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**Immunoregulation in melanoma:
role of IL-10 and TGF- β**

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Summary

IL-10 and TGF- β are immunosuppressive cytokines found in tumors including melanoma and, therefore, deemed as the major cause for failing anti-tumor immune responses. However, conflicting evidence reported IL-10 and TGF- β -mediated immune stimulation urges a re-evaluation of their role in tumors. To clarify this discrepancy in melanoma, their expression was compared by quantitative RT-PCR in melanoma and the skin of healthy individuals. Furthermore, their induction in co-cultures of dendritic and T cells with tumor cells and their effects on immune cells were tested. Both, as well as their receptors were expressed in melanoma but at significantly lower levels than in healthy skin. Consequently, the expressions of SOCS-3 and SMAD-7 as responsive genes of IL-10 and TGF- β respectively were low in tumors but high in healthy skin. T cells co-cultured with a large number of tumor cells developed an anergic state but without a correlation with IL-10 or TGF- β production. In addition, the anergic state of T cells induced *in vitro* was reversed to a certain extent when a high concentration of exogenous IL-10 or TGF- β was added. A combination of IL-10 and TGF- β gave a better result than using either cytokine alone. However, a high concentration of IL-10 could suppress functional T cells, supporting the idea that IL-10 acts differently at different activation and functional stages of T cells. iDCs differentiated in the presence of tumor cells *in vitro* had a mix population of complete and incomplete differentiated iDCs, produced a high IL-10 level and were less efficient in inducing T cell proliferation. However, they could be induced to mature, and blocking IL-10 did not alter the capacity of the resulting mature DCs to induce CD4 T cell proliferation. Increased TGF- β production could only be seen when DCs were generated in direct contact with a large number of tumor cells. Blocking TGF- β during the differentiation of iDCs in the presence of tumor cells did not change the DC phenotype and capacity to induce CD4 T cell proliferation. These results indicate that both cytokines did not alter the capacity of DCs to induce CD4 T cell proliferation. DCs induced into maturation in the presence of tumor cells produced increased IL-10, in opposite to similar or even decreased TGF- β levels and were more efficient in inducing T cell proliferation. The lack of correlation of IL-10 and TGF- β with immune deficits *in situ* and *in vitro* suggests reconsidering their role in cancer.

Zusammenfassung

IL-10 und TGF- β sind immunsupprimierende Zytokine, die in verschiedenen Tumoren, u.a. im Melanom, entdeckt wurden und als Hauptursache für das Versagen der Anti-Tumorimmunantwort angesehen werden. Allerdings wurden divergente Daten auch berichtet. Um diese Diskrepanz zu erklären, wurde die Expression dieser Zytokine mittels quantitativer RT-PCR im Melanom und in Haut gesunder Individuen verglichen. Weiterhin wurde die Induktion beider Zytokine in Kokultorexperimenten mit Dendritische Zellen und T-Zellen zusammen mit Tumorzellen sowie ihr Einfluß auf das Immunsystem untersucht. Beide Zytokine sowie deren Rezeptoren wurden im Melanom exprimiert, aber im Vergleich mit gesunder Haut auf signifikant geringerem Level. Dementsprechend waren die Expressionen von IL-10-induzierbare-SOCS-3 und auch TGF- β -induzierbare-SMAD-7 im Tumor gering und in der gesunden Haut hoch. T-Zellen, die mit einer großen Zahl an Tumorzellen kokultiviert wurden, entwickelten einen anergischen Zustand, aber ohne mit dem IL-10 oder TGF- β Level zu korrelieren. Dieser *in-vitro*-induzierte anergische Zustand der T-Zellen wurde durch Zugabe hoher Konzentrationen an exogenem IL-10 oder TGF- β zu einem gewissen Grad wieder aufgehoben. Eine Kombination von IL-10 und TGF- β resultierte in einem deutlicheren Ergebnis als die Verwendung eines Zytokins alleine. Allerdings konnte eine hohe Konzentration an IL-10 funktionelle T-Zellen unterdrücken, was die Idee unterstützt, dass IL-10 in verschiedenen Aktivierungs- und funktionellen Stadien der T-Zellen unterschiedlich wirkt. Dendritische Zellen, die zusammen mit Tumorzellen kokultiviert wurden, wiesen eine gemischte Population an vollständig und unvollständig differenzierten iDCs auf, produzierten hohe Level IL-10 und konnten die CD4 T-Zellproliferation weniger effizient induzieren. Trotzdem konnten sie zur Reifung induziert werden, wobei die Blockierung von IL-10 nicht die Fähigkeit der resultierenden, reifen DCs veränderte, CD4 T-Zellproliferation zu induzieren. Die erhöhte Produktion an TGF- β wurde ausschließlich beobachtet, wenn DCs durch direkten Kontakt mit einer großen Zahl an Tumorzellen generiert wurden. Die Blockierung von TGF- β während der Differenzierung der tumorassoziierten iDCs veränderte nicht den DC-Phänotyp und die Fähigkeit, CD4 T-Zellproliferation zu induzieren. Diese Ergebnisse zeigen, dass beide Zytokine die Funktion der DCs nicht verändern. DCs, deren Reifung in der Gegenwart von Tumorzellen induziert wurde, produzierten erhöhte Level an IL-10, dagegen gleiche oder verminderte Level an TGF- β und waren effizienter in der Induktion der CD4 T-Zellproliferation. Die fehlende Korrelation von IL-10 und TGF- β mit den Immundefiziten *in situ* und *in vitro* legt den Schluß nahe, ihre Rolle bei Krebs neu zu überdenken.

1. Introduction

1.1. Melanoma

Melanoma is a cancer arisen from melanocytes found predominantly in the skin. It is a very aggressive type of cancer and the mortality rate is the highest among skin cancer related deaths. In the early stages, melanoma can be cured by surgery but at advanced stages, patients show poor responses to therapy (reviewed in Kuphal and Bosserhoff, 2009).

The American Joint Committee on Cancer (AJCC) classification system is based on principal that malignant tumor follows the timely progression. The current AJCC system for cutaneous melanoma classifies patients into primary tumor (T1-4), regional lymph nodes (N1-3), and distant metastasis (M1-3). Besides the TNM staging, anatomic staging group is also used for classifying melanoma into 4 stages. Stages I and II are localized melanoma, stage III is regional metastatic melanoma and stage IV is distant metastatic melanoma (Kim *et al*, 2002; Balch *et al*, 2009).

Several risk factors are linked to the development of melanoma, such as gene mutation, chronic immunosuppression and environmental exposure. Several susceptible genes have been implicated to play a role in the pathogenesis of melanoma. BRAF mutation is the most frequent mutation found in melanoma. Mutations in the BRAF oncogene leads to constitutive activation of mitogen-activated protein kinase (MAPK) signaling pathway, inducing cell proliferation. Mutation of BRAF is reported to promote tumorigenicity in nude mice (Davies *et al*, 2002; Kumar *et al*, 2003). CDKN2A tumor-suppressor gene encodes the negative regulator of cell proliferation, providing the mechanism of holding cell proliferation at the G1/S1 checkpoint to permit repair of DNA damage. Loss of CDKN2A function results in the loss of proliferative and apoptotic control (Goldstein *et al*, 2000). Tumor-suppressor gene PTEN (Phosphatase and Tensin Homolog deleted on Chromosome ten) encodes a PTEN protein which has a lipid phosphatase and a protein phosphatase activity. The lipid phosphatase activity of PTEN arrests the cell cycle progression at G1/S phase, upregulates proapoptotic machinery and downregulates the antiapoptotic proteins. The protein phosphatase activity involves in the cell spreading and migration, as well as the inhibition of MAPK signaling. Therefore, the loss of lipid and protein phosphatase activity of PTEN results in the uncontrolled growth of cells, escape from apoptosis, abnormal cell spreading and migration (Wu *et al*, 2003). Familial melanoma syndrome has been associated with germline mutation of several gene products: p16, alternate reading frame (ARF) and cyclin-dependent kinase-4 (CDK4), which are all involve in the cell cycle control (Goldstein *et al*,

2000; Pho *et al*, 2006). Immunosuppression has also been associated with a higher risk of melanoma since a higher incidence occurs in patients with acquired immunodeficiency syndrome and post-transplant patients receiving immunosuppressive agents (Euvrard *et al*, 2003). A short, intense episode of burning sun exposure, as well as cumulative UV exposure over the years may contribute to the risk factors of melanoma. UV radiation can induce DNA damage. In most cases, cells repair the DNA damage and survive or they undergo apoptosis when the damage is irreparable. However melanocytes produce a high level of prosurvival factor Bcl2 which could help the mutant melanocytes escape apoptosis from which melanoma eventually arises (Gruber *et al*, 2008, Mackie *et al*, 2009, Jhappan *et al*, 2003).

To date, surgery through wide local excision remains the standard procedure (Ingraffea, 2013). However, due to the aggressiveness of the tumor and the limited efficiency of currently applied therapeutics such as chemo-and radio-therapy, the survival rate of patients with metastatic melanoma is very low. Median survival rates after metastasis occurs is 6-9 months, with records of a 5-year survival rate less than 10% (Ingraffea, 2013). A new experimental strategy, such as immunotherapy has been offered as an alternative treatment for the late stage of melanoma patients, with limited success so far. Immunotherapy for malignant melanoma strives to stimulate the patients' immune system to fight the cancer. Several approaches have been applied to treat melanoma, including treatment with cytokines such as Interleukin (IL)-2 and Interferon (IFN)- α . Treatment of IL-2, the first immunotherapy approved by Food and Drug administration (FDA), could only demonstrate a small efficiency range from 6-10% with no clinically meaningful prolongation of overall survival. IFN- α treatment shows a modest antitumor activity but the responses are limited to patients with low-volume disease in cutaneous or soft-tissue sites. Furthermore, concern about high toxicity regarding the high dosage needed to apply has arisen (Markovic *et al*, 2007, Bhatia *et al*, 2009).

Another experimental approach is by using a tumor vaccine. A study showed that a trivalent vaccine against self-melanoma antigen glycoprotein 100 (gp100), tyrosinase and melanoma antigen recognized by T cells 1 (MART-1) demonstrated a promising result in increasing T cell activity against melanoma. Nevertheless, treatment with a peptide vaccine is a very specific approach. Treatment with signal transduction inhibitor which includes BRAF kinase inhibitor or a combination of BRAF and mitogen-activated-ERK (MEK) inhibitor is associated with increased CD8 T cells infiltration in melanoma and increased expression of melanoma antigens. But again, this treatment is limited to the patients with melanoma

containing BRAF mutation. Furthermore, the medications targeting mutated BRAF (Vemurafenib and GSK2118436) are limited to a short duration due to the emerging resistance to therapy and are associated with the increased development of squamous cell carcinomas (Frederick *et al*, 2013, Ingraffea, 2013; Callahan *et al*, 2013; Finn *et al*, 2012). The limitation of immunotherapy against melanoma is caused mainly by tumor escape mechanism. Understanding the biology of melanoma is therefore important to develop new therapeutic approaches.

1.2. Tumor immunology

The immunosurveillance hypothesis states that the immune system surveys the whole body and is able to recognize and eliminate tumor cells. The major key effector cells in fighting tumor cells are T cells and the natural killer (NK) cells (reviewed in Igney and Krammer, 2002).

T cells are able to kill tumor cells in an antigen dependent manner. Effective activation of naïve T cells requires several signals. The first signal is the interaction between T cell receptor (TCR) and MHC (major histocompatibility complex)-peptide complex. The second signal is the interaction between co-stimulatory molecules on T cells (i.e., CD28) and antigen presenting cells (APC) (i.e., B7-1 and B7-2). The third signal is the cytokines produced by dendritic cells (DC) to stimulate T cells. Various tumor antigens can be recognized by T cells. Some of these antigens are expressed exclusively by tumor (tumor specific antigens). These antigens arise from mutations of normal genes. Some other antigens are shared by tumor cells and healthy cells (tumor associated antigens). Overexpressed non-mutated proteins could also serve as tumor antigens for T cells. Upon recognition of tumor antigens, CD8 T cells are activated and become cytotoxic T cells (CTL), which can kill the target cells. CTL can kill the target cells using different mechanisms: perforin/granzyme pathways and Fas-FasL interaction. The release of perforin and granzymes leads to perforation of target cells and subsequent apoptotic death induced by granzymes. The Fas-FasL interaction also induces apoptosis. Besides CD 8 T cells, there is another set of T cells, which is called CD4 T cells. Based on the differences of cytokine production pattern in mouse, CD4 T cells are divided into CD4 helper T cells type I (TH1) and type II (TH2) subsets. TH1 cells help the activation of CTL to kill target cells, while TH2 cells help to stimulate a humoral response and suppress the development of the TH1 response. Human CD4 T cells that exhibit TH1 and TH2-like

phenotype was found in tissue or peripheral blood of patients in different disease states (reviewed in Igney and Krammer, 2002; Shresta *et al*, 1998; Romagnani, 1991).

Activation of NK cells depends on the balance of the inhibitory and activating signals. Cells expressing reduced inhibitory ligands (e.g. MHC class I molecules) and/or increased activating ligands will activate NK cells. The two main groups of inhibitory receptors of NK cells in humans are the killer-cell immunoglobulin-like receptors (KIRs) family that sense the classical MHC class I expression and the CD94/NKG2A receptors that bind to non-classical MHC class I molecule HLA-E. The lack of MHC class I, which sometimes occurs in tumors, will activate NK cells. In humans, the major activating receptors are NCR NKp30, NKp44 and NKp46 and CTLR NKG2D. Upon activation, NK cells release perforin and granzymes. NK cells could also kill the target cells by inducing apoptosis mediated by surface TNF ligand family members (FasL, TNF- α and TRAIL) that interact with specific receptors on the target cell surface. In addition, NK cells also secrete cytokines and chemokines, which will help effector cells to eliminate target cells (reviewed in Waldhauer and Steinle, 2008; Zamai *et al*, 2007).

1.3. Immunesuppressive mechanisms in tumor

Melanoma is highly immunogenic (able to induce immune response) and often found to be heavily infiltrated by immune cells. One offered explanation of the immunogenicity of melanoma is due to the presence of melanoma antigen (Melan-A, NY-ESO-1, MART-1, gp100, gp75, tyrosinase) (Hussein, 2005). But since the tumor persists and can grow progressively in patients, the immune system is clearly ineffective to completely eradicate the tumor. Several possibilities have been proposed to explain the failure of the immune response to completely eliminate cancer. They can generally be classified into the inhibition of anti-tumor priming and the inhibition of effector functions (Frey and Monu, 2008).

In the first category falls the modulation of antigenicity. Tumor cells can escape T cell recognition by modulating the presentation or processing of antigens. Downregulation of MHC class I as well as class MHC class II molecules in malignant cells has been shown. Several mechanisms that alter the MHC molecules in human (called human leukocyte antigen (HLA)) are described, such as defect of beta (2)-microglobulin synthesis, loss of genes encoding or downregulation of HLA heavy chain, defect of regulatory mechanism that control HLA expression, and alteration in antigen processing machinery (reviewed in Campoli and

Ferone, 2008; Yaguchi *et al*, 2011). In some tumor cases, expressions of tumor antigens (e.g., MART-1, Melan-A) are downregulated. Furthermore, mutations in the antigens can result in the escape from tumor recognition by T cells (Igney and Krammer, 2002).

Another suggested mechanism that limits T cell activation in tumor cases is the lack of co-stimulation. B7 co-stimulatory molecules play an important role to activate T cells. The failure of co-stimulatory signaling could lead to an immunological tolerance. Immunohistochemistry and FACS analysis show no expression of B7 on primary and established head and neck squamous cell cancer (Wollenberg, *et al*, 1998).

There is increasing evidence that DCs isolated from cancer patients have functional deficiency, thereby decreasing their capacity to induce immune responses. Mature DCs express high levels of activation markers and produce high level of cytokines, enable them to stimulate T cells to induce immune responses. DCs can be activated via pattern recognition receptors (PRRs) or inflammatory cytokines signaling. PRRs are protein expressed by innate immune cells to identify pathogen associated molecular patterns (PAMPs), which are associated with microbial pathogens and viruses or endogenous danger signal released by damaged cells (Joffre *et al*, 2009, Fujii *et al*, 2004, Le Bon *et al* 2001). It is suggested that the lack of T cell stimulation in cancer is caused by altered DCs with decreased functions. The circulating blood DCs' number decreases in melanoma and the number of total DCs decreases as melanoma progress. There is a minimal recruitment and a low activation of DCs found within renal cell carcinoma (Troy *et al*, 1998). In addition of the low activation, IL-12 production needed by T cell is also impaired in cancer patients' DCs (Della *et al*, 2003). The number and allo-stimulatory activity of myeloid DCs are lower in pancreatic cancer patients than in healthy individuals (Yanagimoto *et al*, 2005). DCs that infiltrate non-small cell lung cancer are blocked in immature state and although they could be induced into maturation by TLR4 stimulation, they display a poor APC function even after the TLR stimulation (Perrot *et al*, 2007).

The second category includes the negative regulation of immune responses. There are several negative regulation mechanisms that are suggested in cancer patients: (1) expansion of immune suppressor cells, (2) expression of co-inhibitory molecules and (3) production of immunosuppressive cytokines or other suppressive factors.

Several immune suppressor cells are found in cancer patients, including regulatory T cells (T-regs), Tumor associated macrophages (TAMs) and Myeloid-derived suppressor cells (MDSCs) (reviewed in Allavena *et al*, 2008; Gajjar and Nagaraj, 2009).

T-regs are defined as a subset of T cells that functionally suppress the immune responses. T-regs suppress the immune responses through (1) contact dependent mechanism involving B7-H4 expression on antigen presenting cells (APCs) which negatively regulate T cell responses, (2) CTLA-4-mediated induction of IDO expression, (3) IL-10 and Transforming Growth Factor- β (TGF- β) expressions or (4) direct killing of effector cells via granzyme and perforin (reviewed in Zou, 2006; Gross and Walden, 2008). There are several possible mechanisms that can explain the increased frequency of T-regs in the tumor microenvironment. Firstly, abundant expression of CCL22 in the tumor microenvironment stimulates T-reg infiltration in tumors. Secondly, tumor microenvironment contains molecules that can alter APC differentiation and function, which will then induce T-reg expansion. Lastly, tumor microenvironment can produce a high level of TGF- β which will convert normal T cells into T-regs. Higher frequencies of T-regs are found in peripheral blood of varieties of cancer patients. Studies of T-regs in humans show a potent immunosuppressive activity *in vitro*. An accumulation of T-regs in patients also indicates a reduced survival in patients (reviewed in Zou, 2006; Curiel, 2007).

Accumulating evidence suggests TAMs actively promotes tumor growth and development. Several animal model experiments suggest TAMs promotes tumor progression and angiogenesis by producing pro-angiogenic cytokines (e.g., TNF- α , IL-1). TAMs isolated from tumors are generally less efficient in presenting antigens. TAMs are unable to produce IL-12 needed in anti-tumor responses mediated by NK cells and T cells. TAMs are also shown to produce immunosuppressive cytokine IL-10 and TGF- β and to express programmed death ligand (PD-L)-1 (reviewed in Quatromoni and Erulanov, 2012; Pollard, 2004).

MDSCs are described as a mixture of cells in immature state of myeloid origin. MDSCs have suppressive capacity on adaptive immune response and these cells were reported to be found in blood of varieties of cancer patients. Several mechanisms of MDSCs to suppress immune responses have been described. MDSCs are reported to upregulate the production of reactive oxygen species (ROS). A combination of ROS and nitric oxide (NO) forms peroxynitrite that could damage proteins, including those regulating MHC-II expression and T cell apoptosis. MDSCs can inhibit NK cell cytotoxicity by inhibiting NKG2D expression and IFN- γ production. Aside from their suppressive effect on adaptive

immune responses, MDSCs are also able to regulate innate immune responses by increasing IL-10 production, suppressing macrophage IL-12 production and activating and enhancing T-regs (reviewed in Gabrilovij and Nagaraj, 2009; Sinha *et al*, 2007; Lindau *et al*, 2013).

To date, two key co-inhibitor molecules have been identified to affect T cell functions: cytotoxic T lymphocyte associated antigen 4 (CTLA-4) and programmed cell death 1 (PD-1). CTLA-4 in resting cells is intracellularly localized to clathryn-associated complexes. By activation, CTLA-4 will be relocated to the cell surface, which is a temporary state, and will be rapidly reinternalized again. CTLA-4 binds CD80 and CD86, with 20-40 folds higher affinity than of CD28. CTLA-4 ligation antagonizes early T cell activation leading to a decrease of some stimulatory cytokines (e.g., IL-2), an increased immunosuppressive cytokines production, a cell cycle arrest, a modulation of TCR signaling and a decreased T cell proliferation. CTLA-4 has also been implicated in the modulation of T-regs by enhancing T-reg immunosuppressive activity (Jago *et al*, 2004; Fife and Bluestone, 2008; Mocellin and Nitti, 2013).

PD-1 is a glycoprotein, which carries immunoreceptor tyrosine-based inhibitory motifs (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM). The binding of PD-1 with PD-L1 and PD-L2 will cause phosphorylation on ITIM and ITSM. PD-1/PD-L1 interaction decreases cytokine synthesis and glucose metabolism, blocks T cell proliferation and survival. Signaling through PD-1 ligation leads to a decreased IFN- γ production, a decreased T cell proliferation, an increased T cell apoptosis and a blockage of CD28-mediated activation (Fife and Bluestone, 2008; Giancchetti *et al*, 2013).

CTLA-4 and PD-1 are also suggested to play a critical role in maintaining peripheral tolerance. In this state, T cells are not deleted but in a state of muted responsiveness to antigen challenge, a state called anergy (Fife and Bluestone, 2008). Observations in mice show that anergic T cells but not functional memory T cells express a high level of negative regulator PD-1 (Barber *et al*, 2006) and a blockade of the negative regulators signaling could reverse the T cell anergy to some extent (Golden-Mason *et al*, 2009).

CTLA-4 and PD-1 are thought to play important roles affecting T cell functions in tumor. Lymphocytes from peripheral blood and tissue samples from oral squamous cell carcinoma patients express higher levels of PD-1 compared with healthy individuals and the expression of PD-L1 is intense in oral squamous cell carcinoma sites (Malaspina *et al*, 2011). Within the tumor microenvironment, PD-1 ligands are often expressed by tumor and tumor

stroma cells and correlated with unfavourable prognosis for patients (Gross and Walden, 2008).

The last mechanism of negative regulation discussed here is carried out by immunosuppressive cytokines or other suppressive factors. Vascular endothelial growth factor (VEGF) is reported to be secreted by many tumors. It is reported to have an angiogenic function and be able to inhibit DC differentiation and maturation (reviewed in Gross and Walden, 2008). Deregulation of the enzyme cyclooxygenase-2 (COX-2) in cancer leads to a production of abundant prostaglandin E₂ (PGE₂). PGE₂ is able to promote proliferation, survival, angiogenesis, migration, invasion, and induce production of IL-10 (Greenhough *et al*, 2009). Intracellular adhesion molecule-1 (ICAM-1) is reported to be shed from tumor cells and able to block the binding of effector cells to their target. Adenosine produced by tumor cells can inhibit IL-12 production and induce IL-10 production. Activation of the enzyme indoleamine 2, 3-dioxygenase (IDO) has been implicated in a variety of primary tumor and demonstrated in tumor cell lines. IDO is an enzyme that catalyzes the first step of tryptophane degradation leading to kynurenine. T cells seem to be sensitive to a shortage of tryptophane and kynurenine is toxic to lymphocytes. IDO-mediated tryptophan deficiency inhibits T cell proliferation, sensitizes T cells to apoptosis and upregulates T-reg activity (reviewed in Gross and Walden, 2008; Frey and Monu, 2008, Hwu *et al*, 2000; Lee *et al*, 2002; Hill *et al*, 2007).

Two well-known suppressive cytokines are IL-10 and TGF- β . Immunosuppressive cytokines are important players in the immune system since a proper balance of pro- and anti-inflammatory reaction is necessary for regulating it (Grütz, 2005). Self-limiting mechanisms are important to prevent an excessive activation of T cells that could cause damage to the host. This immune system homeostasis is necessary to make sure that immune system responds adequately to infections or injuries and then is negatively regulated back to the baseline. These two cytokines are detected in patients' blood, primary tumor and sentinel lymph nodes and therefore have been proposed as major immunosuppressive cytokines in cancer (Polak, 2007).

IL-10 is considered to be the key immunoregulator during inflammation triggered by infection or trauma to ensure the inflammation reaction will not be excessive and in the end damages the host. Human IL-10 is a 35 kD homodimer. IL-10 is produced by immune cells, including monocytes, dendritic cells, macrophages, B cells, T cells, NK cells, mast cells and granulocytes, and it is also produced by non-immune cells, such as epithelial cells and keratinocytes (reviewed in Iyer and Cheng, 2012). Since IL-10 is a cytokine normally

expressed to limit the immune response, it has been suggested to play a role in inducing tolerance (Grütz, 2005).

IL-10 activity is mediated by a heterodimeric IL-10 receptor composed of IL-10R1 and IL-10R2. IL-10 mainly uses the Janus kinase (JAK) family members and Signal Transducer and Activator of Transcription (STAT) transcription factors in its signaling pathway. The binding of IL-10 to its receptor will activate JAKs, which in turn phosphorylate two tyrosines (Tyr 446 and Tyr 496). Afterwards, STAT3 will bind to the receptor via SH2 domain and be phosphorylated. Additionally, STAT1 and STAT5 are activated. These transcription factors will form homo- and hetero-dimers and migrate to the nucleus to activate target genes. The activation of IL-10 leads to large changes of the expression profile of immune modulatory genes, which in effect will inhibit pro-inflammatory cytokine secretions, decrease antigen presentation and phagocytosis, and enhance inhibitory, tolerance and scavenger functions of the cells. The suppressor of cytokine signaling 3 (SOCS-3) is one of the target genes whose expression is induced by IL-10. SOCS-3 induction seems to be responsible for terminating IL-10 activation (reviewed in Sabat *et al*, 2010, Ding *et al*, 2003).

IL-10 is capable to inhibit the activities of T cells, B cells, NK cells, mast cells, monocytes and macrophages (Couper *et al*, 2008; Moore *et al*, 2001) with monocytes and macrophages appeared to be the primary target of IL-10 effect. IL-10 is reported to inhibit CC chemokines (MCP-1, MCP-5, MIP-1 α , MIP-1 β , MIP-3 α , MIP-3 β , RANTES, and MDC) and CXC chemokines (IL-8, IP-10, MIP-2, and KC) synthesis. Thus, IL-10 inhibits the productions of nearly all inducible chemokines involved in inflammatory reaction. IL-10 also inhibits the production of pro-inflammatory cytokines (IL-1, IL-6, IL-18, GM-CSF, G-CSF, M-CSF, TNF, LIF, and PAF). IL-10 also increases the expression of their natural antagonist and induces production of anti-inflammatory mediators such as IL-10 itself, IL-10R antagonist and soluble TNF- α receptor. Furthermore, IL-10 inhibits IL-12, NO production, and expression of MHC class II and co-stimulatory molecules (reviewed in Moore *et al*, 2001; Sabat *et al*, 2010).

IL-10 also has direct effects on T cells. It is reported to inhibit both proliferation and cytokine synthesis of CD4 T cells, which includes the production of IL-12 and IFN- γ by TH1 and IL-4 and IL-5 by TH2. Upon CD4 T cell activation *in vitro*, the presence of IL-10 induces these cells to develop a regulatory phenotype (reviewed in Sabat *et al*, 2010). In contrast, IL-10 has no direct inhibitory effect on the proliferation of CD8 T cells stimulated with anti-CD3 mAb (Groux *et al*, 1998).

Several investigations showed that IL-10 is produced in tumors. IL-10 is strongly expressed in basal cell carcinoma and squamous cell carcinoma (Kim *et al*, 1995). IL-10 mRNA is found in tissues of primary tumors and metastases but not in adjacent normal skin of patients with malignant melanoma (Krüger-Krasagakes *et al*, 1994). Serum IL-10 levels of advanced melanoma patients are higher than normal volunteers (Nemunaitis *et al*, 2001). Addition of exogenous IL-10 in culture enhances melanoma proliferation and prolongs the melanoma survival while blocking IL-10 decreases the proliferation (Yue *et al*, 1997). An *in vitro* study by Garcia-Hernandez and colleagues shows that IL-10 induces tumor proliferation and angiogenesis in the B16-melanoma model (Garcia-Hernandez *et al*, 2002).

Investigators report that IL-10 induces CTL anergy by downregulating the expression of MHC class I and transporter associated with antigen processing (TAP) in human melanoma cells (Kurte *et al*, 2004). Expressed by tumor cells, it is suggested to increase the expression of nonclassical HLA class Ib molecules (i.e. HLA-G), which is thought to inhibit the cytolytic activity of NK cells and CTLs (Urosevic and Dummer, 2003; Gros *et al*, 2008; Le Gal *et al*, 1999). Several groups suggest that it promotes tumor growth by blocking the induction of naïve T cells into CTLs (Steinbrink *et al*, 1999) and maintains T cell anergy by inducing the IDO (Lee *et al*, 2005). IL-10 is reported to hinder antigen presenting properties of DCs, thus impairing T cell responses against tumor (reviewed in Mocellin *et al*, 2005).

Although IL-10 is generally accepted as a major immunosuppressive cytokine, conflicting evidence suggesting IL-10-mediated immune stimulation has also been reported. Injection of IL-10 after a booster vaccine enhances anti-tumor immunity and vaccine efficiency in one *in vivo* study by maintaining CD8 effector function (Fujii *et al*, 2001). Another study demonstrates that transfection of IL-10 gene into melanoma cells results in the loss of tumorigenicity in proportion to IL-10 secreted (Gerard *et al*, 1996). IL-10 is also reported to inhibit melanoma metastasis in mice via NK cells-dependent mechanism (Zheng *et al*, 1996).

The other major anti-inflammatory cytokine suggested to play a role in cancer is TGF- β . TGF- β is a pleiotropic cytokine, involved in numerous physiological and pathological processes, ranging from cell growth and differentiation, embryogenesis, reproduction, bone formation, carcinogenesis and immune response, among others. Targets of TGF- β include CD4 T cells, CD8 T cells, dendritic cells, NK cells and macrophages. In mammals, three TGF- β forms have been identified (TGF- β 1, TGF- β 2 and TGF- β 3), and TGF- β 1 is the predominant isoform in the immune system. TGF- β is synthesized as an inactive form

composed of a TGF- β dimer in association with the latency-associated protein (LAP). This latent form is secreted as such or forms a complex with latent TGF- β binding protein (LTBP). TGF- β is activated by degradation of LAP or alteration of its conformation. The active TGF- β binds to a tetrameric complex of TGF- β R1 and TGF- β R2 and initiates TGF- β signaling (reviewed in Li and Flavell, 2008, Massague', 2008).

When active, TGF- β binds to the receptor, the TGF- β R2 will phosphorylate TGF- β R1 which in turn will phosphorylate SMAD proteins. The receptor-regulated SMADs (SMAD-1, 2, 3, 5, and 8) are directly phosphorylated by TGF- β R1 and will form complexes with the co-mediator SMAD-4. These SMAD complexes will translocate to the nucleus and in conjunction with other nuclear co-factors regulate transcription of target genes. The inhibitory SMADs (i.e. SMAD-6 and 7) negatively regulate TGF- β signaling by competing with receptor-regulated SMAD for receptor or SMAD-4 interaction and by targeting the receptor for degradation (reviewed in Shi and Massague', 2003).

TGF- β plays a complex role during carcinogenesis. TGF- β can be produced by tumor cells and immune cells such as T lymphocytes and DCs (Enk, 2005; Polak *et al*, 2007). In early stages TGF- β can inhibit tumor growth but in later stages it can promote tumor growth (Javelaud *et al*, 2008, Lebrun, 2012). Alteration of TGF- β signaling can have a significant effect on tumor initiation and progression but it can also function as a tumor suppressor (Yang and Moses, 2008). Different points of disruption in TGF- β signaling have been described in cancer. Reduced expression of TGF- β R1 or TGF- β R2 is reported in lung, gastric, prostate and bladder cancer. Loss of SMAD-3 expression is noted in gastric cancer and T cell lymphoblastic leukemia. SMAD-4 mutation is reported in pancreatic carcinoma and pancreatic cancer (reviewed in Massague', 2008). In a mouse model, knockout of TGF- β R resulted in a more aggressive adenocarcinoma tumor (Ijichi *et al*, 2006) and promoting squamous cell carcinoma (Guasch *et al*, 2007). Some human cancer cells express a high level of TGF- β which impacts the tumor microenvironment, angiogenesis and metastasis (Leivonen and Kahari, 2007). Increased expression and secretion of different TGF- β isoforms in melanoma cell lines compared with normal melanocytes are reported by several studies (Javelaud *et al*, 2008). Blocking TGF- β signaling reduces pancreatic adenocarcinoma primary tumor growth and decreases the incidence of metastasis *in vivo* (Gaspar *et al*, 2007). TGF- β can have a pro- or anti-tumor activity by affecting cell differentiation. TGF- β can favor epithelial differentiation into less proliferative states partly through downregulation of

inhibitor of differentiation DNA binding 1 (ID1) but TGF- β may also activate carcinoma progenitor cells to have a high motility and become invasive mesenchymal derivatives (reviewed in Massague', 2008).

Another suggested mechanism to inhibit effector function is the immune modulation by the enhance production of tumor-derived factors. It is reported that chemokine (C-C motif) ligand 2 (CCL2) produced by melanoma resulted in a tumor associated macrophages (TAMs) recruitment and a greater level of angiogenesis. Tumor IL-6 is reported to skew the monocyte differentiation into TAMs (reviewed in Ilkovitch and Lopez, 2008).

1.4. Objectives

This study was designed to investigate the role of IL-10 and TGF- β in melanoma, with focus on the following objectives.

General objective: to clarify the conflicting evidence about the role of IL-10 and TGF- β in melanoma.

Specific objectives:

- To investigate both cytokines *in situ* by measuring the levels of expression of the two immunosuppressive cytokines, in relation to the expression of their responsive genes, in a large number of melanoma samples and compare them to the healthy skin samples since previous studies were done with small numbers of samples and comparisons were not done with healthy skin samples (Dummer *et al*, 1998, Krüger-Krasagakes *et al*, 1994, Itakura *et al*, 2011).
- To study the active role of IL-10 and TGF- β in suppressing immune responses using an anergic T cell *in vitro* model.
- To evaluate the role of IL-10 and TGF- β in the differentiation and maturation of tumor-associated DCs.

2. Materials and Methods

2.1. Clinical materials

Melanoma metastases from tumor surgeries and skin samples from healthy individuals from breast reduction surgeries and foreskins were obtained from the dermatosurgery, dermatohistopathology and the tumor bank of the Department of Dermatology, and from the Gynecological Department of the Charité. The samples were shock-frozen in liquid nitrogen and cryopreserved. Several melanoma cells were developed into cell lines. The melanoma cell lines used in the *in vitro* experiments were ChaMel84 and SK-Mel-28. ChaMel84 was established in our lab from the tumor of one of the patients. SK-Mel-28 was obtained from American Type Culture Collection (ATCC).

This study had been reviewed and approved by the ethic commission of Charité under the title 'Mechanism of immune suppression in tumor' on 18 September 2008 (EA1/157/08).

2.2. Methods

2.2.1. Preparation of human Peripheral Blood Mononuclear Cells

Whole blood was obtained from a buffy coat. Peripheral Blood Mononuclear Cells (PBMCs) were obtained by standard density gradient centrifugation using Ficoll-Paque (density 1.077 g/ml) (Biochrom, Berlin, Germany). Blood was diluted with an equal volume of PBS (Gibco, Darmstadt, Germany). Diluted blood was layered on top of 15 ml Ficoll solution (Biochrom, Berlin, Germany) using a 50 ml tube to create a Ficoll gradient by centrifugation for 20 minutes at 1000xg at room temperature, with the brake off. PBMCs were collected from the Ficoll : plasma interface and washed twice by PBS. Cells were resuspended in RPMI medium (RPMI, 1 mM L-Glutamine-L-Alanine, 1% penicillin/streptomycin, and 10% FBS) (Biochrom, Berlin, Germany).

2.2.2. CD4 and CD8 T cell isolation

T cells were isolated from PBMCs using Dynabeads FlowComp (Invitrogen, Darmstadt, Germany). Isolation was done according to the manufacturer's instruction with few modifications. The product is intended for positive magnetic isolation of CD4 or CD8 T cells from human PBMCs. 1×10^8 PBMCs were incubated with 25 μ l Human biotinylated-CD4

or -CD8 antibodies, which will bind to the target cells, for 10 minutes at 4°C and then washed with isolation buffer (PBS supplemented with 0.1%BSA (Sigma Aldrich, Steinheim, Germany)). Cells that have bound the specific antibodies were incubated with 75 µl Flowcomp Dynabeads (superparamagnetic beads coated with streptavidin) for 15 minutes at the room temperature. Cells bound to Dynabeads through the streptavidin-biotin interaction were separated from other cells by placing the tube containing the mixture of cells on a magnet. Bound cells stayed on the tube and the unbound cells were washed off by an isolation buffer. Beads were later removed from the cells by incubating the cells with 1 ml FlowComp Release Buffer to break the biotin-streptavidin interaction.

2.2.3. Dendritic Cell Generation

Monocytes were sorted from PBMCs using Dynabeads FlowComp (Invitrogen, Darmstadt, Germany). Separation was done according to the manufacturer's instruction. The product is intended for positive magnetic isolation of CD14 cells from human PBMCs. 5×10^7 PBMCs were incubated with 25 µl Human biotinylated-CD14 antibodies for 15 minutes at 4°C and then washed with isolation buffer (PBS supplemented with 0.1%BSA). Cells that have bound the CD14 antibodies were incubated with 75 µl Flowcomp Dynabeads (supermagnetic beads coated with streptavidin) for 20 minutes on ice. Cells bound to Dynabeads through the streptavidin-biotin interaction were separated from other cells by placing the tube containing the mixture of cells on a magnet. Bound cells stayed on the tube and the unbound cells were washed off by an isolation buffer. Beads were later removed from the cells by incubating the cells with 1 ml FlowComp Release Buffer to break the biotin-streptavidin interaction. In some experiments, the adherence method to isolate monocytes was used. When the adherence method was used, 1×10^7 PBMCs were put into a small culture flask (Nunc, Schwerte, Germany) for 1-2 hours, at 37°C. The non-adherent cells were washed off and the adherent cells were washed with cold PBS 4 more times.

Monocytes were cultured at 1×10^5 cells/well in 12 well plates (TPP, Berlin, Germany) in RPMI medium supplemented with 50 ng/ml GM-CSF (Aesca, Traiskirchen, Austria) and 50 ng/ml IL-4 (Promokine, Heidelberg, Germany). The cytokines were added on day 0 and day 4. Maturation of DCs was done by adding cytokine cocktail (10 ng/ml IL-1, 25 ng/ml IL-6, 10ng/ml TNF- α (Miltenyi, Bergisch Gladbach, Germany)) on day 5. Cells were harvested on day 7 with cold PBS.

2.2.4. RNA isolation

RNA isolation was done using RNA isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. Tissue (40-80 mg) was put into a 15 ml tubes with 700 μ l lysis buffer to lyse the cells. Further disruption of tissue was done using ultraturrax homogenizer (IKA, Staufen, Germany) for 5 seconds on ice. Samples then treated with 10 μ l proteinase K solution for 10 minutes at 55°C to remove proteins. Debris was removed by centrifugation and supernatant was mixed with 0.5 volumes ethanol and centrifuged through RNeasy spin column, where RNA will bind to the silica membrane. Traces of DNA were removed by DNase treatment. DNase and any contaminant were washed away by a wash buffer and RNA was eluted in RNase-free water. When cells were used in RNA isolation, the number of cells used was 1×10^6 cells.

RNA concentration was measured with a Qubit fluorometer (Invitrogen, Staufen, Germany) according to the manufacturer's instruction. Sample was diluted 20 times with Quant-it working solution (Invitrogen, Staufen, Germany). The assay was performed at room temperature. Calibration was done using the calibration standard provided by the company.

2.2.5. cDNA synthesis

cDNA generation was done using Super Script III kit (Invitrogen, Staufen, Germany) to synthesize first-strand cDNA from total RNA according to the manufacturer's instruction. The primer used in the synthesis was cDNA-3' Primer (AAG CTG TGG TAA CAA CGC AGA GTC GAC TTT TTT TTT TTT TTT TTT TTT TTT TTT VN). cDNA synthesis mixture (20 μ l) contained 5xRT buffer (4 μ l), 10 mM DTT, 200 u SuperScript III Enzyme, 50 pmol cDNA-3 primer, 1 μ M dNTP mix and 1 μ g RNA. The cycling condition comprised of denaturation at 50°C for 5 minutes, annealing and cDNA synthesis at 50°C for 60 minutes and termination at 72°C for 15 minutes.

2.2.6. Measurement of cytokine production by ELISA

Supernatants were collected at different time points of experiments. Cytokine production was measured by a sandwich ELISA. The procedure was run in duplication. The concentrations of antibodies used in ELISA were as listed:

Cytokine detection	Capture antibody	Detection antibody
IL-10 (eBioscience, Frankfurt, Germany)	2 µg/ml	1 µg/ml
IL-12 (BioLegend, Fell, Germany)	2 µg/ml	2 µg/ml
IFN- γ (Thermo Scientific, Schwerte, Germany)	2 µg/ml	2 µg/ml
TGF- β (eBioscience, Frankfurt, Germany)	6.25 µg/ml	2.5 µg/ml

Capture antibody was diluted in PBS, except for the IL-12 antibody which was diluted in coating buffer (0.1 M NaHCO_3 , 0.03 M Na_2CO_3 , pH 9.5 (Merck, Darmstadt, Germany)), and incubated overnight at 4°C to coat the plate. After washing the plate, blocking solution (1% BSA in PBS) was added into the well for one hour at the room temperature to coat any plastic surface on the well that remained uncoated by the captured antibody. Sample supernatant (50 µl) was then added into the well and incubated for 90 minutes at room temperature to let the antigen binds to the immobilized (capture) antibody. To activate latent TGF- β 1 to immunoreactive TGF- β 1 in the cell culture supernatant, supernatant was incubated with 0.1 ml 1 N HCl (Merck, Darmstadt, Germany) for 10 minutes, followed by neutralization using 0.1 ml 1.2 N NaOH (Carl Roth, Karlsruhe, Germany). Plate was washed to remove the unbound proteins. Detection antibody conjugated with biotin was diluted in reagent diluent (1% BSA in PBS) and incubated for 90 minutes at room temperature to let the antibody binds to the antigen of the immobilized protein bound with the capture antibody. After removal of excess detection antibody, streptavidin conjugated to alkaline phosphatase (AP) (R&D Systems, Wiesbaden, Germany) was used with 1000 times dilution, incubated for 30 minutes at room temperature to let streptavidin bind to biotin-conjugated antibody. As substrate, 1 mg/ml pNPP (Sigma Diagnostics, Steinheim, Germany) diluted in detection buffer (1 M TRIS-Cl, pH 9.5 (Merck, Darmstadt, Germany)) was used. Optical density was determined using an ELISA reader (Dynatech Laboratories, Denkendorf, Germany) set to the wavelength 405 nm.

2.2.7. Flow cytometry analysis

Phenotypic analysis of cell surface molecule expression was done by flow cytometry. Cells were stained with following fluorescent antibodies against: CD3, CD4, CD8, (Biolegend, Fell, Germany), CD11c, CD14, CD25, CD45RO, CD69, CD71, CD80, CD83, CD86, CTLA-4, PD-L1, HLA-DR (BD Bioscience, Heidelberg, Germany), and PD1 (eBioscience, Frankfurt, Germany). Cells were collected and washed one time with FACS buffer (BD Bioscience, Heidelberg, Germany). After washing, cells were stained with antibodies against cells surface molecules (1:25-1:200 in 100 μ l FACS buffer (BD Bioscience, Heidelberg, Germany) for 20 minutes at room temperature. Cells were washed again one time with FACS buffer and then analyzed by FACS Calibur (BD Bioscience, Heidelberg, Germany) using a Cell Quest Pro version 4.0.2 software (BD Bioscience, Heidelberg, Germany).

For Granzyme B measurement, cells were permeabilized using fixation and permeabilization buffer (eBioscience, Frankfurt, Germany). After staining the cells with cell surface molecules' antibody, cells were fixed and permeabilized. For detection, fluorescent antibody against granzyme B (Caltag, Darmstadt, Germany) (1:25 in 100 μ l FACS buffer) was used. Staining was done for 30 minutes at room temperature.

2.2.8. mRNA quantification by qPCR

Quantification of the expression level of targets was done using a SYBR Green real time PCR detection method (SABiosciences, Hilden, Germany). Total mRNA from skin cancer patients and healthy donors were isolated from frozen cells or tissue and reverse transcribed into cDNA. Amplification of target genes by commercially available primers (SABiosciences, Hilden, Germany) using these cDNA as a template was done to quantify the expression level of the target genes. RT-PCR amplification mixtures (13 μ l) contained 50 ng cDNA, 2x SYBR Green Master Mix buffer (6.5 μ l), and 380 nM forward and reverse Primers. The reactions were run in duplication on Applied Biosystem 7500 Real-Time PCR System (Applied Biosystem, Darmstadt, Germany). The cycling condition comprised of 10 minutes polymerization activation at 95°C, 40 cycles of 95°C for 1 minute and 60°C for 1 minute, and dissociation at 95°C for 15 seconds, 60°C for 1 minutes and 95°C for 15 seconds. The following target genes were tested: IL-10, TGF- β , TGF- β R1, and IL-10R, and SMAD-7 and SOCS-3. To study whether the cells in the local microenvironment can respond to these

cytokines, the expression of their receptors, TGF- β R1 and IL-10R1, were measured. Activation of TGF- β was determined by measuring the expression of SMAD-7, since SMAD-7 expression increases when TGF- β signaling is activated. The expression level of SOCS-3 was also measured as a marker of IL-10 induction as IL-10 induces SOCS-3 expression. Quantitative results were normalized to geometric averaging of three internal control genes to get a more accurate normalization than just using one internal control. Nine housekeeping genes were tested to be used as internal control (B2M, HMBS, SDHA, GAPD, RPL13A, HPRT, YWHAZ, UBC, and ACTB). The sequence of primers was listed in Table 1.

Table 1. Primers sequence

Primer	Forward primer	Reverse Primer
ACTB	CTGGAACGGTGAAGGTGACA	AAGGGACCTTCCTGTAACAATGCA
B2M	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT
GAPD	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
HMBS	GGCAATGCGGCTGCAA	GGGTACCCACGCGAATCAC
HPRT1	TGACACTGGCAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
RPL3A	CCTGGAGGAGAAGAGGAAAGAGA	TTGAGGACCTCTGTGTATTTGTCAA
SDHA	TGGGAACAAGAAGGGCATCTG	CCACCACTGCATCAAATTCATG
UBC	ATTGGGTCGCGGTTCTTG	TGCCTTGACATTCTCGATGGT
YWHAZ	ACTTTCCTACATTGTGGCTTCAA	CCGCCAGGACAAACCAGTAT

(Vandesompele *et al*, 2002)

PCR was done to eliminate primers with unspecific results (UBC and ACTB). Three housekeeping genes (SDHA, GAPD, and HPRT) were chosen based on the stability of expression level in different melanoma samples. The stability was calculated using the geNorm software. Prior to measure the expression of target genes in samples, amplification efficiency for each primer was calculated using the equation:

$$E = 10^{(-1/\text{slope})}$$

A five point standard curve of each primer using a 2-fold serial dilution starting from 250 ng was used. PBMC (2×10^6 cells/ml) stimulated with 100 ng/ml LPS, 500 ng/ml Ionomycine and 10 ng/ml PMA (Sigma Aldrich, Steinheim, Germany) was used as a template for positive control. The validity of standard curve was checked by confirming the slope that was falling between -3.3 to -3.8 (critical factors for successful real time PCR, qiagen, 2006).

Amplification efficiency for each primer was shown in Table 2.

Table 2. Amplification efficiency

HPRT	1.9936
GAPD	1.8363
SDHA	1.8205
IL-10	1.8994
IL-10RA	1.8932
SMAD-7	1.8462
TGF- β	1.9376
TGF- β R1	1.8921
SOCS-3	1.8492
IFG- γ	1.8672

The Ct values were transformed into quantities. Calculation on relative expression value was calculated using the equation:

$$Q = E^{(\min Ct - \text{sample Ct})}$$

where Q : quantity

$$E : \text{amplification efficiency} = 10^{(-1/\text{slope})}$$

minCt : lowest Ct value = Ct value of the sample with the highest expression

Relative quantification of target gene was then normalized to housekeeping genes:

$$\text{Normalization of GOI} : Q_{GOI}/NF$$

$$\text{where NF} : \text{Geomittel of 3 HKG} = (Q_{GAPD} + Q_{HPRT} + Q_{SDHA})/3$$

2.2.9. Proliferation assay

Proliferation assay was done using flow cytometric analysis of cell division by CFSE dilution. CD4 T Cells were washed with PBS then incubated with 3-4 mM CFSE (Invitrogen, Darmstadt, Germany) in PBS for 10 minutes at 37°C. Staining was stopped by adding cold RPMI medium and incubating for 5 minutes on ice. Cells were washed 3 times with RPMI medium and used for proliferation assay. 2×10^5 CD4 cells/well were used in proliferation

assay. Stimulation for proliferation was done by adding 1×10^4 cells/well DC or 4×10^4 cells/well tumor cells into the well. The flow cytometry analysis was done 5 days later. The MFI and percentage of low-CFSE-stained cells of each group were calculated. The percentages of low-CFSE-stained cells represented the percentages of proliferating cells. The stimulation index represents the number of cells proliferating in response to stimulation divided by the number of cells undergoing background proliferation in un-stimulated condition. It is calculated by dividing the value of low-CFSE stained cells of stimulated T cells with the value of low-CFSE-stained cells of un-stimulated T cells. Expansion index represents the fold expansion of overall culture. It was calculated by dividing the total MFI of un-stimulated T cells with MFI of stimulated T cells.

2.2.10. Statistical analysis

The Student's one-tailed independent *t*-test with unequal variances was used to assess statistical differences between two groups. The *p* value below 0.05 and 0.10 are considered significant.

3. Results

3.1. IL-10 and TGF- β in melanoma and healthy skin

The expression of IL-10 and TGF- β in 21 melanoma samples was compared with 12 skin samples from healthy individual by quantitative PCR (qPCR). IL-10 was detectable in both melanoma and healthy skin samples, showing a variety level of expression in both populations. Two melanoma samples had a medium level of IL-10 expression but most samples had a low expression level. On the contrary, most skin samples from healthy individuals had a high expression level of IL-10 with only two samples had a low expression level. Student *t*-test showed that the average expression of IL-10 in melanoma was 4.7-fold significantly lower than the expression in healthy skin (p value: 0.0051) (Fig. 1a). TGF- β was also detectable in melanoma and healthy skin with heterogeneous results. Two melanoma samples had a high expression level of TGF- β , eight samples had a moderate level and 11 samples showed a low expression. Two healthy skin samples had a high expression of TGF- β and ten samples had a moderate level. The average expression of TGF- β was 1.7-fold lower in melanoma than in healthy skin samples (p value: 0.0190) (Fig. 1b).

The expression of IL-10 and TGF- β receptors were measured to analyze whether cells were able to respond to IL-10 and TGF- β stimulation. IL-10R expression in melanoma and healthy skin samples showed heterogeneous expression levels. Two melanoma samples had a high expression of IL-10R, one sample had a moderate level and 18 samples had a low level of expression. Four skin samples from healthy individual had a high expression of IL-10R, three had a moderate level and five samples had a low level of expression. The average expression of IL-10R was 2.3-fold lower than the expression in healthy skin (p value: 0.0687) (Fig. 1c). TGF- β R expression in melanoma and healthy skin also demonstrated a high variety. Two melanoma samples had a high expression level of TGF- β R but 19 samples showed a low expression. One healthy skin showed a high expression of TGF- β R, three had a moderate level and eight samples had a low level of expression. The average expression of TGF- β R in melanoma was 1.7-fold lower than the expression in healthy skin (p value: 0.0953) (Fig.1d).

Suppressor of Cytokine Signaling-3 (SOCS-3) is reported to be one of IL-10 responsive genes as IL-10 induces SOCS-3 synthesis (Donnelly *et al*, 1999), while SMAD-7 expression is reported to be induced by TGF- β stimulation (Zhao *et al*, 2000). Therefore the expressions of SOCS-3 and SMAD-7 as reporter genes were measured to study the response

of cells to IL-10 and TGF- β production. SOCS-3 expression in melanoma and healthy skin samples was various. One melanoma sample had a moderate level of SOCS-3 expression and 20 other samples had a low expression level. Five skin samples from healthy individual had a high expression level of SOCS-3, two samples had a moderate level and five samples had a low expression level. The average expression of SOCS-3 in melanoma was 5.9-fold significantly lower than the expression in healthy skin (p value: 0.0036) (Fig. 1e). SMAD-7 expression in melanoma and healthy skin samples was also heterogeneous. Three melanoma samples had a moderate level of SMAD-7 expression and 18 samples had a low expression level. Seven samples from healthy individual had a high expression of SMAD-7, two had a moderate level and three samples had a low expression level. The average expression of SMAD-7 was 8.3-fold significantly lower in melanoma than in healthy skin samples (p value: 0.0003) (Fig. 1f).

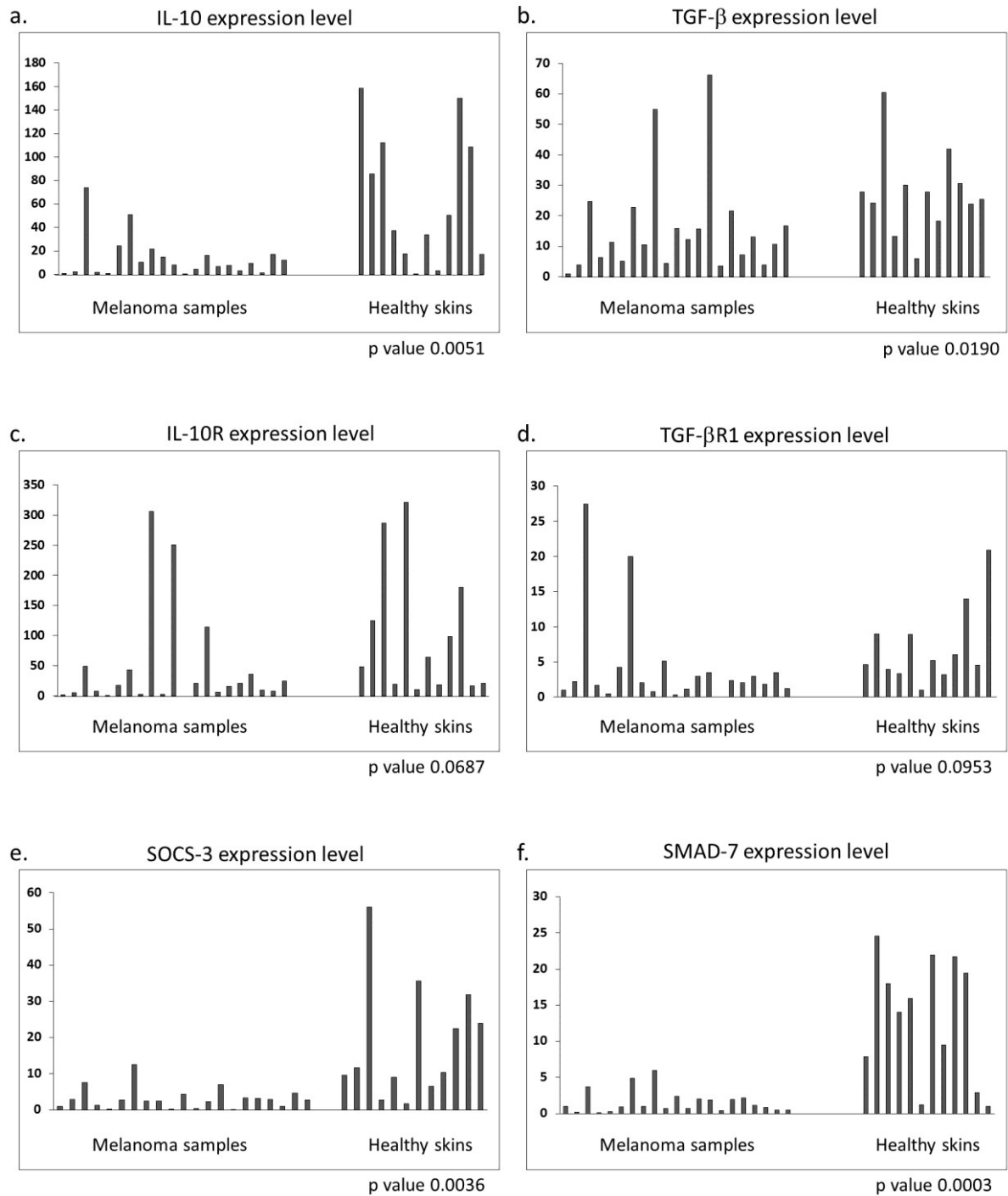


Fig.1. qPCR of 21 melanoma and 12 healthy skin samples. (a) IL-10, (b) TGF- β , (c) IL-10R, (d) TGF- β 1, (e) SOCS-3 and (f) SMAD-7 expression levels were quantified by qPCR in duplicate. Quantitative results were normalized to geometric average of three internal control genes (SDHA, GAPD and HPRT). Student's *t*-test analysis (melanoma vs healthy skin samples); the p values are shown in the graphs.

3.2. *In vitro* model for anergic T cells

3.2.1. Establishing an *in vitro* anergic T cells model

To test the active role of IL-10 and TGF- β in suppressing immune response in melanoma, an *in vitro* model was designed to generate a model of anergic T cells found in tumors. Anergic T cells were defined as dysfunctional T cells which were unable to respond to stimuli. Factors that need to be determined in generating anergic T cells model were the ratio between T cells and tumor cells in culture and the time needed by the T cells to rest to be able to respond to re-challenge. The melanoma cell line used in the *in vitro* experiments was ChaMel84 which had been established in our lab from one of the patients. ChaMel84 was chosen since a large number of tumor cells were needed in the experiments and ChaMel84 grows rapidly in culture. Anergic T cells were induced *in vitro* by incubating allogenic T cells with irradiated melanoma (30 Gy) in the presence of IL-2 (50 U/ml) in several steps.

Titration experiments were done to determine the ratio of T cells to tumor cells needed to induce the optimal response and failed response of CD8 T cells. T cell proliferation was used to measure T cell responses. Different numbers of tumor cells were used to induce 5×10^5 T cells. The ratios were: 1:1, 5:1, 10:1 and 20:1 (T cells: tumor cells). The numbers of T cells were counted every week for three weeks. T cell number decreased continually when T cells were co-cultured with the same number of tumor cells (1:1). T cell number decreased at the first 2 weeks of co-culturing for the other combinations (5:1, 10:1 and 20:1) and started increasing after 2 weeks. The 5:1 ratio showed the highest T cell number (5 folds compared with the beginning of co-culture) after 3 weeks, while 10:1 ratio tripled and 20:1 ratio doubled the original number (Fig. 2). For further experiments, the 5:1 group would be used as a control group which represented functional T cells and the 1:1 group as a model of anergic T cells.

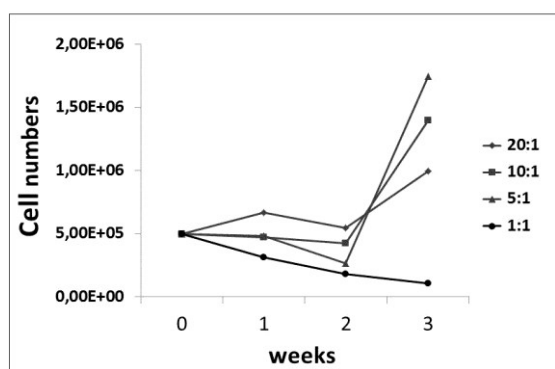


Fig. 2. Titration experiments of T cells co-cultured with tumor cells. Different numbers of tumor cells were added into wells to determine the optimal and failed induction. The ratios were 1:1, 5:1, 10:1 and 20:1 (T cells: tumor cells). The numbers of T cells were counted every week for three weeks. The figure was a representative of 3 different independent experiments.

Kinetics study was done to determine the time needed by T cells to rest to enable them to respond upon re-challenge. Firstly, tumor-specific T cells were selected and enriched by co-culturing T cells with melanoma at 10:1 ratio for 10-11 days. Secondly, T cells were driven into anergic T cells by co-culturing T cells with the same ratio of melanoma (1:1 ratio). Functional T cells as a control group were generated by co-culturing T cells with melanoma at 5:1 ratio. During the anergy induction, different co-culturing times were used (9, 12 or 18 days). Lastly, T cells were re-challenged with melanoma at 5:1 ratio for 5 days to measure the ability of T cells to respond upon re-challenge. T cell proliferation, and Granzyme B and Interferon- γ (IFN- γ) productions were used to measure T cell responses. T cell proliferation was calculated as a stimulation index. The stimulation index represents the numbers of cells proliferating in response to stimulation divided by the numbers of cells undergoing background proliferation in un-stimulated condition.

The results showed that the difference between donors was quite large. In 9 days co-culture, donor 3 showed the biggest difference of T cell responses between control (5:1 group) and anergic T cells (1:1 group), where the difference in proliferation rate was 30 fold, Granzyme B expression had a 1.5-fold difference and IFN- γ production had an 80-fold difference (Fig. 3a). Donor 2 had the least difference between control and anergic T cells, where proliferation had a 4-fold difference, Granzyme B expression a 1.4-fold difference and IFN- γ a 2-fold difference (Fig. 3a). In 12 days co-culture, donor 3 again showed the largest difference between control and anergic T cells, but with decreasing gap, with a 12-fold difference of proliferation, a 3-fold difference of Granzyme B and a 60-fold difference of IFN- γ production (Fig. 3b). Granzyme B production from 12 days culture (Fig. 3b) showed a different tendency with 9 and 18 days cultures (Fig. 3a and c), where donor 1 and donor 2 showed no difference between control and anergic T cells. In contrast, stimulation index and IFN- γ production showed similar results on 9, 12 or 18 days co-culture (Fig. 3a-c). In 18 days co-culture, donor 3 showed the largest difference between control and anergic T cells, with a 7-fold difference of proliferation, a 2-fold difference of Granzyme B and a 20-fold difference of IFN- γ production (Fig. 3c). Donor 1 had the least difference of proliferation (1.4-fold) and Granzyme B production (1.2-fold) while donor 2 had the least difference of IFN- γ production (1.9-fold) between control and anergic T cells (Fig. 3c). Nevertheless, all three donors showed the same tendency where all control T cells showed positive responses on all response parameters measured and anergic T cells failed to respond when T cells were co-cultured with tumor cells for 9 days. Thus, 9 days co-culture was chosen in further experiments.

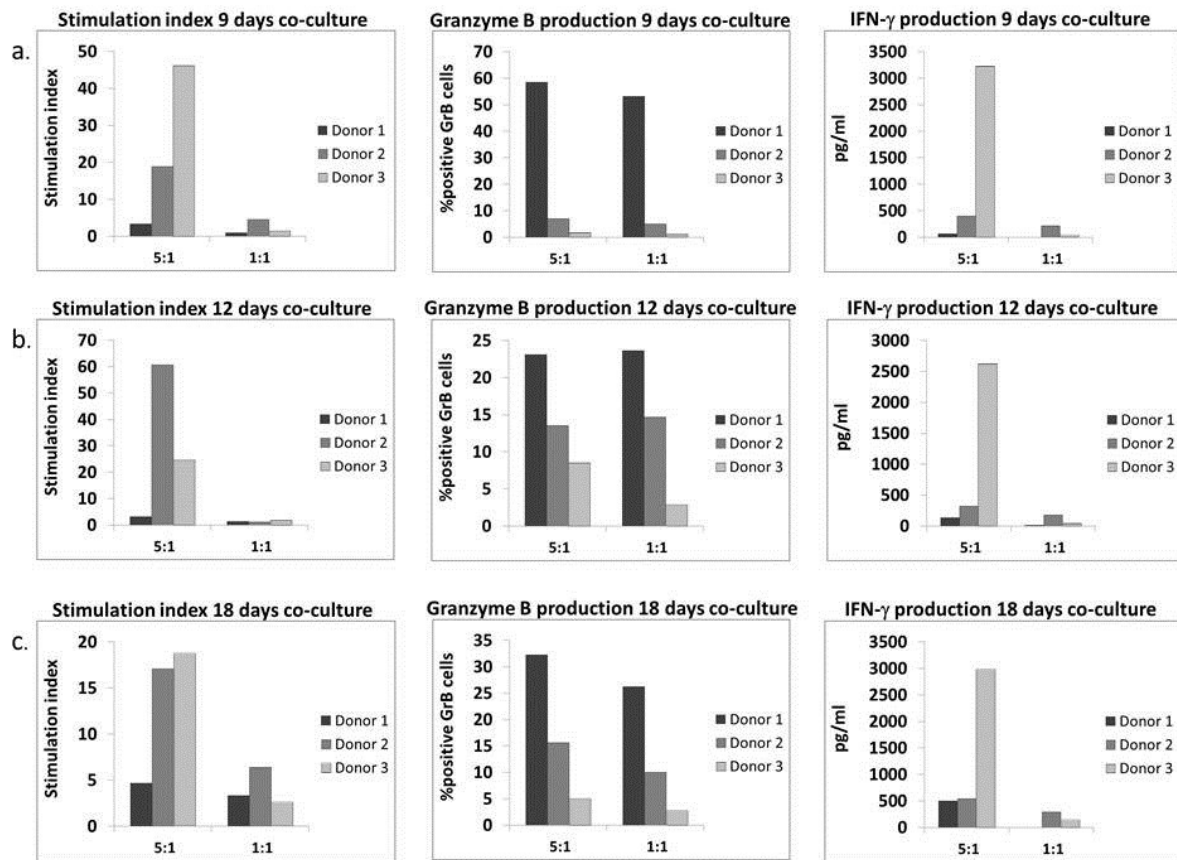


Fig. 3 Kinetics experiments of T cells co-cultured with tumor cells. Anergic T cells were induced *in vitro* by stimulating T cells with irradiated melanoma (30 Gy) supplied with IL-2 (50 U/ml) in several steps. Firstly, tumor-specific T cells were selected and enriched by co-culturing T cells with melanoma at 10:1 ratio for 10-11 days. Secondly, T cells were driven into anergic T cells by co-culturing T cells with the same ratio of melanoma (1:1 ratio) in different culture times: (a) 9, (b) 12 and (c) 18 days. Functional T cells as a control group was generated by co-culturing T cells with melanoma at 5:1 ratio. Lastly, T cells were re-challenged with melanoma at 5:1 ratio for 5 days to measure the ability of T cells to respond upon re-challenge. Data show results from three independent experiments. Stimulation index, Granzyme B and IFN- γ productions were used to measure T cell responses.

To confirm that the anergic T cell *in vitro* model was not exclusive for the ChaMel84 cell line, *in vitro* experiments using a different melanoma cell-line (SK-Mel-28) were done. SK-Mel-28 is a melanoma cell lines deposited at ATCC which has tumorigenic potential in nude mice. The selection process was done by co-culturing T cells with irradiated SK-Mel-28 cells (10:1) for 10-11 days. To induce anergy, different numbers of SK-Mel-28 cells were used to stimulate T cells (T cells: SK-Mel-28 cells = 1:2, 1:1 and 5:1) for 9 days. The re-challenge was done by co-culturing T cells with SK-Mel-28 cells (5:1) for 5 days.

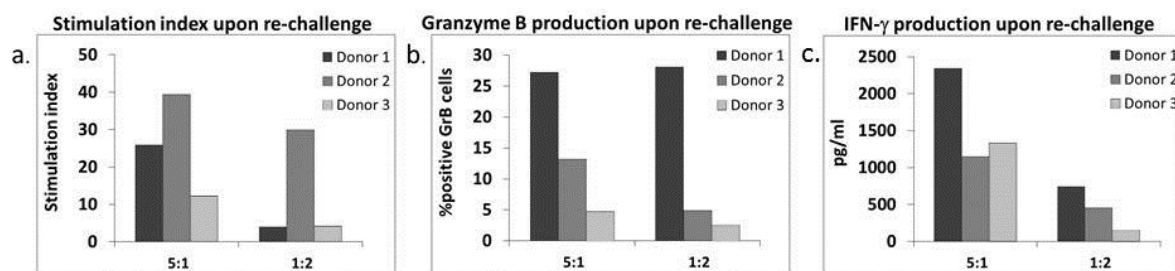


Fig. 4. T cell responses upon SK-Mel-28 stimulation. Firstly, tumor-specific T cells were selected and enriched by co-culturing T cells with melanoma at 10:1 ratio for 10-11 days. Second stimulation to induce anergy was done by co-culturing 1:2, 1:1 and 5:1 T cells vs tumor cells for 9 days. Lastly, T cells were re-challenged with melanoma at 5:1 ratio for 5 days to measure the ability of T cells to respond upon re-challenge. (a) Stimulation index, (b) Granzyme B and (c) IFN- γ production were used to measure T cell responses. Only results from 1:2 and 5:1 groups are shown. Data show results from three independent experiments.

The results showed that T cell proliferation was reduced in anergic T cells, where donor 1 had a 7-fold difference, donor 2 had a 1.3-fold difference and donor 3 had a 3-fold difference of proliferation between control and anergic T cells (Fig. 4a). Granzyme B production showed a different tendency where donor 2 and 3 showed less production on anergic T cells (2.7- and 1.9-fold difference, respectively) but donor 1 showed no decrease production of Granzyme B (Fig. 4b). IFN- γ production was also reduced after re-challenge in anergic T cells, with a 3.15-fold difference in donor 1, a 2.5-fold difference in donor 2 and a 9-fold difference in donor 3 (Fig. 4c). One important thing to be noted was that the number of tumor cells needed to induce anergic T cells using this cell line was different. A double numbers of tumor cells compared with T cells were needed to induce anergy using SK-Mel-28 cell lines. These experiments showed that the anergic T cells model could be generated *in vitro* using different melanoma cell lines.

3.2.2. IL-10 and TGF- β in the *in vitro* anergic T cell model

Based on kinetics experiments, *in vitro* experiments to study the active role of IL-10 and TGF- β in suppressing immune response against melanoma were done. In order to support the immunosuppression hypothesis, a positive correlation between IL-10 and TGF- β production with immune suppression was expected, thus a higher expression of IL-10 and TGF- β was expected to be found in the anergic T cell culture. Firstly, tumor-specific T cells were selected and enriched by co-culturing T cells with melanoma at 10:1 ratio for 10-11 days. Secondly, T cells were driven into anergic T cells by co-culturing T cells with the same ratio of melanoma (1:1 ratio) for 9 days. Functional T cells as a control group was generated by co-culturing T cells with melanoma at 5:1 ratio. Lastly, T cells were re-challenged with melanoma at 5:1 ratio for 5 days to measure the ability of T cells to respond upon re-challenge. T cell responses were normalized to the control group since variations between donors were high. By normalization, the variations could be minimalized and the difference between groups would be clearer. The results are shown as fold changes compared with the control group.

The results of the *in vitro* experiment showed that anergic T cells (1:1 group) had significantly lower Granzyme B production (fold change: 0.72 ± 0.23), lower proliferation (fold change: 0.53 ± 0.28), and in particular lower IFN- γ production (fold change: 0.06 ± 0.05) upon tumor cells re-challenge compared with the control T cells (5:1 group) (Fig. 5a). Three out of five donors showed a higher IL-10 production in the anergic T cell culture (1:1 group) compared with the control T cells (5:1 group) but two donors showed a lower IL-10 productions in the anergic T cell group compared with the control group (Fig. 5b). IL-10 production during anergy induction showed that there was no uniform tendency of increasing IL-10 production in the anergic T cell culture. Likewise, three out of five donors showed a higher TGF- β production in anergic T cell culture compared with the control T cell culture but two donors showed a lower TGF- β production in the anergic T cell group compared with the control group (Fig. 5c). Thus, there was no co-linearity between IL-10 and TGF- β productions with anergic T cell induction.

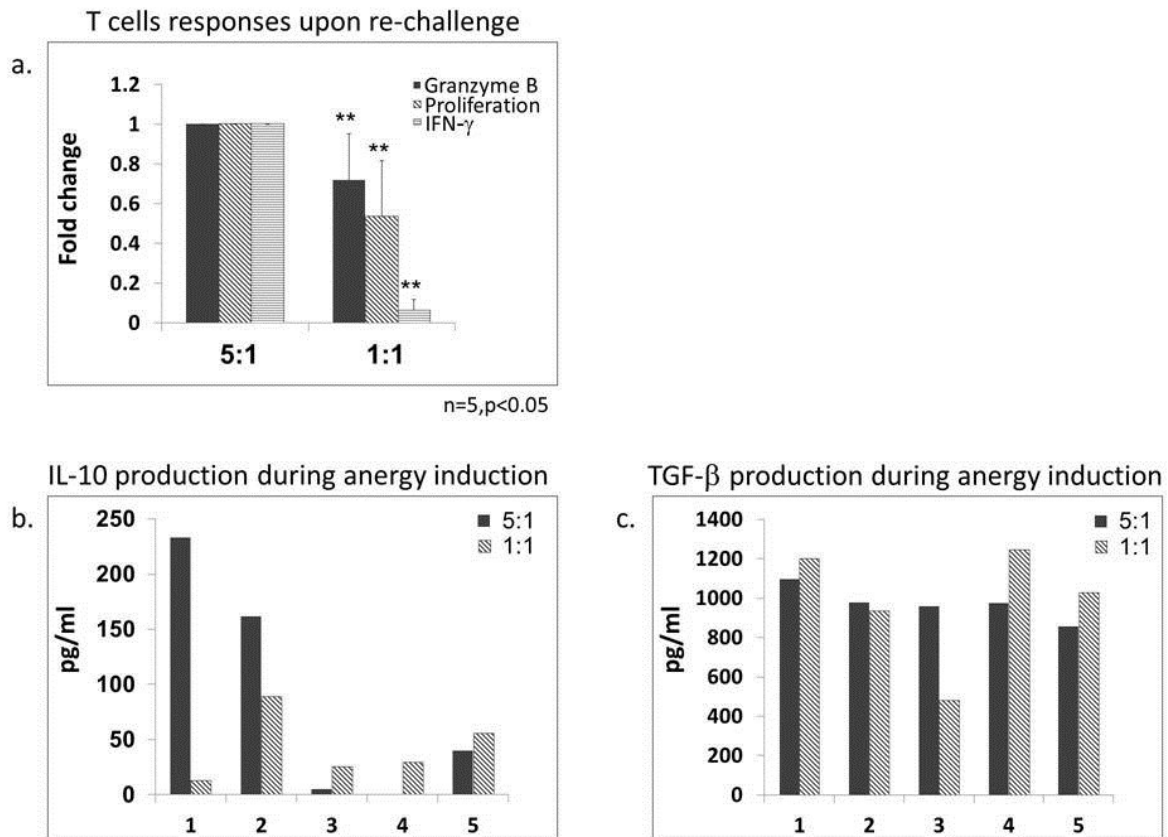


Fig. 5. IL-10 and TGF- β productions during anergic T cell induction. After tumor-specific T cell selection process, T cells were co-cultured with different numbers of tumor cells (5:1 as control and 1:1 as anergic T cell model). (a) Five days after re-challenge, the percentage of low-CFSE-stained cells and percentage of Granzyme B positive cells were calculated by flow cytometry, while IFN- γ production was measured by ELISA. Results from 5 different donors are shown as fold changes compared with the control group (5:1 group). Statistical analysis was done by student *t*-test (5:1 group vs 1:1 group) $p < 0.05$ is considered significant. (b) IL-10 and (c) TGF- β produced during anergic T cell induction were measured by ELISA.

3.2.3. T cell activation in the anergic T cell model

To analyze the T cell subsets in the culture, CD45RO expressions were measured at the end of anergy induction. Around 70% T cells were found to be memory T cells expressing CD45RO. Anergic T cell culture (1:1 group) had slightly more naïve T cells, expressing CD45RA, compared with the control group (%CD45RA⁺ cells: 26.72±13.46 vs 22.94±10.32) (Fig. 6a).

To investigate the activation state of the anergic T cells induced *in vitro*, the expression of well-known activation and proliferation markers CD25, CD69 and CD71 and the IFN- γ production were analyzed. The results are shown as fold changes normalized to the control. Anergic T cells and control T cells had similar expression of CD71, however anergic

T cells had a higher expression of the activation marker CD25 and CD69 compared with the control (fold change: 1.32 ± 0.45 and 1.22 ± 0.32 , respectively) (Fig. 6b). Moreover, anergic T cells produced a significantly increased level of IFN- γ compared with the control (fold change: 1.46 ± 0.37) (Fig. 6c). No suppression of anergic T cells could be observed.

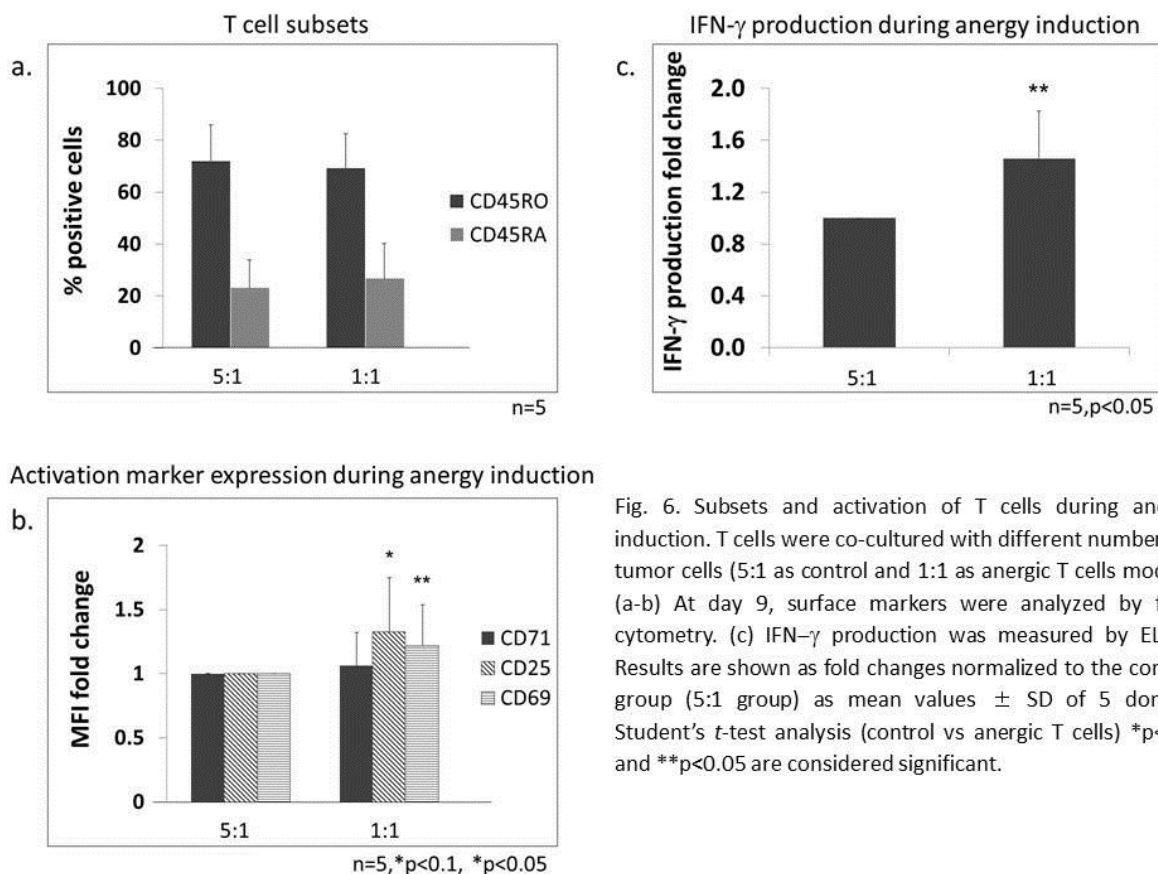


Fig. 6. Subsets and activation of T cells during anergy induction. T cells were co-cultured with different numbers of tumor cells (5:1 as control and 1:1 as anergic T cells model). (a-b) At day 9, surface markers were analyzed by flow cytometry. (c) IFN- γ production was measured by ELISA. Results are shown as fold changes normalized to the control group (5:1 group) as mean values \pm SD of 5 donors. Student's *t*-test analysis (control vs anergic T cells) *p<0.1 and **p<0.05 are considered significant.

Kinetics experiments to further investigate the activation of T cells during anergy induction were done by co-culturing T cells with different numbers of tumor cells (T cells: tumor cells = 1:1, 2:1, 5:1 and 10:1) for 9 days. T cells were then re-challenged with tumor cells (5:1) for 5 days. IFN- γ production was used to measure the activation of T cells during anergy induction. T cell proliferation was used to determine T cell responses upon re-challenge. Proliferation is shown as a stimulation index (SI).

T cells co-cultured with tumor cells at 1:1 ratio produced 12,417 pg/ml IFN- γ during anergy induction. IFN- γ production was lower (mean value: 10,386 pg/ml) when T cells were co-cultured with tumor cells at 2:1 ratio compared with the 1:1 ratio and the lowest

production reached when the 5:1 ratio was used (mean value: 7,925 pg/ml). IFN- γ production of T cells co-cultured with tumor cells at 10:1 ratio was higher (mean value: 14,479 pg/ml) than at 1:1 ratio. T cell responses upon re-challenge showed the exact opposite results where T cells co-cultured with tumor cells at 1:1 ratio had the lowest proliferation capacity (SI mean value: 2.1). The proliferation capacity was higher at 2:1 ratio culture (SI mean value: 11.8) and at 10:1 ratio (SI mean value: 4.8) compared with the 1:1 ratio. The highest proliferation capacity could be observed at 5:1 ratio (SI mean value: 14.5) (Fig. 7).

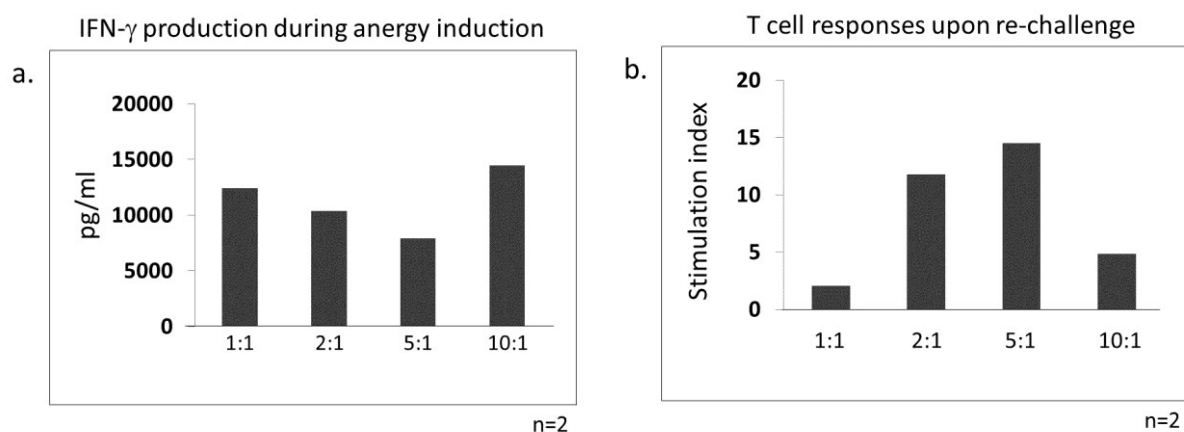


Fig. 7. Kinetics experiments of anergic T cell induction. After tumor-specific T cell selection process, T cells were co-cultured with different ratios of tumor cells (1:1, 2:1, 5:1 and 10:1) for 9 days. (a) IFN- γ production during anergy induction was measured by ELISA. (b) T cell proliferation was measured upon re-challenge by flow cytometry. Proliferation is shown as a stimulation index. Results are shown as mean values of 2 different donors.

3.2.4. Co-inhibitor molecules on T cells co-cultured with tumor cells

The expression of the co-inhibitor molecules PD-1 and CTLA-4 was investigated to study whether these molecules were involved in causing T cell anergy in this *in vitro* model. After tumor-specific T cell selection process, T cells were co-cultured with tumor cells at different ratios (1:1 and 5:1). Flow cytometry analyses of PD-1 and CTLA-4 expression were done at the end of anergy induction. Results are shown as fold changes compared with the control.

PD-1 and CTLA-4 expressions on anergic T cells and control T cells showed no difference (Fig.8). No negative regulation by co-inhibitors molecules could be observed on anergic T cells generated *in vitro*.

Co-inhibitor molecule expression during anergy induction

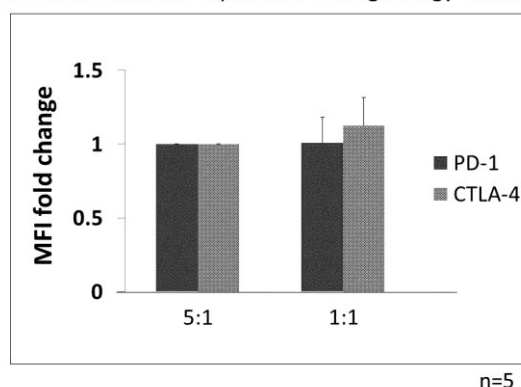


Fig. 8. Co-inhibitor molecule expression of T cells during anergy induction. T cells were co-cultured with different numbers of tumor cells (5:1 as control and 1:1 as anergic T cell model). At the end of anergy induction, surface markers were analyzed by flow cytometry. Results are shown as fold changes compared with the control group (5:1 group) as mean values \pm SD of 5 donors.

3.2.5. Effect of IL-10 and TGF- β on T cells co-cultured with tumor cells

To answer whether IL-10 and TGF- β could assist or interfere with anergic T cell induction, exogenous IL-10 or TGF- β or antibodies against IL-10 or TGF- β were added into T cells co-cultured with tumor cell at 5:1 ratio during anergic T cell generation. After tumor-specific T cell selection process, T cells were further co-cultured with tumor cells (5:1) for 9 days in the presence or absence of exogenous IL-10 (20 ng/ml) or TGF- β (5 ng/ml) or antibody against IL-10 (5 μ g/ml) or TGF- β (10 μ g/ml). A 5:1 ratio T cells to tumor cells culture without any addition of cytokine or antibody was used as a control group. A 1:1 ratio T cells to tumor cells without any addition of cytokine or antibody was used as a control of anergic T cell induction. T cells were then re-challenged with tumor cells (5:1) for 5 days.

IFN- γ production and T cell proliferation were used to measure T cell responses. Results of T cell responses were normalized and compared with the control group. Results are shown as fold changes compared with the control group.

As was shown in previous experiments, co-culturing T cells with tumor cells at 1:1 ratio increased the IFN- γ production during anergy induction (fold change: 2.27 ± 1.14) compared with the 5:1 ratio (control), and decreased the IFN- γ production and proliferation (fold change: 0.37 ± 0.16 and 0.72 ± 0.07 , respectively) upon re-challenge. Adding exogenous IL-10 to the 5:1 group did not make any change to the IFN- γ production during anergy induction or upon re-challenge but it decreased the proliferation upon re-challenge (fold change: 0.90 ± 0.7). However, adding TGF- β into the 5:1 culture significantly decreased the IFN- γ production during anergy induction (fold change: 0.69 ± 0.22) and gave a better response upon re-challenge, with a 3-fold increase of IFN- γ production (fold change: 3.01 ± 3.03) and a 1.3-fold increase of proliferation (SI fold change: 1.31 ± 0.18). Adding a combination of IL-10 and TGF- β to the 5:1 group did not change the IFN- γ production during anergy induction, but a higher IFN- γ production (fold change: 1.40 ± 0.65) and proliferation (SI fold change: 1.53 ± 0.51) were observed upon re-challenge. Blocking IL-10 production in T cells co-cultured with tumor cell at 5:1 ratio during anergy induction made no change to the IFN- γ production during anergy induction or to the T cell responses upon re-challenge. On the other hand, blocking TGF- β in T cells co-cultured with tumor cells at 5:1 ratio during anergy induction led to a decreased IFN- γ production during anergy induction (fold change: 0.90 ± 0.09), and an increased IFN- γ production (fold change: 1.77 ± 0.82) upon re-challenged even though no increase of proliferation upon re-challenge was observed (SI fold change: 0.98 ± 0.09) (Fig. 9a and b).

To study the effect of IL-10 and TGF- β on anergic T cells, exogenous IL-10 or TGF- β or antibodies against IL-10 or TGF- β were added into T cells co-cultured with tumor cell at 1:1 ratio during anergic T cell generation. After tumor-specific T cell selection process, T cells were further co-cultured with tumor cells (1:1) for 9 days in the presence or absence of exogenous IL-10 (20 ng/ml) or TGF- β (5 ng/ml) or antibody against IL-10 (5 μ g/ml) or TGF- β (10 μ g/ml). T cells were then re-challenged with tumor cells (5:1) for 5 days to determine T cell responses upon re-challenge using IFN- γ production and T cell proliferation as parameters. The results from measurement of T cell responses were normalized and compared

with the 1:1 group (shown as fold changes) without any addition of cytokines or antibodies. As a positive control, the results of the measurement from the 5:1 group are shown.

Co-culturing T cells with tumor cells at 5:1 ratio resulted in a lower IFN- γ production during anergy induction (fold change: 0.51 ± 0.21), and a higher IFN- γ production and proliferation upon re-challenge (fold change: 2.98 ± 1.13 and 1.40 ± 0.14 , respectively). Addition of IL-10 into the 1:1 group slightly increased the IFN- γ production during anergy induction and upon re-challenge (fold change: 1.15 ± 0.15 and 1.27 ± 0.82 , respectively) but no changes on proliferation could be detected. Adding exogenous TGF- β to the 1:1 group significantly reduced the IFN- γ production during anergy induction (fold changes: 0.54 ± 0.12), opposed to a higher IFN- γ production upon re-challenge (fold change: 1.53 ± 0.46) even though no change on proliferation was observed. Adding a combination of IL-10 and TGF- β to the 1:1 group decreased the IFN- γ production during anergy induction (fold change: 0.71 ± 0.32) and increased the production upon re-challenge (fold change: 3.59 ± 2.73) with no effect on proliferation. Blocking IL-10 production in T cells co-cultured with tumor cells at 1:1 ratio decreased the IFN- γ production during anergy induction (fold change: 0.71 ± 0.34), but no changes on T cell responses upon re-challenge was observed. Blocking TGF- β in T cells co-cultured with tumor cells at 1:1 ratio significantly lowered the IFN- γ production during anergy induction (fold change: 0.87 ± 0.08), but no significant changes on T cell responses upon re-challenge was detected (Fig. 9c and d).

The above results showed large standard deviations. To confirm the results above, the experiments were repeated with fewer groups done in each experiment due to the limitation of resources and time in handling the experiments. In addition to IFN- γ production and T cell proliferation, Granzyme B production was also included to determine T cell responses. Results are shown as fold changes compared with the control group.

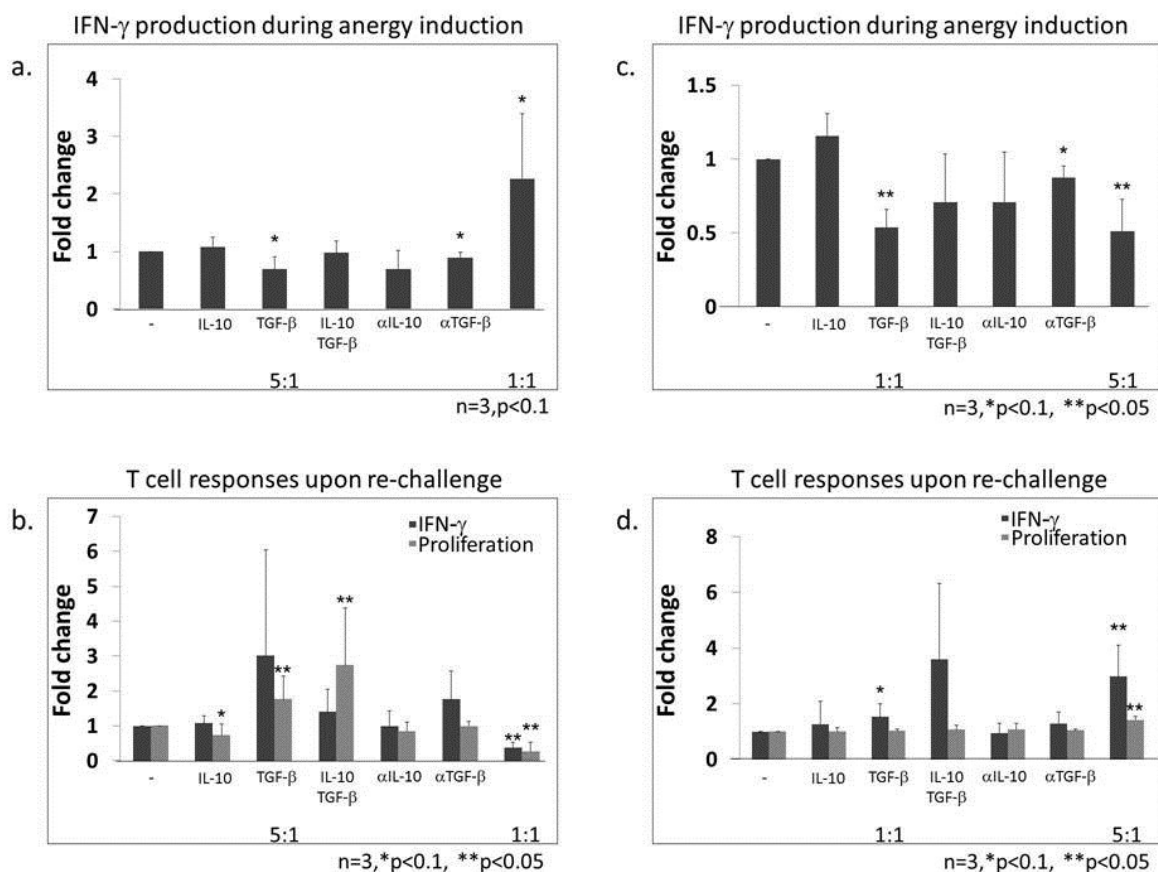


Fig. 9. Effect of IL-10 and TGF- β to T cells co-cultured with tumor cells. After tumor-specific T cell selection process, T cells were further co-cultured with different numbers of tumor cells (5:1 and 1:1) for 9 days in the presence or absence of exogenous IL-10 (20 ng/ml) or TGF- β (5 ng/ml) or antibody against IL-10 (5 μ g/ml) or TGF- β (10 μ g/ml). T cells were then re-challenged with tumor cells (5:1) for 5 days. (a) IFN- γ production during energy induction in the 5:1 ratio culture was measured by ELISA. (b) T cell responses upon re-challenge from the 5:1 group were determined by IFN- γ production measured by ELISA and cell proliferation measured by flow cytometry. (c) IFN- γ production during energy induction in the 1:1 ratio culture was measured by ELISA. (d) T cell responses upon re-challenge from the 1:1 group were determined by IFN- γ production measured by ELISA and cell proliferation measured by flow cytometry. Results are shown as fold changes normalized to the control group as mean values \pm SD of 3 donors. Student *t*-test analysis (control vs treatment), *p<0.1 and **p<0.05 are considered significant.

In the first group of experiments, exogenous IL-10 (20 ng/ml) and TGF- β (5 ng/ml) was added into T cells co-cultured with tumor cells at 5:1 ratio to study whether these cytokines could assist inducing T cells to become anergic T cells (Fig. 10). Addition of exogenous IL-10 during anergy induction increased slightly the IFN- γ production (fold change: 1.14 ± 0.21), opposed to a decrease capacity to respond upon re-challenge, which was observed by a lower Granzyme B production (fold change: 0.80 ± 0.33) and proliferation (SI fold change: 0.87 ± 0.06), even though no change was detected on the IFN- γ production. Addition of exogenous TGF- β during anergy induction decreased significantly the IFN- γ production to around 66% of the control's level (fold change: 0.66 ± 0.20). However, enhancement of the IFN- γ production to around 45% upon re-challenge was observed (fold change: 1.44 ± 0.49) even though there was almost no difference in the Granzyme B production (fold change: 0.96 ± 0.09) or proliferation (SI fold change: 1.01 ± 0.15). Combining IL-10 and TGF- β decreased the IFN- γ production during anergy induction (fold change: 0.70 ± 0.23). A double production of IFN- γ (fold change: 2.14 ± 1.11) and a 13% increased of proliferation (fold change: 1.13 ± 0.07) was observed upon re-challenge but there was no changes in Granzyme B production (fold change: 0.94 ± 0.76) (Fig 10).

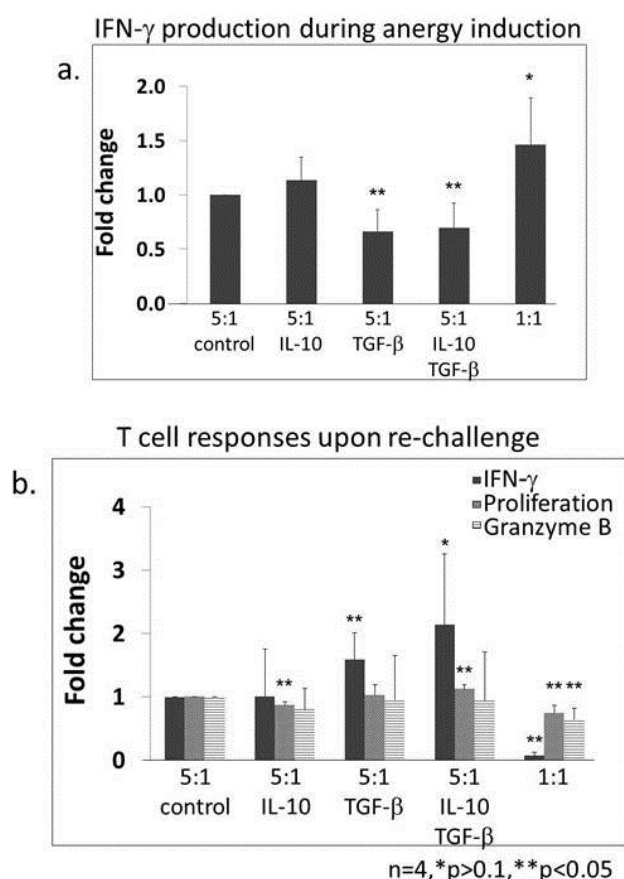


Fig. 10. Effect of exogenous IL-10 and TGF- β on functional T cells. After tumor-specific selection process, T cells were further co-cultured with tumor cells (5:1) for 9 days in the presence or absence of exogenous IL-10 (20 ng/ml) or TGF- β (5 ng/ml). T cells were then re-challenged with tumor cells (5:1) for 5 days. (a) IFN- γ production during anergy induction was measured by ELISA. (b) IFN- γ production measured by ELISA, Granzyme B production and cell proliferation measured by flow cytometry were used to determine T cell responses upon re-challenge. Results are shown as fold changes compared with the control group as mean values \pm SD of 4 donors. Student's *t*-test analysis (control vs exogenous cytokines), **p*<0.1 and ***p*<0.05 are considered significant.

In the second group of experiment, exogenous IL-10 (20 ng/ml) and TGF- β (5 ng/ml) were added to T cells co-cultured with tumor cells at 1:1 ratio to investigate whether addition of these immunosuppressive cytokines to the culture would help suppressing the T cell responses or on the contrary, interfering with the anergy induction. Results were normalized to T cells co-cultured with tumor cells at 1:1 ratio without any addition of cytokines and shown as fold changes compared with the control group. Addition of IL-10 only slightly decreased the IFN- γ production during anergy induction (fold change: 0.96 ± 0.34), but could enhance T cell responses with a 4-fold increase of IFN- γ (fold change: 4.06 ± 5.21), a 50% increase of Granzyme B production (fold change: 1.48 ± 0.34) and a 5% increase of proliferation (SI fold change: 1.04 ± 0.04) upon re-challenge. Addition of TGF- β significantly decreased the IFN- γ production during anergy induction (fold change: 0.59 ± 0.17) but doubled the IFN- γ production (fold change: 1.97 ± 1.32) upon re-challenge although the proliferation significantly lowered (SI fold change: 0.67 ± 0.29). No change was observed in the Granzyme B production (Fig. 11).

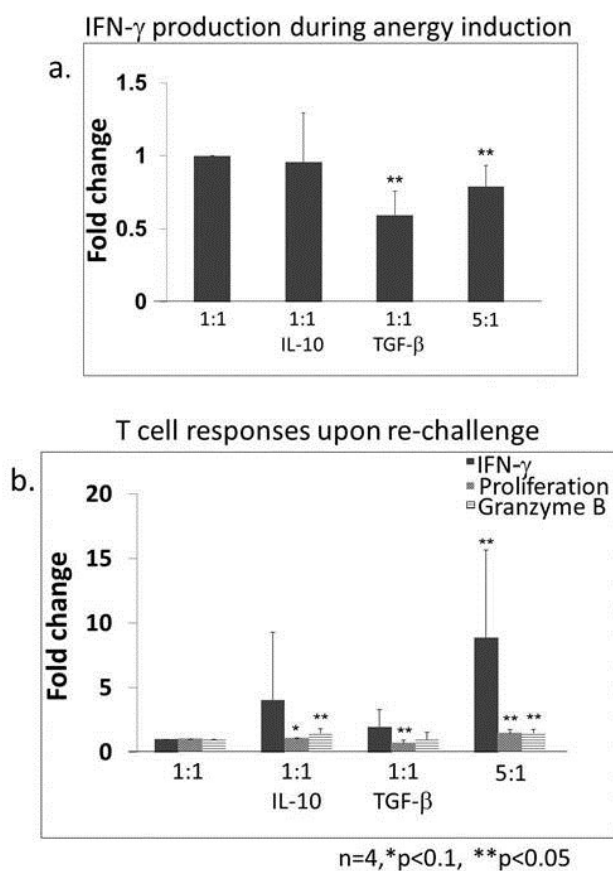


Fig. 11. Effect of exogenous IL-10 and TGF- β on anergic T cells. After tumor-specific selection process, T cells were further co-cultured with tumor cells (1:1) for 9 days in the presence or absence of exogenous IL-10 (20 ng/ml) or TGF- β (5 ng/ml). T cells were then re-challenged with tumor cells (5:1) for 5 days. (a) IFN- γ production during anergy induction was measured by ELISA. (b) IFN- γ production measured by ELISA, Granzyme B production and cell proliferation measured by flow cytometry were used to determine T cell responses upon re-challenge. Results are shown as fold changes compared to the control group as mean values \pm SD of 4 donors. Student's *t*-test analysis (control vs exogenous cytokines), **p*<0.1 and ***p*<0.05 are considered significant.

In the third group of experiment, antibody against IL-10 (5 $\mu\text{g/ml}$) was added to T cells co-cultured with tumor cells at 1:1 ratio to further examine the role of endogenous IL-10 in inducing T cell anergy. Results were normalized to T cells co-cultured with tumor cells at 1:1 ratio without any addition of antibodies and shown as fold changes compared with the control group. IFN- γ production was lower when T cells were co-cultured with tumor cells at 1:1 ratio in the presence of antibody against IL-10 (fold change: 0.83 ± 0.05). There was an increase of Granzyme B production (fold change: 1.50 ± 0.23) but no difference of IFN- γ production or proliferation upon re-challenge (Fig. 12).

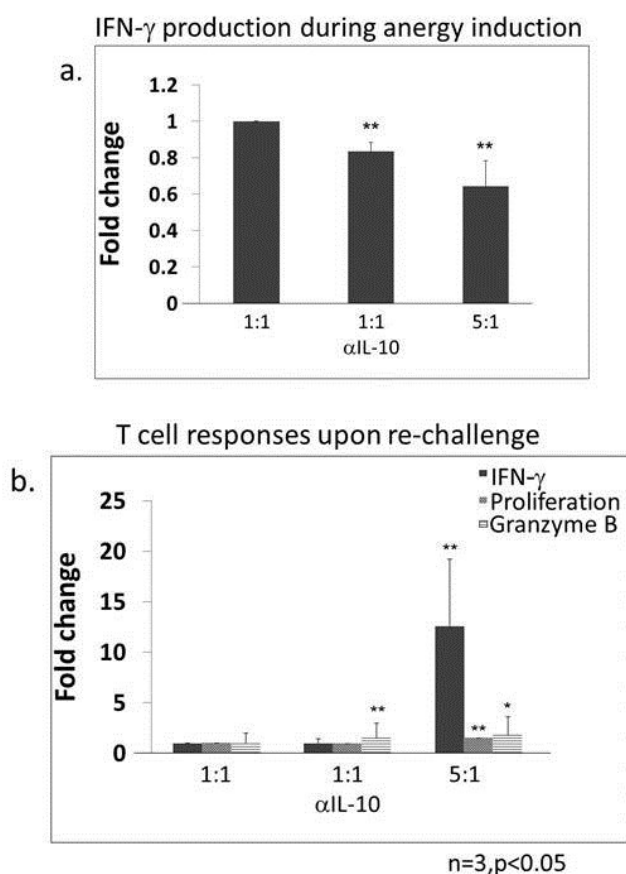


Fig. 12. Effect of antibody against IL-10 on anergic T cells. After tumor-specific selection process, T cells were further co-cultured with tumor cells (1:1) for 9 days in the presence or absence of antibody against IL-10 (5 $\mu\text{g/ml}$). T cells were then re-challenged with tumor cells (5:1) for 5 days. (a) IFN- γ production during anergy induction was measured by ELISA. (b) IFN- γ production measured by ELISA, Granzyme B production and cell proliferation measured by flow cytometry were used to determine T cell responses upon re-challenge. Results are shown as fold changes compared to the control group as mean values \pm SD of 3 donors. Student's *t*-test analysis (control vs addition of $\alpha\text{IL-10}$), * p <0.1 and ** p <0.05 are considered significant.

3.3. Tumor-associated DCs *in vitro*

3.3.1. Generation of tumor-associated DCs *in vitro*

In addition to T cell *in vitro* experiments, melanoma cells were also co-cultured with monocytes to study the effect of melanoma cells on DC generation since melanoma has been suggested to induce defects on DCs via IL-10 and TGF- β productions. To establish the optimal cell ratio for generating tumor-associated DCs *in vitro*, monocytes were differentiated into immature dendritic cells (iDCs) for 5 days and then 1×10^5 cells/well iDCs were further co-cultured with different ratios (1:1, 1:5 and 1:10) of irradiated melanoma cells (ChaMel84) in the presence of cytokine cocktail (IL-1, IL-6, TNF- α). At the end of experiments, cells were collected and the MFI of CD14 was calculated by flow cytometry. Monocytes and macrophages express CD14 while DCs do not express CD14 therefore the loss expression of CD14 signifies the differentiation of DCs from monocytes. Measurements were gated on CD11c to exclude the tumor cells in analysis since in the culture set up only myeloid cells express CD11c. To study whether tumor cells alter DC capacity to induce T cell proliferation, DCs were co-cultured with allogenic CFSE-stained T cells (1:20) for 5 days. The percentages of low-CFSE-stained cells represented the percentages of proliferating cells. On day 5, low-CFSE-stained T cells were calculated by flow cytometry. Data are shown as stimulation index fold changes compared with the control.

CD14 expression on tumor-associated DCs was not altered (Fig. 13a). Tumor-associated DCs cultured at 1:1 ratio had a slight increase capacity to induce CD4 T cell proliferation (SI mean value: 1.16) compared with the control. Tumor-associated DCs cultured at 1:5 ratio showed further increased DC capacity to stimulate CD4 T cells (SI mean value: 1.33). Tumor-associated DCs at 1:5 ratio and 1:10 ratio had similar capacity to induce T cell proliferation (Fig. 13b). Based on these results, 1:1 and 1:5 ratios were chosen for further experiments.

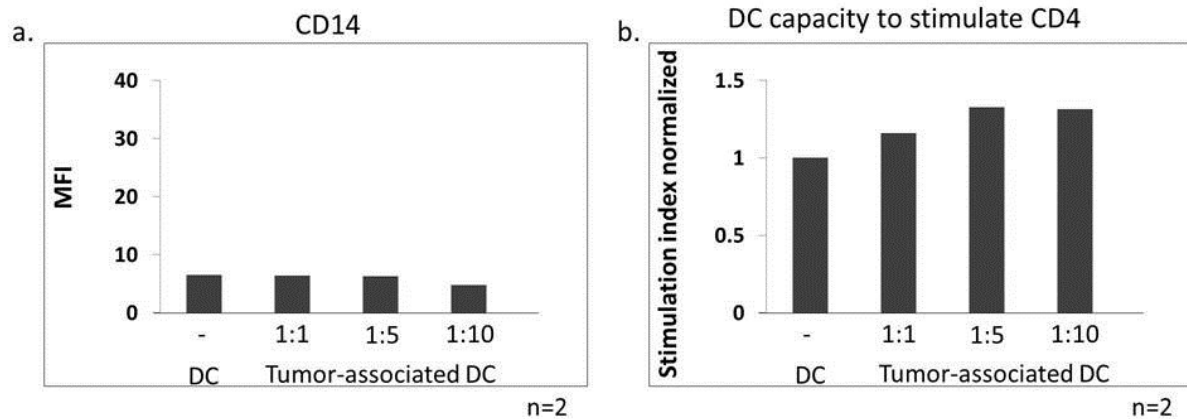


Fig. 13. Tumor-associated DCs generation. Monocytes were differentiated into iDCs for 5 days, and then further co-cultured with different numbers of tumor cells (1:1, 1:5 and 1:10). (a) CD14 expression, gated on CD11c, was measured by flow cytometry. (b) On day 7, DCs were co-cultured with allogenic CFSE-stained CD4 T cells. The stimulation index represents the number of cells proliferating in response to stimulation divided by number of cells undergoing background proliferation in un-stimulated condition. Stimulation index are shown as fold changes normalized to control DCs. Results are shown as mean values of 2 donors.

To confirm the robustness of DC generation *in vitro* setting, a different melanoma cell lines (SK-Mel-28) was used. To clarify the effect of melanoma cells on DC generation in differentiation and maturation stages, melanoma cells were added at the beginning of these two stages. Different ratios of melanoma cells were used in culture (monocytes : melanoma cells = 1:1 and 1:5) to study the effect of tumor burden on DC generation. To study the effect of melanoma cells on DC differentiation, 1×10^5 cells/well monocytes were cultured in the presence or absence of irradiated melanoma cells (SK-Mel-28) in a direct contact for 5-7 days. Immature dendritic cells (iDCs) which were differentiated in the presence of tumor cells were called tumor-associated iDCs. To investigate the effect of melanoma cells on DC maturation, iDCs (1×10^5 cells/well) were generated from monocytes for 5 days and at day 5, induced into maturation by adding cytokine cocktail (IL-1, IL-6, TNF- α) in the presence or absence of irradiated melanoma cells at ratios 1:1 and 1:5 (iDCs : melanoma). DCs induced into maturation in the presence of tumor cells were called tumor-associated mDCs. On day 7, cells were collected and the percentage of positive cells or MFI was calculated by flow cytometry. Measurements were gated on CD11c to exclude the tumor cells in analysis. DC capacity to induce T cell proliferation was investigated by co-culturing DCs with allogenic CFSE-stained CD4 T cells for 5 days.

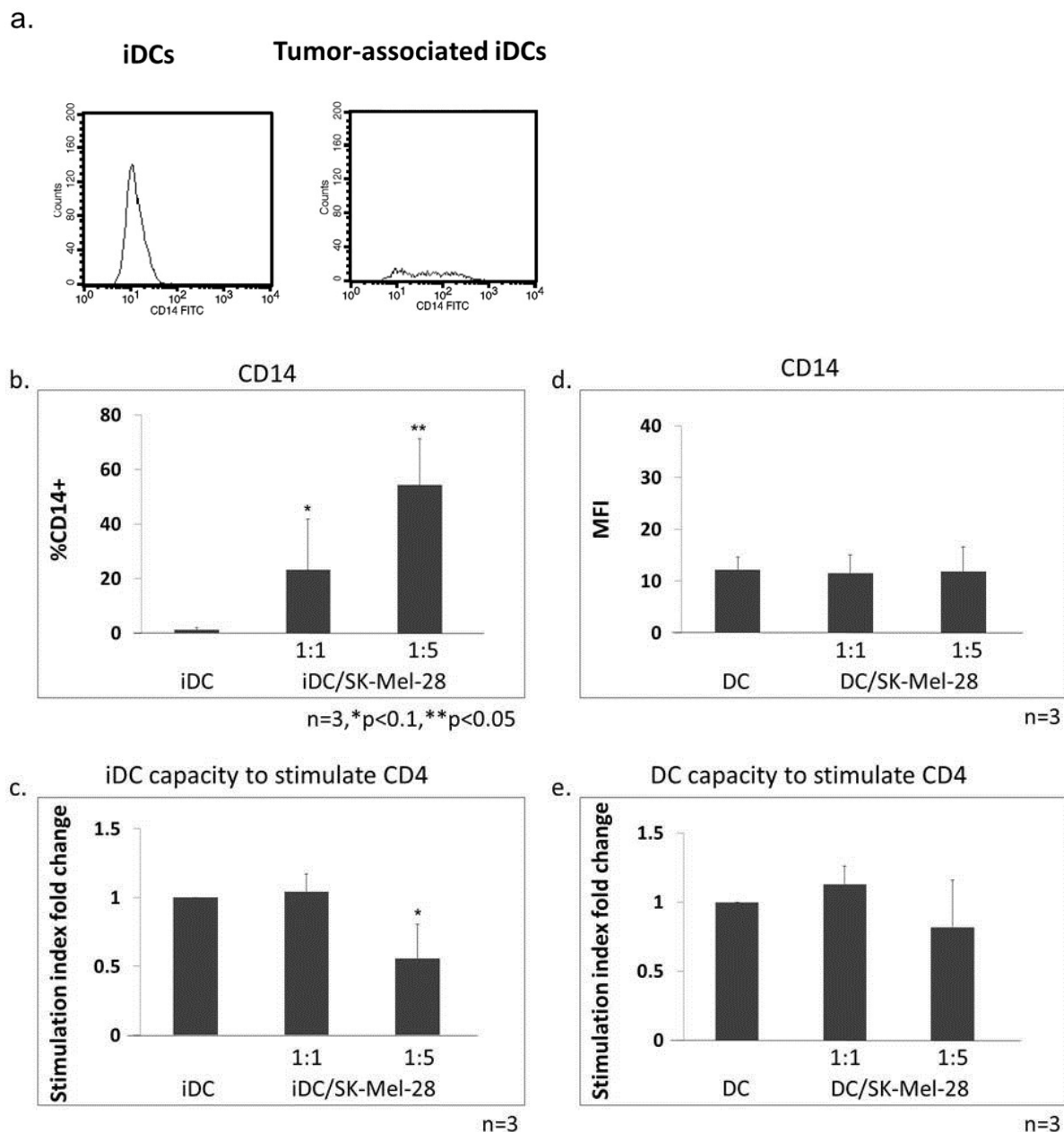


Fig. 14. DC generation in the presence of melanoma cells (SK-Mel-28). (a-b) Different numbers of tumor cells were used in co-culture in a direct contact. In the 1:1 group, 1×10^5 monocytes were cultured with 1×10^5 irradiated tumor cells (SK-Mel-28) and in the 1:5 group, 1×10^5 monocytes were cultured with 5×10^5 irradiated tumor cells. CD14 histogram of control iDCs and tumor-associated iDCs is shown as representative of 3 donors. %CD14⁺ is shown as mean values \pm SD of 3 donors. (d) iDCs (1×10^5 cells/well) were generated for 5 days and on day 5, cytokine cocktail (IL-1, IL-6 and TNF- α) together with irradiated tumor cells (1:1 and 1:5) were added. (c and e) On day 7, DCs were co-cultured with CD4 T cells (1:20). The stimulation index represents the number of cells proliferating in response to stimulation divided by number of cells undergoing background proliferation in un-stimulated condition. Stimulation index is shown as fold changes normalized to control DCs. Results are shown as mean values \pm SD of 3 donors. Student's *t*-test analysis (control vs tumor-associated iDCs or tumor-associated mDCs), **p<0.05 and *p<0.1 are considered significant.

In the control group, one peak showing a CD14 negative group could be observed from the histogram. However, in the tumor-associated iDCs, there was a shift of the CD14 expression toward the positive value (Fig. 14a). Co-culturing monocytes with SK-Mel-28 altered the CD14 expression as the percentage of CD14⁺ cells was higher on tumor-associated iDCs in corresponding to tumor burden (1:1 group: 23.22±18.63 and 1:5 group: 54.34±16.91) compared with the control iDCs (1.30±0.63) (Fig. 14b). However, CD14 expression level was not altered on the tumor-associated mDCs (Fig. 14d). The tumor-associated iDC capacity to induce CD4 T cell proliferation showed a significant 2-fold decrease in the 1:5 group (SI fold change: 0.56±0.25) compared with the control (Fig. 14c). The capacity to stimulate CD4 T cell proliferation of tumor-associated iDCs in the 1:1 group (Fig. 14c) or tumor-associated mDCs (Fig. 14e) was similar to the control DCs. These experiments showed that DC generation in the presence of different melanoma cell lines gave similar results.

3.3.2. DC maturation induced by tumor cells

To study whether tumor cells alone are sufficient to induce DC maturation, iDCs (1x10⁵ cells/well) were generated for 5 days and then tumor cells (ChaMel84) were added (iDCs : tumor = 1:5) into the culture. As a control, cytokine cocktail (IL-1, IL-6, TNF- α) was added to stimulate DC maturation. These experiments were set up in a cell culture well plate where a direct contact between iDCs and tumor cells occurred or in a transwell system where iDCs had no direct contact with tumor cells. In a no contact group, a transwell membrane with 0.4 μ m pores was used. Using the transwell system, there would be no direct contact between tumor cells and iDCs but soluble factors and smaller molecules could still be exchanged in the culture. On day 7, MFI of CD80, and percentages of CD14⁺, CD83⁺ and HLA-DR^{high} cells were calculated by flow cytometry. CD80, CD83 and HLA-DR are used to measure DC activation and maturation as these markers are upregulated on mature DCs. Measurements were gated on CD11c to exclude the tumor cells in analyses. MFI and percentages of activation and maturation markers were normalized to control DCs.

The percentage of CD14⁺ in both direct contact and transwell assays increased when iDCs were induced with tumor directly in the absence of pro-inflammatory cytokines (33.09±28.59 and 26.60±26.50, respectively) compared with control DCs (2.80±2.24) (Fig. 15a). The standard deviation was high because one donor had a very low percentage of CD14⁺ cell compared with the other two donors. The expression level of CD83 of DCs stimulated into maturation by tumor cells alone in direct contact and transwell assays were

significantly lower compared with the control DCs (%CD83⁺: 0.26±0.14 and 0.19±0.07, respectively). The expression level of HLA-DR of DCs stimulated by tumor cells was lower in the direct contact assay and transwell assay (%HLA-DR^{high}: 0.54±0.02 and 0.78±0.59, respectively) compared with the control but the difference was found to be statistically significant only in the direct contact assay. CD80 expression levels of DCs stimulated by tumor in both direct and transwell assays were significantly lower compared with the control DCs (MFI: 0.51±0.13 and 0.57±0.07) (Fig. 15b).

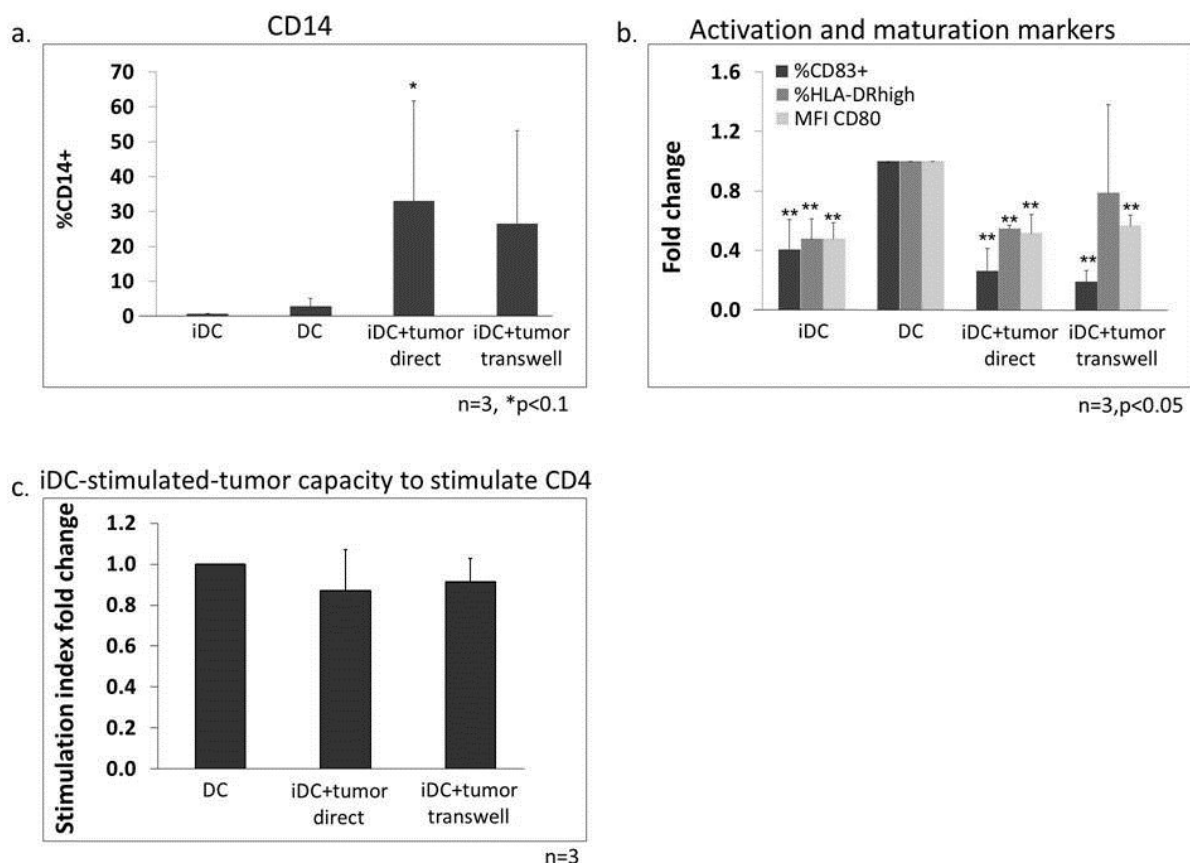


Fig. 15. DC maturation induced by tumor cells. Monocytes (1×10^5 cells/well) were generated into iDCs and at day 5, tumor cells (1:5) were added with or without direct contact to stimulate DC maturation. As a control, cytokine cocktail (IL-1, IL-6, TNF- α) were given to stimulate DC maturation. On day 7, percentages of (a) CD14⁺, (b) percentage of CD83⁺ and HLA-DR^{high} cells, and MFI of CD80 were calculated by flow cytometry. Measurements were gated on CD11c to exclude tumor cells in the analysis. (c) On day 7, DCs were co-cultured with CD4 T cells. The stimulation index represents the number of cells proliferating in response to stimulation divided by number of cells undergoing background proliferation in un-stimulated condition. Results are shown as mean values \pm SD of 3 donors. Data are shown as fold changes normalized to control DCs. Student's *t*-test analysis (control vs tumor associated DCs), **p<0.05 is considered significant.

The capacity of DCs to induce T cell proliferation was also measured. Results are shown as stimulation index fold changes compared with the control. iDCs cultured with tumor cells had lower capacity to induce T cells in both direct contact and transwell assays but the differences between altered iDCs and control DCs were not statistically significant (SI fold change: 0.87 ± 0.19 and 0.91 ± 0.11) (Fig. 15c).

3.3.3. Maturation of tumor-associated iDCs

Previous experiments showed that tumor cells altered DC differentiation, leading to the next question whether the altered iDCs can still mature when given the necessary stimulation. To answer this question, 1×10^5 cells/well monocytes were differentiated into iDCs in the presence or absence of 5×10^5 cells/well tumor cells (1:5) for 5 days. On day 5, iDCs were stimulated with cytokine cocktail (IL-1, IL-6, TNF- α) to induce DC maturation.

The percentage of CD14⁺ cells increased 65-fold (27.79 ± 1.25) on resulting matured tumor-associated DCs generated in the transwell assay and increased 163-fold (66.94 ± 848) in the direct contact assay compared with the control iDCs (0.41 ± 0.37) (Fig. 16a). The HLA-DR expression level was similar on control iDCs and tumor-associated DCs in the transwell assay but the HLA-DR expression decreased significantly when tumor-associated-DCs were co-cultured in direct contact assay (MFI fold change: 0.68 ± 0.22). Expression level of CD83 decreased significantly on tumor-associated DCs in both direct contact and transwell assays (fold change: 0.29 ± 0.17 and 0.33 ± 0.11 , respectively) compared with the control DCs (Fig. 16b).

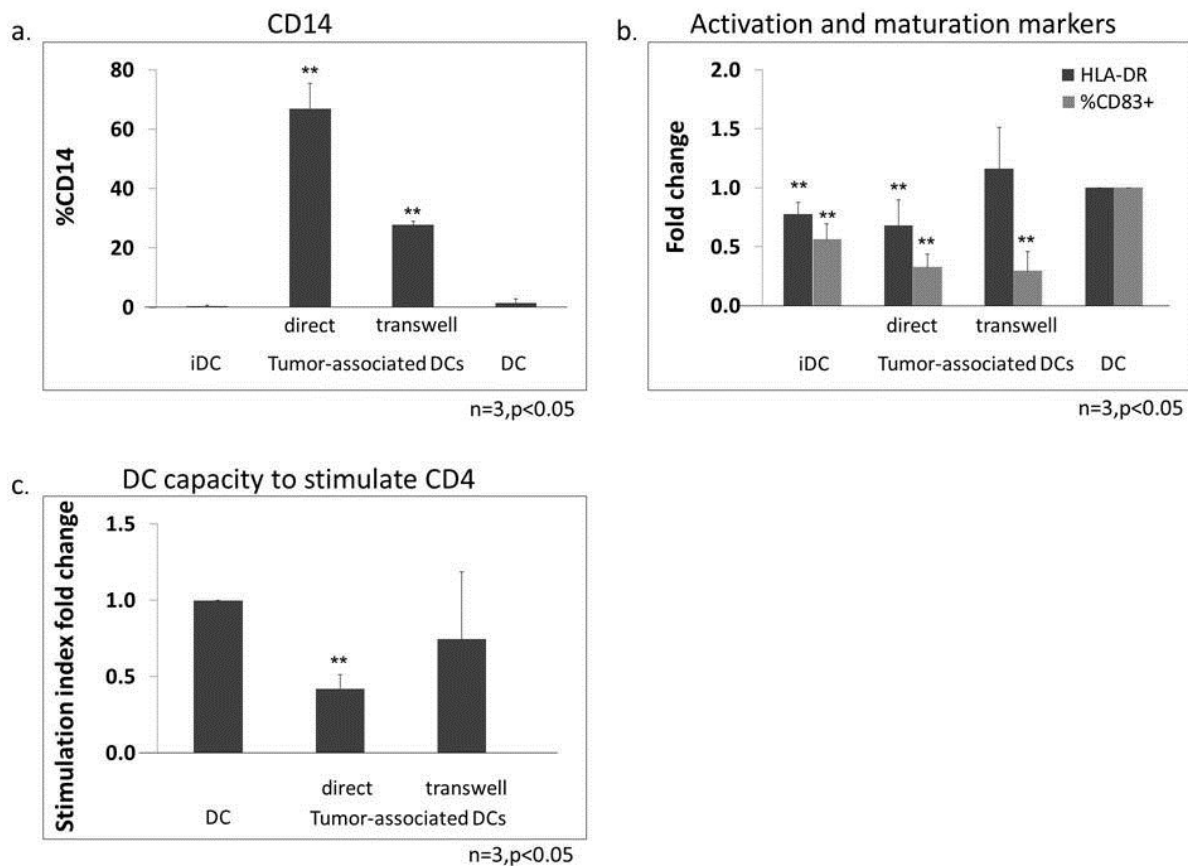


Fig. 16. Maturation of tumor-associated iDCs. iDCs (1×10^5 cells/well) were generated in the presence or absence of tumor cells (1:5). On day 5, cytokine cocktail (IL-1, IL-6, TNF- α) were added to induce DC maturation. On day 7, (a) percentage of CD14⁺ and (b) MFI of HLA-DR and percentage of CD83⁺ cells were calculated by flow cytometry. Measurements were gated on CD11c to exclude the tumor cells in analysis. (c) On day 7, DCs were co-cultured with CD4 T cells. The stimulation index represents the number of cells proliferating in response to stimulation divided by number of cells undergoing background proliferation in un-stimulated condition. Data are shown as mean values \pm SD of 3 donors. Data of activation markers and T cell proliferation are shown as fold changes normalized to control DCs. Student's *t*-test analysis (control vs tumor-associated iDCs), ** $p < 0.05$ is considered significant.

The capacity of DCs to induce T cell proliferation was measured by co-culturing DCs with allogenic CD4 T cells for 5 days. Tumor cells were not removed from DCs co-culture in the direct contact assay but they were removed when transwell system were used. Results are shown as stimulation index fold changes compared with the control DCs. Tumor-associated DCs in the direct contact assay still had a low capacity to induce T cell proliferation even though they have been induced into maturation (SI fold change: 0.42 ± 0.09). In the transwell system assay, the difference of capacity to stimulate T cell proliferation between tumor-associated DCs and control DCs was statistically not significant (SI fold change: 0.74 ± 0.44) (Fig. 16c).

3.3.4. Modulation of DCs by tumor cells

To investigate the effect of melanoma cells on DC differentiation, in relation to the expression levels of IL-10 and TGF- β during co-culture, monocytes were cultured for 5-7 days in the presence or absence of irradiated melanoma cells (ChaMel84) with or without direct contact. Different numbers of melanoma cells were used in culture to investigate the effect of tumor burden on DC differentiation. In 1:1 group, 1×10^5 monocytes were co-cultured with 1×10^5 melanoma cells and 1:5 group, 1×10^5 monocytes were co-cultured with 5×10^5 melanoma cells. At the end of experiments, cells were collected and the percentage of CD14⁺ cells was calculated by flow cytometry. Measurements were gated on CD11c to exclude the tumor cells from the analyses. The morphology of cells was analyzed under microscope. Supernatants were also collected and IL-10 and TGF- β production (pg/ml) were measured by ELISA.

In the control group, one peak showing a CD14 negative group was observed from the histogram. However, in the tumor-associated iDCs, there was a shift of the CD14 expression toward the positive value (Fig. 17a). The morphology of the tumor-associated iDCs also changed. A mixture of cell population was observed, consisted of the typical round iDCs cells and macrophage-like cells which attached to the plate (Fig. 17b). In the 1:1 direct contact assay, the percentage of CD14⁺ cells on the tumor-associated iDCs increased 32 times and in the 1:5 group increased 97 times compared with the control (%CD14⁺: 14.85 ± 3.97 and 44.67 ± 26.76 vs 0.46 ± 0.25). In the transwell assay, the percentages of CD14⁺ cells were also significantly higher compared with the control iDCs but the difference between the 1:1 group and 1:5 group was not large (%CD14⁺: 9.96 ± 4.74 and 15.93 ± 10.08 , respectively) (Fig. 17c). The experiments also showed that no cell contact was needed to affect the differentiation although the DC alteration was more pronounced in the direct contact assay.

Control iDCs did not produce IL-10 in contrast to the tumor-associated iDCs. IL-10 production in the direct contact assay increased in proportion to the number of tumor cells added into the culture (1:1 group: 190 ± 117 pg/ml and 1:5 group: 285 ± 161 pg/ml). IL-10 production in the transwell assay also increased although not as much as in the direct contact assay (1:1 group: 126 ± 67 pg/ml and 1:5 group: 201 ± 94 pg/ml) (Fig. 17e).

While control iDCs did not produce IL-10, they produced TGF- β (568 ± 25 pg/ml). In the tumor-associated iDCs 1:1 group direct contact assay, TGF- β production was similar to the control (564 ± 123.05 pg/ml) but in the 1:5 group, TGF- β production was doubled

(953±216 pg/ml). In contrast, TGF- β production decreased around 50% in tumor-associated iDCs in both 1:1 and 1:5 groups in the transwell assay (255±300 and 332±414 pg/ml, respectively) (Fig. 17f).

The capacity of iDCs to induce T cell proliferation was measured by co-culturing iDCs with CFSE-stained T cells (1:20) for 5 days. Tumor cells were not removed from iDCs co-culture in the direct contact assay but they were removed when transwell system were used. Results are shown as stimulation index fold changes compared with the control iDCs. Tumor-associated iDCs in the direct contact assay had significantly lower capacity to induce T cell proliferation, both in the 1:1 group (SI fold change: 0.69±0.18) and the 1:5 group (SI fold change: 0.58±0.25). Tumor-associated iDCs in the transwell assay also only had half the capacity of control iDCs to stimulate CD4 T cell proliferation (SI fold change 1:1 group: 0.51±0.10 and 1:5 group: 0.52±0.47) (Fig. 17d).

To investigate the effect melanoma cells on DC maturation, in relation to the expression levels of IL-10 and TGF- β during co-culture, monocytes were generated into iDCs for 5 days, and then induced into maturation by adding cytokine cocktail (IL-1, IL-6, TNF- α) in the presence or absence of melanoma cells (ChaMel84) for 2 days. Different numbers of melanoma cells were also used during DC maturation to study the effect of tumor burden on DC maturation. In the 1:1 group, 1×10^5 iDCs were co-cultured with 1×10^5 melanoma cells and in the 1:5 group, 1×10^5 iDCs were co-cultured with 5×10^5 melanoma cells. At the end of experiments, cells were collected and the expression of CD14, CD83 and HLA-DR were measured by flow cytometry. Measurements were gated on CD11c to exclude the tumor cells from the analyses. Data of activation and maturation markers are shown as MFI fold changes normalized to MFI of the control DCs generated in the absence of tumor cells. The morphology of the cells was analyzed per microscopy. Supernatants were collected and the productions of IL-12, IL-10, and TGF- β were measured by ELISA. IL-12 production was measured as a marker of DC maturation since mature DCs produce IL-12.

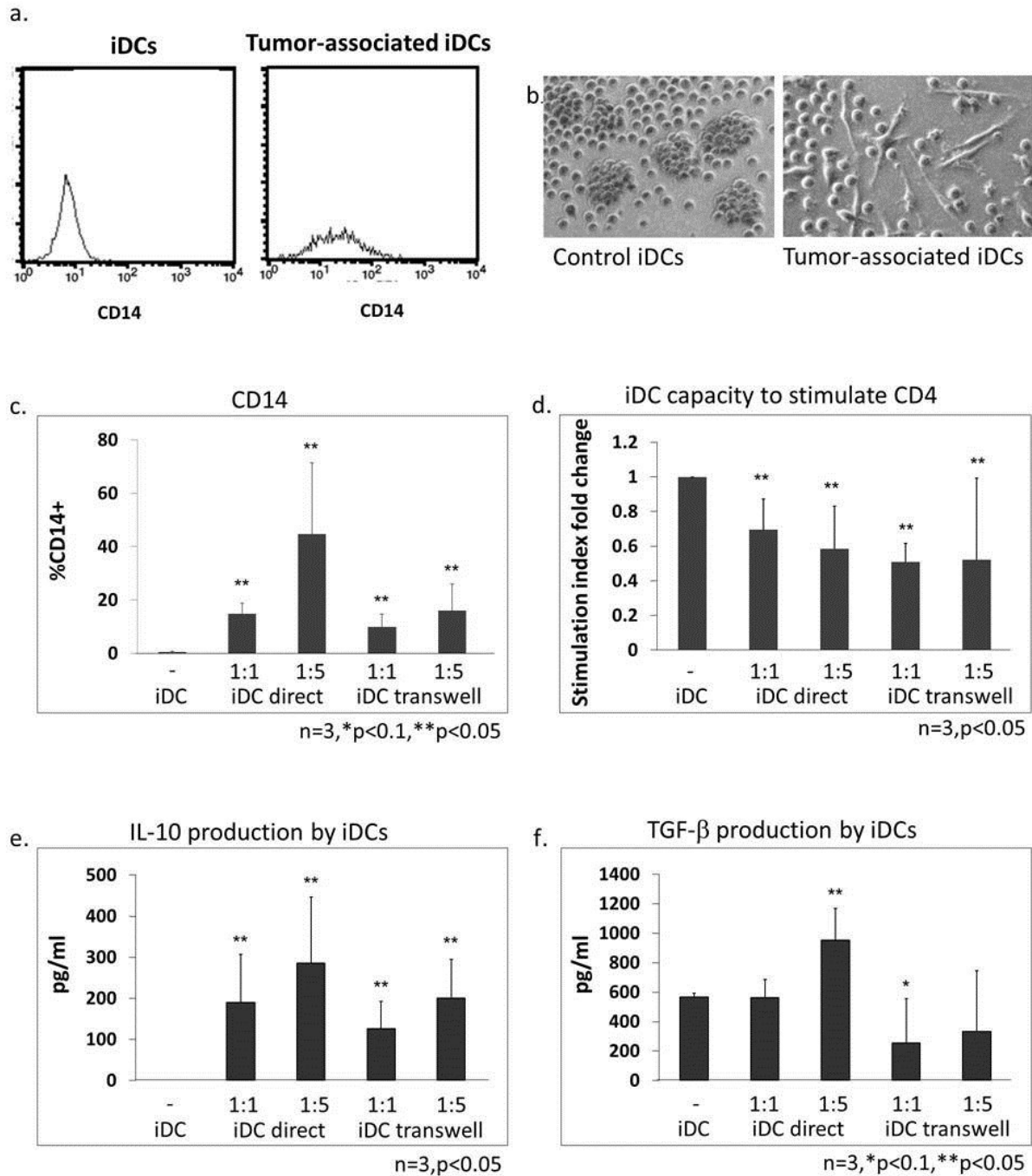


Fig. 17. DC differentiation in the presence of melanoma cells. Monocytes were co-cultured with tumor cells (ChaMel84) in ratio 1:1 and 1:5 for 7 days. On day 7, iDCs were co-cultured with CFSE-stained CD4 T cells. (a) CD14 histogram of control iDCs and tumor-associated iDCs. (b) iDC morphology under microscope. The figure is representative of 3 different experiments. (c) %CD14⁺ cells were calculated by flow cytometry. (d) iDC capacity to stimulate T cells was shown by a stimulation index. The stimulation index represents the number of cells proliferating in response to stimulation divided by number of cells undergoing background proliferation in un-stimulated condition. Stimulation index is shown as fold changes normalized to control iDCs. Results are shown as mean values \pm SD of 3 donors. (e-f) IL-10 and TGF- β productions were measured by ELISA. Student's *t*-test analysis (control iDCs vs tumor-associated iDCs), *p<0.1 and **p<0.05 are considered significant.

CD14 expression level on tumor-associated mDCs was not altered when tumor cells were added during DC maturation (Fig. 18a). The morphology of tumor-associated mDCs also did not change (Fig. 18b).

Tumor-associated mDCs in the direct contact assay showed a significant increase of CD83 expression level in proportion to the number of tumor cells added into culture. In the 1:1 group the expression level increased 1.4 times (MFI fold change: 1.39 ± 0.04) and in the 1:5 group the expression level increased 1.9 times (MFI fold change: 1.94 ± 0.56) compared with the control DCs. On the other hand, tumor-associated mDCs in the transwell assay showed a 1.3-fold increase of CD83 expression level only in the 1:5 group (MFI fold change: 1.28 ± 0.13), while the 1:1 group showed no difference in comparison with control DCs. HLA-DR expression level showed a similar tendency in the direct contact assay. In the 1:1 direct contact assay, the HLA-DR expression level increased 1.2 times (MFI fold change: 1.19 ± 0.08) and in the 1:5 group the expression level increased 1.3 times (MFI fold change: 1.33 ± 0.26). In the 1:1 transwell assay, no difference of HLA-DR expression level could be observed. A slight increase of 1.1 times of HLA-DR expression level could be observed only in the 1:5 group (MFI fold change: 1.14 ± 0.12) (Fig. 15c). In the 1:1 direct contact assay, a double amount of IL-12 was produced compared with the control (132.67 ± 33.50 vs 59 ± 52.16 pg/ml) while the 1:5 group could produce 3 times more IL-12 (180 ± 25.51 pg/ml). In the transwell assay, tumor-associated mDCs only showed a slight increase of the IL-12 production (1:1 group: 63.33 ± 55 pg/ml and 1:5 group: 66.33 ± 58.04 pg/ml) (Fig. 18d).

IL-10 production changed when tumor cells were added during DC maturation. In the 1:1 direct contact assay, tumor-associated mDCs produced two times more IL-10 compared with control DCs (57 ± 31 vs 24 ± 42 pg/ml). The IL-10 production from tumor-associated mDCs in the 1:5 direct contact assay increased 7 times (181 ± 123 pg/ml) compared with control DCs. In the transwell assay, similar results could be observed as the 1:5 group produced more IL-10 (160 ± 50 pg/ml) compared with the 1:1 group (71 ± 51 pg/ml) (Fig. 18f).

TGF- β production increased slightly when DCs were co-cultured with high numbers of tumor cells in the direct contact assay in comparison with control DCs (496 ± 153 vs 427 ± 129 pg/ml). DCs co-cultured with a low number of tumor cells in the direct contact assay showed a similar TGF- β production (314 ± 114 pg/ml) with control DCs. Similar observation were made in the tumor-associated iDCs differentiation and maturation, where TGF- β production decreased when transwell assay were used (1:1 group: 291 ± 318 pg/ml and 1:5 group: 266 ± 80 pg/ml) (Fig. 18g).

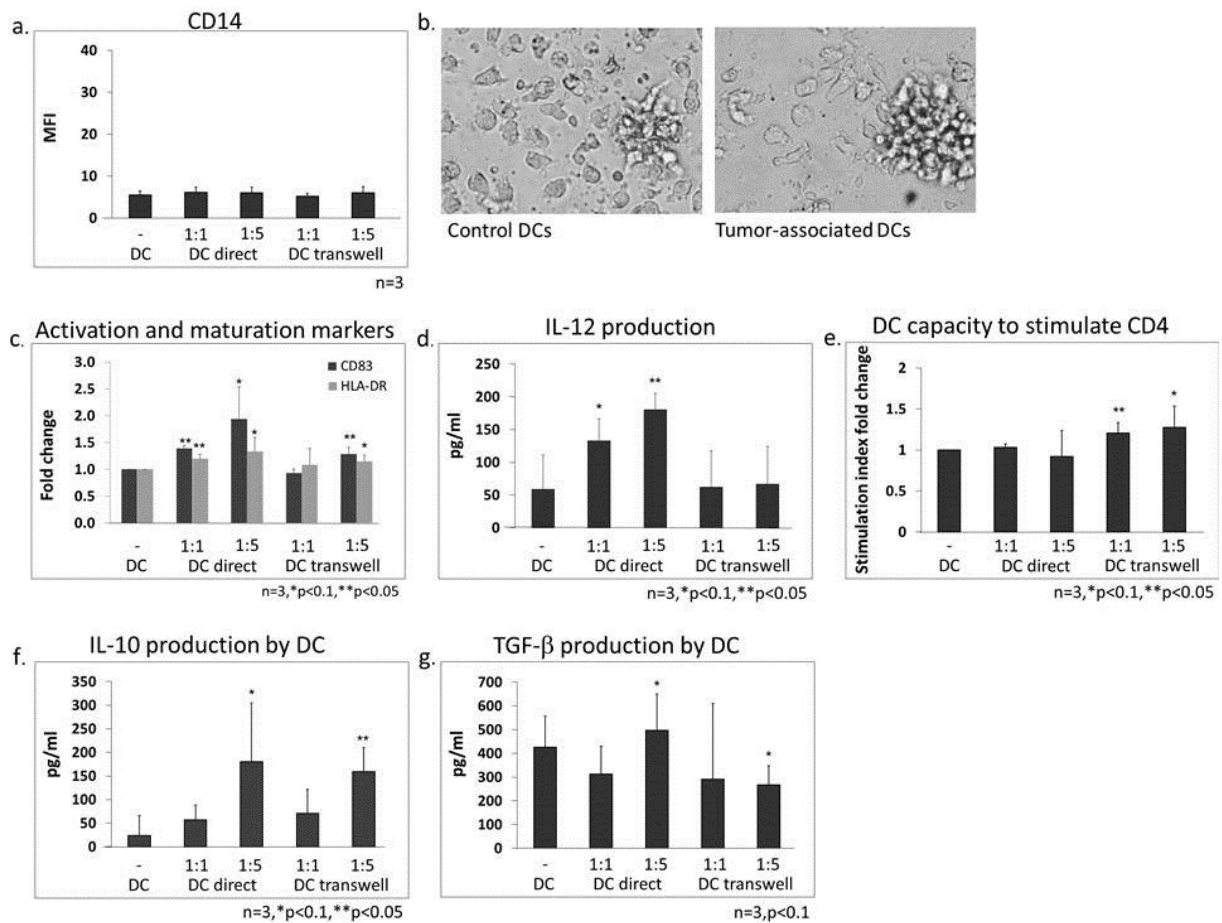


Fig. 18. DC maturation in the presence of melanoma cells. iDCs were generated for 5 days and induced into maturation by cytokine cocktail (IL-1, IL-6, TNF- α) in the presence or absence of tumor cells (ChaMel84) (iDCs: tumor cells = 1:1 and 1:5) at day 5 in a direct contact or transwell system. On day 7, mDCs were co-cultured with CFSE-stained CD4 T cells. (b) mDC morphology under microscope. The figure is representative of 3 different experiments. (a) CD14 and (c) activation and maturation markers expression level was measured by flow cytometry. Measurements were gated on CD11c. (d) IL-12 and (f-g) IL-10 and TGF- β productions were measured by ELISA. (e) DC capacity to stimulate T cells was shown by a stimulation index. The stimulation index represents the number of cells proliferating in response to stimulation divided by number of cells undergoing background proliferation in un-stimulated condition. Data are shown as mean values \pm SD of 3 donors. Results are shown as fold changes normalized to control DCs. Student's *t*-test analysis (control DCs vs tumor associated mDCs), * p <0.1 and ** p <0.05 are considered significant.

The capacity of DCs to induce CD4 T cell proliferation was measured by co-culturing DCs with CFSE-stained T cells (1:20) for 5 days. Tumor cells were not removed from DCs co-culture in the direct contact assay but they were removed when transwell system were used. Results are shown as stimulation index fold changes compared with the control DCs. The capacity to induce CD4 T cell proliferation of tumor-associated mDCs in the direct contact assay from the 1:1 group (SI fold change: 1.03 ± 0.04) and the 1:5 group (SI fold change: 0.91 ± 0.32) was similar with the control group. On the other hand, the capacity to stimulate CD4 T cells of tumor-associated mDCs from the transwell assay from both the 1:1 group (SI fold change: 1.21 ± 0.13) and the 1:5 group (SI fold change: 1.28 ± 0.26) increased slightly (Fig. 18e).

Previous experiments showed that DCs were altered when they were generated in the presence of tumor cells, including in the experiments using the transwell system. To investigate whether exosomes, which can flow through the transwell membrane affect the DC differentiation and maturation, tumor supernatant which was free from exosomes was used to generate DCs. Monocytes (1×10^5 cells/well) were cultured in medium only or medium containing 10% of exosome-free supernatant and 50% of exosome-free supernatant for 7 days to study the effect of exosome on DC differentiation. Different percentages of exosome-free supernatant were used to study the dose effect on DC generation. To study the effect of exosome on DC maturation, iDCs were generated for 5 days and then induced into maturation by cytokine cocktail (IL-1, IL-6, TNF- α) on day 5 in the medium only or medium containing 10% or 50% of exosome-free supernatant. At the end of experiments, cells were collected and the expression of CD14, CD83 and HLA-DR were measured by flow cytometry. Data of activation and maturation markers are shown as % positive cells fold changes normalized to % positive cells of control DCs generated in the absence of tumor cells.

The results showed that iDCs generated in the exosomes-free supernatant had a higher percentage of CD14⁺ cells compared with the control iDCs in a dose dependent manner (10%: 4.18 ± 0.95 and 50%: 11.94 ± 5.93 vs control: 3.77 ± 2.79) (Fig. 19a). CD14 expression level was not altered in the DCs induced into maturation in the exosomes-free supernatant (Fig. 19b). No difference on CD83 and HLA-DR expression levels were observed when DCs were induced into maturation in 10% of exosome-free supernatant, but a slight increase of CD83 expression (fold change: 1.32 ± 0.56) and a significant increase of 60% of HLA-DR expression (fold change: 1.60 ± 0.35) were observed when DCs were induced into maturation in 50% of exosome-free supernatant (Fig. 19c). From these results, it could be concluded that exosomes

could be exempted in this study since exosomes-free medium used to differentiate monocytes were still able to alter the DCs.

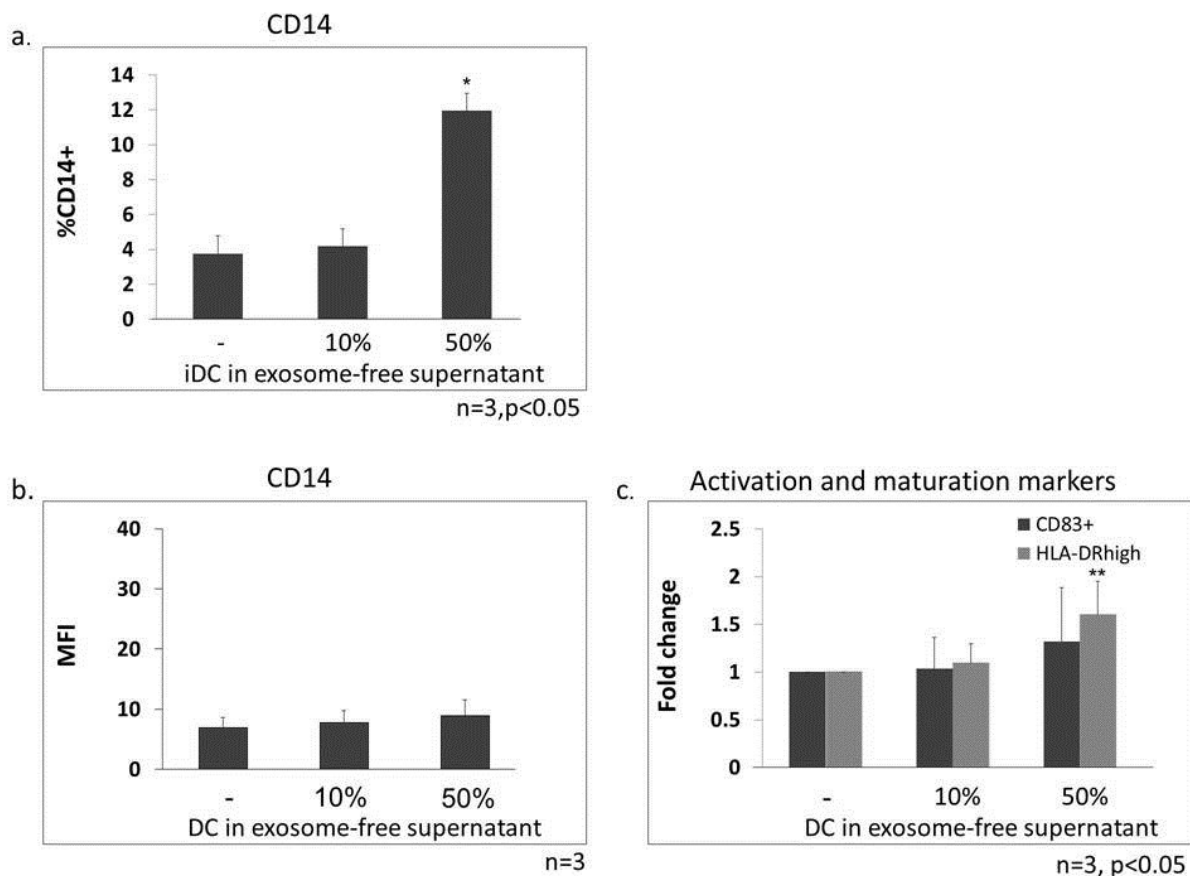


Fig. 19. DC generation in exosome-free supernatant. (a) Monocytes (1×10^5 cells/well) were cultured in medium only or medium containing 10% of exosome-free supernatant and 50% of exosome-free supernatant for 7 days to study the effect of exosome on DC differentiation (b) iDCs were generated for 5 days and then on day 5 induced into maturation by cytokine cocktail (IL-1, IL-6, TNF- α) in the medium only or medium containing 10% and 50% of exosome-free supernatant. Data are shown as mean values \pm SD of 3 donors. Data of activation and maturation markers are shown as fold changes normalized to control DCs. Student's *t*-test analysis (control vs iDCs or Dcs cultured in exosome-free supernatant), * $p < 0.1$, ** $p < 0.05$ are considered significant.

Previous experiments showed that IL-10 and TGF- β were produced during the DC generation in the presence of tumor cells. To investigate which cells actually produced the cytokines during tumor-associated iDC generation, 1×10^5 cells/well monocytes were co-cultured with 5×10^5 tumor cells/well (1:5) using a transwell system for 5 days. On day 5, iDCs and tumor cells were separated, and washed. Each cell type was further cultured in separate wells for 24 hours. At the end of experiments, supernatant were collected and then IL-10 and TGF- β productions were measured by ELISA. To investigate which cells produced the cytokines during the generation of tumor-associated mDCs, iDCs were generated and at

day 5, 1×10^5 cells/well iDCs were induced into maturation by cytokine cocktail (IL-1, IL-6, TNF- α) addition for 24 hours in the presence of tumor cells (1:5) using a transwell system. After 24 hours co-culture, mDCs and tumor cells were separated and washed. Each cell type was further cultured in separate wells for 24 hours. At the end of experiments, supernatant were collected and then IL-10 and TGF- β productions were measured by ELISA. IL-10 and TGF- β productions in the supernatant of tumor cell-line alone were also measured as a control.

During the generation of tumor-associated iDCs, tumor cells produced IL-10 (161 ± 115 pg/ml) while tumor-associated iDCs did not produce IL-10. As a control, IL-10 in tumor cell line supernatant alone was also measured (17 pg/ml). TGF- β was produced by both tumor cells (531 ± 75 pg/ml) and tumor-associated iDCs (334 ± 113 pg/ml). TGF- β could also found in the tumor cell line supernatant (285 pg/ml) (Fig. 20a). During the generation of tumor-associated mDCs, both tumor cells and tumor-associated mDCs produced IL-10 (101 ± 84 and 42 ± 49 pg/ml, respectively). Tumor cells also produced 4 times more TGF- β (460 ± 56 pg/ml) than DCs (118 ± 203 pg/ml) (Fig. 20b).

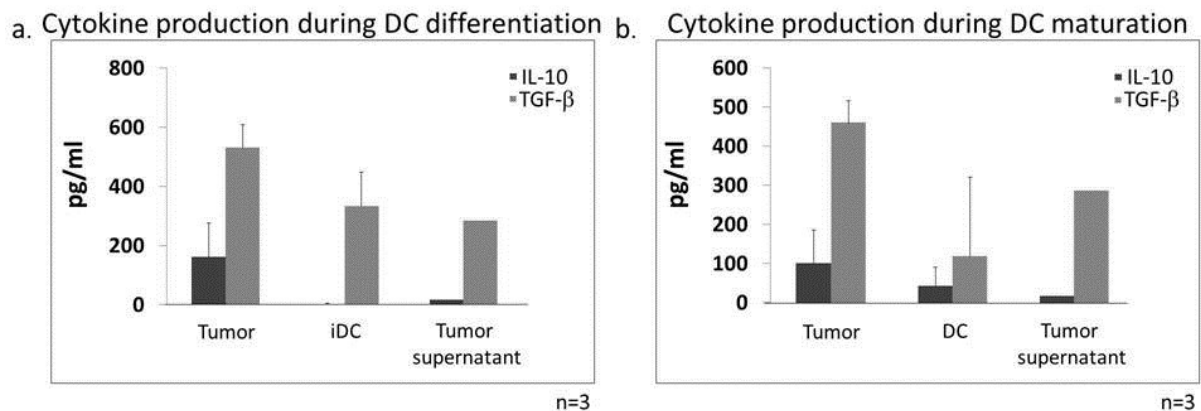


Fig. 20. Cytokine production during tumor-associated iDC and mDC generation. (a) Monocytes (1×10^5 cells/well) were co-cultured with tumor cells (1:5) in a transwell system for 5 days. On day 5, cells were separated, washed and further cultured in separate wells for 24 hours. (b) iDCs were generated for 5 days and then on day 5, iDCs and tumor cells were co-cultured in a transwell system for 24 hours. Cells were then separated, washed and further cultured in separate wells for 24 hours. Supernatant were collected and IL-10 and TGF- β productions were measured by ELISA. Data are shown as mean values \pm SD of 3 donors.

To confirm that interaction of the two cell types was needed to induce IL-10 production during the generation of tumor-associated iDCs, iDCs were generated in tumor supernatant. To study the IL-10 production during tumor-associated iDC differentiation, 1×10^5 cells/well monocytes were cultured in medium only or medium containing 10% or 50% tumor supernatant. To study the IL-10 production during tumor-associated mDC generation, monocytes were generated into iDCs for 5 days, and then 1×10^5 iDCs were further cultured for 2 days in medium only or medium containing 10% or 50% tumor supernatant in the presence of cytokine cocktail (IL-1, IL-6, TNF- α). At the end of experiments, IL-10 was measured by ELISA.

Control iDCs generated in medium alone produced 78 ± 79 pg/ml IL-10. There was no difference of IL-10 produced by tumor-associated iDCs generated in 10% or 50% tumor supernatant compared with the control (52 ± 45 and 80 ± 116 pg/ml, respectively). A large standard deviation could be detected in this experiment because one sample produced a high amount of IL-10 while the other samples produced very little to none IL-10 (Fig. 21a). Similarly, no difference of IL-10 production could be detected between the control DCs (451 ± 373 pg/ml) and tumor-associated DCs cultured in 10% or 50% tumor supernatant (399 ± 376 and 368 ± 16 pg/ml, respectively) (Fig. 21b).

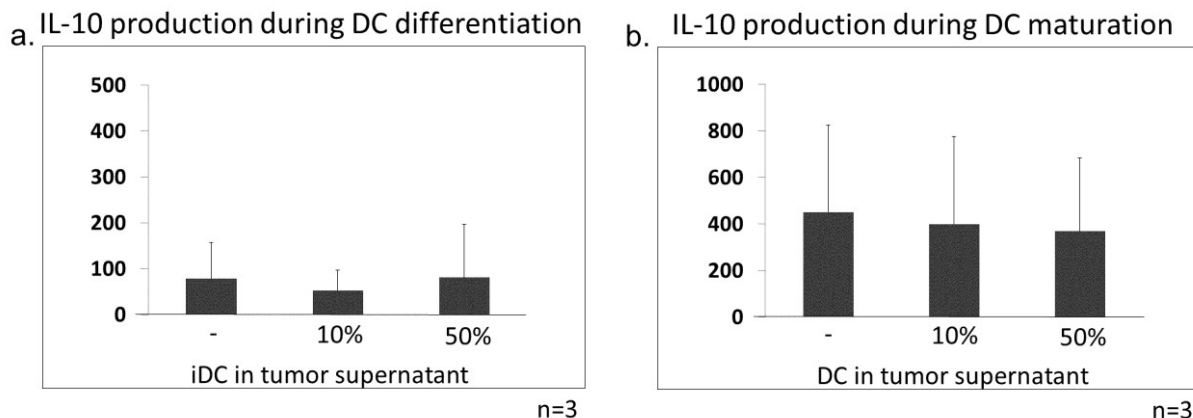


Fig. 21. IL-10 production during DC generation in tumor supernatant. (a) 1×10^5 monocytes were cultured in medium only or medium containing 10 % or 50% tumor supernatant. (b). iDCs were generated for 5 days and then on day 5, 1×10^5 iDCs induced into maturation by cytokine cocktail (IL-1, IL-6, TNF- α) in medium only or medium containing 10% and 50% tumor supernatant. At the end of experiments, supernatant were collected and IL-10 production was measured by ELISA. Data are shown as mean values \pm SD of 3 donors.

To evaluate the effect of endogenous immunosuppressive cytokine produced during tumor-associated iDC or mDC generation, neutralizing antibodies against IL-10 and TGF- β were added into the culture. 1×10^5 cells/well monocytes were co-cultured with 5×10^5 tumor cells/well (1:5) in the presence or absence of antibody against IL-10 (5 $\mu\text{g/ml}$) or TGF- β (10 $\mu\text{g/ml}$) for 5 days in a transwell system. On day 5, cytokine cocktail (IL-1, IL-6, TNF- α) was added to induce the maturation of iDCs. Matured DCs were then analyzed by flow cytometry for phenotypic changes and further co-cultured with allogenic CD4 T cells to investigate the DC capacity to stimulate T cell proliferation. Data of activation and maturation markers, as well as stimulation index, are shown as fold changes normalized to the control DCs generated in the absence of tumor cells.

The percentage of CD14⁺ cells in tumor-associated iDCs culture was higher compared with the control iDCs (38.22 ± 12.04 vs 0.70 ± 0.52). Adding antibodies against IL-10 or TGF- β into the culture did not change the percentage of the CD14⁺ cells (33.43 ± 9.77 and 39.54 ± 14.01 , respectively) in the tumor-associated iDCs culture (Fig. 22a). The HLA-DR expression level of matured tumor-associated iDCs was similar (fold change: 1.09 ± 0.28) with the control DCs. The presence of antibodies against IL-10 and TGF- β did not change the expression level of HLA-DR (fold change: 1.04 ± 0.17 and 0.99 ± 0.27 , respectively) on the matured tumor-associated iDCs. The CD83 expression level on matured tumor-associated iDCs was significantly lower (fold change: 0.48 ± 0.49) compared with the control DCs. Addition of antibodies against TGF- β did not make any change to the CD83 expression level (fold change: 0.45 ± 0.19) on matured tumor-associated iDCs but addition of antibody against IL-10 significantly increased the CD83 expression level (fold change: 0.60 ± 0.41) on matured tumor-associated iDCs compared with the matured tumor-associated iDCs without antibody (fold change: 0.48 ± 0.29) (Fig. 22b).

The capacity of matured tumor-associated iDCs to induce CD4 T cell proliferation was similar (SI fold change: 1.19 ± 0.27) with control DCs. Addition of antibody against TGF- β into the culture did not change the capacity of matured tumor-associated iDCs to stimulate CD4 T cell proliferation (SI fold change: 1.06 ± 0.11). Addition of antibody against IL-10 into the culture significantly increased the capacity of matured tumor-associated iDCs to induce CD4 T cell proliferation (SI fold change: 1.26 ± 0.28) compared with the control DCs but compared with the matured tumor-associated iDCs cultured without antibody no significant changes were observed (Fig. 22c).

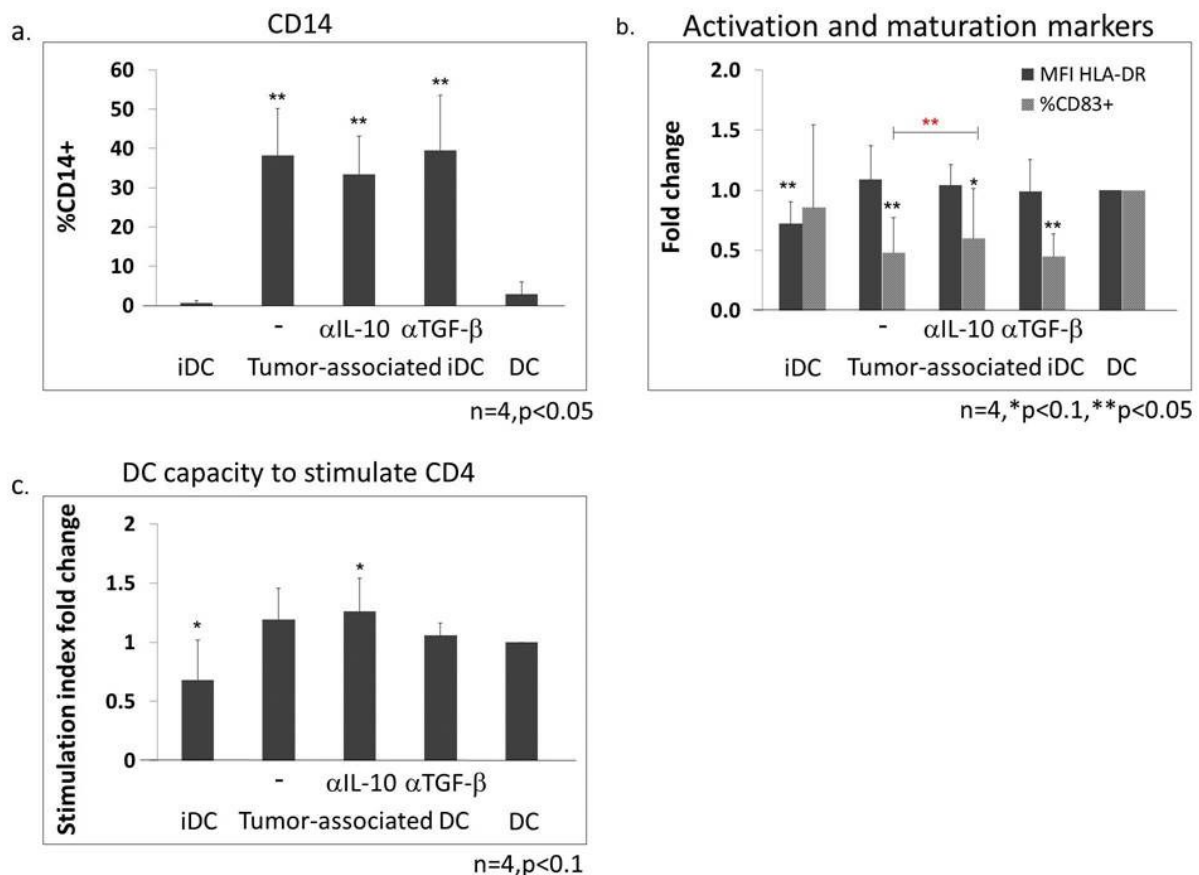


Fig. 22. Effect of endogenous immunosuppressive cytokines production on tumor-associated iDC. iDCs (1×10^5 cells/well) were generated in the presence of tumor cells (1:5), in the presence or absence of α IL-10 (5 μ g/ml) or α TGF- β (10 μ g/ml) in a transwell system. On day 5, cytokine cocktail was added (IL-1, IL-6, TNF- α). DCs were further co-cultured with allogenic CD4 T cells for 5 days. (a) Percentage of CD14⁺ cells (b) CD83⁺ and MFI of HLA-DR were calculated by flow cytometry. Measurements were gated on CD11c. Data are shown as mean values \pm SD of 4 donors. Results are shown as fold changes normalized to control DCs. (c) DC capacity to induce T cell proliferation was determined by flow cytometry. The stimulation index represents the number of cells proliferating in response to stimulation divided by number of cells undergoing background proliferation in un-stimulated condition. Student's *t*-test analysis (control vs tumor-associated DCs; culture with vs without antibodies), *p<0.1 and **p<0.05 are considered significant.

The effect of immunosuppressive cytokines on the tumor-associated mDCs was investigated by generating iDCs for 5 days, then adding the cytokine cocktail (IL-1, IL-6, TNF- α) along with tumor cells (1:5) into culture in the presence or absence of antibody against IL-10 (5 μ g/ml) or TGF- β (10 μ g/ml) in a transwell system. DCs then phenotypically characterized by flow cytometry and then further co-cultured with allogenic CD4 T cells to investigate the DC capacity to stimulate CD4 T cell proliferation. Results of activation and maturation markers, as well as stimulation index, are normalized and shown as fold changes compared with the control DCs.

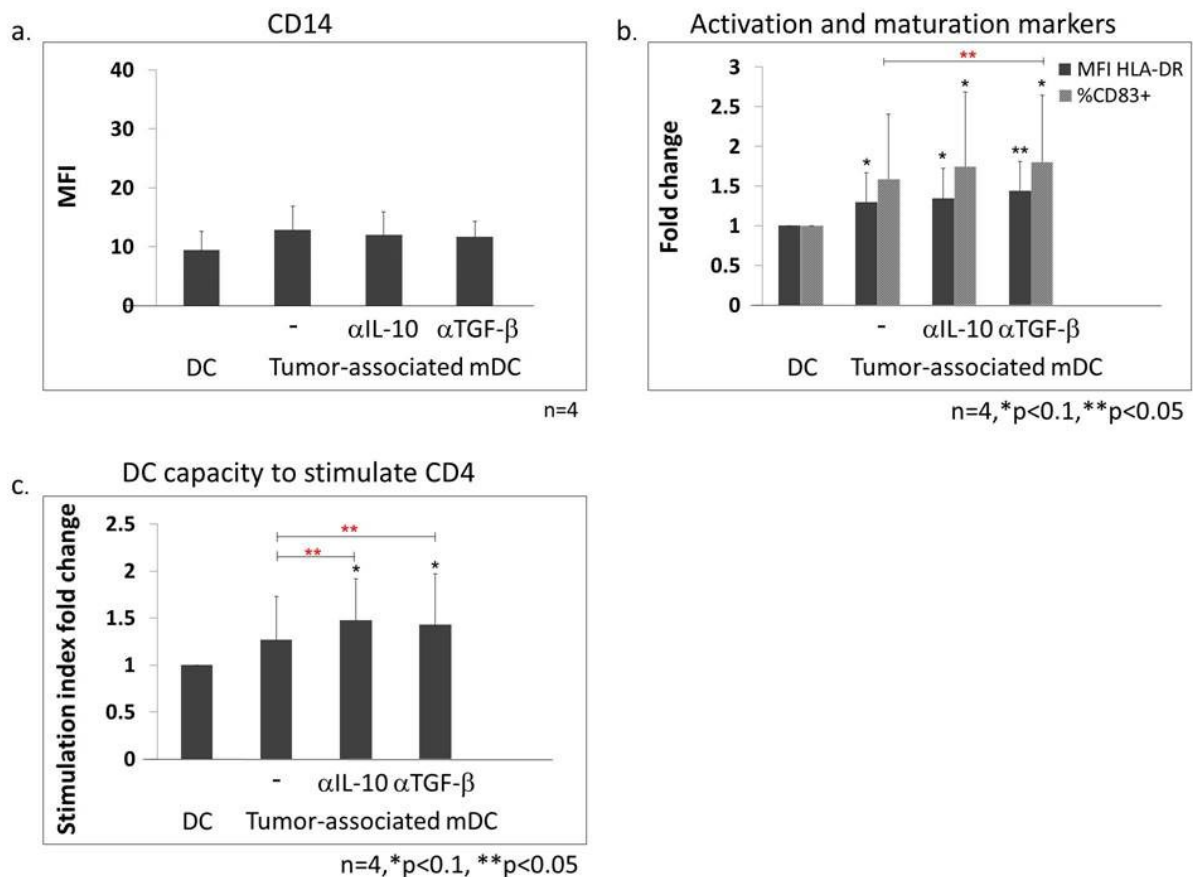


Fig. 23. Immunosuppressive cytokine effect on tumor-associated mDC. iDCs (10^5 cells/well) were generated. On day 5, cytokine cocktail (IL-1, IL-6, TNF- α) along with tumor cells (1:5) were added in a transwell system in the absence or presence of α IL-10 (5 μ g/ml) or α TGF- β (10 μ g/ml). DCs were further co-cultured with allogenic CD4 T cells 5 days. (a) CD14 (b) HLA-DR and CD83 expression level were calculated by flow cytometry. Measurements were gated on CD11c. (c) DC capacity to induce CD4 T cell proliferation was shown by a stimulation index. The stimulation index represents the number of cells proliferating in response to stimulation divided by number of cells undergoing background proliferation in un-stimulated condition. Results are shown as fold changes normalized to control DCs. Data are shown as mean values \pm SD of 4 donors. Student's *t*-test analysis (control vs tumor-associated DCs; culture with vs without antibodies), * p <0.1 and ** p <0.05 were considered significant.

The CD14 expression level of tumor-associated mDCs showed no alteration compared with the control DCs (MFI: 12.90 ± 3.96 vs 9.44 ± 3.15). Addition of antibody against IL-10 or TGF- β did not alter the CD14 expression level on the tumor-associated mDCs (MFI: 12.00 ± 3.97 and 11.59 ± 2.59 , respectively) (Fig. 23a). In contrast, the HLA-DR expression level was significantly enhanced on tumor-associated mDCs (fold change: 1.30 ± 0.37). Addition of antibody against IL-10 and TGF- β did not change the expression level of HLA-DR of the tumor-associated mDCs (fold change: 1.35 ± 0.37 and 1.44 ± 0.37 , respectively)

compared with the tumor-associated mDCs cultured without antibodies. The CD83 expression level was enhanced on tumor-associated mDCs (fold change: 1.58 ± 0.82) compared with the control DCs. Addition of antibody against IL-10 did not make any significant changes to the CD83 expression level on tumor-associated mDCs (fold change: 1.74 ± 0.94) but the addition of antibody against TGF- β increased the CD83 expression level on tumor-associated mDCs (fold change: 1.80 ± 0.84) compared with the tumor-associated mDCs cultured without antibodies (Fig. 23b).

The capacity to induce CD4 T cells proliferation was shown to be not significantly different between control DCs and tumor-associated mDCs (SI fold change: 1.27 ± 0.46). However, antibody against IL-10 and TGF- β increased the capacity of tumor-associated mDCs to induce CD4 T cell proliferation (SI fold change: 1.48 ± 0.44 and 1.43 ± 0.54 , respectively) (Fig. 23c).

3.3.5. Modulation of DCs by exogenous IL-10 and TGF- β

Since the results of this study contradicting the studies emphasizing the importance of IL-10 in modulating DCs, exogenous IL-10 and TGF- β were added into the culture during the DC generation. To investigate the effect of the cytokines on DC differentiation, monocytes were cultured in medium supplied with different concentration of exogenous IL-10 (0.2, 2 and 20 ng/ml) or TGF- β (0.5, 1 and 5 ng/ml) for 7 days. To study the effect these cytokines on DC maturation, iDCs were generated for 5 days and then at day 5, cytokine cocktail (IL-1, IL-6, TNF- α) and different concentration of IL-10 (0.2, 2 and 20 ng/ml) or TGF- β (0.5, 1 and 5 ng/ml) were added. The DCs were then phenotypically characterized by flow cytometry and further co-cultured with allogenic CD4 T cells to investigate the DC capacity to stimulate T cell proliferation.

The percentage of CD14⁺ cells of iDCs generated in 0.2 ng/ml IL-10 were similar to control iDCs (1.20 ± 0.46 vs 1.11 ± 0.69). The percentage of CD14⁺ cells increased slightly when iDCs differentiated in 2 ng/ml IL-10 (5.89 ± 7.66) but enhanced significantly in 20 ng/ml IL-10 culture (22.02 ± 5.49) (Fig 24a). When IL-10 was added during the DC maturation, CD14 expression level was not altered (Fig. 24b). The expression level of CD83, CD86 and HLA-DR on DCs induced into maturation in the presence of 0.2 and 2 ng/ml IL-10 were similar with control DCs. In contrast, the expression of CD86 was only 50% of the control level (MFI: 39.08 ± 5.68 vs 70.57 ± 31.09 , the HLA-DR expression decreased to 75%

(MFI: 31.94 ± 3.25 vs 44.81 ± 8.04) and the CD83 expression level decreased to 84% (MFI: 10.73 ± 0.46 vs 12.85 ± 0.83) when 20 ng/ml IL-10 was added into the culture (Fig. 24c).

The capacity of iDCs to induce CD4 T cell proliferation did not change when 0.2 and 2 ng/ml IL-10 was added into culture but a decreased capacity (SI fold change: 0.73 ± 0.07) was observed when 20 ng/ml IL-10 was added into culture during DC differentiation (Fig. 22d). There was no change on DC capacity to induce CD4 T cell proliferation when IL-10 was added into culture during DC maturation (Fig. 24e).

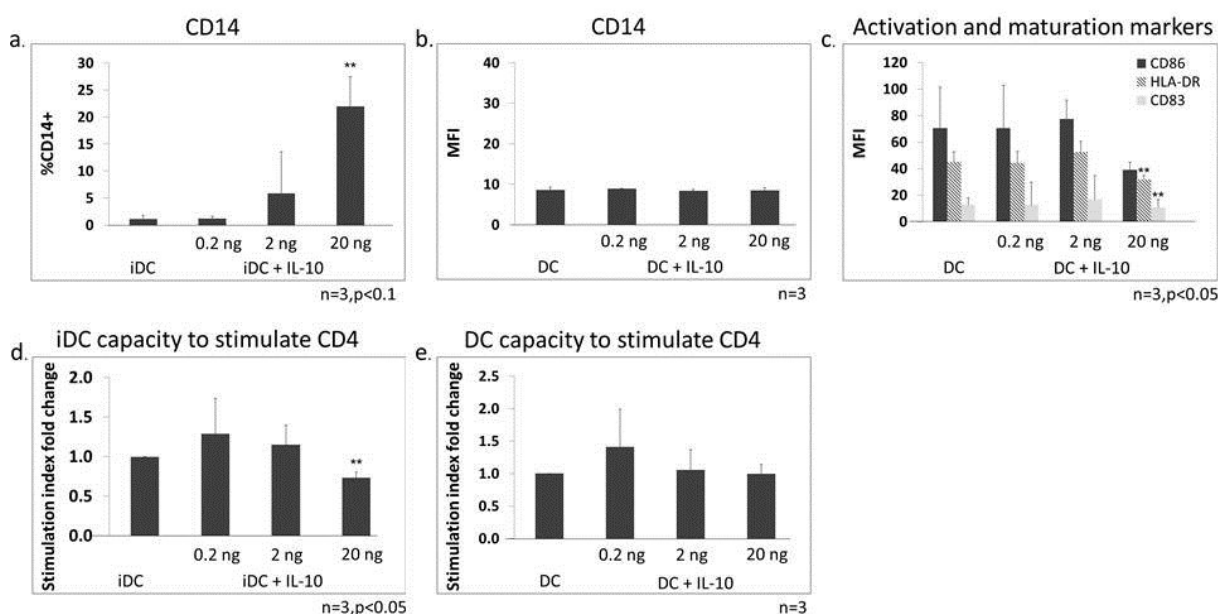


Fig 24. Effect of exogenous IL-10 on DC differentiation and maturation. (a) Monocytes were cultured in the absence or presence of different concentration of IL-10 (0.2, 2 and 20 ng/ml) for 7 days. CD14⁺ cells were calculated by flow cytometry (b) iDCs were generated for 5 days, IL-10 (0.2, 2 and 20 ng/ml) was added on day 5 along with cytokine cocktail (IL-1, IL-6, TNF- α). CD14, CD86, HLA-DR and CD83 expression level were calculated by flow cytometry. (d-e) DCs were further co-cultured with allogenic CD4 T cells for 5 days. Results are shown as stimulation index fold changes normalized to the control DC. The stimulation index represents the number of cells proliferating in response to stimulation divided by number of cells undergoing background proliferation in un-stimulated condition. Data are shown as mean values \pm SD of 3 donors. Student's *t*-test analysis (control vs DCs cultured in different concentration of IL-10), ** $p < 0.05$ is considered significant.

Addition of exogenous TGF- β into culture during DC differentiation, as well as during DC maturation, did not change the CD14 expression level of iDCs or DCs (Fig. 25a and b). The changes was observed though on the HLA-DR expression level, where addition of 0.5 ng/ml TGF- β into the culture during DC maturation showed a two-fold decrease compared with the control (MFI: 14.54 vs 27.07). The decrease of HLA-DR expression was not dose-dependent as addition of a higher concentration of TGF- β (1 and 5 ng/ml) did not decrease the expression further (MFI: 15.18 and 12.65, respectively). Similar observation were made for the CD86 expression level, where addition of 0.5 ng/ml TGF- β was enough to cause a two-fold decrease compared with the control (MFI: 13.64 vs 25.61). Addition of 1 and 5 ng/ml TGF- β into culture during the DC maturation also showed a two-fold reduced expression level of the CD86 compared with the control (MFI: 14.08 and 13.97, respectively). The expression of CD83 showed a decrease level when 0.5, 1 and 5 ng/ml TGF- β was added into the culture (MFI: 8.27, 7.94 and 8.42, respectively) compared with control DCs (MFI: 9.55) (Fig. 25c).

iDC capacity to induce CD4 T cell proliferation increased when TGF- β was added into culture, where 0.5 ng/ml TGF- β could increase the capacity 1.28 times, 1 ng/ml TGF- β 1.18 times and 5 ng/ml of TGF- β 1.70 times compared with control iDCs (Fig. 25d). The DC capacity to induce CD4 T cell proliferation also increased when TGF- β was added into the culture during DC maturation. The addition of 0.5 ng/ml TGF- β increased the capacity 1.29 times, 1 ng/ml TGF- β 1.47 times and 5 ng/ml of TGF- β 1.48 times compared with control DCs (Fig. 25e).

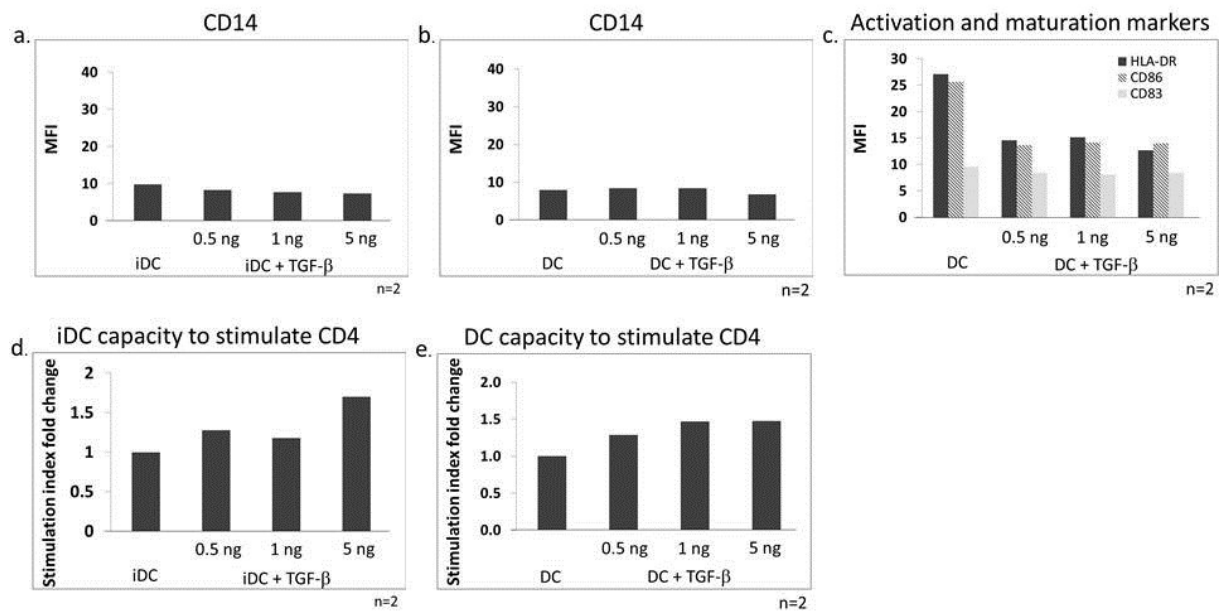


Fig 25. Effect of exogenous TGF- β on DC differentiation and maturation. (a) Monocytes were cultured in the absence or presence of different concentration of TGF- β (0.5, 1 and 5 ng/ml) for 7 days. (b) iDCs were generated for 5 days, TGF- β (0.5, 1 and 5 ng/ml) was added on day 5 along with the cytokine cocktail (IL-1, IL-6, TNF- α). DCs were further co-cultured with allogenic CD4 T cells for 5 days. Data of activation and maturation markers are shown as fold changes normalized to control DCs. The stimulation index represents the number of cells proliferating in response to a stimulation divided by number of cells undergoing background proliferation in an un-stimulated condition. Stimulation index is shown as a fold change normalized to control iDCs or DCs. Results are shown as a mean value of 2 donors.

3.3.6. Co-inhibitory molecules on tumor-associated DCs

In addition to immunosuppressive cytokines, expressions of the co-inhibitory molecules PD-L1 on DCs were measured on DCs generated in cultures contained melanoma supernatant. To investigate the expression of PD-L1 during the DC differentiation, monocytes (1×10^5 cells/well) were cultured in medium only or medium containing 10% or 50% melanoma supernatant for 5 days and then expression of PD-L1 was measured by flow cytometry. To investigate the changes of PD-L1 expressions during DC maturation, iDCs (1×10^5 cells/well) were generated and then induced into maturation by a cytokine cocktail (IL-1, IL-6, TNF- α) addition in medium only or medium containing 10% and 50% melanoma supernatant for 2 days.

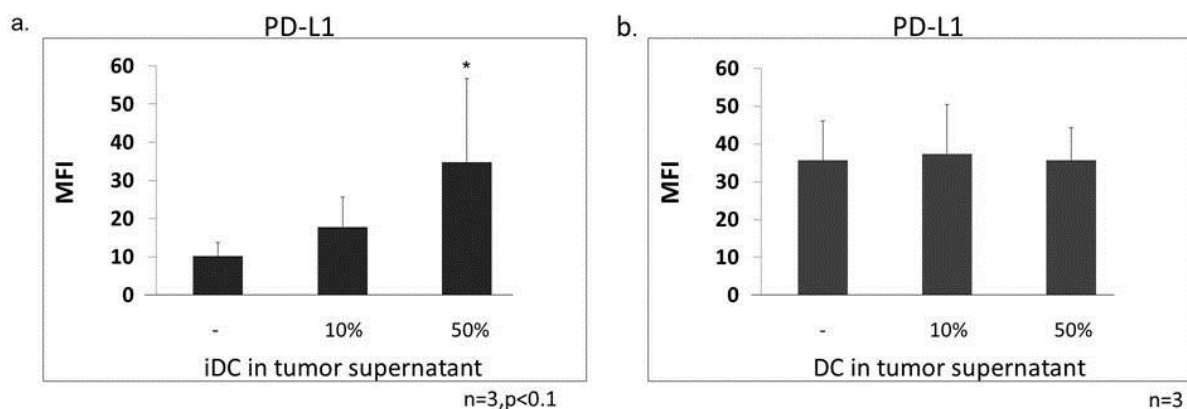


Fig. 26. Co-inhibitory molecule expression on DCs cultured in the medium containing melanoma cell lines supernatant. (a) Monocytes (1×10^5 cells/well) were cultured in the medium containing 0%, 10% and 50% melanoma supernatant for 5 days and then PD-L1 expressions were measured by flow cytometry. (b) iDCs (1×10^5 cells/well) were generated and further cultured in the medium containing 0%, 10% and 50% melanoma cell-lines supernatant for 2 days in the presence of a cytokine cocktail (IL-1, IL-6, TNF- α). Data are shown as mean values \pm SD of 3 donors. Student's *t*-test analysis (control vs DCs cultured in tumor supernatant), * $p < 0.1$ and ** $p < 0.05$ are considered significant.

PD-L1 expression level increased when iDCs were generated in the medium containing 10% tumor supernatant (MFI: 17.75 ± 7.93) compared with the control (MFI: 10.26 ± 3.42). iDCs cultured in the medium containing 50% of tumor supernatant showed a significant three-fold increase of the PD-L1 expression level (MFI: 34.86 ± 21.82) compared with the control (Fig. 25a). PD-L1 expression levels did not differ when DCs were induced into maturation in the medium containing 10% or 50% tumor supernatant (MFI: 37.37 ± 13.18 and 35.62 ± 8.76 , respectively) compared with the control (MFI: 35.66 ± 10.55) (Fig. 26b).

3.4. Results summary

In situ experiments showed:

- No correlation between IL-10 and TGF- β productions, their receptors and responsive genes expressions, with the failure of immune response to eradicate melanoma cells.

T cell anergy model demonstrated:

- Anergic T cells could be induced *in vitro* by co-culturing T cells with an equal number of tumor cells (ChaMel84).
- No co-linearity between IL-10 and TGF- β productions and anergic T cell induced *in vitro*.
- Addition of exogenous IL-10 to T cells co-cultured with a low number of tumor cells led to a slightly reduced T cell responses (shown by slightly reduced Granzyme B production and cell proliferation) capacity upon re-challenged compared with the control group.
- Blocking the endogenous IL-10 did not make any differences to T cell responses upon re-challenge in both functional and anergic T cells induced *in vitro* compared with the control group.
- T cell responses upon re-challenge were better compared with the control when exogenous IL-10 was added into T cells co-cultured with an equal number of tumor cells.
- Addition of exogenous TGF- β to the anergic or functional T cell cultures caused better T cell responses upon re-challenge compared with the control.
- Blocking the endogenous TGF- β made no significant changes in T cell responses upon re-challenged in both functional and anergic T cell cultures compared with the control.
- Better T cell responses upon re-challenge were observed when a combination of IL-10 and TGF- β was applied than using either IL-10 or TGF- β alone.

Tumor associated DCs *in vitro* showed:

- Tumor-associated iDCs were a mixed population of cells (consisted of round cells and macrophage-like cells), less efficient in inducing CD4 T cell proliferation and produced more IL-10 compared with the control iDCs.
- No correlation between the amount of IL-10 produced by tumor-associated iDCs and their reduced capacity to induce T cell proliferation.

- Blocking IL-10 during tumor-associated iDC generation did not change their capacity to induce CD4 T cell proliferation.
- Tumor-associated mDCs were more activated and had a similar or even an enhanced capacity to induce CD4 T cell proliferation.
- Tumor-associated iDC and mDC cultures in transwell assays showed a reduced TGF- β production.
- Only tumor-associated iDCs and mDCs co-cultured with a high number of tumor cells in direct contact assays showed a higher TGF- β production compared with the control.
- Endogenous TGF- β did not reduce tumor-associated mDC capacity to induce CD4 T cell proliferation.
- Exogenous TGF- β addition during DC differentiation did not suppress the DC function, instead an enhanced capacity could be observed.
- Tumor-associated iDCs generated in the transwell assay could still be induced into maturation and the resulting matured DCs had similar capacity to stimulate CD4 T cell proliferation with the control DCs, in contrast to the tumor-associated iDCs differentiated in direct contact assays where maturation was failed to induce and consequently, had less capacity to induce CD4 T cell proliferation.

4. Discussion

IL-10 and TGF- β are suggested to actively suppress anti-tumor immune responses and thereby facilitating tumor growth and development since a high expression of IL-10 and TGF- β was found in varieties of cancers (Kim *et al*, 1995; Leivonen and Kahari, 2007). The IL-10 expression was reported in melanoma samples but not in adjacent healthy skin of patients. Moreover, serum IL-10 level of advanced melanoma patients was higher than normal volunteers (Krüger-Krasagakes *et al*, 1994; Nemunaitis *et al*, 2001). All this data led to an interpretation of an upregulation of the immunosuppressive cytokines in tumor cases to actively suppress the immune system. Contrary to this suggestion, based on *in situ* results from this study, there is no correlation between IL-10 and TGF- β production and the failure of the immune response to eradicate melanoma cells. Furthermore, no active suppression of T cells or DCs functions *in vitro* by IL-10 and TGF- β produced endogenously in culture was found.

Krüger-Krasagakes and colleagues (1994), as well as Itakura and colleagues (2011), have studied IL-10 expression in melanoma samples. However, they did not compare melanoma with the skin of healthy individuals. Here for the first time, IL-10 and TGF- β expressions, together with their receptors and responsive genes, were analysed in both melanoma and the skin of healthy individuals. The results demonstrated that while the expression of both immunosuppressive cytokines to be still detectable in melanoma but the expressions were significantly lower in melanoma samples than in the healthy skin samples. Likewise, the expression levels of IL-10 receptor and TGF- β receptor in melanoma were lower than in the healthy skin although the p values > 0.05 , indicating a larger variation in both sample groups. Despite the observation, lower expression levels in the responsive genes of the two cytokines in melanoma and the highly significant difference compared to the healthy skin is strong evidence against the active role of these two immunosuppressive cytokines in preventing effective anti-tumor immune responses in melanoma.

This conclusion also hold true for the one melanoma case (ChaMel45) that had a higher level of IL-10 expression than the healthy skin (mean value: 73.98 vs 64.56). Here, the SOCS-3 expression, as the responsive gene marker for the IL-10 signaling, was still very low compared with the mean value in the healthy skin (mean value: 7.61 vs 18.47), indicating a low response to the IL-10 induction in this melanoma sample, which is in line with the low expression of IL-10R in this sample compared with the healthy skin samples (mean value:

48.74 vs 100.67). Similarly, in two melanoma cases (ChaMel57 and ChaMel109) where higher levels of TGF- β expression were found compared with the healthy skin (mean value: 66.15 and 54.84 vs 27.47), the TGF- β R expression levels were lower compared with the healthy skin (mean value: 3.47 and 0.80 vs 7.05). As a result, a lower expression of SMAD-7, a responsive gene of the TGF- β signaling, in these two melanoma samples in comparison with the healthy skin (mean value: 1.85 and 6.01 vs 13.18) was observed.

Thus, IL-10 and TGF- β were not upregulated in melanoma samples and even in the cases of high expressions of immunosuppressive cytokines, no upregulation of the corresponding receptors was observed. Consistently, the cytokine and cytokine receptors expressions translated into a low level to no signal transduction as indicated by the low expressions of SOCS-3 and SMAD-7 as the responsive gene markers for IL-10 and TGF- β respectively. It can therefore be concluded that these two immunosuppressive cytokines are not important for melanoma development and progression.

In the *in vitro* model, anergic T cells, which were un-responsive to tumor re-challenge due to the over-stimulation by tumor cells, could be generated. When anergic T cells were re-challenged, all parameters of T cell responses (T cell proliferation, Granzyme B and IFN- γ production) were low. However, no enhancement of IL-10 or TGF- β production was observed in the anergic T cell cultures compared with the functional T cell cultures. Thus, there was no correlation between T cell anergy and IL-10 or TGF- β production. On the contrary, anergic T cells seemed to be more activated, shown by the increased expression of CD25 and CD69 activation markers and enhanced IFN- γ production compared with the control, leading to a conclusion that there was no active suppression by IL-10 or TGF- β in the anergic T cell model.

Previous studies reported that chronic immune activation, such as happen during chronic HIV-infection, is characterized by an increased expression of activation markers of T cells and an enhanced pro-inflammatory cytokines production (Haas *et al*, 2011). These reports matched the own observation of enhanced IFN- γ production and activation markers (CD25 and CD69) expression levels by anergic T cells relative to the control. Kinetics experiments also supported the idea as an enhanced production of IFN- γ , indicating the increasing activation, during the anergy induction showed a positive correlation with the number of tumor cells added into the T cell culture. In tumor, as in persistent virus inflammation and autoimmune disease, which all are associated with a chronic inflammation,

impaired T cell function was reported (Baniyash M, 2004). The activation state of anergic T cells in the *in vitro* model was in line with the finding of previous study in the group. Analysis of TILs from 148 melanoma patients had shown that 80% of tumor-infiltrating T cells were memory T cells and about 55% expressed CD69, suggesting a continuous active response against the tumor. Similarly, T cells in the *in vitro* anergic T cell model were mostly memory T cells. In previous studies in the group, the active T cells state among TILs was also indicated by the expressions of PD-1 and CTLA-4, where about 20% expressed CTLA-4 and 35% expressed PD-1. The expressions of PD-1 and CTLA-4 could not be detected in anergic T cells in the *in vitro* model though. However PD-1 expression was also different in tumor site and peripheral blood, which is in line with the tissue specific differences in PD-1 and PD-L1 expressions reported by Blackburn (Blackburn *et al*, 2010). This could explain the differences between TILs and anergic T cells which were generated from T cells isolated from peripheral blood. Observations from *in vitro* anergic T cell model and analyses of TILs from melanoma patients suggest that anergic T cells are in an active state instead of a suppressed state.

Several experimental studies aim to induce effector T cells against melanoma. A study using a trivalent vaccine against self-melanoma antigen glycoprotein 100 (gp100), tyrosinase and melanoma antigen recognized by T cells 1 (MART-1) were done to increase T cell activity against melanoma (Ingraffea, 2013). As the data from the present and earlier studies in the group implicate an active state of TIL and anergic T cells induced *in vitro*, strategies for melanoma therapy that aim at inducing T cell activation need to be reconsidered.

Recent reports suggesting the importance of IL-10 in monocytes or macrophages functions, and therefore further experiments using monocytes and DCs co-cultured with tumor cells were carried out. The experiments showed that tumor-associated iDCs were a mixed population of cells, consisted of round cells and macrophage-like cells, had a reduced DC capacity to induce T cell proliferation, and an enhanced IL-10 production. But again there was no correlation between the amount of IL-10 produced by tumor-associated iDCs with their reduced capacity to induce CD4 T cell proliferation. Although tumor-associated iDCs cultured with a high excess of tumor cells produced around twice as much IL-10 than the tumor-associated iDCs cultured with an equal numbers of tumor cells, the capacity of tumor-associated iDCs in these two groups to induce CD4 T cell proliferation was similar. Blocking IL-10 during tumor-associated iDCs generation also did not change their capacity to induce CD4 T cell proliferation. IL-10 production by tumor-associated mDCs also increased around

sevenfold compared with the control DCs but the capacity of tumor-associated mDCs to stimulate CD4 T cell proliferation was not suppressed, rather an enhanced capacity up to 30% was observed.

One study supporting the idea of IL-10 mediated immune suppression showed that exogenous IL-10 could inhibit DC function (Allavena *et al*, 1998), however the concentration of IL-10 added to the culture was very high (20 ng/ml). A high concentration of IL-10 could indeed inhibit DC function as confirmed by the *in vitro* experiment in this study, however a low concentration of exogenous IL-10 alone, which was similar to the endogenous IL-10 produced by tumor-associated iDCs could not inhibit DC function. These results showed that DC modulation by IL-10 was dependent on the IL-10 concentration and earlier studies had exceeded by far the naturally induced level.

Both iDCs and tumor cells used in this study (ChaMel84) did not produce IL-10 when cultured alone. However, when cultured together, tumor cells produced IL-10, suggesting that an interaction between tumor cells and iDCs was needed to induce the IL-10 production. The necessity of cell-cell interaction was confirmed by the results of experiments with tumor supernatant. In these experiment, IL-10 produced by DCs generated in the medium alone or in the medium containing tumor supernatant was similar even when increasing amount of tumor supernatant was used, indicating that cell-cell contact was needed. This result also implied that the enhancement of IL-10 production found in the tumor-associated iDCs culture was a direct consequence of increasing number of tumor cells used in the culture.

All this evidence demonstrates that even though IL-10 could be detected in the culture of tumor-associated DCs, there was no correlation between IL-10 endogenous production and the altered DC phenotype and capacity to stimulate CD4 T cell proliferation. This result implies that while DCs altered by tumors may still be important for mediating tumors escape, IL-10 production is not important for modulating DCs. Although IL-10 does not seem to be involved in altering DCs phenotypes and functions, IL-10 could mediate other mechanisms. An *in vivo* renal carcinoma study showed that IL-10 is able to promote M2 polarization of Tumor associated macrophages, a subset of macrophages that have poor antigen-presenting capacity and suppress T cell activity (Lee *et al*, 2013). Further studies to investigate the role of IL-10 in macrophage polarization are therefore of interest.

TGF- β produced in the tumor-associated DC culture did not translate to an active immune suppression. Interestingly, tumor-associated iDC and mDC cultures in transwell

assays showed a reduced TGF- β production. The increase tumor-associated DC capacity in inducing CD4 T cell proliferation could be explained by this finding. Blocking TGF- β during tumor-associated mDC maturation further increased the capacity of tumor-associated mDCs to stimulate CD4 T cell proliferation. Exogenous TGF- β addition during DC differentiation also did not suppress the DC function, instead an enhanced capacity could be observed. Similar results were observed when exogenous TGF- β was added during DC maturation, where the DC function was enhanced although the expressions of activation and maturation markers were reduced. DC modulation by TGF- β was concentration dependent and an increasing TGF- β concentration led to an increased DC capacity.

IL-10 and TGF- β were shown to be expressed in tumor with conflicting results supporting the role of these cytokines in both immune suppressive and immune stimulation. Active suppression of immune responses by IL-10 and TGF- β could not be demonstrated in this study, leading to the questions about the role of these two cytokines in anergy induction.

Addition of exogenous IL-10 to T cells co-cultured with a low excess of tumor cells seemed to lead to reduced T cell responses capacities upon re-challenged, whereas blocking the endogenous IL-10 during the anergy induction did not make any differences in all ratios of T cells to tumor cells tested when compared with the control groups. Exogenous IL-10 seemed to assist the induction of anergic T cells when IL-10 was added into the functional T cell culture but the effect was not strong even when a high concentration was applied. Only around a 20% reduction of T cell proliferation and Granzyme B production along with no reduction of IFN- γ production were observed. These results could explain why blocking the endogenous IL-10 did not have any effect since the endogenous IL-10 production was far below the concentration of exogenous IL-10 added into the culture. The opposite effect of exogenous IL-10 was observed when IL-10 was added during anergy induction. Here, IL-10 seemed to interfere with anergy induction as the T cell responses were better when exogenous IL-10 was added into the culture.

The addition of TGF- β did not drive T cells to anergy. On the contrary, TGF- β enhanced the capacity of T cells to respond to tumor re-challenged. These experiments also showed that adding TGF- β during anergy induction did not support the anergy induction process nor further suppressed T cell responses since no changes of T cell responses were detected, suggesting that exogenous TGF- β did not interfere with anergy induction. Blocking endogenous TGF- β caused no significant changes on T cell responses upon re-challenged,

indicating that the low concentration of endogenous TGF- β was not important for inducing T cell anergy.

As mentioned above, anergic T cells seemed to be in an active state. Kinetics experiment showed increasing activity when larger numbers of tumor cells were added into the culture, suggested an over-activation state of the T cells when co-cultured with high numbers of tumor cells. Exogenous TGF- β may protect T cells from over-activation, indicated by the low production of IFN- γ during anergy induction, preserving the capacity of T cells for responses upon re-challenged. This proposal is in agreement with a study showing a reduced TGF- β 1 and TGF- β receptor expressions in patients with chronic dermatitis compared with normal skin which may lead to the retention of inflammation and chronic skin inflammation (Khareshi *et al*, 2011).

Combining exogenous IL-10 and TGF- β in T cells co-cultured with tumor cells during anergy induction seemed to weaken the effect of TGF- β to suppress the over-activation of T cells as IFN- γ production during the anergy induction was higher compared with the effect of using TGF- β alone. Unexpectedly, IFN- γ production, but not T cell proliferation, was higher upon re-challenge when combined cytokines were applied instead of using TGF- β alone. These results suggest that combining these two cytokines could rescue the anergic T cells to some extent. When the combination of IL-10 and TGF- β was added into the functional T cell culture, better T cell responses were observed upon re-challenge compared with adding either IL-10 or TGF- β alone, suggesting that TGF- β could counter the IL-10 effect in promoting anergy. These data implied that TGF- β did not actively suppress T cell responses. On the contrary, it could interfere with the T cell anergy induction.

With the *in vitro* model for anergy induction, pleiotropic effects of IL-10 and TGF- β could be demonstrated in both immune stimulation and suppression. IL-10 and TGF- β were shown to function differently depending on types and different stages of target cells. The results urge not to simply categorize these cytokines as targets of anti-immune escape therapies. On the contrary, TGF- β application to interfere with T cells anergy may add a new aspect to the development of immune therapies against cancer.

The *in vitro* experiments showed decreased responses of anergic T cells upon re-challenge but there was no evidence for negative regulation by the immunosuppressive cytokines IL-10 and TGF- β or the co-inhibitor molecules PD-1 and CTLA-4 in causing this

impair T cell function. Another possible explanation was offered by *in vivo* experimental demonstrating a downregulation of TCR-zeta chain and an induced impaired T cell function upon excessive inflammation (Baniyash M, 2004). An earlier study in melanoma had already shown a decrease of TCR-zeta and tyrosine kinases relating to TCR signaling (Zea *et al*, 1995).

The generation of tumor-associated iDCs *in vitro* showed that tumor cells altered the differentiation of monocytes into iDCs. The alteration was more apparent in direct contact assay, shown by the higher CD14 expression and the lower DC capacity to stimulate T cell proliferation, compared with the transwell assays. However, alteration of iDCs could still be observed in the transwell assay, suggesting that tumor-derived molecules or tumor-induced soluble factors impact DC differentiation.

Exosomes are membranous particles released from cells. They can contain a variety of molecules, including peptides, mRNA, microRNA, lipids. Exosomes have been described as an alternative pathway used by tumor cells to suppress immune system (Zhang and Grizzle, 2011). Exosomes could be exempted in this study since exosomes-free medium used to differentiate monocytes was still able to alter DCs. This experiment also showed that DC alteration was dose dependent since alteration was more extensive when higher concentrations of the tumor supernatant were used in the experiments.

In transwell assays, tumor-associated DCs generated in the absence of activation signal (inflammatory cytokines) had a lower capacity to stimulate CD4 T cells compared with the control iDCs. However, in the presence of activation signal, the capacity of tumor-associated DCs was similar to control DCs. This result is in line with the concept that the DC system is in flux and dependent on inflammatory reaction (reviewed by Shortman and Naik, 2007). However, when tumor-associated DCs were generated in direct contact with tumor cells, the presence of activation signal did not induce the maturation of altered iDCs (CD83 expression remained low) and the capacity to stimulate CD4 T cells was reduced. This result suggested that although tumor-derived soluble factors can modulate DC phenotypes and their capacity to stimulate T cell proliferation, a direct contact with tumor cells was needed to completely determine the alteration of DCs.

In contrast to the effect of tumor cells during DC differentiation, tumor cells do not seem to have a negative effect on DC maturation or activation. In transwell assays, tumor-associated mDCs even had an increased activation and enhanced capacity to induce T cell

proliferation. IL-10 production also increased in the tumor-associated mDC culture, suggesting that IL-10 production may thus be used as an indicator of activation. This idea was also supported by the results demonstrating that mature DCs produced IL-10 but immature DCs did not.

To answer whether tumor cells alone are sufficient to induce DC maturation, iDCs were generated and tumor cells were added. The results showed low expressions of activation markers and low capacity to induce CD4 proliferation, indicating that tumor cells alone are insufficient to induce DC maturation. This result is in line with studies done by Joffre and colleagues (Joffre *et al*, 2008), which showed that tissue-derived signals are not sufficient to activate DCs in chimeric mice. The experiments in this study also showed that tumor-associated iDCs were more activated than control iDCs but the capacity to induce T cell proliferation remained low. The results were in agreement with previous reports showing that DC activation could be tolerogenic as iDCs co-cultured with tumor supernatant displayed enhanced activation markers but had a decreased capacity to stimulate CD4 T cells (Kiertscher *et al*, 2000).

Baumgartner and colleagues found that DC maturation and function are not altered by melanoma-derived immunosuppressive soluble factors (Baumgartner *et al*, 2012). The difference of results could relate to the use of LPS by Baumgartner and colleagues to induce DC maturation. LPS is a strong stimulant and probably the effect is too strong so that the difference between groups could not be detected. That was the rationale for using a cytokine cocktail to induce DC maturation in this study as the cytokine cocktail is a far weaker stimulant than LPS.

IL-10 and TGF- β productions were shown to have no correlation with the DC alteration in this study although tumor-associated iDC culture produced IL-10. Aside from IL-10 and TGF- β , other soluble factors have been suggested in different studies to be able to modulate immune response. DNA chip analysis of ChaMel84, which was used in this study, had been done before and had shown upregulation relative to melanocytes of some soluble factors that were correlated with a tumor progression. ChaMel84 expressed 23 times upregulation of IL-8 compared with melanocytes. Neutralizing IL-8 is shown to inhibit angiogenesis, tumor growth and metastasis of human melanoma in nude mice (Huang *et al*, 2002). It also expressed 12 times more of CXCL16 relative to melanocytes. Increasing CXCL16 is correlated with poor prognosis in colorectal cancer (Verbeke *et al*, 2011) and it seems to be important in the bladder cancer progression (Lee *et al*, 2013). A seven-fold

increase of IL-33 expression was also found in ChaMel84 compared with melanocytes. Increased level of IL-33 is correlated with poor prognosis in gastric cancer (Sun *et al*, 2011). The mechanism by which these cytokines modulate immune responses and support tumor progression is still unknown. The correlation between these cytokines and DC modulation or immune modulation in general, is an important aspect to be explored.

Since a negative regulation of immune response by immunosuppressive cytokines could not be found, a negative regulation by co-inhibitor molecules was investigated as well. A dose-dependent increased expression of PD-L1 was detected when iDCs were generated in the presence of tumor supernatant. This could explain the decreased tumor-associated iDC capacity to induce T cell activation. This result supported the idea of addressing the PD-1/PD-L1 axis in the cancer therapy. Clinical trials evaluating the anti PD-L1 in cancer treatments are still on going. However several open issues are still unresolved, such as: the heterogeneity of PD-L1 expression and the prognostic role of TILs in addition to PD-1/PD-L1 to identify patients who will be benefit from such therapy (Merelli *et al*, 2013).

In addition to the PD-1/PD-L1 axis, a treatment to apply antibodies against CTLA-4 is also interesting to pursue as an increased CTLA-4 expression was observed in melanoma in previous studies in the group, as well in cancer tissue of non-small-cell-lung cancer patients compared with healthy individual (Antczak *et al*, 2013). One study showed that dual blocking of PD-1 and CTLA-4 leads to a reversal of dysfunctional TILs and to tumor rejection in a mice model for carcinoma (Duraismwamy *et al*, 2013). Ipilimumab, an antibody targeting cytotoxic T cells, was approved by FDA in 2011 to be applied against un-resectable melanoma. Blockage of CTLA-4 can lead to durable cancer regression and phase III studies show a survival benefit for melanoma patients. However, ipilimumab can lead to immune-related adverse events, which can be serious and life threatening (Frederick *et al*, 2013, Ingraffea, 2013; Callahan *et al*, 2013; Tarhini, 2013; Finn *et al*, 2012).

DCs are shown to play an important role in anti-tumor responses as it has been reported that tumors infiltrated by DCs are associated with a good prognosis. On the contrary, some reports also conclude that DCs can exert pro-tumorigenic functions by blocking the anti-tumor responses and support angiogenesis and metastasis (reviewed in Ma *et al*, 2013). Experiments done in mice shows DCs in early tumorigenesis are immunocompetent while DCs in the late stages are immunosuppressive (Scarlett *et al*, 2012). The *in vitro* model developed in this study showed that DCs co-cultured with tumor cells indeed response differently in different stages and the changes were related to the tumor burden, resembling

the changes of DCs during the tumor progression. Exposure of tumor cells during DC differentiation would alter DC phenotypes and render DC capacity to induce CD4 T cell proliferation in line with the tumor burden. On the contrary, exposure to tumor cells had no negative effect on DC maturation and could even enhance the activation of DCs. This *in vitro* model provided a mean to study the effect of tumor cells on DCs and observe what tumor cells do or do not do to DCs. The study of DC modulation by tumors is important as DCs are the main APCs to present tumor-associated antigens to T cells. DC-based tumor vaccines have been extensively tested but the results of the respective clinical trials were mostly disappointing. Many explanations have been offered for the ineffectiveness of DC-based tumor vaccines, including inefficient antigen loading, altered DC maturation states, heterogeneous DC populations, and tumor-mediated immune suppression (reviewed in Evel-Kabler and Chen, 2006). This *in vitro* model can help to further unravel DC modulation by tumors in human.

Analysis of iDC morphology showed that tumor-associated iDCs were a mixture of round-unattached-cells and macrophage-like cells. Flow cytometry analysis showed a mixture of CD14⁻ and CD14⁺ cells in the culture. Since only unattached cells were harvested to be analyzed by flow cytometry, the high expression of CD14 on these unattached cells indicating the incomplete differentiation from monocytes into iDCs. The DCs modulated by tumor cells during their differentiation resembles myeloid-derived suppressor cells (MDSC) described in patients with different cancers. MDSCs are described as a population of mixed cells that stay in immature state, have myeloid origin and ability to suppress T cell responses. MDSCs are found in blood of patients with various cancers (Gabrilovich and Nagaraj, 2009; Simpson *et al.*, 2012).

Aside from MDSCs, iDCs co-cultured with tumor cells in this experiment might also fit the model of regulatory dendritic cells. Tumor cells are suggested to be able to differentiate iDCs into a subset of DCs, so called regulatory dendritic cells. Tumor-associated regulatory dendritic cells are suggested to directly and indirectly maintain antigen specific and non-specific T cell responses by secreting TGF- β , controlling T cell polarization, promoting T-reg differentiation and activity, and promoting MDSCs. The expression of immunoregulatory molecules (i.e. PD-L1, PD-L2, B7-H3, B7-H4), increased production of immunoregulatory cytokines (i.e. IL-10, TGF- β), or decreased production of pro-inflammatory cytokines (i.e. IL-6, IL-12, TNF- α) have been described in various regulatory dendritic cell models. In addition,

tumor-induced regulatory dendritic cells show a reduced expression of MHC class II and the co-stimulatory molecules CD80 and CD86 (reviewed in Ma *et al*, 2012; Shurin *et al*, 2013).

The *in situ* studies showed that not only no upregulation of IL-10 and TGF- β was detectable, but the expression levels from these two cytokines were also considerably lower compared with the expression in the healthy skin. This finding suggests a relatively high expression of these cytokines in healthy skin which is not surprising considering skin acts as the first barrier against infections. Skin is exposed to microorganisms all the time and it needs immunosuppressive mechanism to suppress the excessive immune reaction. These results suggest that IL-10 and TGF- β are important to suppress the excessive immune reaction in healthy skin. This suggestion is also in line with the finding of relatively low expression of IL-10 in psoriasis lesions compared with healthy skin (Cheng *et al*, 2001). Psoriasis is an inflammatory disease of skin with a complex pathogenesis. The low expression of IL-10 in psoriasis lesions could contribute to the disease progress. High systemic level of IL-10 was correlated with poor survival of some cancer patients (reviewed in Mocellin *et al*, 2005) and in connection to the result reported herein, the high level of IL-10 might just reflect the extent of the disease and of the T-cellular immune responses against the tumor rather than the immune suppression.

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Appendix

Table 3. Patients and Tumor Status

Patient ID	Stage	MM subtype	Metastasis location	Treatment
ChaMel44	3	NM	SK/ ST/ BO	S, C, A
ChaMel53	3	NM	SK/ ST/ BO	S, C, A
ChaMel74	3	NM	LU, LI	S, C, A
ChaMel84	3	NM	SK/ ST/ BO	S
ChaMel87	3	NM	CNS, SK/ ST/ BO	S, C, R
ChaMel96	3	NM	LU	S, C
ChaMel41	4	SSM	LI, SK/ ST/ BO, CNS	S, C, R
ChaMel21	3	SSM	LU, SK/ ST/ BO	S, C, A
ChaMel39	3	SSM	LI, SK/ ST/ BO	S, C, A
ChaMel45	3	SSM	LU	S
ChaMel47	4	SSM	LU, CNS, SK/ ST/ BO	S, C
ChaMel57	3	SSM	nd	S
ChaMel60	3	SSM	CNS, SK/ ST/ BO	S, C
ChaMel80	3	SSM	CNS, SK/ ST/ BO	S, C
ChaMel109	3	SSM	SK/ ST/ BO	S, C, A
ChaMel121	4	SSM	CNS, SK, ST, BO	S
ChaMel50	3	SSM	CNS, SK/ ST/ BO	S
ChaMel59	3	nd	LU, SK/ ST/ BO	S, C, A
ChaMel114	3	nd	LU	S, C
ChaMel105	3	nd	LN, ST	S
ChaMel5	4	nd	LU, LI	S, C, A

(extracted from Stefani Gross' dissertation)

NM : nodular melanoma
SSM : superficial spreading melanoma
nd : not described

LU : lung
LI : liver
SK : multiple skin metastases
ST : soft tissue
BO : bone
CNS : central neuron system

S : surgery
C : chemotherapy
A : adjuvant
R : radiotherapy

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und nur mit den angegebenen Hilfsmitteln erstellt habe.

Berlin, Januar 2014

Hiermit erkläre ich, dass ich Kenntnis von der dem angestrebten Verfahren zugrunde liegenden Promotionsordnung habe.

Berlin, Januar 2014

List of Publications

In preparation:

1. IL-10 and TGF- β regulation in melanoma. (first author)