

Education in immunotherapy part 2

Summary of the pathogenesis and treatment of cancer

Simplified version

Abstract

Intrinsic tumour suppression. Every normal cell has the capability to eliminate itself when its DNA is damaged. This, amongst others, to prevent the development of cancer cells. But, sometimes the genes that are involved in intrinsic tumour suppression are damaged, the intrinsic tumour suppression mechanism fails and cancer cells may survive.

Tumour cells have certain properties: the hallmarks of cancer. These are: 1) Unlimited replicative potential and replicative immortality; 2) Resisting cell death; 3) Self-sufficiency in growth signals; 4) Insensitivity to anti-growth signals; 5) Evasion of contact inhibition and the ability to invade and migration (metastasis); 6) The ability to attract and sustain angiogenesis for nutrient supply ; 7) The ability to suppress the immune system.

The immune reaction to eliminate cancer cells is called the extrinsic tumour suppression. This type of tumour suppression engages only after the intrinsic tumour suppression has failed. Cancer immunoediting is the process of cancer elimination by the immune system. This process is complex and can be simplified into three phases: 1) The elimination phase (= the immunosurveillance); 2) The equilibrium phase (= the immunoselection); 3) The escape phase (= the immunosubversion). In the latter case, the tumour mass is uncontrollable by the immune system and cancer cells may grow, invade the surroundings and metastasise. The tumour mass consist of tumour stem cells, more or less matured tumour cells, cancer associated fibroblasts (CAFs), tumour associated macrophages (TAMs), regulatory T-cells (Tregs), myeloid derived suppressor cells (MDSCs or MSCs), etc. This tumour matrix protects the tumour cells from being destroyed by the immune system. In other words: the patient's own immune system protects the tumour cells within the tumour mass. Apparently, an individual tumour cell is far more vulnerable to the immune system than a tumour mass with its defensive tumour matrix. Chronic inflammation plays a crucial role in the development of the tumour matrix.

The best treatment option is to remove all tumour masses completely by means of radical curative surgery and treatment of the underlying chronic inflammation. Unfortunately, this is not always possible. In these cases, modern cancer therapy consists of: the combination of debulking surgery, treatment of the underlying chronic inflammation, immunotherapy to specifically attack the tumour cells, and immunotherapy to weaken the tumour protection by the tumour matrix.

The pathogenesis of cancer

Intrinsic tumour suppression

Normal cells have intrinsic tumour suppression properties. During the evolution, innate tumour-suppressive mechanisms in the proliferative programmes of mammalian cells have developed. This mechanism triggers apoptosis, autophagy, or senescence, in case of aberrant proliferation. Apoptosis may be triggered by two major mechanisms: binding of death ligands to death receptors in the extrinsic pathway or cytotoxicity that initiates the intrinsic "mitochondrial" pathway. Autophagy is responsible for nutrient homeostasis, energy salvage and degradation of old, malfunctioning organelles within a cell (recycling). In normal cells, autophagy inhibits their transformation to premalignant tumour cells by reducing reactive oxygen species (ROS), DNA damage, protein aggregation, and mitochondrial abnormality etc. In established tumour cells, autophagy promotes their growth by serving as a cell survival mechanism. Autophagy inhibits the regression of malignant tumour cells to benign tumours. Additionally, autophagy is also a type II programmed cell death and can initiate cell death in different circumstances. Senescence stands for irreversible state of dormancy, due, amongst others, to telomere shortening, see "*Failure of the intrinsic tumour suppression*" [Lowe SC, et al., Nature 2004, Campisi J, et al., Nat Rev Mol Cell Biol 2007; Koff JL, et al., Int J Mol Sci 2015; Zhi X, et al., F1000 Prime Rep2015]. Intrinsic tumour suppression includes:

1. Mechanisms within the cell, for example:
 - a. Tumour suppressor genes that protect the cells from tumour formation, for example: PTEN, p53, p63, p73, LKB1 (Liver kinase B1), etc.
 - b. Oncogenes may have apoptotic and/or anti-apoptotic functions: E1A, Myc, E2F, growth factors, receptor tyrosine kinases, cytoplasmic tyrosine kinases, RAS, etc.
 - c. Oncogene checkpoints and cell cycle checkpoints: pre-set and autonomous sensors for aberrant proliferative signalling to deal with elevated or sustained fluxes of mitogenic signalling. These checkpoints generally respond

through the p53 pathway [Mombach LCM, et al., *Bmc Genomics* 2014; Lowe SC, et al., *Nature* 2004, Campisi J, et al., *Nat Rev Mol Cell Biol* 2007; Koff JL, et al., *Int J Mol Sci* 2015].

d. Tumour suppressive microRNA-198 (miR-198) [Marin- Muller C, et al. *Clin Cancer Res* 2013].

2. Social signals that keep the cells to respect their supportive microenvironment:

Micro-environmental signals: Contact inhibition of proliferation [McClatchey AI, et al., *Curr Opin Cell Biol* 2012] and contact inhibition of locomotion [Batson J, et al., *J Microsc* 2013] involve the protein E-cadherin, the transmembrane receptors integrins and the non-canonical Wnt/calcium pathway.

Failure of the intrinsic tumour suppression

Multiple mutations of the tumour suppression genes and/or of oncogenes may lead to a failure of the intrinsic tumour suppression mechanisms. Mutations may occur by oncogenic stimuli such as: chronic sunburns, radioactive radiations, certain chemical compounds, cigarette smoke, toxic micro-environment of chronic inflammation, oncogenic viruses, etc.

Failure of the intrinsic tumour suppression leads to the development of tumour cells. Generally, these cells have the following properties (**the 6 (+1) hallmarks of cancer**) [Hanahan D, et al., *Cell* 2011; Kanterman J, et al., *Semin Cancer Biol* 2012; Schreiber RD, et al., *Science* 2011; Zitvogel L, et al., *Nat Rev Immunol* 2006]:

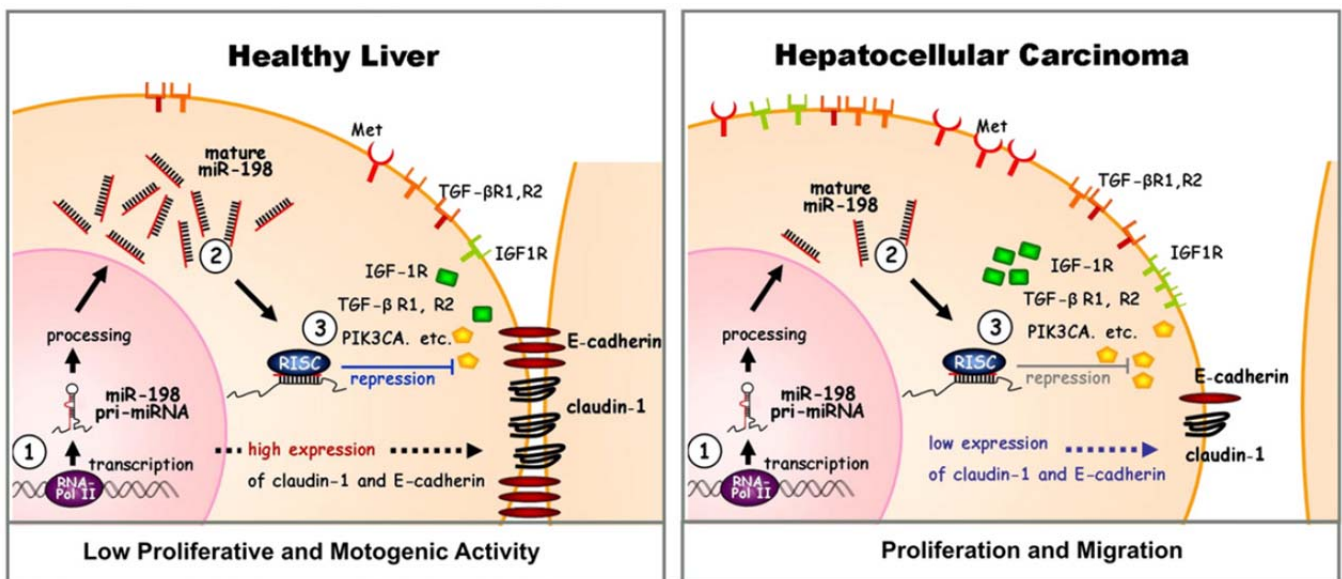
1. Unlimited replicative potential and replicative immortality: *“The telomeres, composed of multiple tandem hexanucleotide repeats, shorten progressively in non-immortalized cells propagated in culture, eventually losing the ability to protect the ends of chromosomal DNAs from end-to-end fusions; such fusions generate unstable dicentric chromosomes whose resolution results in a scrambling of karyotype that threatens cell viability. Telomerase, the specialized DNA polymerase that adds telomere repeat segments to the ends of telomeric DNA, is almost absent in non-immortalized cells but expressed at functionally significant levels in the vast majority (~90%) of spontaneously immortalized cells, including human cancer cells. By extending telomeric DNA, telomerase is able to counter the progressive telomere erosion that would otherwise occur in its absence.”* [Hanahan D, et al., *Cell* 2011].
2. Resisting cell death.
 - a. Evasion of apoptosis and avoidance of oncogene induced senescence. This is due, amongst others, to loss of the p53 tumour suppressor function, loss of the action of anti-apoptotic oncogenes, and increased expression of anti-apoptotic Bcl-2 family regulatory proteins [Hanahan D, et al., *Cell* 2011].
 - b. Evasion of autophagy by the loss of function of Beclin-1 protein. Beclin-1 (a member of BH-3 only subfamily of apoptotic regulatory protein) is necessary for the induction of autophagy [Hanahan D, et al., *Cell* 2011].
 - c. Acquisition of multiple drug resistance (MDR) [Kathawala RJ, et al., *Drug Resist Update* 2015].
 - ATP-binding cassette (ABC) transporters use energy derived from the hydrolysis of ATP to adenosine di-phosphate (ADP) to transport their substrates across the membrane against a concentration gradient. Forty-nine members of the ABC transporter family have been identified.
 - The ABC transporter family is divided into seven subfamilies, ABCA through ABCG. Structurally, ABC transporter proteins have two nucleotide-binding domains (NBDs) and two transmembrane binding domains (TMDs). The NBDs are proteins consisting of conserved ABC and are responsible for binding and extruding harmful substrates out of the cell.
 - Certain ABC transporters genes are clearly overexpressed in tumours with MDR.
 - d. Tumour cells are resistant to hypoxia and preferably metabolize glucose by anaerobic glycolysis. Therefore, tumour cells utilise masses of glucose because the anaerobic glycolysis is 18 times more inefficient to produce ATP compared to the aerobic metabolism [Kato Y, et al., *Cancer Cell Int* 2013; Michelakis ED, et al., 2008]. This makes tumours detectable by FDG PET imaging. In addition, tumour cells have carbon anhydrase enzyme CA IX, this is a hypoxia-induced protein that is located at the cell membrane with an extracellular orientation [Swietach P, et al., *J Biol Chem* 2009; Chiche J, et al., *Cancer Res* 2009; Svastová E, et al., *FEBS Lett* 2004]. This enzyme converts CO₂ into acid. The low pH paralyses the immune response against tumour cells [Kato, et al., 2013; Michelakis ED, et al., *Br J Cancer* 2008].
 - e. Shifting metabolism away from mitochondria (aerobic glycolysis) and towards the cytoplasm (anaerobic glycolysis), suppresses apoptosis according to the intrinsic pathway, a form of cell death that is dependent on mitochondrial energy production. This improves tumour cell survival [Kato, et al., *Cancer Cell Int* 2013; Michelakis ED, et al., *Br J Cancer* 2008; Koff JL, et al., *Int J Mol Sci* 2015].
3. Self-sufficiency in growth signals. [Zitvogel L, et al., *Nat Rev Immunol* 2006].
 - a. Heterotypic signalling is the process of production of soluble mitogenic growth factors by one cell type to stimulate proliferation of other cells. Many cancer cells acquire the ability to synthesize growth factors to which they are responsive.

- This creates a positive feedback signalling loop that is known as autocrine stimulation.
 - Some of the autocrine growth factors that are produced by tumour cells not only provide growth stimulatory signals but also subvert the immune response simultaneously (see next table).
- b. Overexpression of growth-factor receptors might enable cancer cells to become hyper-responsive to ambient amounts of growth factors that would not normally trigger proliferation. On the other hand, such overexpression might cause ligand-independent signalling. For example:
- The epidermal-growth-factor receptor (EGFR; also known as Erb-B1) and Erb-B2 is upregulated in some cancers. Both EGFR and Erb-B2 are important tumour antigens for the induction of T-cell responses, and patients with tumours that overexpress either of these growth-factor receptors often mount immune responses to EGFR- or Erb-B2-derived peptides.
 - The activating mutation of signal transducers such as Ki-RAS, which is frequently mutated in colon carcinoma. Mutated Ki-RAS has been assessed as a potential antigen for incorporation in anti-tumour vaccines.
4. Insensitivity to anti-growth signals. In contrast to TP53 that receives inputs from stress and abnormality sensors that function within the cell's intracellular operating systems, retino-blastoma associated protein (RB) transduces growth-inhibitory signals that originate largely outside of the cell. Defects in the RB pathway function may lead to persistent cell proliferation [Hanahan D, et al., Cell 2011; McClatchey AI, et al., Curr Opin Cell Biol 2012; Batson J, et al., J Microsc 2013].
5. Evasion of contact inhibition and the ability to invade and migration (metastasis) [Hanahan D, et al., Cell 2011].

The mechanisms that are involved in contact inhibition, invasive growth and metastasis:

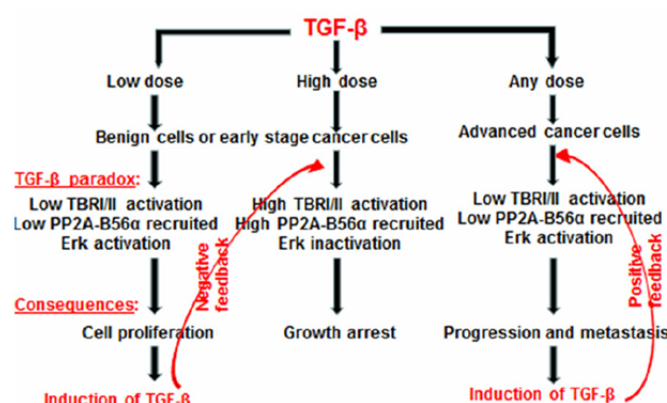
a. microRNA-198 (miR-198) is suppressed in many types of cancer cells. Possible consequences are:

- High expression of mitogenic signal transducers and of the TGF- β receptors as illustrated below [Elfimova N, et al., Biochim Biophys Acta 2013].

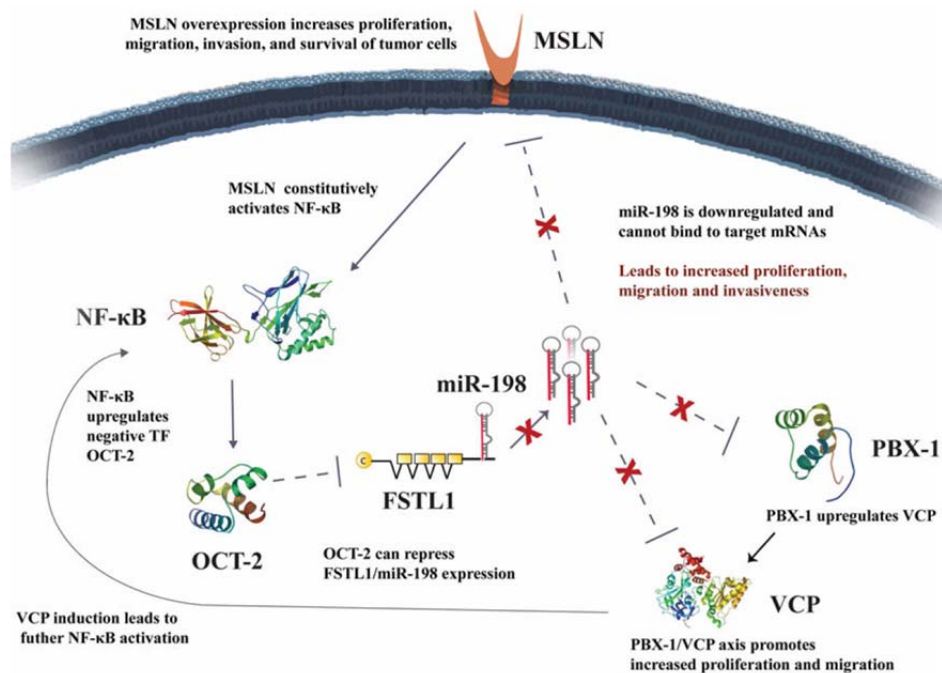


TGF- β is known to inhibit cell growth in benign cells but promote progression in cancer cells. The TGF- β paradox: In cancer cells TGF- β promotes invasive progression and metastasis as illustrated below [Zhang Q, et al., Am J Clin Exp Urol 2014].

TGF- β paradox between benign & malignant cells:



- Mesothelin (MSLN) is a cell surface glycoprotein overexpressed in ~90% of human pancreatic adenocarcinomas. MSLN overexpression leads to increased cell proliferation, invasion, and migration in vitro and increased tumour growth in vivo, and constitutively activates NF- κ B to promote cell survival. Down-regulation of miR-198 is involved in a complex reciprocal regulatory loop with MSLN, which represses miR-198 even further through NF- κ B-mediated OCT-2 induction (NF- κ B = nuclear factor kappa-light-chain-enhancer of activated B cells, is a protein complex that is involved in the control of the transcription of DNA). This increases tumorigenesis as illustrated below [Marin-Muller C, et al. Clin Cancer Res 2013].



- Decreased expression of miRNA-429 (miR-429) leads to promotion of bone metastasis of breast cancer cells. Overexpression of miR-429 remarkably suppresses invasion in vitro. Targets for decreased expression of miR-429 are: overexpression of ZEB1 gene (Zinc finger E-box-binding homeobox) and CRKL gene (CRK-like protein). Both genes have oncogenic properties [Ye ZB, et al., In J Oncol 2015].
- Merlin (neurofibromin), the cytoplasmic NF2 gene product, orchestrates contact inhibition via coupling cell-surface adhesion molecules (e.g., E-cadherin) to transmembrane receptor tyrosine kinases (e.g., the EGF receptor). Merlin strengthens the adhesivity of cadherin-mediated cell-to-cell attachments. Additionally, by sequestering growth factor receptors, Merlin limits their ability to efficiently emit mitogenic signals. Loss of the NF2 gene is observed in neurofibromatosis [Hanahan D, et al., Cell 2011].
- Liver kinase B1 (LKB1), an epithelial polarity protein, which organizes epithelial structure and helps maintain tissue integrity [Hanahan D, et al., Cell 2011].
 - LKB1 can overrule the mitogenic effects of the powerful Myc oncogene when the latter is upregulated in organised, quiescent epithelial structures. Suppression of LKB1 leads to destabilisation epithelial integrity is destabilised, and epithelial cells become susceptible to Myc-induced transformation.
 - LKB1 has also been identified as a tumour suppressor gene that is lost in certain human malignancies.
- Activation of invasion and metastasis [Hanahan D, et al., Cell 2011; Eccles SA, Int J Dev Biol. 2011]:
 - Involves the loss of E-cadherin, a key cell-to-cell adhesion molecule. By forming adherence junctions with adjacent epithelial cells, E-cadherin helps to assemble epithelial cell sheets and maintain the quiescence of the cells within these sheets. Increased expression of E-cadherin was well established as an antagonist of invasion and metastasis, whereas reduction of its expression was known to potentiate these phenotypes. The frequently observed down-regulation and occasional mutational inactivation of E-cadherin in human carcinomas provided strong support for its role as a key suppressor of this hallmark capability.
 - By redirecting TGF- β signalling away from suppressing cell proliferation to activate a cellular program: the epithelial-to-mesenchymal transition (= EMT).
 - The epidermal growth factor receptor (EGFR) and the closely related HER2 (human epidermal growth factor 2 = c-Erb-B2), Erb-B3 and Erb-B4 are type 1 transmembrane receptor tyrosine kinases (RTK)[Eccles SA, Int J Dev Biol 2011]. Mutations in the GRB7 gene leading to overexpression of EGFR will activate several pathways within the cells, for example:

1. Promoting cell growth and opposing apoptosis activities: mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K/Akt), phospholipase C γ , protein kinase C (PKC), Janus kinase (JAK) signal transducer and activator of transcription (STAT) pathway.
2. Promote metastasis by:
 - a. Upregulation of VEGF resulting in stimulation of angiogenesis,
 - b. alteration of cell-cell adhesion, cell matrix adhesion, upregulation of proteases stimulating tumour cell detachment and rapid migration resulting in local spread and distant metastasis through lymph nodes and blood stream.
6. The ability to attract and sustain angiogenesis for nutrient supply. Fast growing tumours have high needs for nutrients and oxygen and for the ability to evacuate metabolic wastes and carbon dioxide. Tumour angiogenesis addresses these needs [Hanahan D, et al., Cell 2011; Eccles SA, Int J Dev Biol. 2011]:
 - a. During tumour development the “angiogenic switch” is typically activated causing the vasculature to continually sprout new vessels. The gradation of the “angiogenic switch” depends on the type of the tumour.
 - b. The most important element in this process is the VEGF-A gene, the VEGF signalling runs via three receptor tyrosine kinases (VEGFR-1–3) and is a complex system regulated at multiple levels.
 - c. VEGF gene expression can be upregulated by hypoxia, by oncogene signalling and EGFR activation.
 - d. Pericytes are important components of tumour vasculature.
 - e. There is evidence for suppressed expression of endogenous angiogenesis inhibitors such as plasmin (angiostatin) and type 18 collagen (endostatin) in tumour tissue.
7. **The ability to suppress the immune system.**

Extrinsic tumour suppression mechanism: cancer immunoediting

Cancer immunoediting engages only after cellular transformation has occurred and intrinsic tumour suppressor mechanisms have failed as illustrated by the next figure [Zitvogel L, et al., Nat Rev Immunol 2006; Dunn GP, et al., Nat Immunol 2002; Schreiber RD, et al., Science 2011; Mittal D, et al., Curr Opin Immunol 2014].

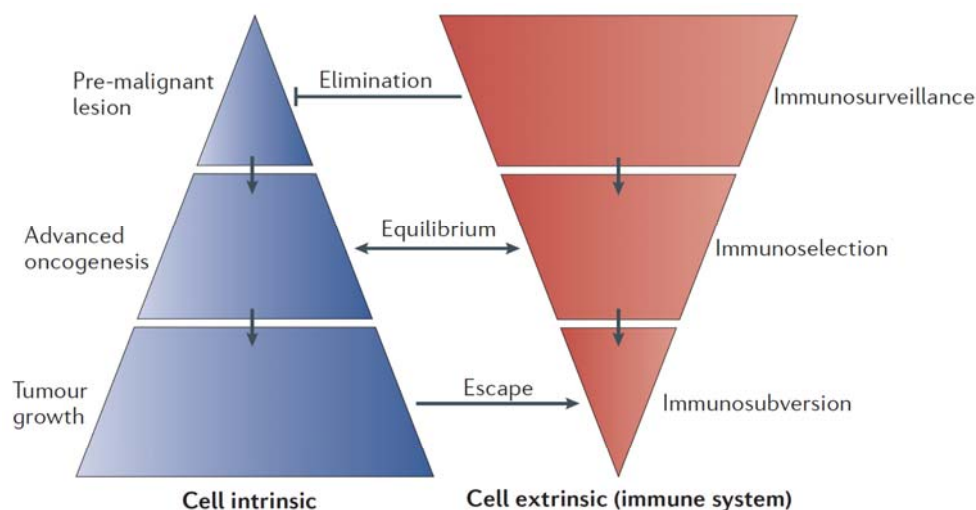
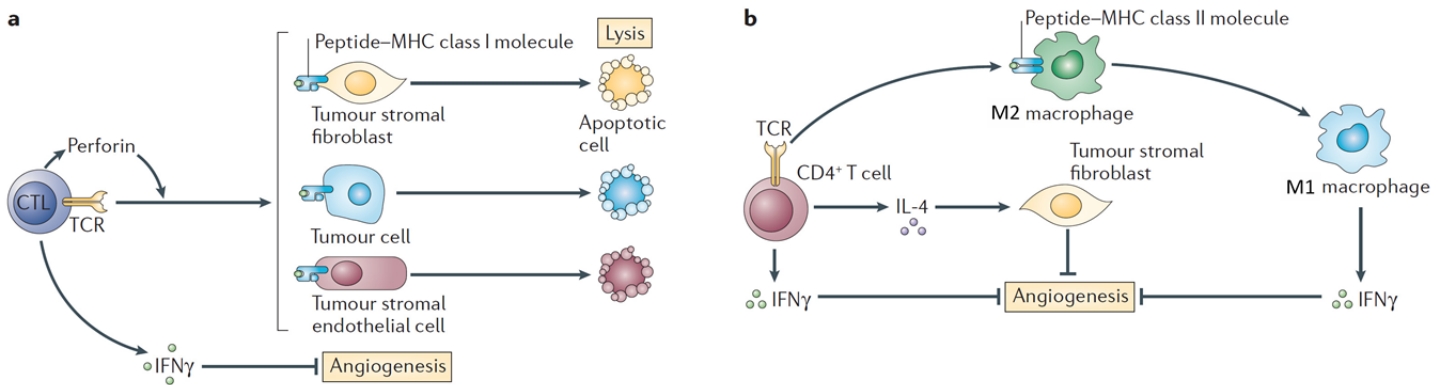


Figure 1 | **Relationship between cell-intrinsic and cell-extrinsic aspects of tumour progression.** This figure illustrates the central concept that multistep carcinogenesis results from crosstalk of cancer-cell-intrinsic factors and host immune system (cell-extrinsic) effects.

[Zitvogel L, et al., Nat Rev Immunol 2006]

The process may be simplified into three phases:

- 1. The elimination phase (= immunosurveillance)** [Mittal D, et al., Curr Opin Immunol 2014, Zitvogel L, et al., Nat Rev Immunol 2006]: Elimination is the phase where the innate and adaptive immune systems co-operate to detect and destroy the individual tumour cells before they become a mass.

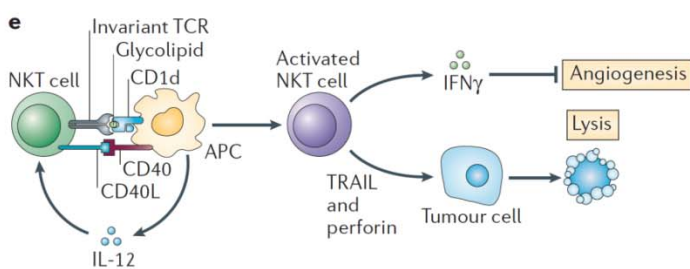
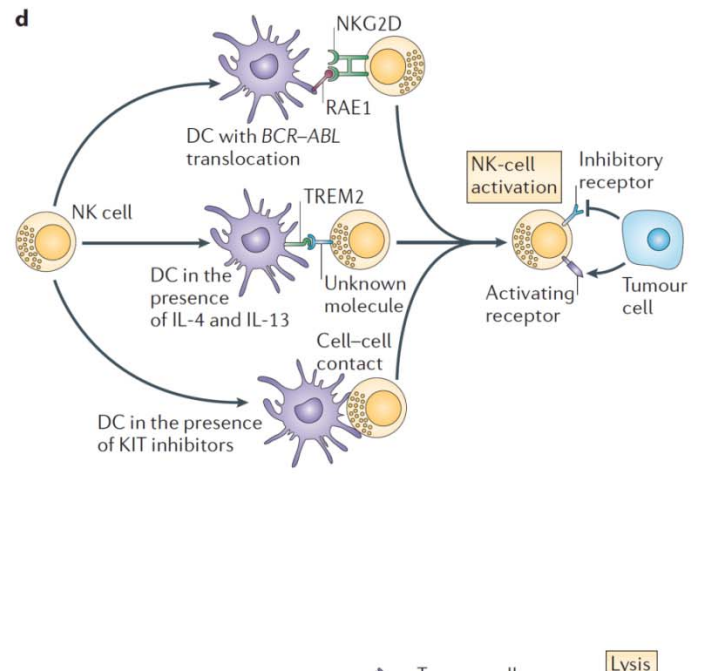
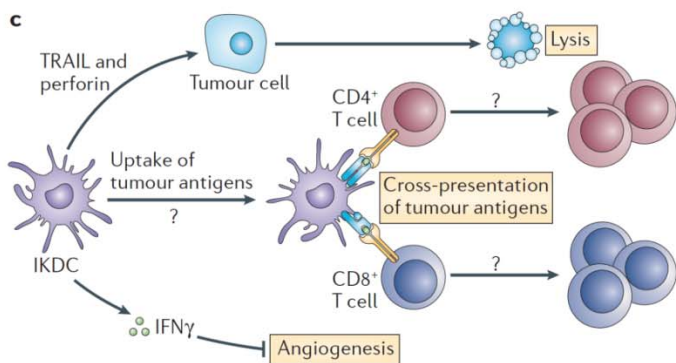


a. CD8+ cytotoxic T lymphocytes (CTLs) (see figure above):

- Recognize and kill stromal and/or tumour cells in a MHC-restricted and perforin-dependent manner.
- In addition, they secrete the anti-angiogenic cytokine interferon- γ (IFN γ).

b. Activated CD4+ T helper cells (Th) (see figure above):

- Recognize tumour-infiltrating macrophages in a MHC-class-II-dependent manner. Converting interleukin-10 (IL-10)-producing M2 macrophages into IFN γ -producing M1 macrophages.
- TH1 cells can also release IFN γ , which inhibits angiogenesis.
- TH2 cells Produce IL-4 and block neo-angiogenesis indirectly, through an effect on stromal fibroblasts.



c. IFN-producing killer dendritic cells (IKDCs) (see figure above):

- Kill tumour cells in a TRAIL (tumour-necrosis factor (TNF)-related apoptosis-inducing ligand)-dependent and perforin-dependent manner.
- IKDCs are also a major source of IFN γ .
- IKDCs might also cross-present tumour antigens to T cells.

d. Natural killer cells (NKs) (see figure above):

- Can be activated in a NKG2D (NK group 2, member D)-dependent manner by dendritic cells (DCs) with a BCR-ABL (breakpoint-cluster region fused with Abelson leukaemia-virus protein) translocation.

- During chronic inflammation and in the presence of IL-4 and IL-13, DCs upregulate TREM2 (triggering receptor expressed on myeloid cells 2), which is required for NK-cell triggering.
 - Inhibitors of KIT (growth factor receptor associated with tyrosine kinase activity) promote NK-cell activation through cell-cell contact between DCs and NK cells.
- e. Natural killer T-cells (NKTs) recognize glycolipids bound to CD1d at the surface of antigen-presenting cells (APCs) (see figure above). Activated NKTs secrete IFN γ in a CD40- and IL-12-dependent manner and can lyse tumour cells through a MHC-unrestricted, TRAIL or perforin-dependent pathway.
2. **The equilibrium phase (= immunoselection)** [Dunn GP, et al., Nat Immunol 2002]. The immune system holds the tumour in a state of functional dormancy. This is probably a very prolonged process. The essentials are:
- The balance of elimination promoting cytokine (IL-12) and persistence promoting cytokine (IL-23) maintain the tumour in equilibrium. These cytokines share the common subunit IL-12p40.
 - A minor tumour-promoting role for IL-10 was also shown.
 - Many other pathways (e.g. IL-4, IL-17A, TNF, IFN $\alpha\beta$) do not appear to play an important role.
 - Important immune resistance mechanisms involve, amongst others, immunosuppressive myeloid derived suppressor cells (MDSCs) and immune-inhibitory pathways, termed **immune checkpoints**, which normally (in inflammatory processes other than tumour environment) mediate immune tolerance and mitigate collateral tissue damage [Kanterman J, et al., Semin Cancer Biol 2012; Pardoll DM, Nat Rev Cancer 2012; Ceeraz S, et al., Trends Immunol 2013]
- Comparison of the cellular environment of tumours in equilibrium versus those that escape: higher proportions of CTLs, NKs, $\gamma\delta$ T-cells and lower proportions of NKT-cells, Foxp3+ Treg cells, and MDSCs are associated with the equilibrium phase. This means that tolerance and rejection of the tumour cells (which are regulated by the **immune checkpoints**) are more or less in balance.
- e. Coordination of the interaction between IFN- γ and TNF by the tumour antigen-specific T-cells. In the absence of either TNFR or IFN- γ , the tumour antigen-specific T-cells promote angiogenesis and multistage carcinogenesis. While the combination of IFN- γ and TNF drives cancers into senescence by inducing permanent growth arrest.

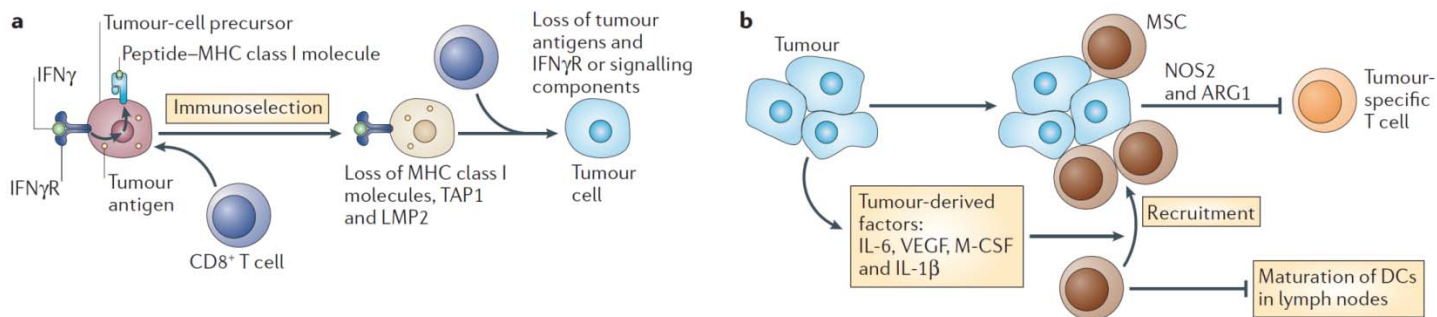
Table 1 | **Impact of cancer-cell-intrinsic characteristics on the immune system**

Cancer hallmark	Factor involved or characteristic	Effect on immune response	Refs
Self-sufficiency in growth signals	IL-4	• Promotes differentiation into T _H 2 cells	50
	IL-6	• Inhibits inflammatory response, through aberrant JAK-STAT3 signalling	51,53
	IL-10	• Promotes differentiation into T _H 2 cells	50,52,53
Insensitivity to antigrowth signals	TGF β resistance	• Inhibits antigen presentation • Inhibits T-cell proliferation • Inhibits NK-cell cytotoxicity • Activates T _{Reg} cells	23,57,58
Evasion of apoptosis	BCL-2-family proteins	• Tumour antigens	66
	MUC1	• Subverts DC function • Tumour antigen	68,69
	Survivin	• Promotes T-cell apoptosis, through inducing CD95L expression by tumour cells • Tumour antigen	71,72
	Reduced caspase activation	• Reduces tumour immunogenicity	82
Limitless replicative potential	Mutated p53	• Tumour antigen	86
	TERT	• Tumour antigen	84,85
Sustained angiogenesis	COX2	• Promotes T _H 2-cell responses • Inhibits T-cell cytotoxicity	90-92
	VEGF	• Inhibits T-cell activation • Inhibits NF- κ B activation in DCs	87,88
Tissue invasion and metastasis	Reduced NECL2 expression	• Inhibits NK-cell cytotoxicity, through CRTAM • Inhibits CD8 ⁺ T-cell IFN γ production, through CRTAM • Leads to loss of adherence at epithelial cell-cell junctions	93
	Altered lysosome trafficking	• Increases immunogenicity, through increasing cell-surface HSP70	95,96
	MMPs	• Induce cleavage of CD25 • Activate TGF β • Promote shedding of ICAM1 and ICAM2	98

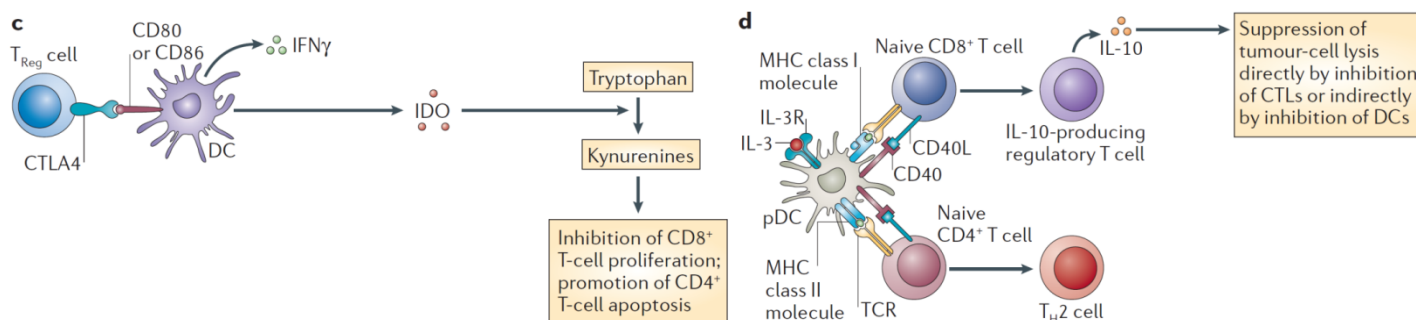
BCL-2, B-cell lymphoma 2; CD95L, CD95 ligand; COX2, cyclooxygenase-2; CRTAM, MHC-class-I-restricted T-cell-associated molecule; DC, dendritic cell; HSP70, heat-shock protein 70; ICAM, intercellular adhesion molecule; IFN γ , interferon- γ ; IL, interleukin; JAK, Janus kinase; MMP, matrix metalloproteinase; MUC1, mucin-1; NECL2, nectin-like 2; NF- κ B, nuclear factor- κ B; NK cell, natural killer cell; p53, tumour-suppressor protein p53; STAT3, signal transducer and activator of transcription 3; TERT, telomerase reverse transcriptase; TGF β , transforming growth factor- β ; T_H2 cell, T helper 2 cell; T_{Reg} cell, CD4⁺CD25⁺ regulatory T cell; VEGF, vascular endothelial growth factor.

[Zitvogel L, et al., Nat Rev Immunol 2006]

3. **The escape phase (= immunosubversion)** [Zitvogel L, et al., Nat Rev Immunol 2006; Mittal D, et al., Curr Opin Immunol 2014; Obermayer N, et al., Immunol Invest 2011]. In this phase the immune system fails to restrict tumour growth causing clinically apparent disease.



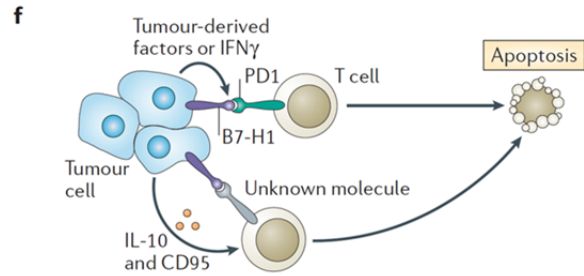
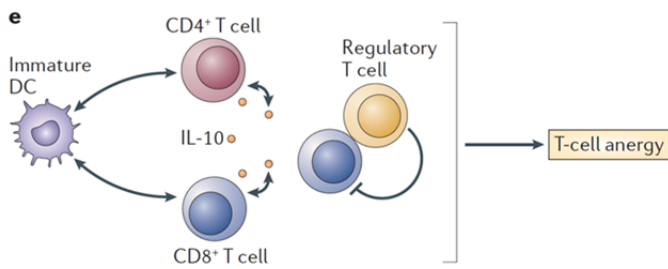
- As a result of immunoselection, tumour variants lose the antigen-processing machinery, antigens and sensitivity to immune effectors such as interferons (IFNs). The tumour cells evade immune recognition with loss of tumour antigens, MHC class I or co-stimulatory molecules (see figure above).
- Tumour-derived factors recruit myeloid suppressor cells (MSCs = MDSCs) and prevent their differentiation into mature dendritic cells (DCs), in a STAT3 (signal transducer and activator of transcription 3)-dependent manner. MSCs inhibit tumour-specific T cells (CTLs, NKTs) through arginase-1 (ARG1) or nitric-oxide synthase 2 (NOS2). The tumour cells express molecules of increased resistance (STAT-3), survival (anti-apoptotic molecule Bcl2) and immunosuppression (IDO, TDO, PD-L1, galectin-1/3/9, CD39, CD73, adenosine receptors) (see figure above).



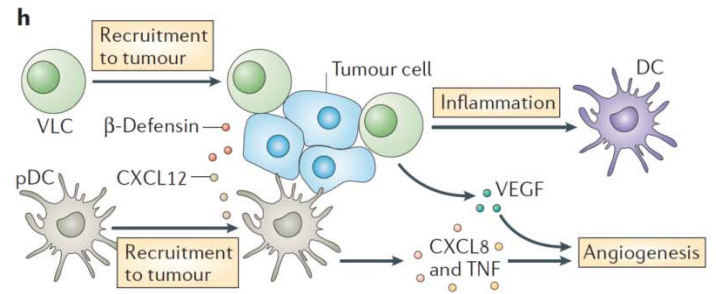
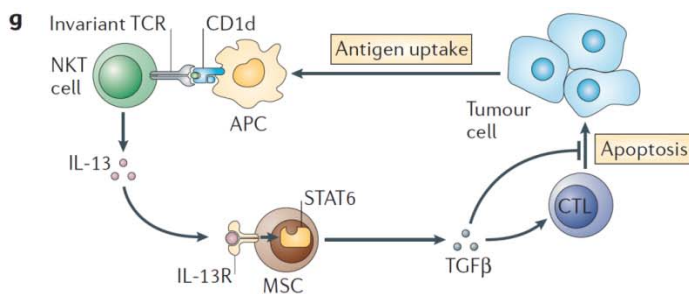
- MDSCs (MSCs) and IDO expressing DCs induce the generation of regulatory T-cells. T-cells including Tregs (Tregs = Foxp3 expressing cells), which are regulated by the **immune checkpoints**) may express co-inhibitory receptors such as PD-1, CTLA-4, etc. that may suppress anti-tumour immune response and favour tumour outgrowth. [Pardoll DM, Nat Rev Cancer 2012; Okazaki T, et al., Nat Immunol 2013; Ellestad KK, et al., Eur J Immunol 2014; Walker LS, et al., Trends Immunol 2015] (see figure above).

Regulation of the immune response by CTLA-4 receptors [Walker LS, et al., Trends Immunol 2015]:

- >90% of CTLA-4-expressing cells also express Foxp3, suggesting that CTLA-4 regulates immune responses mainly by modulating the function of Foxp3+ T-cells (Tregs).
 - <10% of CTLA-4 receptors are transiently expressed on the surface of T-cells within 24–48 h after activation
 - The interaction between CD80 or CD86, at the surface of DCs, and CTLA4 (cytotoxic T-lymphocyte antigen 4) at the surface of T cells or CD4+CD25+ Tregs, induces the production of IFN γ and the immunosuppressive factor indoleamine 2,3-dioxygenase (IDO) by DCs.
 - IDO, arginase, CD39 and CD73 are immunoregulatory enzymes. IDO catabolizes tryptophan to kynurenine, arginase catabolises L-arginine to ornithine and urea, CD39 metabolises ATP to AMP, which can further be metabolised to adenosine by CD73. Adenosine can bind to adenosine receptors (A2aR and A2bR) expressed on tumour cells, endothelial cells and immune cells. This results in a reduction of the amount of tryptophan, which is T-cell tropic, and in the generation of kynurenines, which suppresses the effector cells activity through the action of Tregs (extrinsic effector cells suppression).
- Plasmacytoid DCs (pDCs) activated by interleukin-3 (IL-3) and CD40 ligand (CD40L) promote the differentiation of naive CD4+ and CD8+ T cells into T helper 2 (TH2) cells and anergic IL-10-producing CD8+ Tregs, respectively. This state of anergy (with respect to tumour-cell lysis) is mediated by IL-10, either directly (by interaction with cytotoxic T lymphocytes, CTLs) or indirectly (by inhibition of DCs). (TCR=T cell receptor) (see figure above).



- e. Repetitive stimulation of naive T cells with immature DCs results in T-cell anergy, together with IL-10 production and IL-10-independent, cell-contact-dependent (Tregs) regulatory activity (see figure above).
- f. B7-H1 (and B7-H4) is expressed by some tumours (for example, in response to IFN γ), and it directly promotes T-cell INACTIVATION through programmed cell death 1 (PD1)-dependent pathways or by PD1-independent pathways (which are mediated by IL-10 or CD95) (see figure above).
- Regulation of the immune response by PD-1 receptor [Okazaki T, et al., Nat Immunol 2013; Ellestad KK, et al., Eur J Immunol 2014; Franceschini D, et al., J Clin Invest 2009]
 1. PD-1 is expressed on effector cells such as CD4–CD8– double-negative $\alpha\beta$ and $\gamma\delta$ T-cells in thymus and on activated T-cells, B cells, natural killer cells, natural killer T-cells, CD4+ CD25+ Foxp3 Tregs and myeloid cells in the periphery.
 2. On B-cells, PD-1 is essential for the generation and selection of high-quality, high-affinity antibodies.
 - The PD-1 receptor has two ligands [Okazaki T, et al., Nat Immunol 2013; Ellestad KK, et al., Eur J Immunol 2014; Walker LS, et al., Trends Immunol 2015]
 1. PD-L1, being highly expressed in non-lymphoid cells, including parenchymal cells, tumour cells and virus infected cells, which allows PD-1 to directly inhibit effector cells against these target cells. In other words: PD-L1 expressing tumour cells inhibit T-cell mediated cytotoxicity against them.
 2. The other ligand of PD-1 is PD-L2. This expression of PD-L2 is restricted to professional APCs. In contrast to other APCs, professional APCs display a fragment of the antigen, bound to a class II MHC molecule, on their membrane (dendritic cells, B-cells, etc.).
 - As with the interaction between CTLA-4 receptors and CD80/CD86 ligands, PD-1 and PDL-1 interaction is also regulated by the **immune checkpoints**. PD-1 regulates the **threshold of immune response** and **peripheral tolerance**. The action of PD-1 can be compared to a rheostat or a potentiometer [Okazaki T, et al., Nat Immunol 2013; Ellestad KK, et al., Eur J Immunol 2014; Walker LS, et al., Trends Immunol 2015]. Engagement of PD-1 by either PD-L1 or PD-L2 results in decreased proliferation of effector T-cells and decreased IFN- γ , IL-10, IL-4, and IL-2 secretion from anti-CD3-stimulated T-cells without an increase in cell death. Indeed, in contrast to what has been claimed in previous publications, engagement of PD-1 by one of its ligands does not increase the chance for apoptosis of the PD-1 containing cell. This indicates that the nomenclature for this receptor and its ligands is perhaps misleading [Ellestad KK, et al., Eur J Immunol 2014].



- g. CD4+ natural killer T (NKT) cells produce IL-13, which suppresses CTL-mediated tumour rejection through a pathway that involves the α -chain of the IL-13 receptor (IL-13R α) and STAT6. IL-13 produced by NKT cells can also activate MSCs to produce transforming growth factor- β (TGF β), which suppresses CTLs (see figure above).
- h. Vascular leukocyte cells (VLCs) and pDCs (plasmacytoid DCs) are attracted to tumour beds through β -defensins and CXC-chemokine ligand 12 (CXCL12), respectively. The subsequent angiogenic effect is mediated by CXCL8 in the case of pDCs and by vascular endothelial growth factor (VEGF) in the case of VLCs, which can differentiate into endothelial cells or into bona fide DCs during acute inflammation. Tumour cells secrete cytokines VEGF, TGF- β , IL-6, M-CSF that enhance angiogenesis (see figure above).

Summary of escape phase

In the escape phase, the balance is skewed towards tumour progression due to the presence of immunosuppressive cytokines and molecules such as IL-10, TGF- β , VEGF, IDO, PD-L1.

Tumour mass may well consist of tumour stem cells (10% up to 90% depending on the type of tumour), more differentiated tumour cells resemble the original tissue which was under repair during chronic inflammation, and the so called tumour matrix (90% to 10% depending on the type of tumour). The tumour matrix includes, amongst others, immunosuppressive T lymphocytes (Tregs), MDSCs, cancer associated fibroblasts (CAFs), and tumour associated macrophages (TAMs). The tumour matrix is in fact an important defence mechanism. Obviously, single tumour cells are more vulnerable to immune attacks than large tumour masses [Lu P, et al., J Cell Biol 2012].

The important role of chronic inflammation in Intrinsic and extrinsic tumour suppression

Chronic inflammation may be induced by repetitive extended exposure to sunburns, radioactive radiations, certain chemical compounds, cigarette smoke, oncogenic viruses, etc.

There is a high concentration of free radicals in the chronically inflamed environment. These free radicals are produced by the innate and adaptive immune systems in their action of killing the infected or damaged cells. The toxic microenvironment considerably increases the risk of multiple point mutations, deletions or rearrangements of DNA. The damaged cells are normally destroyed by the **intrinsic tumour suppression** mechanism [Houghton J, et al., Science 2004; Li HC, et al., World J Gastroenterol 2006]. But, when **intrinsic tumour suppressor** mechanisms have failed, the mutated cells remain alive and **cancer immunoediting** becomes engaged.

In some areas of the inflamed tissue, the balance between inflammation and immunosuppression will tip towards immunosuppression to mitigate collateral damage by the chronic inflammation. The activation of the Tregs by CTLA-4 and PD1 receptors regulated by the **immune checkpoints** play a crucial role. **As a consequence, in these areas, the tumour may get into the escape phase** [Kanterman J, et al., Semin cancer Biol 2012; Pardoll DM, Nat Rev cancer 2012; Obermajer, et al., Immunol Invest 2012; Dunn GP, et al., Nat Immunol 2002; Schreiber RD, et al., Science 2011; Mittal D, et al., Curr Opin Immunol 2014]. There are many types of tumours that are associated with chronic inflammation (see next table) [Shacter E, et al., Oncology 2011; Rook GA, Dalglish A, Immunol rev 2011].

Table 1. The major infectious agents that trigger cancer

Mechanism	Organism	Cancer	References	
Infect and transform lymphoid cells	Epstein-Barr virus (EBV)	Burkitt's lymphoma*	(4)	
	Human Herpesvirus 8 (HHV8)	Kaposi sarcoma		
	Human T-lymphotropic virus I (HTLV-1)	T-cell leukemia		
Transformation	HPV	Cervical cancer	(2)	
Inflammation and partial integration	HBV	Hepatocellular carcinoma	(2)	
Chronic inflammation and oncogenic proteins	Hepatitis C virus (HCV)	Hepatocellular carcinoma	(4)	
Chronic stimulation of lymphocytes by pathogen antigens and/or autoantigens	<i>Helicobacter pylori</i>	Gastric lymphoma	(3)	
	<i>Campylobacter jejuni</i> †	Immunoproliferative small intestinal dis.	(29)	
	<i>Borrelia burgdorferi</i>	Cutaneous lymphoma	(3)	
	<i>Chlamydia psittaci</i>	Ocular lymphoma	(30)	
	Hepatitis C virus (HCV) ‡	Spleen lymphoma	(3)	
	<i>Bacteroides fragilis</i> (enterotoxigenic)	Colorectal cancer	(146, 147)	
Chronic inflammation	Malaria (cofactor)	Burkitt's lymphoma	(7)	
	Tuberculosis	Lung cancer	(9, 10)	
	Schistosomiasis	Bladder cancer	(2, 5)	
	Liver flukes	Cholangiocarcinoma	(5)	
	<i>Salmonella</i> spp.	Gallbladder cancer	(8)	
	<i>Helicobacter pylori</i>	Gastric and esophageal adenocarcinoma	(113, 115)	
	Chronic inflammation; possible associations	<i>Propionibacterium acnes</i> ?	Prostate	(148)
		<i>Mycoplasma</i> spp. ?	Prostate	(149)
		?? unknown prostatitis ??	Prostate	
Immunosuppression	HIV	EBV+ CNS lymphomas	(4)	
		HHV8+ sarcoma (Kaposi)		
		HPV+ ano-genital cancers		

*EBV also associated with nasopharyngeal carcinoma, Hodgkin lymphoma and non-Hodgkin lymphoma.

†*C. jejuni* produce CdtB (cytolethal distending toxin B) that causes direct DNA damage.

‡HCV also encodes oncogenic proteins.

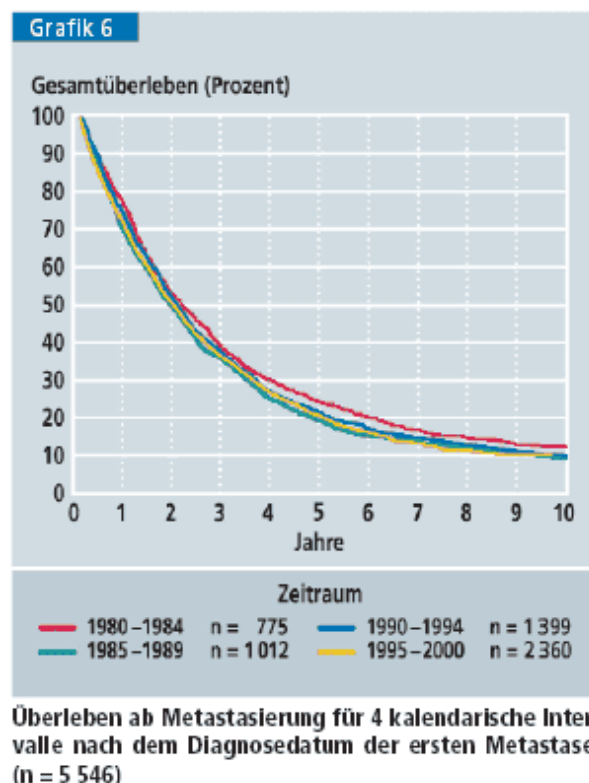
The treatment of cancer

The best treatment of cancer is: **radical curative surgery**. Unfortunately, often enough, radical surgery is not possible because of infiltration of the tumour cells in the surrounding tissues, presence of tumour cells in the lymph nodes and/or distant metastasis. In these cases several other options are available:

1. Modern therapy of advanced tumour consists of the combination of non-curative surgery and immunotherapy. Additionally, treatment of the underlying chronic inflammation is mandatory. An example of a rather effective tumour therapy, with a high probability of durable remission is given below:
 - a. Tumour mass debulking to minimize the ultra-defensive tumour matrix with its strong immunosuppressive effect.
 - b. Eliminate chronic inflammation to prevent new tumour mass formation (for example antibiotic treatment against *Helicobacter Pylori* in case of gastric cancer, non-Hodgkin lymphoma, etc.) and to reduce resistance to apoptosis (for example due to cytomegalovirus infection [Goldmacher VS, et al., Apoptosis 2005; Cai Q, et al., PLoS Pathol 2011]) and to reduce multiple drug resistance (MDR, for example Epstein-Barr virus infection and cytomegalovirus infection [Banerjee AS, et al., Virology 2013; Cinatl J Jr, et al., Cancer Res 1998; Allart S, et al., J Biol Chem 2002; Lu J, et al., Virology 2011]).
 - c. Attack the tumour cells by means of autologous primed dendritic cells, with the help of an oncolytic virus [Nesselhut J, et al., J Clin Oncol 2011, 2013, 2014; Schirmacher V, et al., Front Oncol 2014] or without the help of an oncolytic virus [Dillman RO, et al., J Immunother 2012; Nesselhut J, et al., J Clin Oncol 2009, 2010]. Dendritic cell therapy in combination with New Castle Disease Virus (NDV) as performed at the “Praxisgemeinschaft für Zelltherapie Duderstadt” increases the efficacy of the dendritic cell therapy.
 - To specifically attack the tumour and leave the normal tissue alone, one marks the tumour by administering New Castle Disease Virus (NDV, oncolytic virus) to the patient. Cytokine production (IFN- α) of the normal cells will neutralize the virus immediately. Cancer cells are lacking IFN- α and a certain proportion of the cancer cells will be killed by the virus. The remaining cancer cells survive the attack, but are infected by the virus and present the NDV antigen on their surface. Moreover, on the surface of tumour cells the p2X7 receptor (a purigenic P2 receptor) are mutated and show an open configuration exposing the p2X7 antigens [Sluyter R, et al., Recent Pat DNA Gene Seq 2011].
 - The autologous monocytes of the patient are transformed, using appropriate stimuli, to dendritic cells in cultures at the lab. During this process we expose the dendritic cells to NDV, P2X7 antigen and, when available, to tumour antigens. When the dendritic cells have almost matured, they were administered back to the patient. The dendritic cells will instruct the NKTs and CTLs in the body to attack the tumour. In this way an effective and specific (adaptive) immunity against cancer cells is achieved.
 - This method resulted in 100% success in killing the tumour cells in vitro, but in patients with metastatic disease or glioblastoma, the response rate is approximately 40% with a median 1-year survival of approximately 30%. In postoperative colono-rectal cancer with a non-detectable disease on imaging after tumour removal: 100% response rate and 80% survival after 5 years [*personal communication by Dr. Thomas Neßelhut, data are not published*]. This is due to the protection of cancer cells by immunosuppressor cells (Tregs, MDSCs, and others) [Nesselhut J, et al., J Clin Oncol 2009]. The T-regs are, amongst others, controlled by the **immune checkpoints** through PD-1 (programmed cell death protein), PD-2, and CTLA-4 (cytotoxic T-lymphocyte-associated protein) receptors [Pardoll DM, et al., Nat Rev Cancer 2012].
 - d. It makes sense to combine DC treatment with anti-immunosuppression therapy. These two therapy modalities are complementary. The most effective way to destroy the immunosuppression, appeared to block the PD-1 receptors of the effector cells of the immune system with anti-PD1 receptor antibody nivolumab (1-year overall survival of 73%) [Robert C, et al., New Engl J Med 2014]. As PD-1 is expressed on CD4-CD8- double-negative $\alpha\beta$ and $\gamma\delta$ T-cells in thymus and on activated T-cells, B cells, natural killer cells, natural killer T-cells and myeloid cells in the periphery, blocking the PD-1 receptor activates these cells. The combination of DC with NDV and the PD-1 receptor antibody nivolumab (= Opdivo, Bristol-Myers Squibb, see below) promises to significantly increase the efficacy of each individual treatment when administered separately, especially in pancreatic cancer, where PD-1 receptor antibody treatment has shown little effect on tumour growth and overall survival of the patients [Winograd R, et al., Cancer Immunol Res 2015].
2. Other cancer therapy with dendritic cells:
 - a. Dendritic cell therapy primed with tumour cell antigens in advanced melanoma resulted in 2-year survival rates of 72% (DC arm) versus 31% (control arm) (P=0.007) [Dillman RO, et al., J Immunother 2012].
 - b. Provenge (sipuleucel-T, Dendreon Corporation) are dendritic cells, produced from peripheral blood monocytes primed with recombinant antigen consisting prostate acid phosphatase. The 36-month survival probability of this treatment was 31.7% in the sipuleucel T group versus 23.0% in the placebo group [Kantoff PW, et al., N Engl J Med 2010].
3. Anti-immunosuppression therapy as monotherapy:
 - a. Certain immune checkpoint inhibitors are very effective in producing durable remissions in advanced melanoma:
 - Opdivo (nivolumab, Bristol Myers Squibb) anti-PD-1 monoclonal antibody: approved by the FDA and in Japan, and expected to be approved by EMEA (Europe) in the beginning of 2015 for metastasised melanoma: **1- year overall survival rate of 73%** [Robert C, et al., N Engl J Med 2014].

- Yervoy (ipilimumab, Bristol Myers Squibb) antibody against CTLA-4 receptors: approved by EMEA (Europe) and FDA (USA) for metastasised melanoma: **1- year overall survival rate of 46%**, drug related mortality of 2.1%, some patients develop thrombocytopenia, neutropenia, or autoimmune pancytopenia [Hodi FS, et al., N Engl J Med 2010].
 - Keytruda (pembrolizumab; Merck) anti-PD-1 monoclonal antibody, approved by FDA for metastasised melanoma: **1- year overall survival rate of 58% in the 2 mg/kg group and 63% in the 10 mg/ group** [Robert, et al., Lancet 2014].
- b. Decrease the activity of the myeloid derived suppressor cells (MDSCs)
- Cox-2 inhibitor is beneficial in the treatment of advanced cancers but with increased risk of cardiovascular events [Chen, et al., Clin Ther 2014].
4. Traditional therapy of advanced cancer consisting of the combination of non-curative surgery, radiation and/or chemotherapy.
- a. Chemotherapy and radiation therapy disrupt the immunosuppression and the tumour matrix. This renders the tumour cells vulnerable to immune attacks. Tumour antigens from damaged tumour cells will enter the lymphatic system and can be phagocytised by dendritic cells and other APCs. These APCs will present the antigens to the NKTs and CTLs and these cells will attack the tumour cells. The problem is that the traditional high dose chemotherapy also destroys the immune system. This annihilates the positive effects of chemotherapy.
- b. Almost all tumour stem cells have MDR (multiple drug resistance) properties. This makes them invulnerable to chemotherapy. The first cycle of chemo wipes out the tumour matrix and leads to unjustifiably high expectations. The composition of a regrown tumour after chemotherapy changes and has a higher proportion of tumour stem cells. The following cycles are predictably less efficient in reducing the tumour mass size.
- c. In the 2013 NHS report (320 pages!!) on “Clinical effectiveness and cost-effectiveness of first-line chemotherapy for adult patients with locally advanced or metastatic non-small cell lung cancer: a systematic review and economic evaluation”, it is made clear that conventional chemotherapy is neither clinically effective, nor cost effective [Brown T, et al., Health Technol Assess 2013].
- d. According to the analysis of the data of Munich Cancer Registry (1980-2000), survival of patients with metastasised breast cancer has not been influenced by the time of diagnosis of the primary tumour or by the time of the metastasis in 20 years. Chemotherapy was an important treatment of metastasised breast carcinoma in this period. In other words, the development of chemotherapy in advanced breast cancer did not result in the improvement of the overall survival in the period 1980-2000 (see next figure) [Schlesinger-Raab A, et al., Dtsch Arztebl 2005].

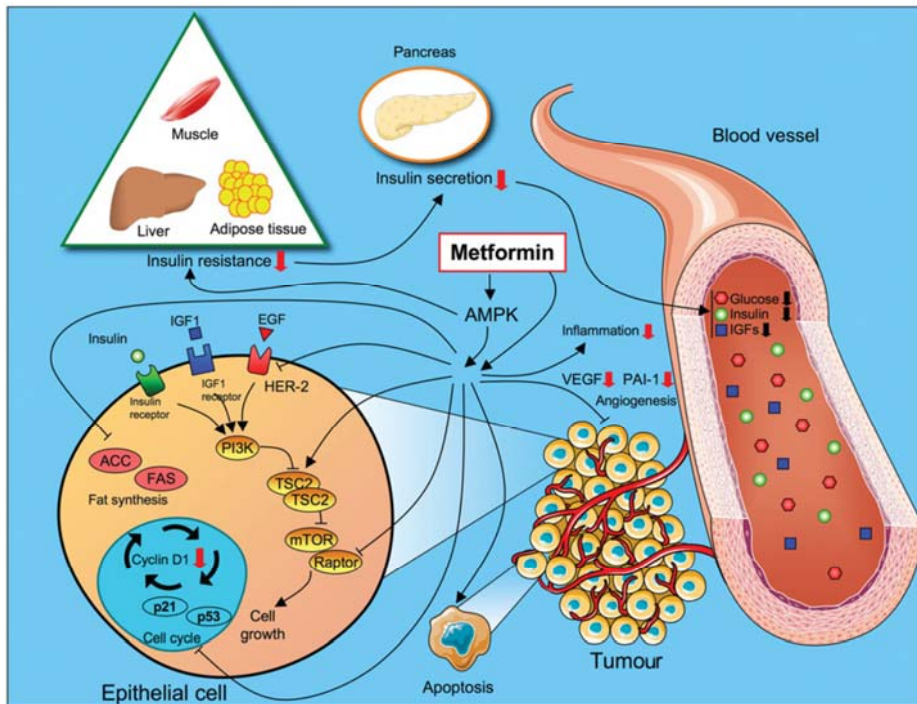
Overall survival of patients with metastasised breast cancer, stratified to the calendar date of the first diagnosis (n=5546) [Schlesinger-Raab A, et al., Dtsch Arztebl 2005].



- e. Despite the above mentioned facts, this therapy proved to be very effective in: gestational trophoblastic neoplasia, testicular cancers, acute lymphoblastic leukaemia (ALL) in children, non-Hodgkin lymphoma in young adults, etc. But in general, chemotherapy is inefficient to produce durable remission and clinically significant overall survival in most **advanced** solid tumours [Morgan G, et al. Clin Oncol (R Coll Radiol) 2004].
- f. In addition, massive and serious side effects of chemotherapy make this therapy unbearable to a considerable proportion of patients [Braun MS, et al., Ther Adv Med Oncol 2011].
5. Low dose and metronomic chemotherapy. When PD-1 antibody is not available, one may prescribe low dose chemotherapy as an adjuvant therapy to the combination of debulking operation and DC therapy in advanced cancer. The rationale for this strategy is given below:
- a. Low dose chemotherapy decreases immunosuppression, tumour growth, angiogenesis and metastasis, and leaves NKTs intact.
- Experimental administration of Cyclophosphamide 10 mg/kg instead of 40 mg/kg resulted in the down-regulation of natural and inducible Tregs while leaving the NKT I cells intact [Rico, MJ et al., Exp Oncol 2012].
 - Experimental low dose cyclophosphamide down-regulated the production of anti-inflammatory IL-10 by T-cells, NO by macrophages and TGF- β by both cell types [Matar P, et al., Int Immunopathol 2001].
 - Experimental low dose cyclophosphamide has an anti-metastatic effect through a switch from the anti-inflammatory Th2 response to inflammatory Th1 response(Th1/Th2 switch). The spleen production of IL-2 and IFN- γ is increased [Matar P, et al., Cancer Immunol Immunother 2002].
 - Experimental low dose gemcitabine also depletes Tregs and increases survival in the orthotopic Panc02 model of pancreatic cancer [Shevchenko, et al., Int J Cancer 2013].
 - Experimental low dose Cyclophosphamide selectively decreases Tregs and inhibits angiogenesis in dog soft tissue sarcoma [Burton JH, et al., J Vet Intern Med 2011].
- b. Low dose cisplatin increases autophagy and apoptosis in experimental lung cancer cell lines [Cho K-H, et al., Oncol Rep 2014].
6. Activation of macrophages
- Gc-MAF monotherapy: The good results of one study in low staged prostate cancer [Yamamoto N, et al., Transl Oncol 2008] proved proven to be ineffective in advanced cancer.
7. Epidermal growth factor receptor (EGFR) family consists of at least 4 tyrosine kinases: EGFR, human epidermal growth factor receptor 2 (HER2),HER3, HER4, etc. The EGFR family controls the intracellular signalling pathways that promote cell growth, proliferation, differentiation, migration, MDR and angiogenesis [Luo M, et al., Am J Cancer Res 2014].
- Gefitinib (Iressa, AstraZeneca and Teva), erlotinib (Tarceva, Genentech and OSI Pharmaceuticals, and Roche), and afatinib (Gilotrif in the US and Giotrif in Europe) out-performed chemotherapy in terms of progression-free survival, overall response rate, and disease control rate. However, no durable remission and/or clinically significant overall survival have been achieved with tyrosine kinase inhibitors [Haaland B, et al., J Thorac Oncol 2014; Li J, et al., Curr Med Res Opin 2013].
- Ruxolitinib is a Janus kinase inhibitor with selectivity for subtypes JAK1 and JAK2 of this enzyme. Ruxolitinib inhibits dysregulated JAK signalling associated with myelofibrosis. JAK1 and JAK2 recruit signal transducers and activators of transcription (STATs) to cytokine receptors leading to modulation of gene expression.
- Sunitinib (marketed as Sutent by Pfizer) is an oral, small-molecule, multi-targeted receptor tyrosine kinase (RTK) inhibitor that was approved by the FDA for the treatment of renal cell carcinoma (RCC) and imatinib-resistant gastrointestinal stromal tumour (GIST) both JAK1 and JAK2. It is the first approved JAK2 inhibitor for the treatment of myelofibrosis.
8. The use of oncolytic viruses as monotherapy [Lichty BD, et al., Nat Rev Cancer 2014]:
- a. Several clinical trials on oncolytic virus tumour therapy as monotherapy are ongoing.
- b. Different oncolytic viruses are of interest: measles, NDV, etc.
9. Metformin as adjuvant therapy combined with cancer immunotherapy. The anti-neoplastic action of metformin appears to be exerted by several pathways (see next figure) [Violett B, et al., Clin Sci (Lond) 2012; Kasznicki J, et al., Ann Transl Med 2014].
- a. Metformin inhibits the growth of cancer cells by the reversal of hyperglycaemia, insulin resistance and hyperinsulinaemia, resulting in reduced levels of glucose, insulin and IGFs and the activation of growth signalling pathways through their respective receptors.
- The anti-tumour effects of metformin appear to be regulated by both AMPK-dependent or -independent mechanisms, leading to inhibition of mTOR signalling, cell cycle by a decrease in cyclin D1 level, stimulation of

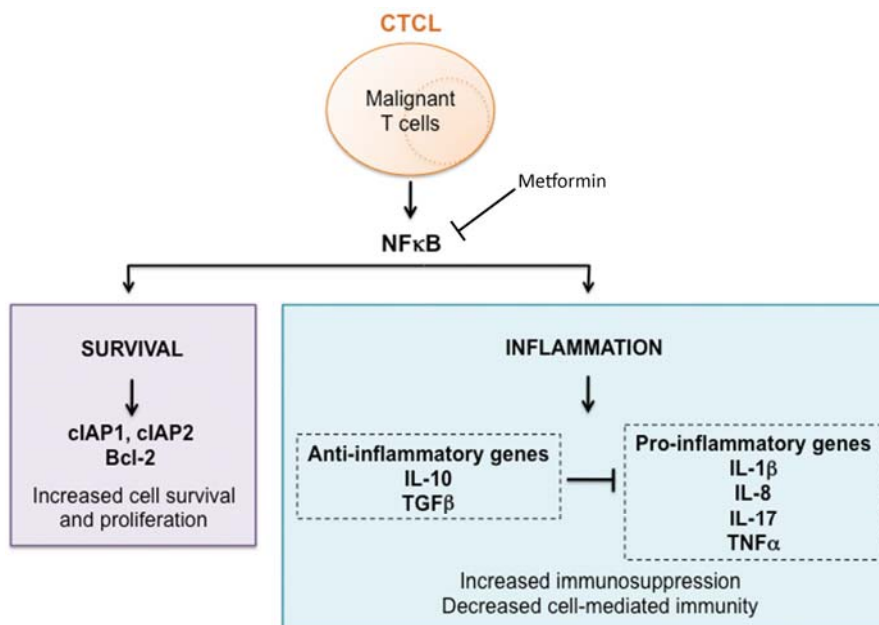
p53/p21 axis (tumour suppressor genes), fatty acid synthesis, angiogenesis and inflammation. EGF = epidermal growth factor; PI3K = phosphoinositide 3-kinase (see figure below).

- Several studies identified that LKB1 (another tumour suppressor gene), a major upstream kinase of AMPK, may be involved in anticancer action of metformin associated with inhibition of mTOR (see figure below).



- Moreover, metformin suppresses the activity of transcription factor nuclear factor-kappaB (NF-κB) and inhibits the induction of the expression of anti-apoptotic genes cIAP1, cIAP2 and Bcl-2, resulting in decreased survival of tumour cells (see figure below) [Chang T-P, et al., Am J Cancer Res 2013; Koh S-J, et al., J Gastroenterol Hepatol 2014].

- b. In addition, suppression of NFκB by metformin also induces the expression of anti-inflammatory genes (IL-10, etc.) and suppresses chronic inflammation (see figure below) [Chang T-P, et al., Am J Cancer Res 2013; Koh S-J, et al., J Gastroenterol Hepatol 2014].



10. Many researchers are investigating the possibility to correct the failed intrinsic tumour suppression mechanism:

a. B-RAF enzyme inhibitors

- Vemurafenib (Zelboraf; Genentech/Daiichi Sankyo), is a B-Raf enzyme inhibitor. Aimed at melanoma patients with V600E mutated BRAF.
- Dabrafenib (Tafinlar; GSK) is a B-Raf enzyme inhibitor. Aimed at melanoma patients with V600E mutated BRAF.

- b. MEK1 and MEK2 (mitogen-activated protein kinase kinase) inhibitor in melanoma patients with V600E mutated BRAF.
 - Trametinib (MEK 1 and MEK 2 inhibitor) was approved as a single-agent by the Food and Drug Administration in 2013 for the treatment of patients with V600E mutated metastatic melanoma.
 - Cobimetinib (GDC-0973, XL-518) by Exelixis and Roche
- c. **The combination of vemurafenib and cobimetinib in BRAF mutated melanoma are quite effective: 9-month overall survival rate of 81%** [Larkin, et al., N Engl J Med 2014].
- d. Bevacizumab (Avastin, by Genentech/Roche) is an angiogenesis inhibitor (monoclonal anti-VEGF antibody) that slows down the growth of new blood vessels. This drug is approved by FDA in 2008 for several types of cancer. The approval for breast cancer was withdrawn by FDA in 2011, because of lacking efficacy and safety data in breast cancer. In addition, bevacizumab appeared to be ineffective in glioblastoma [Chinot OL, et al. N Engl J Med 2014; Gilbert MR, et al., N Engl J Med 2014].

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