCs selectively home to human tumor xenotransplants and reject them on the basis of specific recognition of TAs on tumor cells and on tumor-resident DCs.

Further details about DC migration (85) and the response of MTCs to antigen stimulation *in vivo* (86) have been reviewed.

11. Clinical studies with re-activated BM derived cancer-reactive MTCs

A pilot clinical study was conducted involving metastasized breast cancer patients to investigate whether *ex vivo* re-activation of cancer-reactive MTCs from the BM and their adoptive transfer is feasible and increases the frequencies of cancer-reactive T cells in the blood. The study protocol involved one i.v. transfusion of T cells which had been re-activated in short-term cultures with autologous DCs pulsed with lysate from MCF7 breast cancer cells as source of TAs (87).

Twelve late-stage patients were included. They had received standard therapy and had cancer-reactive MTC in their BM according to respective pre-tests. In all cases, the treatment was feasible and well tolerated. Six patients (immunological responders) showed by ELISPOT assay *de novo* TA-specific, IFN-γ secreting T cells in the blood after 7 days. In contrast, 6 other patients (immunological non-responders) showed in the blood TA-induced IL-4 responses (87).

Responder patients received $>6.5x10^3$ TA-reactive cells while non-responders had received lower numbers from the stimulation cultures. This was due to reduced activation of MTCs, to increased amounts of $CD4^{+}CD25^{hi}$ Treg cells in their BM and to increased TA-induced IL-10 secretion. The latter was prevented by preceding depletion of Treg cells (87).

Following a single adoptive transfer of low numbers of MTCs from the BM, in responder patients these cells must have extensively expanded *in vivo* to reach the numbers that were detected by ELISPOT 7 days later in the peripheral blood. A follow-up analysis revealed that responder patients had a significantly higher overall survival compared to the nonresponder patients (58.6 vs. 13.6 months) (88).

12. Hypothesis: A role of stem memory T cells (TSCMs)?

Several observations from the reported studies are remarkable and deserve an explanation: i) Tumor dormancy: MTCs were found to control tumor dormancy for long times selectively in the BM; ii) Dynamics: MTCs with their different subsets and localization showed great flexibility and, in a resting state, returned to BM. iii) Longevity: TA-specific MTCs from the BM could be recruited to the periphery, exerted effector functions and then reverted back to the BM. Such process was repeated in *nu/nu* mice several times over a period of at least 8 months. iv) Turnover rate: in spite of their high turnover rate, MTCs from BM could transfer long-term protective antitumor immunity. v) Expansion capacity: In the GvL model system as well as in the clinical study, a small number of transferred BM MTC cells was capable to expand *in vivo* and to exert powerful protective antitumor functions.

We hypothesize that MTCs from BM contain a fraction of stem cell-like MTCs (TSCM) which, like BM resident hematopoietic stem cells (HSC), divide asymmetrically to provide self-renewal and differentiational capacity. BM can be considered as a central organ for maintenance of immunological B- and T-cell memory. TSCM cells could play an important role in this context. The center of bones seems to be well protected and specifically organized to preserve cells of importance such as HSCs, memory B cells and MTCs. Other lymphoid organs might support such a function also but BM is suggested to play a central role. This hypothesis would allow to explain the above main observations.

What do we know about TSCM cells? Here we can only mention a few of the recently discovered facts. TSCM have been described in mice, nun-human primates and in man. Approximately 2-4% of the total CD4 and CD8 T cell population in the periphery represents TSCM. They appear as preactivated owing to their expression of CD69 and CD25. They display stem cell-like properties and exhibit a gene profile between naïve T cells and CMTs (89). They are in the G0 phase and their transcriptome is that of resting T cells (90). Extrinsic signals for stemness of MTCs include IL-7 and IL-15 (91). Quiescent early memory $CD8^{+}$ T cells were characterized by high CD127 expression and by efflux of Rhodamine 123, a typical property of stem cells (92).

With their self-renewal capacity and long-term survival, TSCM represent the earliest and long-lasting developmental stage of MTCs. They can rapidly differentiate into more mature CMT, EMT and effector T cells, while maintaining their own pool size through homeostatic self-renewal. TSCM also play a role in T-cell reconstitution following haploidentical allogeneic transplantation (93).

High-resolution tracking of T cells in humans recently unveiled that TSCM are able to persist and to preserve their precursor potential for up to 12 years after infusion of genetically modified lymphocytes (94). Human CMV virus-specific TSCM were defined with the phenotype

$CD8^+CD45RA^+CCR7^+CD127^+CD95^+$. They could be detected at frequencies of ~1/10,000, similar to frequencies seen in other subsets (95). People vaccinated against yellow fever (YF) contained YF-specific $CD8^+$ TSCM cells that were stably maintained for more than 25 years and showed self-renewal capacity *ex vivo* (96).

The repertoire of $CD4^+$ BM MTCs compared to MTCs from the blood was significantly enriched for T cells specific for CMV, tetanus toxoid, measles, mumps and rubella (97).

13. Concluding remarks

Who could have predicted in 1994 when we discovered tumor dormancy and immune control in the BM (9) that this organ is in fact at the center stage of immunological memory? Because of this early observation and recognition of its importance, it is not surprsising that we were among the first in discovering the following: i) in 2001 that cancer-reactive MTCs are enriched in the BM of breast cancer patients (5) and exert therapeutic potential against autologous human tumor xenotransplants in NOD/SCID mice (78), ii) in 2003 that BM is a priming site for T-cell responses to blood-borne antigen (2), iii) in 2005 that small numbers of cancer-reactive MTCs from the BM of allogeneic donor mice can transfer potent GvL reactivity to MHC identical recipient mice with established metastasized cancer (61,62) and, also in 2005, that BM MTCs show longevity (>8 months) and dynamics upon repeated grafting to tumor challenged immunoprotected *nu/nu* mice (44). Support came in 2005 from Di Rosa and Pabst (19) who proposed that BM is a central organ in mature T-cell traffic and contributes greatly to long-term cytotoxic memory.

We now hypothesize that MTCs from the BM are superior to MTCs from other sites in terms of cancer therapeutic potential because they contain TSCM which are especially important for maintaining longevity of the memory. We also suggest that TAs from residual dormant tumor cells play a role in the maintenance of long-term tumor-specific T-cell memory.

BM may be superior to tumor-draining lymph nodes with respect to initiation of TA-specific T cell responses: the presence of $CD69^+$ and $CD25^+$ TSCM and of co-stimulatory CD80 on BM stroma cells might augment the likelihood of an immunogenic versus tolerogenic outcome of cognate T-APC interactions. TA-specific T cell responses in tumor-draining lymph nodes seem to be more dependent on adjuvant activity than those in BM. That is why cancer vaccines combine TAs with adjuvants (98). Our studies in mouse (chapters 2-9) and man (chapters 10 and 11) suggest that in both species the BM allows for spontaneous induction of cancer-reactive MTCs. Furthermore, BM MTCs from both species appear to possess potent therapeutic capacity.

Is it not fascinating then to conclude with the statement 'Immunity: it's in our bones' (99)?

14. Perspectives

Considering the importance of MTCs from the BM for the treatment of diseases like cancer and others, the question for the future is, how to exploit this precious source of cells from the immune system. The pioneering experimental and clinical studies from this review, suggesting potent therapeutic capacity of cancer-reactive BM MTCs from mouse and man will hopefully lead to a new research field and to new effective cancer therapies.

Still, questions remain. Will adoptive cellular therapy (ADI, ACT), for instance with BM derived and *ex vivo* re-activated cells (87) be the way to go or are there other better means? Should one try to recruit and re-activate BM MTCs *in situ*, for instance by active specific immunization (ASI) with appropriate vaccines?

A priori, we hypothesized that ASI would be appropriate in a post-operative adjuvant situation in which the patient's immune system is likely still intact, while ADI would be the method of choice in a more advanced situation with a more or less defective immune system. In both situations we favor the combination with an oncolytic virus, in our case the avian Newcastle disease virus (NDV). With its tumor-selective replication, its induction of immunogenic cell death (ICD) and its broad immunostimulatory properties, this biologic agent can provide a cutting edge between tumor and host (100). This future perspective view rests on the following findings: i) A prospective randomized-controlled study of post-operative ASI with the autologous NDV-modified tumor vaccine ATV-NDV in stage IV colon cancer patients revealed, after a follow-up period of 10 years, a >35% improvement of long-term survival. This result has been explained by recruitment and activation of a pre-exising repertoire of tumor-reactive MTCs from the BM (101). ii) In a further advanced situation of stage IV colon cancer patients with unoperable liver metastases, a phase I ASI study with ATV-NDV vaccine to which anti-CD28 costimulatory bispecific antibodies had been attached revealed that this treatment was safe and could reactivate anergized T cells (102).

Thus, ASI under certain conditions apparently can provide a perspective even for late-stage disease.

This may be particularly true if it is combined with other modalities, for instance: i) with checkpoint blocking antibodies (103) or ii) with conditioning the tumor microenvironment by low-dose X-irradiation (67) or iii) by radiofrequency hyperthermia (104,105). For example, we recently reported from the IOZK two unusual cases, one prostate cancer (104) the other breast cancer (105), of long-term survival of late-stage patients with extensive metastases following RHT combined with immuno- and viro-therapy.

ACT using large quantities of naturally-occurring tumor-reactive lymphocytes has mediated durable, complete regressions in patients with melanoma (106) and metastatic cervical cancer (107). Which ever way (ASI or ADI/ACT or others) will prevail is difficult to predict.

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