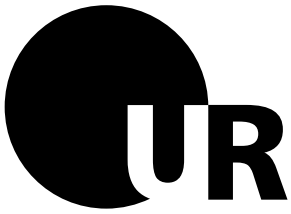
Characterization and functional analysis of the transfer of cell components from human antigen-presenting cells onto T cells via antigen-specific trogocytosis



Dissertation

zur Erlangung des Doktorgrades

der Naturwissenschaften (Dr. rer. nat.)

der Naturwissenschaftlichen Fakultät III

Biologie und Vorklinische Medizin der Universität Regensburg

vorgelegt von

Regina Gary

aus

Pfaffenhofen / Ilm

November 2010

Promotionsgesuch wurde eingereicht am:
09.11.2010

Die Arbeit wurde betreut von:
Prof. Dr. Andreas Mackensen

Prüfungsausschuss:

Vorsitzender:	**Prof. Dr. rer. nat. R. Wirth**
1. Gutachter:	**Prof. Dr. rer. nat. S. Modrow**
2. Gutacher:	**Prof. Dr. med. A. Mackensen**
3. Prüfer:	**Prof. Dr. med. R. Warth**
4. Ersatzprüfer:	**Prof. Dr. rer. nat. H. Tschochner**

Die vorliegende Arbeit entstand in der Zeit von Dezember 2006 bis Oktober 2010
in der Abteilung für Hämatologie und Internistische Onkologie am Klinikum der
Universität Regensburg sowie
in der Medizinischen Klinik 5 – Hämatologie und Internistische Onkologie am
Universitätsklinikum Erlangen

Was auch immer ein Mensch an Gutem
in die Welt hinaus gibt,
geht nicht verloren
(Albert Schweitzer)

Per aspera ad astra

1. Introduction

1.1. The immune system

1.1.1. Innate and adaptive immunity

The human immune system is a highly complex system consisting of many components which play an important role in the defense against the myriad potentially pathogenic mirco-organisms as well as in the rejection of malignant tumors. It is composed of the innate and adaptive immune system: innate immunity mediates a non-specific, immediate first-line defense whereas adaptive immunity is specialized and characterized by specific recognition and long-lasting protective immunity.

The innate immunity consists of different mechanisms to protect the body from harmful micro-organisms. A first mechanic barrier is the surface epithelium. Macrophages and neutrophil granuloyctes are critical in the defense against both intracellular and extracellular bacteria as well as against fungi by phagocytosis of these micro-organisms. Furthermore, the acute phase response and the complement system opsonize pathogens for phagocytic uptake and have direct antimicrobial activities. Host cells infected by viruses or other intracellular pathogens as well as tumor cells can be eliminated by natural killer cells (NK cells). Pathogens are detected by germline-encoded conserved pathogen pattern receptors (PPRs) which recognize motifs called PAMPs (pathogen-associated molecular patterns) or by missing self recognition (Medzhitov 2007). In addition to the important role as first defense against micro-oranganisms, innate immune recognition is also critical for activation of the adaptive immune system.

Adaptive immunity is divided in humoral immunity mediated by B cells and cellular immunity mediated by T cells. In contrast to innate immunity, the concept of enormous diversity of B and T cell receptor (BCR and TCR) repertoires is based on somatic recombination and gene rearrangement. Professional antigen-presenting cells (APC) such as dendritic cells (DC) link innate and adaptive immunity (Steinman and Hemmi 2006). As professional APC, DC are usually located in the skin and mucosal epithelia, the sites of contact with external environment. Immature (i)DC are highly phagocytic and take up microbial antigens in the periphery. After phagocytosis, DC migrate to T cell rich areas in lymph nodes where DC-maturation is induced by cytokines. Mature DC (mDC) process

antigens and present them via Major Histocompatibility Complex (MHC) class I and II molecules to T cells, thereby initiating the cellular immune response (Abbas and Janeway 2000). The humoral part of adaptive immunity is characterized by secretion of antibodies. T cells expressing CD154 and T cell-derived cytokines control the division and differentiation of activated B cells into antibody-secreting plasma cells. Antibodies acting in different ways support the removal of pathogens by phagocytes via opsonization, activate the complement system, and inhibit the entrance of pathogens into cells via binding to pathogens. Plasma memory B cells are capable to mediate lifelong immunity against once encountered pathogens.

1.1.2. The adaptive cellular immune response

The adaptive cellular response is mediated by T lymphocytes. The majority of T cells is characterized by TCRs with αβ-heterodimers mainly represented in the lymphoid organs and responding to peptide antigens. Only a small subset of T cells which is located in the mucosal and epithelial barrier expresses TCRs with γ and δ chains recognizing lipid antigens. In 1974, Zinkernagel and Doherty discovered that the recognition of foreign protein antigens in combination with self-MHC by TCRs is essential for T cell activity (Zinkernagel and Doherty 1974; Zinkernagel and Doherty 1997). Antigens of intracellular origin are typically presented on MHC class I molecules activating cytotoxic T cells which express the co-receptor CD8 whereas extracellular antigens are presented after endocytosis on MHC class II molecules stimulating T helper cells characterized by the co-receptor CD4. However, extracellular antigens can be also presented on MHC class I molecules giving rise to a cytotoxic response via a mechanism which is called cross-presentation. Two independent signals are commonly acknowledged to be critical for the activation of naïve T cells: the peptide-MHC-complex (pMHC) has to be recognized by the TCR followed by the additional binding of co-stimulatory molecules. Most of activated $CD4^+$ helper cells move to the site of infection mobilizing and activating other immune cells. However, some $CD4^+$ T cells remain in the lymph node providing a cytokine environment which supports B cell functions (Campbell, Kim et al. 2001). $CD4^+$ T cells are known to differentiate in one of many diverse subsets, depending on the kind of pathogens and the surrounding cytokines. Th1, Th2, Th17 and Treg are the most prominent $CD4^+$ subsets. Th1 cells play an important role in inflammation processes and activate macrophages, NK cells and T cells via

interferon (IFN)-γ secretion whereas the most described function of Th2 cells is the recruitment of mast cells, basophil and eosinophil granulocytes by interleukins (IL) as IL-4, IL-13 and IL-25 as defense at mucosal and epithelial surfaces (Szabo, Sullivan et al. 2003; Voehringer, Reese et al. 2006). Th17 are amongst others able to activate neutrophils by secretion of IL-17, IL-6 and tumor necrosis factor (TNF), thereby regulating acute inflammation (Langrish, Chen et al. 2005; Mangan, Harrington et al. 2006). A further subset of $CD4^+$ T cells is formed by naturally occurring and induced regulatory T cells (T_{regs}). Todays most prominent population of $CD4^+$ T_{regs} are characterized by high expression of CD25 and the transcription factor Foxp3 and exert their regulatory function by suppression of other T cells as well as modulation of DC (Vignali, Collison et al. 2008; Josefowicz and Rudensky 2009). $CD4^+CD25^+Foxp3^+$ T_{regs} are known to use different mechanisms to suppress responder T cells: the release of suppressive cytokines as IL-10, IL-35, and transforming growth factor (TGF)-β leads to inhibition of effector T-cell (T_{eff}) differentiation and activation. Moreover, the consumption of IL-2 in competition with responder T cells limits T_{eff} proliferation, and secreted or surface molecules as galectin-1 can directly inhibit responder T cells (Shevach 2008). Furthermore, T_{regs} suppress the maturation of DC by LAG-3 binding to MHC class II and decrease co-stimulation by CTLA-4 interaction with CD80 or CD86 (Shevach 2008). Another described regulatory T-cell subpopulation is represented by $TCR\alpha\beta^+CD4^-CD8^-$ double negative (DN) T cells (Zhang, Yang et al. 2000; Fischer, Voelkl et al. 2005). Murine DN T cells were already identified in 2000 to exhibit a strong immuneregulatory function (Zhang, Yang et al. 2000). Recently, our group could show that human DN T cells excert a very potent suppressive activity towards $CD4^+$ and $CD8^+$ T cells (unpublished data).

$CD8^+$ T cells are important players in the clearance of intracellularly infected cells and in the rejection of malignant tumors. Naïve $CD8^+$ T cells can face different fates: In the case of steady state antigen recognition, peripheral tolerance in $CD8^+$ T cells can result in the two distinct differentiation states, apoptosis and anergy. In both cases T cells generally fail to develop effector functions (Hernandez, Aung et al. 2001; Schwartz 2003). Under these circumstances, programmed death 1 (PD-1) is required for both, anergy and deletion of $CD8^+$ T cells (Goldberg, Maris et al. 2007; Tsushima, Yao et al. 2007). The outcome seems to be largely dependent on the overall level of TCR signaling (Parish and Kaech 2009). High antigen levels typically cause anergy whereas lower antigen levels lead to apoptosis (Redmond, Marincek et al. 2005). Antigen-presentation by APC in the immunological context of infection activates $CD8^+$ T cells which then will expand and differentiate into

effector and memory T cells. Effector $CD8^+$ T cells, also called cytotoxic T lymphocytes (CTL), can clear infected cells by release of effector molecules as perforin and granzyme or by stimulation of cell surface bound molecules such as Fas. Furthermore, CTL produce high amounts of antiviral cytokines such as IFN-γ and TNF upon TCR ligation (Williams and Bevan 2007). Cytokines secreted by the innate immune system, in particular IL-12 and type 1 interferons, also promote CTL expansion (Cousens, Peterson et al. 1999; Mescher, Curtsinger et al. 2006). Of importance, in certain chronic infections $CD8^+$ T cells fail to exert their normal effector functions such as IFN-γ, TNF and IL-2 production and cytotoxicity. Exhaustion, as this specific state of dysfunction is called, seems to be driven by up-regulation of a network of inhibitory receptors such as PD-1 and LAG-3 (Barber, Wherry et al. 2006; Blackburn, Shin et al. 2009). After successful clearance of the pathogens, the immune reaction is stopped by elimination of most of activated T cells by apoptosis (Hildeman, Zhu et al. 2002). However, a small percentage persists for a long period of time as memory T cells. This subpopulation has a unique ability for self-renewal and survival, and is therefore able to mediate fast and effective immune reactions at reinfections with the same pathogens once encountered.

1.1.3. Interactions between T cells and antigen-presenting cells

A rapid, flexible and dynamic immune system is required to protect the host. Although T cells need to be primed by DC, they represent a major force in eliminating pathogens which needs to be carefully regulated to avoid excessive immune reactions.

This regulation can be mediated by interaction of molecules on the surface of APC like the C-type lectin DC-specific ICAM-3 grabbing non-integrin (DC-SIGN, also CD209). DC-SIGN has a broad range of functions: it is an adhesion receptor responsible for clustering of naïve T cells through binding of intercellular adhesion molecule (ICAM)-3, interacts with ICAM-2 on endothelial cells to induce transendothelial migration and can function as antigen-receptor (Geijtenbeek, Krooshoop et al. 2000; Engering, Geijtenbeek et al. 2002; van Gisbergen, Paessens et al. 2005). The cellular interaction between DC-SIGN and ICAM-3 facilitates the formation of low-avidity lymphocyte functional-associated antigen 1 (LFA-1) interaction with ICAM-1 on the DC as well as the screening of antigen-MHC repertoire (van Kooyk and Geijtenbeek 2002). The importance of ICAM-1 for effective CTL priming could be demonstrated by the fact that ICAM-1 deficient DC could not form

long-lasting contacts with $CD8^+$ T cells resulting in reduced IFN-γ production *in vitro* and *in vivo* (Scholer, Hugues et al. 2008).When the TCR recognizes an antigen presented via MHC molecules, this binding between T cells and APC is getting stronger through high avidity LFA-1-ICAM-1 and CD2-LFA-3 interactions (McDowall, Leitinger et al. 1998; Bleijs, Geijtenbeek et al. 2001). TCR engagement is followed closely by the migration of LFA-1 from the central contact site to a more peripheral location, whereas the TCR complex is moved to the center of the immunological synapse forming the central supramolecular activation cluster (cSMAC) surrounded by the peripheral supramolecular activation cluster (pSMAC) (Monks, Freiberg et al. 1998; Bromley, Burack et al. 2001).

The TCR is critical for antigen recognition and is always co-expressed with CD3 chains which are essential for the signal transduction into the cell via immunoreceptor tyrosine-based activation motifs (ITAMs) (Irving and Weiss 1991; Wegener, Letourneur et al. 1992). The TCR co-receptors CD4 and CD8 interact with conserved residues of MHC molecules and are bound to the protein tyrosine kinase lck at their cytoplasmic tails (Rudd 1990). Upon engagement of the TCR with antigen-MHC complex, the co-receptor-lck complex comes into proximity of the CD3 ITAMs, thus starting the TCR signal cascade. As further required positive regulator for T cell activation, CD45 is one of the most abundant surface antigens on immune cells and characterized as transmembrane molecule with potent protein tyrosine phosphatase activity (Tonks, Diltz et al. 1991; Trowbridge and Thomas 1994). After first TCR engagement, the cell is primed to respond to other activating stimuli, e.g. through the increased affinitiy as well as the higher avidity of integrins for the interaction with further APC.

One very special feature of T-cell activation is that TCR engagement with pMHC-complex on its own is not sufficient for a proper T-cell response as mentioned above (Schwartz 2003). In addition to TCR engagement as signal 1, T cells need also a signal 2 initiated by binding of one of several co-stimulatory receptors. This second signal transduces independent signals or enhances the signaling cascades initiated by the engagement of the TCR complex which is critical for T-cell proliferation and effector differentiation. The most physiologically relevant co-stimulatory receptor on T cells seems to be presented by the cell surface homodimer CD28 (Acuto and Michel 2003). Ligands of CD28 that are present on APC and belong to the B7 familiy are CD80 (B7-1) and CD86 (B7-2). They are up-regulated together with MHC molecules in presence of pathogens after signals of the innate immune system guaranteeing a better delivery of both important signals to T cells (Sharpe and Freeman 2002). Another member of the CD28 family is inducible co-

stimulator (ICOS): ICOS expression on naïve T cells is low but increases after TCR stimulation (Hutloff, Dittrich et al. 1999; Yoshinaga, Whoriskey et al. 1999). In contrast to CD28, ICOS does not bind to B7-1 and B7-2, but to yet another member of the B7 family called ICOS ligand which is expressed more broadly than B7-1 and B7-2 (Coyle and Gutierrez-Ramos 2001). Both ICOS and CD28 activation leads to enhanced cytokine production by T cells but ICOS signaling does not up-regulate IL-2 expression. Instead, ICOS seems to play a more important role for T cell mediated B cell help, for Ig class switching as well as for formation of germinal centers (Coyle, Lehar et al. 2000). These small differences in the co-stimulatory outcome have important regulatory functions in orchestrating the immune response. In regard to the growth of T cells, IL-2 receptor signaling plays a major role in the regulation of proliferation. Prior to stimulation, naïve T cells express only a moderate affinity IL-2 receptor consisting of the β and γ chains. A third IL-2 receptor component, the α chain (CD25), is expressed upon TCR ligation resulting in an increased affinity for IL-2 (Minami, Kono et al. 1993).

After the target cells are cleared, it is essential to stop proliferation and effector functions to prevent damage to uninfected tissue. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is a member of the CD28 family and is competitive to CD28 but has a much higher affinity to B7-1 and B7-2 than does CD28 by itself (Perkins, Wang et al. 1996). CTLA-4 is co-expressed on T cells after an initial T cell activation. Thus, high expression of CTLA-4 on activated T cells prohibits the activating co-stimulatory receptors and impairs T-cell effector responses by prevention of further T-cell activation and by termination of additional expansion of antigen-specific clones (Carreno, Bennett et al. 2000). Another inhibitory molecule also belonging to the CD28 and CTLA-4 family is PD-1 (Zhang, Schwartz et al. 2004). PD-1 shows a much broader expression – namely on T cells, B cells, NK cells, activated monocytes as well as DC – as compared to CTLA-4 expression suggesting a widespread role in immune regulation (Greenwald, Freeman et al. 2005). PD-1 is not expressed on resting T cells but is much like CTLA-4 inducibly expressed after activation, also emphasizing its immunoregulatory function (Nishimura, Agata et al. 1996). The known ligands of PD-1 are PD-L1 and PD-L2, which can both be found on DC. PD-1 counteracts TCR signaling with greater inhibition at low levels of TCR stimulation. PD-1 signaling is proposed to antagonize cell survival signals mediated by anti-apoptotic Bcl-xL, as well as effector differentiation mediated by CD28 and IL-2 (Keir, Butte et al. 2008). However, CD28 and IL-2 can overcome the negative impact of PD-1 signaling (Freeman, Long et al. 2000; Carter, Fouser et al. 2002). Along with CTLA-4 and PD-1, activation

induced cell death (AICD) is mediated by Fas (CD95)-Fas ligand interactions between T cells themselves. The components of the machinery necessary for AICD get up-regulated by TCR ligation, thereby making activated T cells more sensitive to AICD (Arnold, Brenner et al. 2006). Further death receptors called TNF-related apoptosis-inducing ligand (TRAIL)-R1 and TRAIL-R2 can be found on activated T cells whereas their ligand TRAIL is expressed on APC (Guicciardi and Gores 2009). In summary, interactions between DC and T cells play an important role in starting, increasing and finishing T-cell responses.

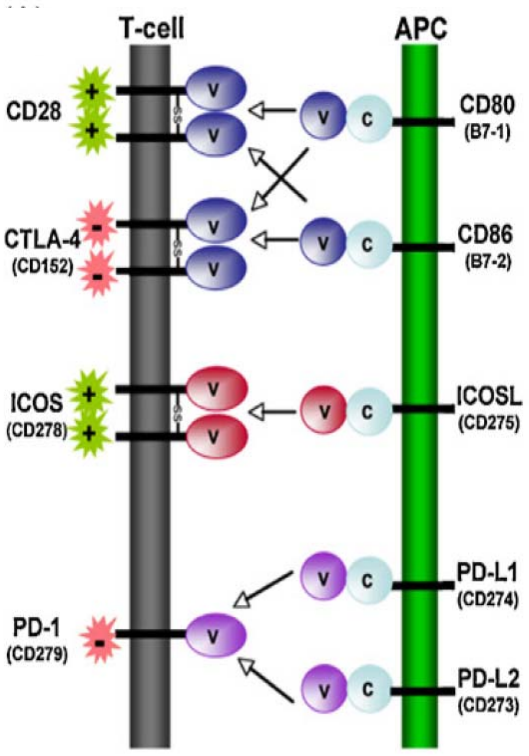


Figure 1: B7-CD28 superfamily costimulatory molecules. This figure shows stimulatory (green stars) and inhibitory (red stars) receptors on T cells and their ligands on DC (Leitner, Grabmeier-Pfistershammer et al.)

1.2. Tumor immunology

Immune cells can be effective in controlling and eliminating malignant tumor cells. Nevertheless, chronic inflammation also can promote tumor growth because immune cells express many factors which favor tissue growth and renewal, angiogenesis and facilitate

metastasis (Coussens and Werb 2002). The fact that T cells can recognize tumor antigens is an important prerequisite for the rejection of tumor cells by T cells. Many factors playing a role in cellular tumor immunology have been identified: 1. the existence of tumor antigens by the use of T cells recognizing intact cancer cells, 2. tumor-specific T cells by cytokine release and lysis of the target cells and 3. immunogenic peptides by elution from cancer cells. In the case of melanoma, a prominent model self-antigen was identified and isolated after screening of melanoma cDNA libraries in 1994 as Melan-A, also called Mart-1 which is expressed on normal melanocytes and belongs to the family of melanocyte differentiation antigens (Coulie, Brichard et al. 1994; Kawakami, Eliyahu et al. 1994). The Mart-1 gene encodes for a 118 amino-acid protein expressed in 80% to 90% of melanoma as well as in cultured melanoma cell lines (Marincola, Hijazi et al. 1996). As highly immuno-dominant antigen, Mart-1 bearing HLA-A2 restricted epitopes, e.g. the naturally processed nonamer AAGIGILTV (Mart-1:27-35), is recognized by the majority of melanoma reactive T cells (Skipper, Gulden et al. 1999). Another well known antigen of the melanocyte differentiation family is gp100 which is involved in melanin synthesis bearing different T-cell epitopes such as YLEPGPVTA (gp100:280-288), ITDQVPTSA (gp100:209-217) or VLYRYGSFSV (gp100: 476-485) (Cox, Skipper et al. 1994; Kawakami, Eliyahu et al. 1995). Interestingly, high frequencies of Mart-1 reactive T cells are not only found in melanoma patients but also in healthy individuals. These T cells show differences in their phenotypes: in healthy individuals the naïve phenotype dominates whereas in melanoma patients T-cell populations are composed of both, naïve and effector T cells (Romero, Dunbar et al. 1998).

Even though the immune system has many alternatives to fight against cancer, tumors have evolved mechanisms to evade the immune response. One problem for the immune system in elimination of tumor cells is the often immunosuppressive tumor microenvironment. T cells within melanoma and tumor-draining lymph nodes were shown to be functional impaired, but not the T cells of peripheral blood (Zippelius, Batard et al. 2004). Expression of arginase, nitric oxide synthetase or indoleamine-2,3-dioxygenase by human solid tumors have been demonstrated to mediate suppressive effects towards immune cells (Munn, Sharma et al. 2004; Bronte, Kasic et al. 2005). To enhance tumor-specific immune response, different strategies were evaluated in clinical trials to enhance tumor immunity. Many therapies aim for T-cell activation and expansion by administration of recombinant IL-2 and prevention of T-cell exhaustion by administration of anti-CTLA-4 blocking antibody (Rosenberg, Yang et al. 1994; Attia, Phan et al. 2005). Other approaches have

focused on active immunization using peptide, protein or recombinant viral cancer vaccines (Eder, Kantoff et al. 2000; Slingluff, Yamshchikov et al. 2001). Immunization with multiple peptides was applied to circumvent the escape of antigen-loss variants in therapies targeting just one single peptide (Valmori, Dutoit et al. 2003). Although an expansion of T cells could be observed in these trials, only a minority of patients showed clinical responses.

The adoptive transfer of tumor-reactive T cells seems to be another promising therapeutic option for cancer patients. Autologous $CD8^+$ T cells as potent effector cells can be activated and expanded *ex vivo* to large numbers with the aim of being transferred back into the patients. The combination of lympho-depletion followed by administration of antigen-specific T cells in combination with recombinant IL-2 provided strong evidence that antigen-specific immunotherapies can result in cancer regression when appropriate treatment and host factors are combined. However, various factors as high levels of tumor infiltrating $T_{regs,}$ NKT cells and $CD11b^+Gr^+$ myeloid suppressor cells have been shown to be unfavorable for efficient CTL responses (Terabe, Matsui et al. 2000; Curiel, Coukos et al. 2004; Makarenkova, Bansal et al. 2006). Other studies showed that terminally differentiated $CD8^+$ T cells are less effective *in vivo* than T cells in an early stage of differentiation. Early effector $CD8^+$ T cells also express lower levels of pro-apoptotic and higher levels of anti-apoptotic molecules and co-stimulatory CD28 (Gattinoni, Klebanoff et al. 2005). Furthermore, tumor infiltrating lymphocytes (TILs) with the highest CD28 expression revealed the longest persistence after adoptive transfer (Powell, Dudley et al. 2005). Moreover, expression of pro-inflammatory cytokines such as IFN-γ are essential to overcome effects of other immune-inhibitory factors such as TGF-β and IL-10, or receptors expressed in T cells such as CTLA-4 and PD-1 that act to limit anti-tumor responses (Wahl, Wen et al. 2006).

Another therapeutic approach is based on the administration of antibodies to induce antibody dependent cellular cytotoxicity (ADCC) as well as complement activation. Rituximab, an antibody binding CD20, has shown effective anti-cancer activity against B cell lymphoma (Held, Poschel et al. 2006). Recently, bi-specific antibodies for functionally connecting target cells with effector cells play a promising role in the treatment against cancer (Agrawal, Garg et al. 2010; Thakur and Lum 2010).

Despite the existence of functional immune responses, tumors can dodge the immune system evolving so called immune escape mechanisms. Tumor cells carrying mutations which lead to the loss of recognition by T cells e.g. by loss of the human leukocyte antigen

(HLA) I expression after β-2-microglobulin (β2m) mutation or deletion can selectively grow out (Restifo, Esquivel et al. 1993). In theory, NK cells should be able to recognize these cells, as they can act independently of HLA I expression. NK cells express activating receptors such as NKG2D which bind to stress-induced ligands (MICA, MICB) which are up-regulated in a variety of tumors (Gonzalez, Groh et al. 2006). One possible explanation to why NK cells do not destroy the tumor cells could be because NK cells are only rapidly activated in the presence of IL-12, IL-2, and type 1 interferons. Those cytokines are usually released under conditions of microbial infections, but not in a pathogen-free "sterile" environment as found in the majority of malignant tumors. Moreover, various tumor cells express cFLIP, an inhibitor of caspase-8, rendering those tumors resistant to receptor-mediated apoptosis (Rippo, Moretti et al. 2004).

Another obstacle for an effective T-cell mediated tumor response is the absence of co-stimulatory molecules such as CD80, CD86, and CD40 on tumor cells leading to T-cell anergy. Next, immunosuppressive cytokines are often found in the serum of cancer patients. One important cytokine seems to be represented by vascular endothelial growth factor (VEGF) which is produced by most tumors and inhibits DC differentiation and maturation (Gabrilovich, Ishida et al. 1998). One study showed that increased plasma levels of VEGF correlated with a decreased number of mature (m)DC in tumor patients (Almand, Resser et al. 2000). Furthermore, elevated IL-10 levels were detected in cancer patients' sera (Knauf, Ehlers et al. 1995; Fortis, Foppoli et al. 1996). IL-10 is known to be secreted by tumor cells and may protect them from CTL by downregulation of HLA I, HLA II, or ICAM-1 molecules.

Expression of death receptor ligands such as FasL on tumor cells is known to be another immune escape mechanism, leading to apoptosis in Fas^+ (CD95) susceptible target cells like activated T cells (Griffith, Brunner et al. 1995). PD-L1 is quite likely to have an important role in the evasion of tumor cells as its expression on tumors strongly correlates with the survival of patients (Blank and Mackensen 2007). Recently also trogocytosis was observed to play a role in immune evasion of tumors via shaving of target molecules or the transfer of multidrug-resistance (MDR) proteins as described later (Beum, Mack et al. 2008; Rafii, Mirshahi et al. 2008). In summary, inhibitory cytokines and molecules, T_{regs} and the transfer of target molecules play an important role in the limitation of anti-tumor immune responses and the failure of cancer immunotherapy and limit immune responses.

1.3. Cell-to-cell communication via intercellular exchange of proteins, cytokines and chemokines

Transcriptome as well as proteome are continually shared between interacting cells. Lymphocytes form cell-to-cell-connections during their circulation through the body which is critical for the immune response (Rechavi, Goldstein et al. 2009). In addition to interaction between immune cells, individual immune cells interact tightly with target cells in their environment in an antigen-dependent or –independent manner. During these interactions, connections such as nanotubes, gap junctions, "pores" and plasma membrane bridges are formed (Joly and Hudrisier 2003; Bopp, Becker et al. 2007; Davis 2007; Rechavi, Goldstein et al. 2007). These tight contacts facilitate the exchange of surface molecules between interacting cells (Davis 2007; Rechavi, Goldstein et al. 2009). Exosomes, nanotubes, and trogocytosis are the three best described mechanisms for cells to exchange cell components (Figure 2).

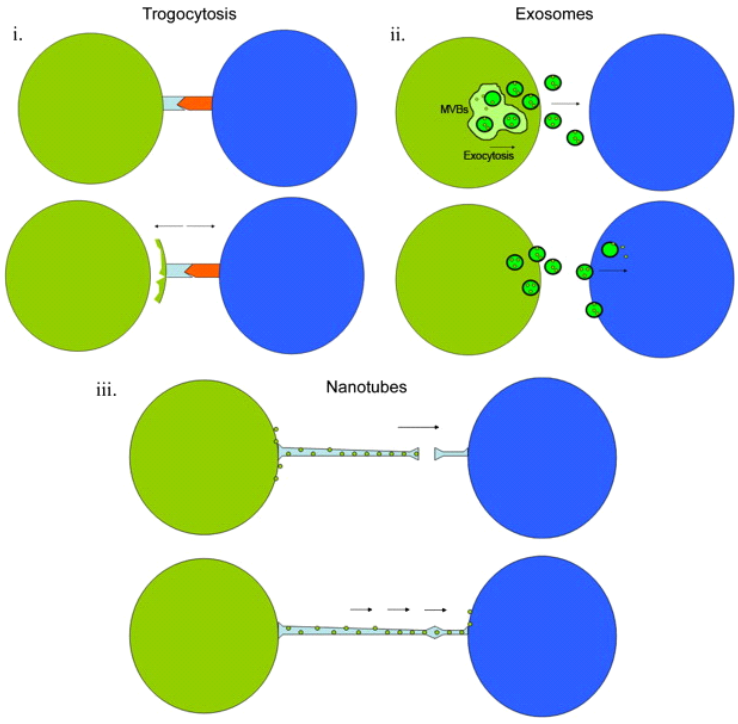


Figure 2: Intercellular exchange via trogocytosis, exosomal protein transport and shuttle of proteins through nanotubes. (Rechavi, Goldstein et al. 2009)

1.3.1. Exosomes and microvesicles

One mechanism for intercellular exchange is the release and subsequent uptake of exosomes. Exosomes are enclosed membrane bodies with a size of 50 to 90 nm. Local disruption is known to result in membrane blebbing, thus interactions between the plasma membrane and the underlying cytoskeleton is important for the release of these membrane vesicles (Hudrisier and Bongrand 2002). This seems to be a quite common mechanism since many immune cells use this strategy of exchange of proteins as well as RNA, especially DC, T cells, and B cells (Raposo, Nijman et al. 1996; Zitvogel, Regnault et al. 1998; Denzer, van Eijk et al. 2000; Fritzsching, Schwer et al. 2002; Andre, Chaput et al. 2004). The release of exosomes can occur spontaneously or can be induced: Epstein-Barr Virus (EBV) transformed B cells, DC, macrophages, as well as tumor cell lines constitutively secret exosomes *in vitro*, while T and B cells release exosomes only upon activation (Peters, Geuze et al. 1989; Raposo, Nijman et al. 1996; Zitvogel, Regnault et al. 1998; Blanchard, Lankar et al. 2002; Bhatnagar, Shinagawa et al. 2007; Arita, Baba et al. 2008). iDC from mice show immediate increases in secretion of exosomes after interaction with antigen-specific T-cell clones, thus proposing a T-cell derived stimulus for induction of exosome secretion (Buschow, Nolte-'t Hoen et al. 2009). Tumor cells revealed an increased release of exosome-like membrane vesicles after radiation and induction of senescence (Yu, Harris et al. 2006; Lehmann, Paine et al. 2008). Some proteins such as p53, diacylglycerol kinase α as well as brefeldin A-inhibited guanine-nucleotide exchange protein 2 (BIG2) are known to play a critical role for the induction of secreted exosomes and exosome-like vesicles (Alonso, Rodriguez et al. 2005; Yu, Harris et al. 2006; Islam, Shen et al. 2007).

Once secreted, exosomes can interact with recipient cells in many different manners. Adhesion via lipids is known as well as ligand-receptor interactions, internalization of the exosomes into endocytic compartments and fusion of the vesicles with the plasma membrane (Thery, Ostrowski et al. 2009). Blocking adhesion molecules by inhibitory antibodies resulted in reduced exosome-capture in co-cultures of DC with exosomes (Morelli, Larregina et al. 2004). In particular ICAM-1 on exosomes has been shown to play a role in the uptake by $CD8^+$ DC expressing LFA-1 and by activated T cells in mice (Segura, Guerin et al. 2007; Nolte-'t Hoen, Buschow et al. 2009). This mechanism may be important for the dissemination of ICAM-1 expressing HIV virions (Fortin, Cantin et al. 1998). Since murine exosomes and microvesicles have been shown to carry exposed

phosphatidylserine (PS) on their surface, PS receptors may be an alternative way for the uptake of exosomes (Miyanishi, Tada et al. 2007).

The regular occurrence of exosomal exchange by numerous cells leads to the question of the potential function of exosomal exchange. A number of studies revealed that exosomes could play a pivotal role in antigen presentation (Tamai, Tanaka et al. 2010). Secreted membrane vesicles can carry both antigenic material and pMHC complexes (Thery, Duban et al. 2002). Exosomes purified from cultured tumor cell lines or from malignant ascites contain tumor antigens and can induce T-cell activation in the presence of recipient's DC (Wolfers, Lozier et al. 2001; Andre, Schartz et al. 2002). Moreover, tumor-derived exosomes were characterized bearing transmembrane proteins such as EGFR2 or endosomal proteins such as Mart-1 and gp100. Macrophages infected by *Mycobaterium sp.* release exosomes containing pathogen-derived antigens (Giri and Schorey 2008); not only bacterial, but also virus antigens are transferred from endothelial cells infected with cytomegalovirus to DC via exosomes (Walker, Maier et al. 2009). Furthermore, exosomes also display preformed pMHC complexes. APC-derived exosomes carry large amounts of MHC class II molecules since they originate from the late endocytic compartments where MHC class II molecules are also located. APC-derived exosomes have been shown to activate human T-cell clones and lines as well as pre-activated murine $CD4^+$ T cells without DC help (Raposo, Nijman et al. 1996; Admyre, Johansson et al. 2006; Muntasell, Berger et al. 2007). However, for activation of murine naïve $CD4^+$ T cells, exosomes need to be captured by recipient DC first (Thery, Duban et al. 2002; Segura, Nicco et al. 2005).

Exosomes can not only modulate immune responses by presenting antigens and MHC molecules but also by further stimulatory or inhibitory molecules. There are reports on CD95 ligand (CD95L, also FasL) or galectin 9 bearing microvesicles derived from tumor cell lines or tumor-bearing patients, that are capable of inducing T-cell apoptosis *in vitro* (Andreola, Rivoltini et al. 2002; Huber, Fais et al. 2005; Klibi, Niki et al. 2009). Tumor-derived exosomes carrying transforming TGF-β have been shown to promote T_{reg} function and impair NK-cell activity (Clayton, Mitchell et al. 2007). Not only APC and tumor cells, but also activated T cells secrete exosomes bearing CD95L which can induce apoptosis of bystander T cells (Monleon, Martinez-Lorenzo et al. 2001). Several studies showed that vesicle secretion by human tumors was described to inhibit anti-tumor immune responses thus proposing exosomes as a mechanism of immune escape (Taylor and Gercel-Taylor 2005; Clayton, Mitchell et al. 2007; Huber, Filipazzi et al. 2008).

Besides immune inhibitory functions also immune-stimulatory properties have been reported for secreted vesicles. Pro-inflammatory exosomes released by macrophages infected by *Mycoplsama sp.* can induce polyclonal stimulation of T and B cells as well as secretion of pro-inflammatory cytokines, if the exosomes contain pathogen-derived pro-inflammatory molecular determinants (Bhatnagar and Schorey 2007; Bhatnagar, Shinagawa et al. 2007; Quah and O'Neill 2007). A quite broad immune response can be triggered by microvesicles released by thrombin-activated platelets: they activate monocytes to secrete pro-inflammatory cytokines and stimulate proliferation, survival, and chemotaxis of other hematopoietic cells (Baj-Krzyworzeka, Majka et al. 2002; Baj-Krzyworzeka, Szatanek et al. 2007). The stimulatory capacity of tumor-derived exosomes observed *in vitro* has opened the field to investigate exosomes as a new immunotherapeutic strategy against cancer. Two phase I clinical trials have been performed in patients with advanced melanoma and non-small cell lung carcinoma using exosomes (Escudier, Dorval et al. 2005; Morse, Garst et al. 2005). Data demonstrate that this novel immunotherapy is safe and can induce at least transient stabilization in 3 out of 6 patients in the melanoma trial and in 3 out of 9 patients in the lung carcinoma trial.

1.3.2. Nanotubes

Intercellular exchange of proteins through membrane tubes between cells provides another possible mechanism of cell-surface protein transfer between cells. Rustom *et al.* were one of the first describing the connections as membranous “tunneling nanotubes” (TNT) between rat pheochromocytoma PC12 cells (Rustom, Saffrich et al. 2004). These membrane bridges were also observed between human immune cells such as T cells, EBV-transformed B cells, primary macrophages, and NK cells, as well as DC (Stinchcombe, Bossi et al. 2001; Onfelt, Nedvetzki et al. 2004; Watkins and Salter 2005). Nanotubes of human immune cells have been described to contain F-actin and to form an average length of about 20 µm and a diameter of 180 – 380 nm regarding nanotubes between T cells (Gurke, Barroso et al. 2008; Sowinski, Jolly et al. 2008). Characterizing the phenotype of nanotubes, three kinds of nanotubes were described as summarized by Gurke *et al*. (Gurke, Barroso et al. 2008).

The one form of nanotubes is presented by membrane bridges forming uninterrupted TNT between cells and enabling the transport of organelles like endocytic vesicles or

mitochondria unidirectionally by an antigen-independent mechanism, as shown for DC and the human acute monocytic leukemia cell line THP-1 (Watkins and Salter 2005). Other nanotubular bridges between cells reveal a junctional border where distinct viral particles can be transported either at the surface of the nanotube by a receptor-dependent mechanism or inside the cellular nanotube by an actin-dependent mechanism (La Boissiere, Izeta et al. 2004; Sowinski, Jolly et al. 2008). This mechanism has been described to play an important role in the spreading of HIV and herpes simplex virus. The third type of cellular nanotubes can extend in direction of the target cell, probably due to chemotaxis, but has no physiologic contact side. This kind of nanotubes may play a role in the delivery of secreted signaling molecules to the target cell and subsequent uptake by a receptor-mediated endocytosis. Onfelt *et al.* demonstrated that endosomes and lysosomes are transported between macrophages by TNT, thus showing the immunological relevance (Onfelt, Nedvetzki et al. 2006). Moreover, the transfer via membrane nanotubes can enable cross presentation of antigen or MHC class II-rich vesicles (Peters, Raposo et al. 1995). Thus, the transport of antigenic signals between myeloid cells demonstrates the immunological communication mediated by nanotubes (Watkins and Salter 2005). Furthermore, nanotubes have been shown to enhance the cytotoxicity of NK cells for lysis of their target cells (Chauveau, Aucher et al.). Despite many *in vitro* data, the *in vivo* relevance of nanotubes remains still unclear.

1.3.3. Trogocytosis

In 1973, there was a first report on a phenomenon of direct transfer of protein molecules to the surface of another cell (Bona, Robineaux et al. 1973). In 2002, this process was termed trogocytosis by Hudrisier and colleagues (Hudrisier and Bongrand 2002). Trogocytosis is derived from the ancient greek word “trogo” what means as much as “to nibble” or “to gnaw” (Joly and Hudrisier 2003). Trogocytosis was then defined as intercellular transfer of membrane patches containing membrane-anchored proteins from a presenting cell to a lymphocyte (Hudrisier and Bongrand 2002). By now, the term “trogocytosis” is used less rigorously and refers to the cell-to-cell contact dependent exchange of plasma membrane and surface molecules between cells. In contrast to exosomes and nanotubes, trogocytosis allows rapid transfer of intact cell-surface proteins between cells in contact with one another (Davis 2007). Trogocytosis has been described for a wide range of immune cells

such as T cells, NK cells, DC, B cells, monocytes and even tumor cells. Nevertheless, the fundamental principles of trogocytosis and its physiological relevance are not yet clear.

Most of the data regarding trogocytosis were generated in mouse models. Of importance, Huang and colleagues reported that murine antigen-specific $CD8^+$ T cells capture pMHC class I complexes from APC after antigen-recognition, rendering them sensitive to peptide-specific lysis by neighboring T cells (fratricide) (Huang, Yang et al. 1999). $CD4^+$ T cells can not only capture pMHC class II complexes from APC with cognate antigen but also co-stimulatory molecules such as CD80 and CD86 (Sabzevari, Kantor et al. 2001; Tsang, Chai et al. 2003; Mostbock, Catalfamo et al. 2007). Capture of membrane components by $CD8^+$ T cells not only occurs *in vitro* but has been also described *in vivo* in mouse models (Riond, Elhmouzi et al. 2007). Furthermore, human $CD4^+$ T cells have been shown to acquire co-stimulatory CD80 after antigen-specific recognition (Tatari-Calderone, Semnani et al. 2002; Game, Rogers et al. 2005). During transendothelial migration human T cells grab adhesion molecules such as CD31, CD49b, CD54, CD61 and CD62E (Brezinschek, Oppenheimer-Marks et al. 1999). Of interest, the transfer of membrane components occurs bidirectional during the interaction between T cells and DC (Busch, Quast et al. 2008). In addition, activated murine B cells transfer antigen-specific BCR to bystander B cells both, *in vitro* and *in vivo*, simultaneously with the transfer of membrane fragments (Quah, Barlow et al. 2008). This process is amplified by the interaction of the BCR with antigen and could play a role in increasing the effective APC pool. Of interest, proteins anchored or recruited to cytoplasmic face of the plasma membrane are more efficiently transferred than those at the luminal side of the cell (Daubeuf, Aucher et al.).

Not only T cells, B cells, and DC but also NK cells can acquire membrane molecules via trogocytosis. Both human T cells and activated NK cells can temporary gain suppressive function after uptake of inhibitory HLA-G molecules from target cells (Caumartin, Favier et al. 2007; LeMaoult, Caumartin et al. 2007). An immediate switch from effector to regulatory function, caused by HLA-G acquisition, has been shown for $CD4^+$ T cells which no longer responded to stimulation as well as for NK cells which transiently stopped both proliferating and being cytotoxic. Monocytes are also able to take up HLA-G by trogocytosis but seemingly without functional consequences (HoWangYin, Alegre et al.). This may be due to the fact that HLA-G disappears quickly from the surface of monocytes and is therefore not able to inhibit on-going T-cell proliferation or cytokine production. In contrast to acquisition of suppressive functions, NK cells also acquire NKG2D activating MHC Class I-related Chain A (MICA) from target tumor cells, thus triggering NK cell

degranulation after NK-NK cell interaction (McCann, Eissmann et al. 2007). In addition, tumor-experienced T cells were shown to acquire MICA supporting NK-cell effector function (Domaica, Fuertes et al. 2009). Trogocytosis not only influences NK cells in their functional outcome, but NK cells can also become sensitive to EBV infection after uptake of CD21 from EBV infected B lymphocytes (Tabiasco, Vercellone et al. 2003).

In a clinical study, isolated stromal cells were observed to confer chemoresistance to epithelial ovarian cancer cells by trogocytosis of multi-drug resistance proteins (Rafii, Mirshahi et al. 2008). Moreover, treatment of CLL patients with rituximab (RTX) leads to the formation of a CD20-RTX complex on target B cells (Beum, Mack et al. 2008). After RTX administration, circulating B cells revealed an essential loss of the target antigen CD20 on their surface. This may be a consequence of trogocytosis, as *in vitro* data show that RTX-CD20 complexes are transferred from B cells to THP-1 monocytes and PBMC via Fcγ receptor-mediated trogocytosis. These two cases show that trogocytosis can have serious implications in cancer immunotherapies.

In contrast, trogocytosis may not only inhibit but also facilitate anti-tumoral immunotherapies. It was described that trogocytosis allows detection and isolation of tumor-specific functional CTL endowed with high functional avidity which in turn may be useful in the clinical application of adoptive immunotherapy (Machlenkin, Uzana et al. 2008). In addition, trogocytosis also facilitates the identification and quantification of pathogen-specific T cells without the need of identifying specific epitopes. These cases suggest that trogocytosis has implications for cancer immunotherapies while the physiological relevance of trogocytosis is still under debate.

In summary, trogocytosis can be a double-edged sword of immunological outcomes: First, trogocytosis can stimulate and increase immune responses by augmenting cytokine secretion, offering co-stimulatory signals or increasing the pool of APC. Second, trogocytosis may induce anergy, turn effector cells into regulatory cells or may even cause fratricide of effector cells. Third, tumors can escape the immune system by acquisition of multi-drug resistance proteins or by loss of the target antigen. Taken together, trogocytosis is a widespread phenomenon in immune cells with diverse immunological and pathological consequences (Ahmed, Munegowda et al. 2008). A better understanding of the transfer process may help to translate this knowledge into therapeutic options in the near future.

1.4. Goals of this study

Trogocytosis is a common way of immune cells to exchange cell components. This phenomenon is well examined for mouse cells but not much data exist about the transfer onto human T cells. This study focuses on trogocytosis of human antigen-specific T cells, especially $CD8^+$ cytotoxic T cells. The goals of this study were to analyze which cell components are transferred from antigen-pulsed DC but also from antigen-presenting tumor cells onto human T cells via trogocytosis. Further investigations aimed for the characterization and for the underlying mechanism of the antigen-specific trogocytosis process. Therefore, factors as kinetics and cell-to-cell contact dependency have been evaluated. By blocking experiments, diverse components playing a role in the T cell interaction with DC and in T-cell activation have been tested for their relevance in the transfer process. Of importance, also functional consequences of trogocytosis was one major focus of these investigations.

2. Material and Methods

2.1. Material

2.1.1. Media, buffer and solutions

AB-Serum, human: PAN Biotech, Aidenbach
Annexin Binding Buffer: Becton Dickinson (BD), Heidelberg
Cytofix fixation buffer: BD, Heidelberg
DC-medium (B´): standard media with 10% FCS
Dimethyl sulfoxide (DMSO): Sigma-Aldrich, Steinheim
Dilution C (DilC) for PKH staining: Sigma-Aldrich, Steinheim
FACSclean: BD, Heidelberg
FACSflow: BD, Heidelberg
FACSrinse: BD, Heidelberg
Freezing medium: 90% fetal calf serum with 10% dimethyl sulfoxide
FACS wash buffer: PBS
FCS (fetal calf serum): PAA, Linz (Austria)
FCS containing medium (B´): standard medium with 10% FCS
Hank´s balanced salt solution (1x): PAA, Linz (Austria)
HSA (human serum albumin): Baxter, Unterschleißheim
Lymphocyte separation reagent (Pancoll): PAN Biotech, Aidenbach
MACS-buffer: PBS + 2mM EDTA (Sigma-Aldrich, Steinheim) + 0.5% HSA
Optimem: Gibco/Invitrogen, Karlsruhe
Paraformaldehyde-solution: Sigma-Adlrich
PBS (Phosphate buffered saline): Gibco/Invitrogen, Karlsruhe
Standard medium (serum free) (M´): 500mL RPMI containing phenol red (PAN Biotech, Aidenbach) with adjuvants: 200mmol/L L-glutamine (Gibco/Invitrogen, Karlsruhe), 2mL vitamins (PAN Biotech, Aidenbach), 5mL non-essential amino acids (PAN Biotech, Aidenbach), 100mmol/L sodium pyruvate (PAN Biotech, Aidenbach), 40U/mL penicillin (Gibco/Invitrogen, Karlsruhe), 40µg/mL streptomycin (Gibco/Invitrogen, Karlsruhe), 50µmol/L β-mercaptoethanol (Gibco/Invitrogen, Karlsruhe)
T-cell medium (A´): standard medium + 10% AB serum

T-cell stimulation medium (A´TF): standard medium + 10% AB serum + TCGF (T cell growth factor; cytokine enriched supernatant described before in (Mackensen, Carcelain et al. 1994))

Tumor-cell medium (B´): standard medium + 10% FCS

2.1.2 HLA-A2-binding peptides

Peptide	Aminoacid-sequence	Solution	Manufacturer
Mart-1 (Melan-A)	ELAGIGILTV	M´ 20% DMSO	Calbiochem, Läufelfingen (Schweiz)
gp100	ITDQVPFSV	M´ 5% DMSO	Bachem, Heidelberg

2.1.3 Peptide-MHC-tetramers

Tetramer	Conjuagation	Manufacturer
Melan-A	PE	Beckman Coulter, Krefeld
gp100	PE	Beckman Coulter, Krefeld

2.1.4. Antibodies

The following monoclonal antibodies conjugated with fluorochromes were used for detection of surface antigens:

Specificity	Isotype	Clone	Source	Conjugation	Manufacturer
CD3	IgG1	SK7	mouse	PerCP	BD, Heidelberg
CD3	IgG1	SK7	mouse	PE-Cy7	BD, Heidelberg
CD4	IgG1	SK3	mouse	PerCP	BD, Heidelberg
CD4	IgG1	SK3	mouse	PE-Cy7	BD, Heidelberg
CD8	IgG1	SK1	mouse	PerCP	BD, Heidelberg

Specificity	Isotype	Clone	Source	Conjugation	Manufacturer
CD8	IgG1	RPA-T8	mouse	APC	BD, Heidelberg
CD8	IgG1	SK1	mouse	APC-H7	BD, Heidelberg
CD14	IgG2b	MoP9	mouse	PerCP	BD, Heidelberg
CD16	IgG1	3G8	mouse	PE	BD, Heidelberg
CD19	IgG1	SJ25-C1	mouse	APC	Caltag, Buckingham. UK
CD25	IgG1	2A3	mouse	PE	BD, Heidelberg
CD25	IgG1	2A3	mouse	PE-Cy7	BD, Heidelberg
CD56	IgG2b	NACM1 6.2	mouse	FITC	BD, Heidelberg
CD69	IgG2a	CH/4	mouse	PE	Caltag, Buckingham. UK
CD80	IgM	BB1	mouse	FITC	BD, Heidelberg
CD80	IgG1	L307.4	mouse	PE	BD, Heidelberg
CD83	IgG1k	HB15e	mouse	APC	BD, Heidelberg
CD86	IgG1	2331	mouse	FITC	BD, Heidelberg
CD107a	IgG1	H4A3	mouse	PE	eBioscience, Frankfurt
CD137	IgG1	4B4-1	mouse	APC	BD,Heidelberg
CD209	IgG2b	120507	mouse	APC	R&D Systems, Wiesbaden-Nordenstadt
HLA-ABC	IgG1	G46-2.6	mouse	PE	BD,Heidelberg
HLA-DR	IgG2a	L243	mouse	PerCP	BD, Heidelberg
MCSP	IgG1	7.1	mouse	PE	Beckman Coulter
PD-1	IgG1k	MIH4	mouse	APC	eBioscience, Frankfurt
PDL-1 (B7-H1)	IgG1	MIH1	mouse	PE	eBioscience, Frankfurt
PDL-1 (B7-H1)	IgG1	MIH1	mouse	Biotin	eBioscience, Frankfurt
PD-L2	IgG1	MIH18	mouse	PE	eBioscience

Specificity	Isotype	Clone	Source	Conjugation	Manufacturer
TCRαβ	IgG2b	BW242/ 412	mouse	PE	Miltenyi, Bergisch-Gladbach

Isotype control staining was carried out with the following immunoglobulins conjugated with fluorochromes:

Isotype	Clone	Source	Conjugation	Manufacturer
IgG1	X40	mouse	FITC	BD, Heidelberg
IgG1	X40	mouse	PE	BD, Heidelberg
IgG1	X40	mouse	APC	BD, Heidelberg
IgG1	MCPC-21	mouse	PE-Cy7	BD, Heidelberg
IgG2a	G155-178	mouse	FITC	BD, Heidelberg
IgG2a	X39	mouse	PE	BD, Heidelberg
IgG2b	MCP-11	mouse	PE	BD, Heidelberg
IgG2b	MOPC-195	mouse	APC	Caltag, Buckingham. UK

Unconjugated antibodies:

Specificity	Isotype	Clone	Source	Manufacturer
CD8	IgG2a	UCHT-4	mouse	Leinco
CD80	IgG1	37711	mouse	R&D Systems, Wiesbaden-Nordenstadt
CD86	IgG1	37301	mouse	R&D Systems, Wiesbaden-Nordenstadt
CD209	IgG2b	120507	mouse	R&D Systems, Wiesbaden-Nordenstadt
ICAM-1	IgG1	BBIG-I1	mouse	R&D Systems, Wiesbaden-Nordenstadt
ICAM-2	IgG	-	goat	R&D Systems, Wiesbaden-Nordenstadt
PD-L1	IgG1	MIH1	mouse	eBioscience, Frankfurt

2.1.5. Chemical Reagents and Dyes

Reagent	Applied concentration	Manufacturer
7AAD	5µL / stain	BD, Heidelberg
AnnexinV- FITC	5µL / stain	Caltag, Buckingham. UK
Bafilomycin	100nM	Sigma-Aldrich, Steinheim
Blebbistatin	50µM	Sigma-Aldrich, Steinheim
Concanamycin A	0.1µg/mL	Sigma-Aldrich, Steinheim
eFluor 710 Streptavidin	30µL / stain	eBioscience, Frankfurt
Golgi-Stop (Monensin)	0.7µL / mL	BD, Heidelberg
Lck inhibitor II	100µM	Calbiochem, Darmstadt
PHA-L	1µg/mL	Sigma-Aldrich, Steinheim
PKH26	1.5µL / 400µL	Sigma-Aldrich, Steinheim
PKH67	1.5µL / 400µL	Sigma-Aldrich, Steinheim
APC Streptavidin	10µL / stain	BD, Heidelberg
Sulfo-NHS-LC-Biotin	5µL / mL	Pierce, Rockford, USA

2.1.6. Beads

Name	Manufacturer
$CD4^+$ T cell isolation Kit	Miltenyi Biotech, Bergisch-Gladbach
$CD8^+$ T cell isolation Kit	Miltenyi Biotech, Bergisch-Gladbach
DN T cell isolation Kit	Miltenyi Biotech, Bergisch-Gladbach
NK cell isolation Kit	Miltenyi Biotech, Bergisch-Gladbach
B cell isolation Kit	Miltenyi Biotech, Bergisch-Gladbach
Anti-PE MicroBeads	Miltenyi Biotech, Bergisch-Gladbach
Anti-CD4 Microbeads	Miltenyi Biotech, Bergisch-Gladbach
Anti-CD8 Microbeads	Miltenyi Biotech, Bergisch-Gladbach
Dynabeads® CD3/CD28 T Cell Expander	Dynal, Invitrogen,

2.1.7. Cell culture flasks, tubes and plates

Name	Manufacturer
Filter Cap Cell Culture Flask, 50mL	Greiner, Frickenhausen
Filter Cap Cell Culture Flask, 250mL	Greiner, Frickenhausen
Filter Cap Cell Culture Flask, 650mL	Greiner, Frickenhausen
Polystyrene Cell Culture Tubes, 16mL	BD, Heidelberg
96-well U- bottom plates	BD, Heidelberg
96-well flat bottom plates	Corning, USA
24-well plates	Corning, USA
24-well tissue transwell culture plate	Corning, USA
culture dishes	Falcon/BD, Franklin Lakes (USA)
15 mL centrifugation tubes	Falcon/BD, Franklin Lakes (USA)
50 mL centrifugation tubes	Falcon/BD, Franklin Lakes (USA)
FACS tubes unsterile	Sarstedt, Nürnbrecht
Cryogenic storage tubes	Greiner, Frickenhausen
1,5 mL cups	Sarstedt, Nürnbrecht
2 mL pipettes	Corning, USA
5 mL pipettes	Corning, USA
10 mL pipettes	Corning, USA
25 mL pipettes	Corning, USA
50 mL pipettes	Corning, USA

2.1.8. Cytokines

Cytokines	Manufacturer
GM-CSF	Bayer, Leverkusen
IFN-γ	PeproTech, Hamburg
IL-1β	Promokine, Heidelberg
IL-4	Promokine, Heidelberg
IL-6	Promokine, Heidelberg
PGE_2	Enzo Life Sciences, Lörrach
TGF-β	Promokine, Heidelberg

Cytokines	Manufacturer
TNF	Promokine, Heidelberg

2.1.9. Cell lines

Cell line	Properties	Reference/Source
Mel1300	HLA-A2$^+$, Melan A$^+$, gp100$^+$	Mackensen A, Carcelain G *et al.*, 1994 J Clin Invest
Na8	HLA-A2$^+$, TAA$^-$	Feder-Mengus C, Ghosh S *et al.*, 2007, Br J Cancer
Laz388	EBV transformed lymphoid B cell line	Piper AA, Tattersall MH, Fox RM, 1980 Biochim Biophys Acta
T2	Hybrid cell line of T- and B-LCL, HLA-A2$^+$, TAP deficient	Salter RD, Howell DN, Cresswell P, 1985 Immunogenetics

2.1.10. Software

GraphPad Prism 5.02 (GraphPad, La Jolla, USA) was used for generation of graphs and calculation of statistics. FACS Data was acquired by a FACSCanto II using BD FACSDiva Software (BD, Heidelberg) for data acquisition. FlowJo software PC version 7.6 (Celeza, Olten, Switzerland) was used for analysis of FACS data. Data of confocal microscopy was processed by the programm ZEN Light Edition (Zeiss, Jena, Germany)

2.2. Methods

2.2.1. Determination of cell numbers using trypan blue staining

The cell number was determined in a solution of 0.4% of trypane blue (Gibco) diluted in distilled water. This life dead staining allows counting of living cells. Only dead cells with a damaged membrane-integrity absorb the blue dye. For calculating the cell number per mL, the following formula was used:

$$\frac{\text{Number of living cells}}{\text{Number of counted quadrants}} \times \text{dilution} \times 10000 = \text{cell number/mL}$$

2.2.2. Cryoconservation of cells

For cryoconservation the cell number was determined and washed once with PBS. Cells were adjusted up to $100x10^6$ per mL in freeze media. The cell suspension was transferred to a pre-cooled cryogenic storage tube and put into a 4°C cooled cryo freezing container (Nalgene Nunc, International Hereford, U.K.). The freezing container was stored at -80°C over night, and then the samples were put into liquid nitrogen for long time storage.

2.2.3. Thawing of cells

A defined amount of medium was added to deep-frozen cells by and by until cells were completely thawed. Cells were subsequently centrifuged and resuspended in fresh medium.

2.2.4. Density gradient centrifugation

The appropriate cells were taken from healthy donors by leukapheresis and diluted 1:1 with PBS. Pancoll was coated with this cell fraction and centrifuged (900g, 4°C, 20'). This density gradient centrifugation leads to a separation of a heavy fraction (erythrocytes), an interphase (mononuclear cells, MNC) and the supernatant containing thrombocytes. The interphase was harvested and washed twice with PBS (300g, 10'). Afterwards, the cell number was adjusted and MNC fraction was frozen or utilized for monocyte extraction.

2.2.5. Isolation of monocytes by countercurrent elutriation

One part of MNC obtained from density gradient centrifugation was adopted for countercurrent elutriation. Therefore, MNC were centrifuged using J-6M/E Elutriator, Beckman Coulter, whereas cells were separated according to size and density. First the system was sterilized using H_2O_2, washed with PBS and the pump was calibrated with Hanks´ buffer. To prevent cells from damage, 6% of autologous plasma was added to Hanks´ buffer for elutriation. The separation of particular cell fractions is carried out by continuously rising flow rate and constant speed of the centrifuge. According to the separation, cells were collected in fractions and the final fraction III (monocytes) was centrifuged (1500rpm, 10', 4°C) and resuspended in fresh media.

2.2.6. Cultivation of cell lines

Adherent cell lines: Tumor cell lines were cultured as monolayers in media containing FCS (B´) in 25cm^2 tissue culture flasks at 37°C, 5% CO_2. To detach the cells from the bottom of the flasks, adherent cells were incubated with trypsin-EDTA (Gibco/Invitrogen, Karlsruhe) and washed with PBS. Afterwards tumor cells were sub-cultured twice a week by diluting 1:10 in fresh medium.

Suspension cell lines: In contrast, Laz and T2 were cultivated in B´ medium in 25cm^2 tissue culture flasks at 37°C, 5% CO_2. B cells were harvested followed by washing with PBS and were sub-cultured twice a week by diluting 1:10 in fresh medium.

2.2.7. Generation of DC from monocytes

Monocytes obtained from elutriation were adjusted to 1x10^6 per mL B´ medium and were differentiated by addition of IL-4 (25U/mL), GM-CSF (800U/mL) and 5ng/mL TGF-β on day 1. On day 6, proinflammatory cytokines IL-1β (10ng/ml), IL-6 (1000U/ml), TNF (10ng/mL), and prostaglandine E2 (1µg/mL) as well as further IL-4 (12.5U/mL) and GM-CSF (400U/mL) were added for another 48h to obtain mDC.

2.2.8. Antigen-pulsing of antigen-presenting cells

mDC (or other kind of APC) were washed in serum free medium M´ and adjusted to a densitiy of 3-5x10^6 cells/mL M´. 30µg/mL peptide and 10µg/mL β–2microglobulin (β-2m) (Scipac, Sittingbourne, UK) stabilizing the MHC-complexes was added to the cell suspension and was incubated at 37°C, 5% CO_2. Tubes were shaken from time to time to avoid adhesion of DC at the plastic-tube. After 2h, APC were washed two times with serum supplemented medium to remove excessive peptide.

2.2.9. Magnetic cell separation

The magnetic cell sorting was carried out using diverse isolation kits by Miltenyi for the isolation of diverse immune cell populations as listed in the table above. MNC were labeled in a two-step-staining procedure. In the first step, Hapten-antibodies (monoclonal mouse anti-X antibody) which are diluted in MACS-buffer were used whereas in the second step anit-Hapten-antibodies (monoclonal AB beads anti-mouse-isotype) were added secondary. As far as applied beads only have a diameter of 50nm (Microbeads), the bead-labeled cell suspension has to pass special columns which are densely packed with iron-particles. Different column sizes are provided by the manufacturer depending on the number of cells and kind of isolation kit (MS-, LS-, LD-columns) (Miltenyi, Bergisch Gladbach). Columns were washed with MACS-buffer before usage. $CD4^+$, $CD8^+$, NK and B cells were isolated via a so-called negative selection meaning that cells of the unwanted phenotype were depleted via the labeling with the Microbeads. Thus, unwanted cells adhered via magnetic forces in the column, whereas cells of the wanted phenotype passed through and were collected afterwards. In contrast, the isolation of DN T cells consisted of a negative selection followed by two positive selection steps via binding of the TCRαβ. In unpurified DN cell cultures, both $CD4^+$ and $CD8^+$ T cells were depleted via Microbeads. Exact information about the particular antibodies against targets on unwanted cell populations for the negative selection is provided in the manufacturer's protocol as well as amounts of used buffers, antibodies, and incubation times. Purity of isolated cell population was checked by flow cytometry.

2.2.10. Cultivation of T cells

Generation of antigen-specific T cells

$CD8^+$ T lymphocytes were enriched by magnetic negative selection by Miltenyi separation system. Enriched T cell population showed a purity of more than 90%. Autologous, peptide-pulsed mDC served as stimulator cells. For antigen-specific expansion, $1x10^5$ $CD8^+$ cells/well were cultured with $2x10^4$ peptide-pulsed mDC/well in 96-well-U-bottom-plates in 225µL/well T-cell stimulation medium which was exchanged every third or fourth day. Restimulation cycles were performed weekly under same conditions. Purity and antigen-specificity of T-cell cultures were analyzed via flow cytometry.

Generation of allo-specific $CD4^+$, $CD8^+$, and DN T-cell cultures

Allogeneic T-cell stimulation was performed in a mixed lymphocyte reaction (MLR). Thereby, differences in MHC restriction between T cells and mDC mediate the stimulatory effect since T cells recognize non-self MHC molecules as target molecules. $CD4^+$, $CD8^+$ and DN T cells were separated out of MNC via magnetic cell separation with the Miltenyi system. T cells were stimulated with mDC from a completely MHC mismatch donor. $7.5x10^4$ $CD4^+$, $CD8^+$, or DN T cells/well were cultured with $2.5x10^4$ mDC/well in 225µL T-cell stimulation medium/well in a 96-well U-bottom plate. Medium was exchanged every third or fourth day. Restimulation cycles were performed weekly under same conditions and purity was checked via flow cytometry.

Cloning of $CD8^+$ T cells

To clone $CD8^+$ T cells, $4x10^6$ allogeneic MNC (irradiated with 30Gy) and $1x10^6$ Laz388 cells (irradiated with 60Gy) were disseminated per 96-well-plate. To assure close cell-to-cell contact, cells were cultured in 150µL T-cell stimulation medium supplemented with 0.225µL PHA-L (1mg/mL) in a V-bottom plate. Cells were stained with antibodies and subsequently sorted resulting in one to three cells per well under sterile conditions. Every fourth day, old medium was exchanged by fresh T-cell stimulation medium. Outgrowing clones were selected and disseminated in 96-well-U-bottom plates to be stimulated every second week with irradiated $6x10^5$ allogeneic MNC/well and $1.5x10^5$ Laz388/well in T-cell stimulation medium supplemented by PHA-L. Medium was exchanged every third or fourth day. Purity and specificity were checked by flow cytometry.

Polyclonal stimulation of $CD4^+$, $CD8^+$, and DN T-cells

For polyclonal expansion of $CD4^+$, $CD8^+$ and DN T-cell populations, cells were cultured with T-cell expansion Dynabeads conjugated with anti-CD3 and anti-CD28 antibodies. Before stimulation, Dynabeads were washed in PBS supplemented with 0.1% BSA and subsequently added in a ratio of 1:1. Every third or fourth day, cells were transferred into fresh medium. Restimulation cycles were performed weekly. Therefore, beads were removed by a magnet, cells were counted and subsequently replaced in fresh medium in U-bottom plates with new anti-CD3/CD28 Beads.

2.2.11. Membrane labeling

PKH is a lipophilic dye incorporating in the membrane bilayer without leaking or toxic side effects. 3-5x10^6 cells were washed with standard medium M´ in polystyrene culture tubes. Cells were resuspended in 200µL DilC and 200µL of staining solution (200µL DilC + 1.5µL PKH dye) was added. Cells were incubated shaking at room temperature (RT) for 20'. After 2h, human serum was added to stop the staining procedure and cells were washed two more times in T-cell medium.

2.2.12. Trogocytosis experiments

For trogocytosis experiments, antigen-presenting cells (mDC, T2 or Mel1300) were pulsed with 10µg/mL relevant or irrelevant peptide and 10µg/mL β-2 microglobulin at 37°C in serum-free standard medium. After 2h, target cells were stained with lipophilic membrane dye PKH67 as described before for 20' at RT and washed three times with complete medium plus 10% human AB serum. Harvested T cell clones and target cells were placed in U-bottom well-plates (E:T ratio unless otherwise noted 3:1 = 7.5x10^5 T cells plus 2.5x10^5 target cells in 200µL standard medium plus 10% human AB serum), incubated at 37°C and harvested at indicated times. Harvested co-cultures were washed with PBS and resuspended before staining with monoclonal antibodies and analyzed by flow cytometry. Doublets were excluded in FACS analysis via FSC-W gating.

2.2.13. Transwell assay

In a transwell-system, cells are separated via a membrane with pores to allow soluble factors of a certain size to pass trough. This system is dedicated to analyze whether a process is cell-to-cell contact dependent or can also be mediated by soluble factors. In this study, antigen-specific $CD8^+$ T cells to be analyzed were added into the bottom chambers (3.0×10^5 per Well) of a 24-well tissue transwell culture plate. Into the insert well, PKH67 labeled antigen-pulsed mDC (1.0×10^5 per insert) with or without antigen-specific PKH26 labeled T cells (2.0×10^5 per insert) were placed as donor cells. Insert and bottom wells were separated by a membrane containing 5µm-pores which allow soluble factors and membrane vesicles but neither DC nor T cells to pass through.

2.2.14. Experiments for inhibition of trogocytosis and apoptosis

Preparation of DC lysates: mDC were antigen pulsed and membrane dye labeled as described before. Lysates were generated by repeated freeze-and-thaw cycles of a defined number of mDC.

Blebbistatin (Inhibitor of Myosin IIa): T cells were pretreated with 50µM Blebbistatin for 30 minutes at 37°C in standard medium. Afterwards, Blebbistatin was washed out and cells were subsequently used in the trogocytosis assay.

Concanamycin A (CMA) / Bafilomycin (Inhibitors of vATPases): T cells or mDC were pretreated with 0.1µg/mL CMA or with 100nM Bafilomycin overnight in T-cell medium. vATPase inhibitor was washed out carefully before cells were put into trogocytosis assay as described before.

Lck-inhibitor: T cells were pretreated with 100µM inhibitor for 1h at 37°C in standard medium. Lck-inhibitor was washed out subsequently before cells were used in the trogocytosis assay.

Fixation of APC: PKH67 labeled, Mart-1 pulsed mDC were incubated for 30’ in 0.05% Glutaraldehyde-solution (Sigma-Aldrich) and were carefully washed with T-cell medium two times before they were used in the trogocytosis assay.

Blocking antibodies: 10µg/mL high purified azide-free blocking antibodies were added to target cells 1h before co-culture in T-cell medium. Antibodies were not washed out but remained in the co-culture during trogocytosis assay.

2.2.15. Flow cytometry and cell sorting

Flow cytometry is a method for detection of cells stained with fluorochrome labeled antibodies to perform phenotypic cell analysis but also cytokines, proliferation, or apoptosis can be detected by fluorescence in flow cytometry. This technique allows counting and examining cells by suspending them in a stream of fluid and passing them by laser beam. Three lasers were available in FASCanto II (BD, Heidelberg) to initiate fluorochromes for detecting the emission in 8 channels. This technique permits the detection of light scattering for each cell; the forward scatter provides information about the size of the cell whereas the side scatter informs about granularity. For the experiments FITC (Fluorescein isothiocyanate), PE (Phycoerythrin), PerCP (Perdinin chlorophyll protein complex), APC (Allophycocyanin), PE-Cy7 and APC-Cy7 were applied. This technique can also be used for fluorescence activated cell-sorting (FACS), thereby the wanted cells are sorted in terms of bound fluorochrome labeled antibodies identifying the wanted phenotype. Cell sorting of $CD3^+CD8^+$ clonal T cells for subsequent analysis after trogocytosis process was performed using a MoFlo cell sorter (Cytomation, Freiburg, Germany).

2.2.16. FACS staining

Direct antibody staining

Surface antigens and intracellular proteins can be directly stained by fluoreochome-linked antibodies. Cells were harvested and transferred to FACS tubes (Sarstedt, Nürnbrecht) for surface staining. After washing twice with PBS, the indicated amount of monoclonal antibody was added to the cells and incubated at 4°C in the dark for 10 minutes. After a further washing step cells were resuspended in 200µL of PBS or fix solution. Analysis was carried out by FACSCanto II flow cytometer (BD, Heidelberg). The software Flowjo (Tree Star, Ashland, USA) was applied for evaluation of the data. Calculated mean fluorescence intensities (MFI) of antibody staining were corrected by subtraction of MFI of isotype background fluorescence.

Annexin/7-AAD staining

Early apoptotic cells expose phosphatidylserine at their surface which can be bound by AnnexinV. 7-Aminoactinomycin (7-AAD) intercalates in double-stranded DNA, thus it can be used for the identification of late apoptotic cells because it only stains cells with

disrupted plasma membrane. After surface antibody-staining, cells were incubated with 5µL Annexin V FITC and 5µL 7-AAD in 150µL Annexin binding buffer at RT for 15' in the dark followed by immediate FACS analysis.

pMHC-tetramer staining

pMHC-tetramer staining is a technique for the identification of specific TCR, thereby antigen-specific T cells can be identified via flow cytometry. For the detection of antigen-specific T cells, cells were washed by PBS and were subsequently incubated with pMHC-tetramers at RT for 30 minutes in the dark. Afterwards, surface antibodies were added without washing out the tretramers. After incubation time, cells were washed with PBS and analyzed by flow cytometry.

2.2.17. Confocal immunefluorescence microscopy

DC were labeled with membrane dye PKH67 as described above and T cells were labeled analogous with membrane dye PKH26. After co-culture for 2 hours at 37°C, cells were harvested, washed two times and incubated with α-PD-L1-Biotin or corresponding IgG-Biotin antibody for 15' at 4°C. Cells were washed two times before incubation with Streptavidin-eFluor710 nanocrystals for 20' at 4°C followed by four washing steps with PBS. Cells were then fixed for 15' at RT with 3% paraformaldehyde. Subsequently, cells were pipetted on adherent SuperFrost Plus microscope slides (Langenbrinck, Emmendingen, Germany). After settling and cell adherence, cells were washed once with PBS. Almost dried slides were mounted with ProLong Gold Antifade Reagent (Invitrogen, Darmstadt, Germany). Slides were analyzed utilizing a Zeiss laser scanning microscope (Zeiss LSM 700, Jena, Germany) with the respective ZEN software (Vers. 2009). Images were captured with a 63x oil objective (NA 1.4) and 12-bit sequential scans. A 5 times zoom was used for high resolution single cell images, this is within the reasonable range of Nyquist Theorem and therefore avoiding loss of resolution by undersampling. Pictures were additionally post-preocessed by linear unmixing.

2.2.18. Transfection of mDC with GFP-RNA

mDC were harvested on day 7 of differentiation and washed with RPMI, then washed with Optimem at RT and resuspended in 100µL Optimem. 5µg of GFP-RNA was put in a

cuvette (Peqlab, Erlangen) and 100µL of the cell suspension was added. The GFP-RNA was provided by Dr. Niels Schaft and Dr. Jan Dörrie, Dept. of Dermatology, University of Erlangen-Nuremberg. Cells were electroporated in a 4mm gap cuvette using a square wave pulse, at 500V for 1ms with a Biorad Gene Pulser. Immediately after transfection, cells were transferred to 10mL DC medium containing IL-4, GM-CSF, IL-1β, IL-6, TNF and PGE_2. After 4h, cells were harvested and incubated for 24h before the use in the trogocytosis assay.

3. Results

3.1. Characterization of T-cell clones specific for Mart-1 peptide and for gp100 peptide

For following experiments analyzing trogocytosis, a human Mart-1-specific and a gp100-specific CD8$^+$ T-cell clone was used in trogocytosis experiments. The specificity of the T-cell clones was regularly checked by tetramer-technology as shown in Figure 3. T-cell clones only with a purity of more than 85% antigen-specific T cells were used in experiments.

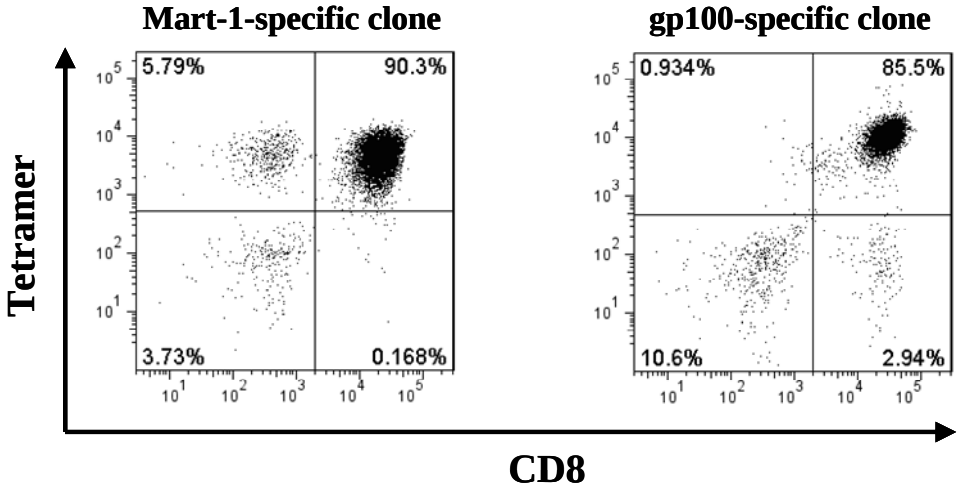


Figure 3: Specificity of human T-cell clones. Mart-1- and gp100-specific T cell clones were stained by TCR-specific tetramers to ensure specific antigen-recognition. Dot plots are gated on vital singlet T cells. Shown is one representative of more than five independent experiments.

3.2. Capture of cell components from mDC by T cells

Trogocytosis is described as intercellular transfer of membrane fragments from presenting cell to lymphocyte (Joly and Hudrisier 2003). To investigate whether membrane components are transferred, acquisition of lipids and proteins was analyzed after antigen-specific recognition using a Mart-1-specific human T-cell clone. After co-culture of Mart-1-specific CD8$^+$ T cells with Mart-1 peptide-pulsed mDC that were either labeled with lipophilic membrane dye PKH26 or with biotinylated proteins for detection by NHS-

Streptavidin, lipid components (PKH26) and proteins (NHS-Streptavidin) were both transferred from mDC onto T cells in an antigen-specific manner (see Figure 4).

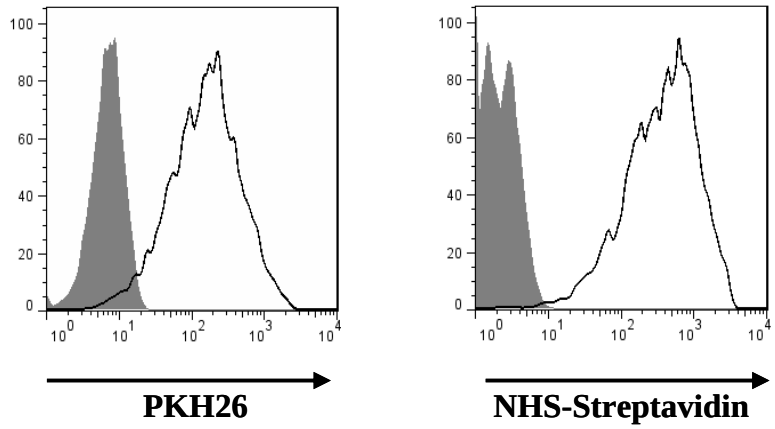
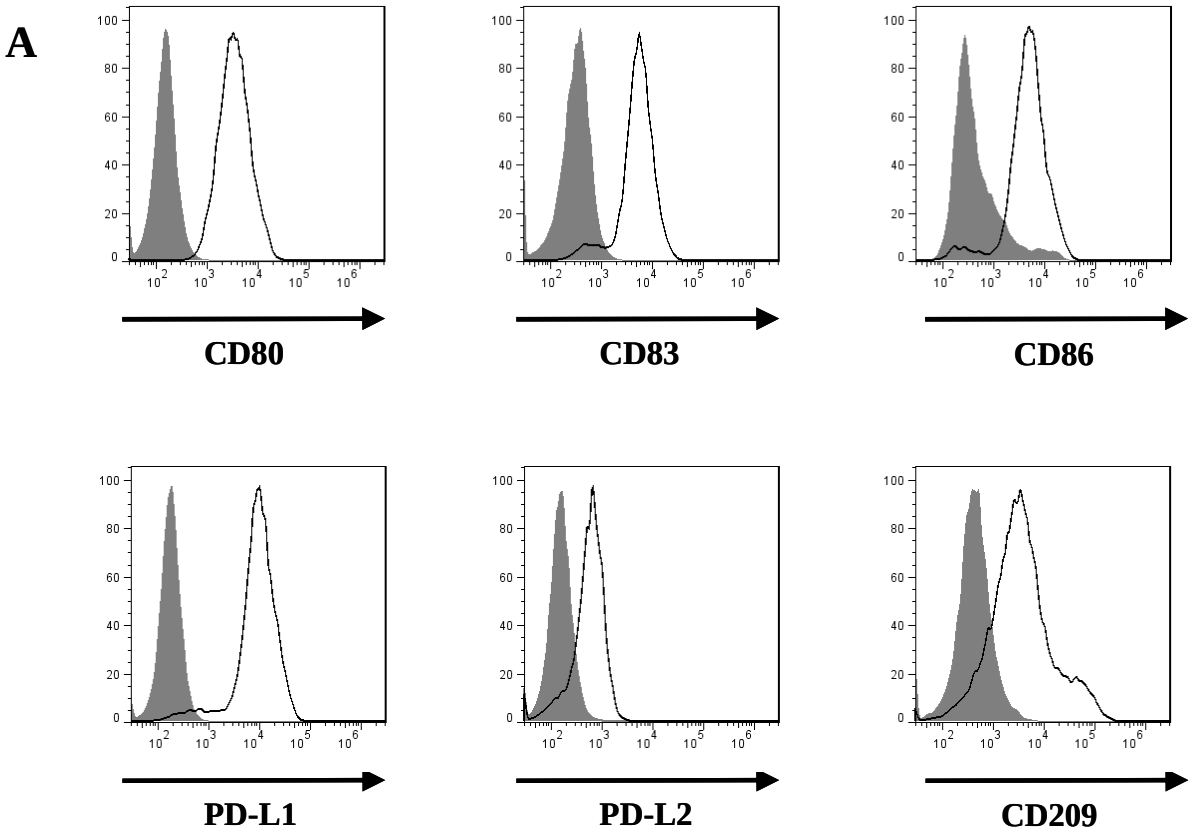


Figure 4: Antigen-specific acquisition of both, lipids and proteins of the plasma membrane from mDC by T cells. Mart-1-specific T cells were co-cultured for 2h with Mart-1 peptide-pulsed, PKH26-labeled or biotinylated mDC. Uptake of either lipid- or protein-labeled membrane fragments was analyzed on T cells by flow cytometry via detection of either PKH26 or NHS-Streptavidin (black solid line). Negative controls are shown as filled histograms. Histograms are gated on $CD8^+$ lymphocytes. Shown is one representative of three independent experiments.

Next, it was analyzed which surface molecules are transferred from mDC onto antigen-specific $CD8^+$ T cells. Antigens that are known to be highly expressed on mDC such as CD209, PD-L1, PD-L2, CD80, CD83, and CD86 were analyzed on monocyte-derived mDC by flow cytometry (see Figure 5A). Of interest, after co-culture of Mart-1-pulsed mDC with Mart-1-specific T cells, PD-L1, CD209, and CD83, but not CD80, CD86, and PD-L2 were transferred onto T cells (see Figure 5B). As control, T cells were analyzed for those molecules before co-culture. Of note, the antigen-specific T-cell clone showed no CD83, CD209, PD-L1, and PD-L2 expression but a low constitutive expression of CD80 and CD86 was documented.


A
CD80
CD83
CD86
PD-L1
PD-L2
CD209

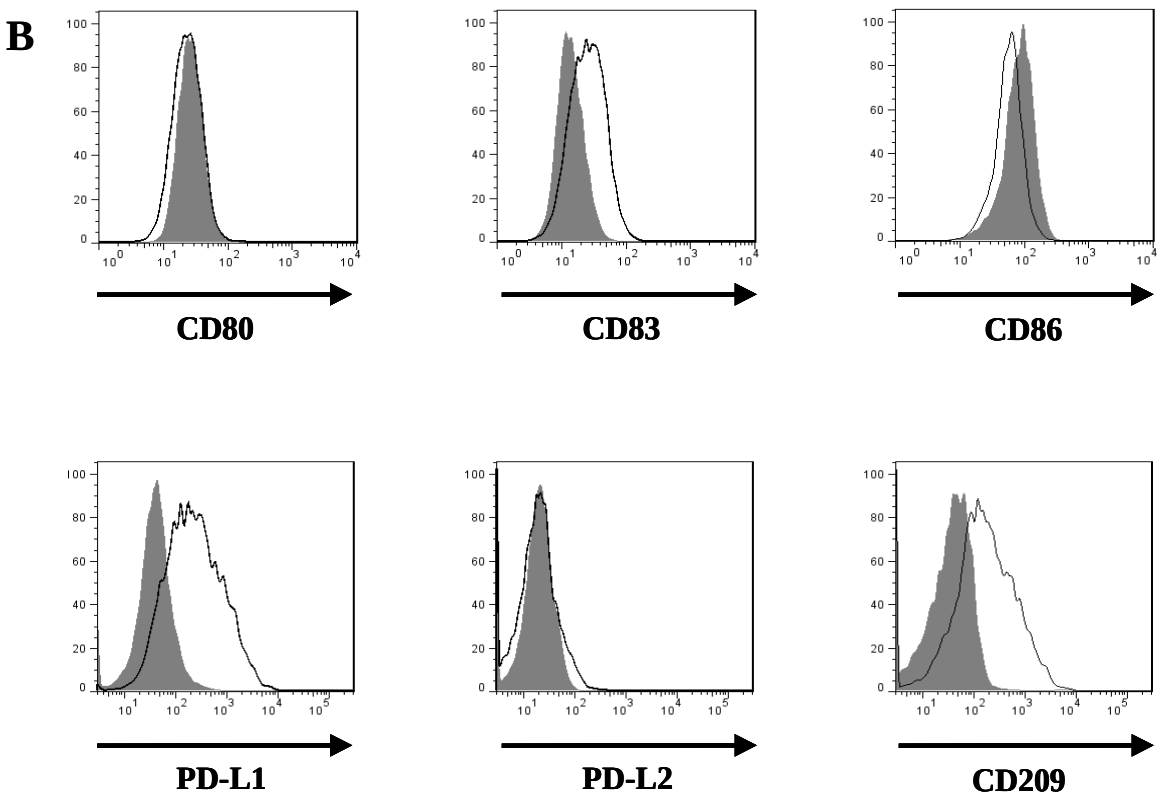

B
CD80
CD83
CD86
PD-L1
PD-L2
CD209

Figure 5: Transfer of DC-specific surface molecules from mDC onto antigen-specific T cells. (A) Expression of surface molecules on mDC. mDC were analyzed by flow cytometry for expression of CD80, CD83, CD86, PD-L1, PD-L2, and CD209 (black solid line). Isotype controls are shown as filled histograms. (B) Mart-1specific $CD8^+$ T-cell clone was screened for uptake of listed molecules after co-culture with Mart-1-pulsed mDC. Transfer was analyzed after 2h of co-culture (black solid line) compared to constitutive surface expression on T cells without contact to mDC (filled histograms). Histograms are gated on $CD8^+$ lymphocytes. Shown is one representative of three (PD-L1, CD209, CD83) or two (PD-L2, CD80, CD86) independent experiments.

Next, the acquisition of green fluorescent protein (GFP) was analyzed as a model protein for cytosolic molecules. mDC were transfected with GFP-encoding RNA, which was transcribed and translated into protein in the cytosol. Of interest, GFP was also transferred from mDC onto $CD8^+$ T cells suggesting that not only surface molecules but also cytosolic components can be acquired by T cells after antigen-specific recognition (see Figure 6). A control vector (mock) served as negative control.

Together, lipids, and proteins of plasma membrane, surface molecules as PD-L1 and CD209 as well as cytosolic components are acquired from human mDC by T cells in an antigen-dependent manner.

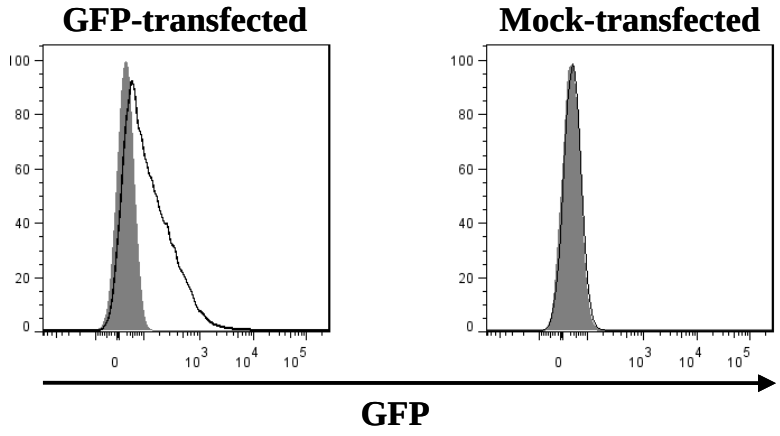


Figure 6: Antigen-specific acquisition of cytosolic GFP from mDC by T cells. mDC were transfected with RNA encoding GFP or with a control vector (mock). Transfer onto $CD8^+$ T cells was analyzed after 2h of co-culture (black solid line). Negative controls are shown as filled histograms. Histograms are gated on $CD8^+$ lymphocytes. Shown is one of four independent experiments.

3.3. Acquisition of membrane fragments and surface molecules by different T-cell subsets

Besides $CD8^+$ T cells, other subpopulations of T cells as $CD4^+$ and DN T cells may also be able to acquire membrane fragments and surface molecules such as CD209 via trogocytosis. To answer this question, purified $CD4^+$, $CD8^+$, and DN T-cell subpopulations were expanded *in vitro* by repetitive stimulation with allogeneic mDC, co-cultured with PKH26-labeled allogeneic mDC and were then analyzed by flow cytometry. As shown in Figure 7, T-cell subpopulations had a similar capacity to take up membrane patches and surface molecules from APC.

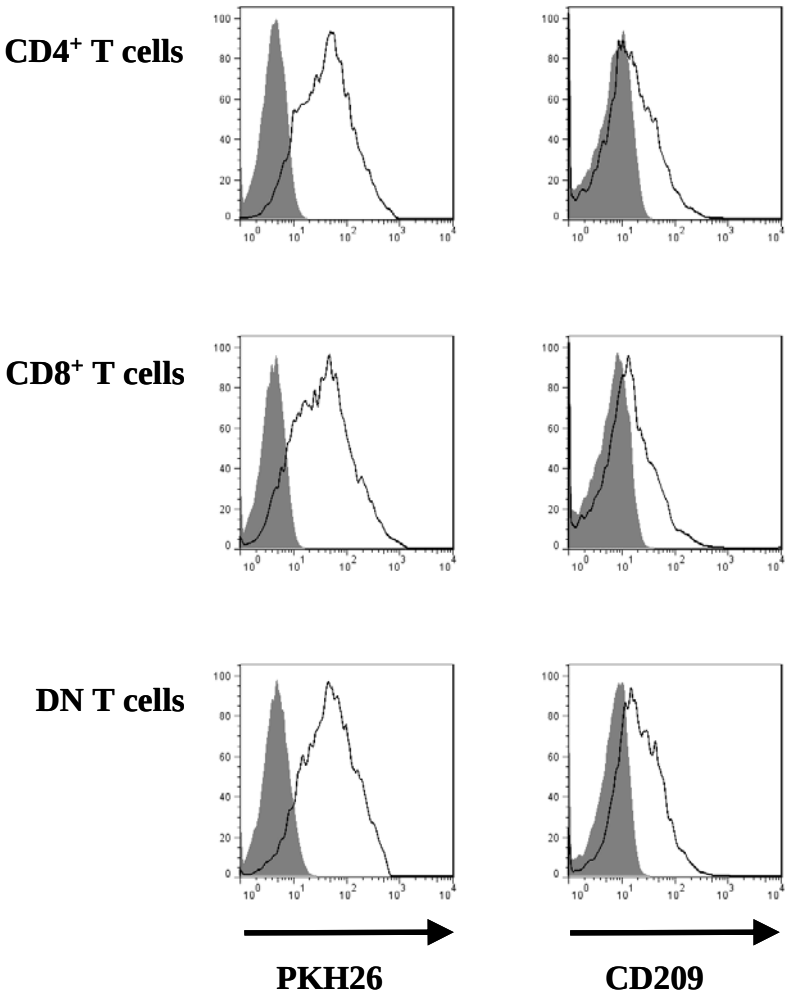


Figure 7: Transfer of membrane fragments and CD209 from DC onto alloreactive T-cell subpopulations. Alloreactive $CD4^+$, $CD8^+$, or DN T-cell lines were co-cultured with PKH26-labeled mDC. T cells were analyzed for transferred membrane fragments and CD209 from mDC via flow cytometry after 2h

of co-culture (black solid line). Negative or isotype controls are shown as filled histograms. Histograms are gated on $CD3^+CD4^+$ lymphocytes (panel 1), $CD3^+CD8^+$ lymphocytes (panel 2), or $TCR\alpha\beta^+$ $CD4^-CD8^-$ DN T cells (panel 3). Shown is one of three representative experiments with similar results.

3.4. Antigen-specificity of the trogocytosis process

Trogocytosis has been described as an antigen-dependent process. Therefore the next question was whether membrane bound PD-L1 and CD209 on mDC could be also acquired by human $CD8^+$ T cells in an antigen-independent manner. Mart-1-specific $CD8^+$ T cells were co-cultured with PKH67-labeled mDC which were either pulsed with the cognate Mart-1 or irrelevant gp100-peptide. As shown in Figure 8A, T cells acquired high amounts of membrane patches, PD-L1, and CD209 after antigen-recognition but neither PD-L1 nor CD209 and just low amounts of membrane fragments after co-culture of T cells with control peptide pulsed DC.

Next, confocal microscopy was performed to visualize the uptake of membrane patches and PD-L1 from APC. PD-L1 expression was detected on mDC but not on T cells by secondary nanocrystal staining in far red (data not shown). After exposure of Mart-1-specific T cells with peptide-pulsed DC, antigen-specific uptake of PD-L1 and membrane fragments by $CD8^+$ T cells was documented (see Figure 8B). Of importance, PD-L1 and membrane patches did not co-localize and were found widespread over the T cells. Activation after antigen-recognition was documented by the increase of activation markers CD25 and CD137 as shown in Figure 9.

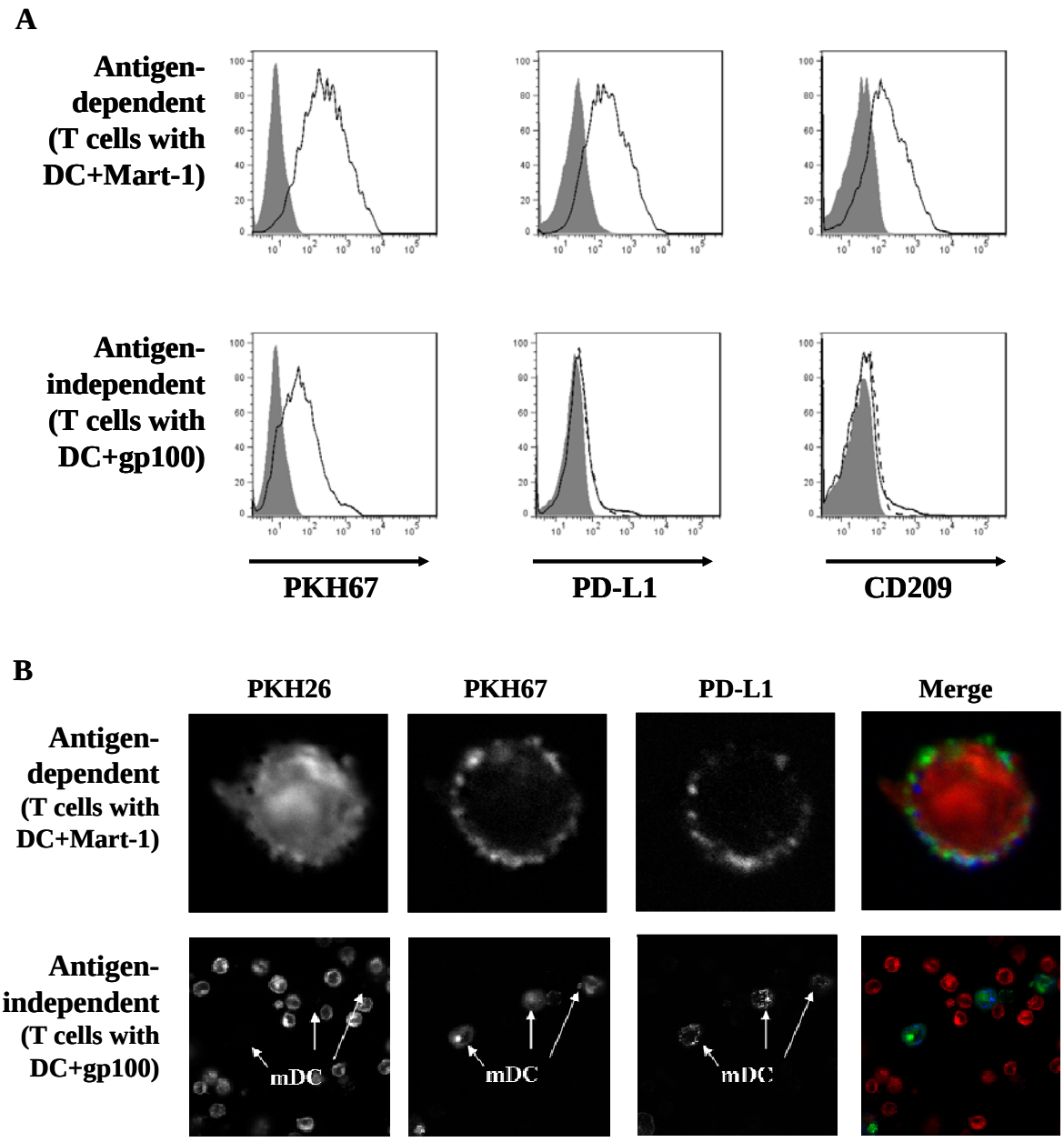


Figure 8: Transfer of membrane fragments, PD-L1 and CD209 from APC on T cells is antigen-specific. (A) Mart-1-specific $CD8^+$ T cells were co-incubated with PKH67-labeled mDC that were pulsed either with Mart-1 peptide or an irrelevant gp100 control peptide. Acquisition of $PKH67^+$ membrane fragments, PD-L1, and CD209 was measured on Mart-1 specific T cells after 2h of co-culture with DC+Mart-1 (upper panel) or with DC+gp100 control peptide (lower panel) by flow cytometry (black solid line). Dashed line in lower panel shows T cells before co-culture as negative control. Unstained or isotype controls are presented as filled histograms. Histograms are gated on $CD8^+PKH67^{low}$ lymphocytes. Shown is one representative of five independent experiments. Filled histograms represent negative control for membrane stain and isotype control for antibody-stain. (B) Visualization of transferred membrane fragments and PD-L1 on T cells by confocal microscopy. mDC were pulsed with specific Mart-1 or control peptide gp100 followed by PKH67-labeling. Mart-1-specific $CD8^+$ T cells were stained with PKH26. After 2h of co-culture with or without antigen-recognition, cells were incubated with anti-PD-L1-Biotin followed by secondary Streptavidin-eFluor staining.

mDC served as positive and T cells as negative control (not shown). In the upper panel, one representative zoomed T cell is shown after antigen-specific trogocytosis. In the lower panel, antigen-unspecific co-culture is shown as control. In the fluorescence overlay (merge), PKH26-labeled T cells are shown in red, PKH67 is shown in green and PD-L1 in blue.

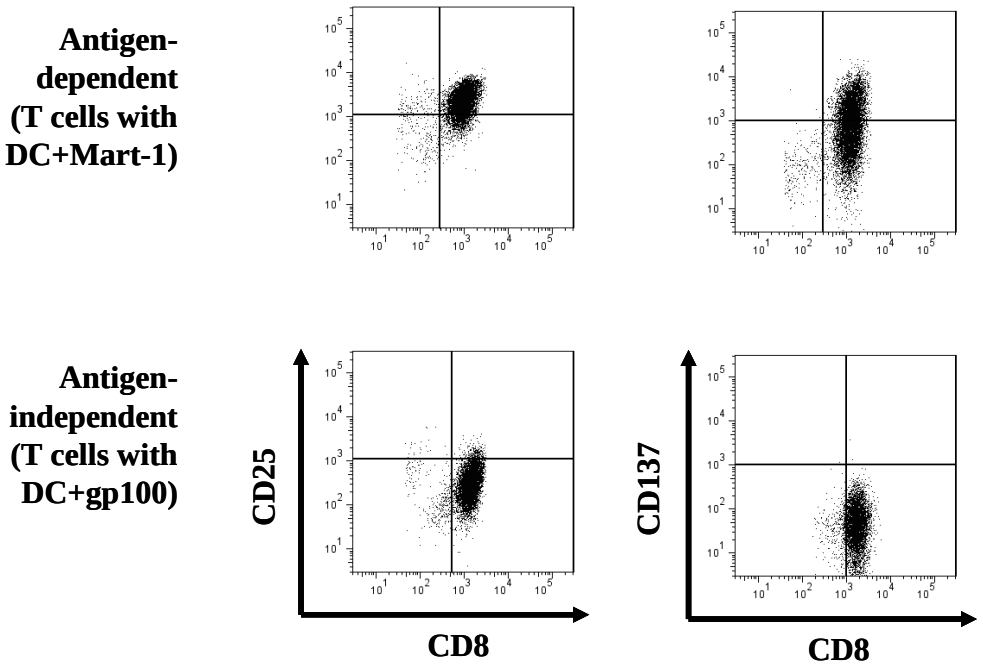


Figure 9: Upregulation of activation markers on T cells after antigen-specific or –non-specific recognition. Mart-1-specific $CD8^+$ T cells were co-incubated with PKH67-labeled mDC which were pulsed either with Mart-1 peptide or with control gp100 peptide. After 22h, expression of activation markers CD25 and CD137 was measured on T cells. Dot plots are gated on $CD8^+PKH67^{low}$ lymphocytes. Shown is one representative of three independent experiments.

To investigate whether T cells can also perform trogocytosis from tumor cells in an antigen-specific manner, the HLA-$A2^+$, tumor associated antigen negative (TAA^-) melanoma cell line Na8 either or not pulsed with Mart-1 peptide and labeled with PKH67 was co-cultured with Mart-1-specific T cells. T cells interacting with antigen-pulsed Na8 cells acquired more membrane fragments than from the negative control without antigen (see Figure 10A). Moreover, activation markers such as CD25 and CD137 were only up-regulated after antigen-specific interaction (see Figure 10B). This shows that also in the interaction with target cells as tumor cells, trogocytosis is triggered by antigen-recognition.

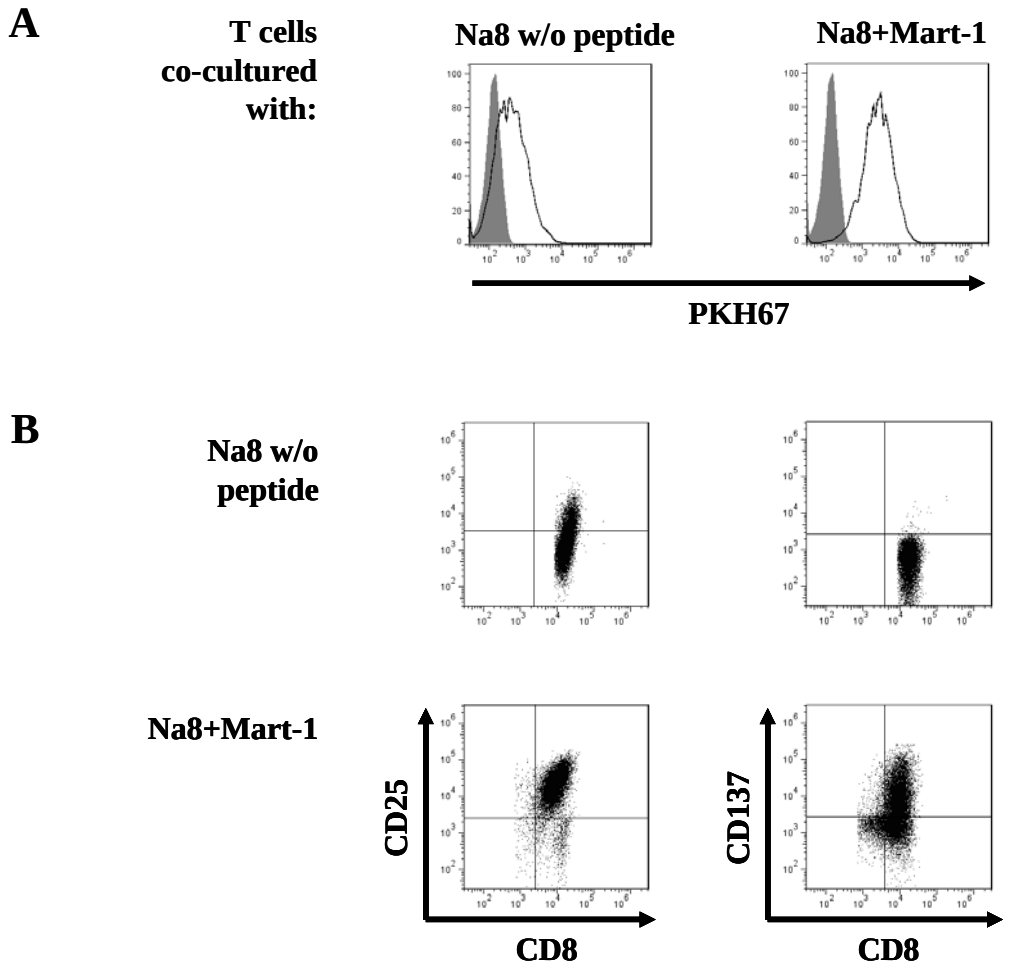


Figure 10: Trogocytosis from melanoma cell line is triggered by antigen-recognition. Na8 melanoma cells either (+Mart-1) or not (w/o peptide) pulsed with antigen Mart-1 peptide and labeled with PKH67 were co-cultured with Mart-1-specific T cells. (A) After 2h, uptake of membrane fragments was analyzed by flow cytometry (black solid line). Negative controls are presented as filled histograms. (B) After 22h, expression of activation markers CD25 and CD137 was measured on T cells. Histograms and dot plots are gated on $CD8^{+}PKH67^{low}$ lymphocytes. Shown is one representative of three independent experiments

3.5. Role of antigen-presenting cells for the transfer of membrane fragments and surface molecules onto T cells

3.5.1 Capacity of different APC sources in the trogocytosis process

Next, the potential of $CD8^{+}$ T cells to acquire membrane patches from different APC sources via trogocytosis was analyzed. Purified peptide-pulsed and PKH67-labeled

monocytes, iDC, mDC, NK cells, or B cells were co-culture with Mart-1-specific T cells. As shown in Figure 11, T cells were able to acquire only low amounts of membrane fragments from monocytes, NK cells, and B cells. In contrast, DC are the most potent donor cells with the capacity of transferring high amounts of membrane fragments onto $CD8^+$ T cells.

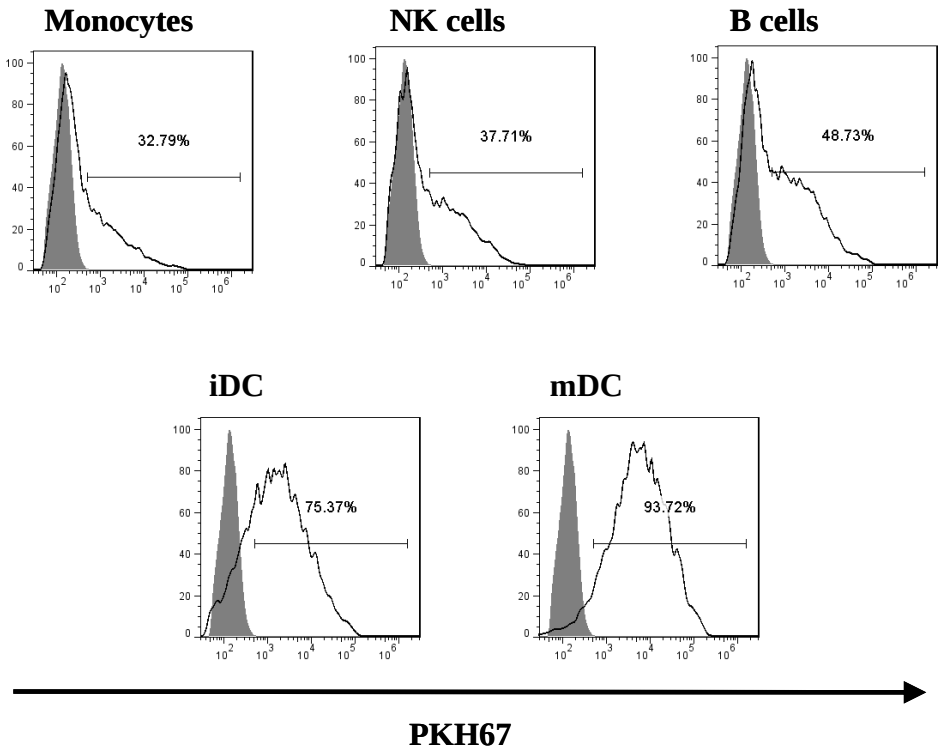


Figure 11: Transfer of membrane patches from different different APC sources onto T cells. Purified monocytes, NK cells, B cells, iDC and mDC were pulsed with Mart-1 peptide and labeled with PKH67. Mart-1-specific T cells were co-cultured with the different APC for 2h and analyzed for acquisition of membrane fragments (black solid line). Negative controls are shown as filled histograms. Histograms are gated on $CD8^+$ $PKH67^{low}$ lymphocytes. Shown is one representative of three independent experiments with similar results.

To compare the transfer of surface molecules and membrane fragments from iDC and mDC onto $CD8^+$ T cells in more detail, expression of surface molecules such as CD80, CD86, PD-L1, and CD209 was analyzed. FACS analysis showed that mDC express higher levels of PD-L1, CD80, and CD86 (see Figure 12), whereas CD209 was expressed on iDC and mDC at similar levels.

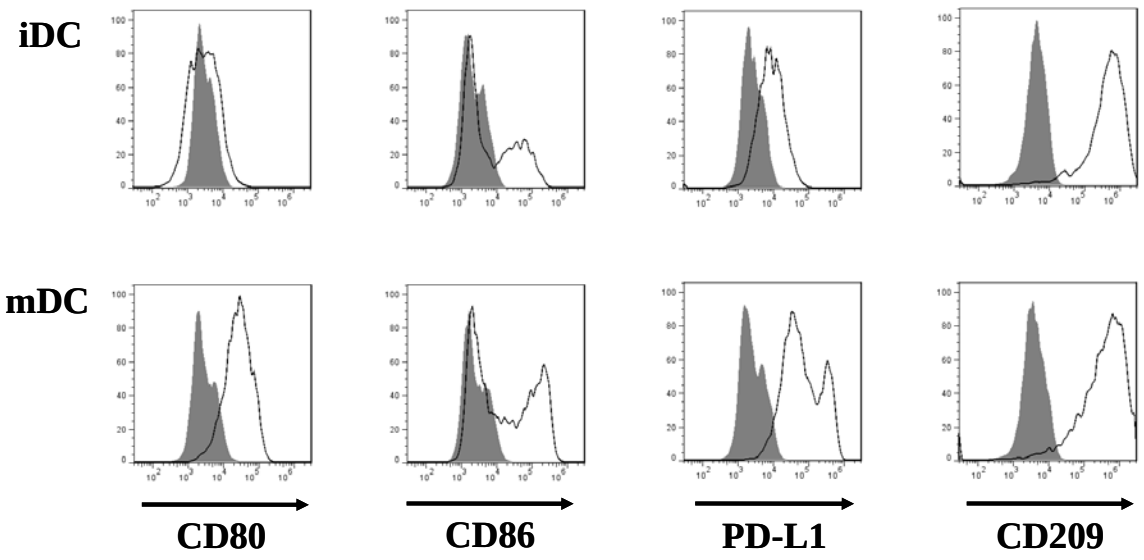


Figure 12: Expression of CD80, CD86, PD-L1 and CD209 on iDC versus mDC. mDC and iDC were analyzed by flow cytomtery for expression of co-stimulatory, co-inhibitory and DC-specific molecules (black solid line). Isotype controls are presented as filled histograms. Shown is one representative of three independent experiments.

Since iDC and mDC have comparable expression levels of PD-L1 and CD209, these antigens were used for further studies. As shown in Figure 13, $CD8^+$ T cells acquired higher amounts of membrane fragments on their cell surface, but also significantly higher levels of surface PD-L1 and CD209 molecules from mDC compared to iDC.

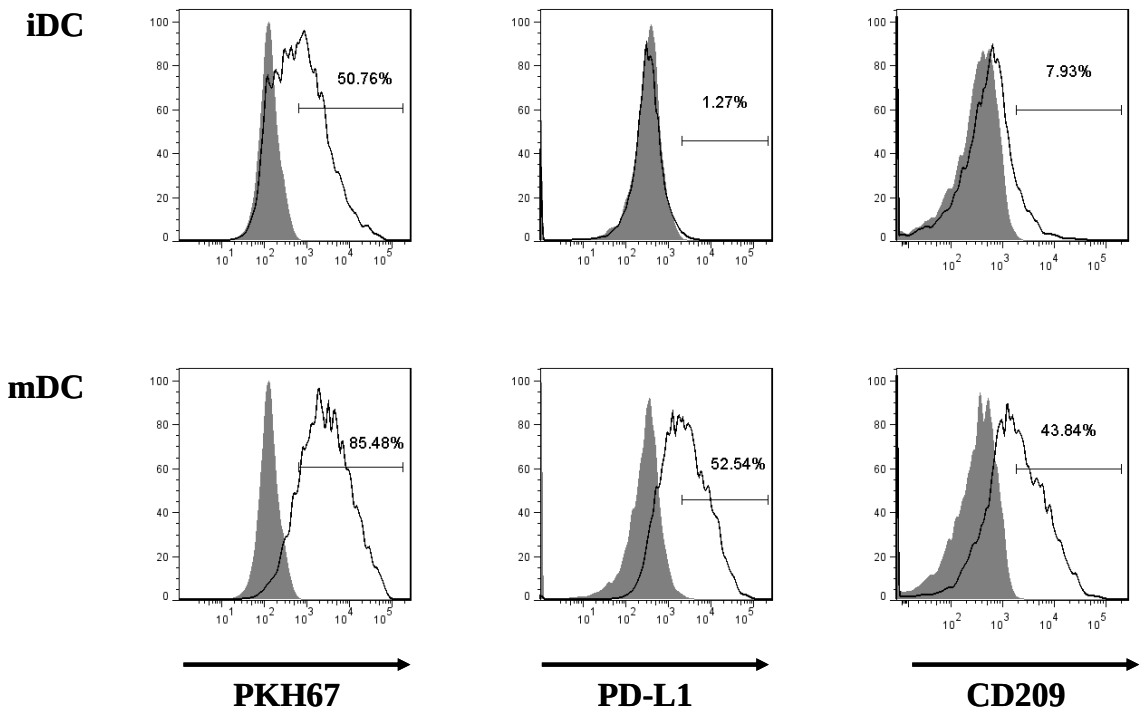


Figure 13: mDC are more efficient in transferring both, membrane patches and surface molecules onto T cells. iDC and mDC were pulsed with Mart-1 peptide and labeled with PKH67. After 2h of co-culture, Mart-1-specific $CD8^+$ T cells were analyzed for acquisition of PKH67, PD-L1, and CD209 by flow cytometry (black solid line). Negative or isotype controls are shown as filled histograms. Histograms are gated on $CD8^+PKH67^{low}$ lymphocytes. Shown is one representative of three independent experiments.

3.5.2. Transfer of membrane fragments and molecules from melanoma cells

Next, it was determined whether tumor antigen-specific $CD8^+$ T cells are also capable of acquiring surface molecules from tumor cells upon antigen-specific recognition. The co-inhibitory molecule PD-L1 is up-regulated on many melanoma cell lines after IFN-γ exposition, whereas the surface antigen melanoma chondroitin sulphate proteoglycan (MCSP) is known to be constitutively expressed (Dong, Strome et al. 2002). The $HLA\text{-}A2^+$ $Mart\text{-}1^+$ melanoma cell line Mel1300 was analyzed for PD-L1 and MCSP before and after IFN-γ exposure and revealed a high MCSP expression that was not modulated by IFN-γ (see Figure 14). As expected, PD-L1 was not constitutively expressed on Mel1300 but was induced up to 90% $PD\text{-}L1^+$ melanoma cells after pretreatment with IFN-γ.

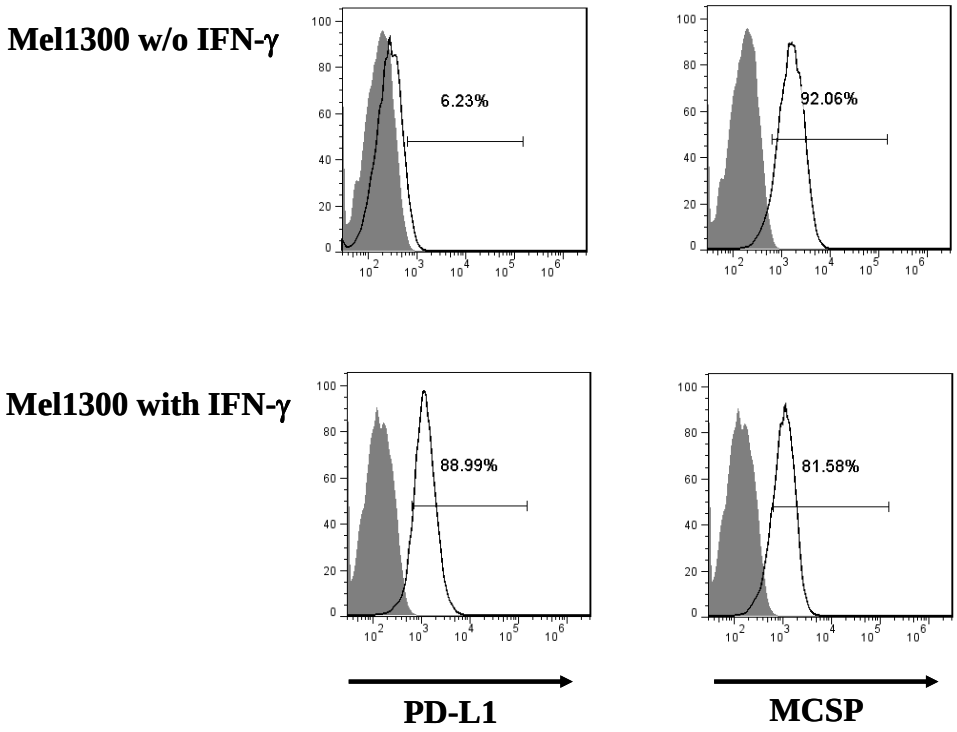


Figure 14: Expression of PD-L1 and MCSP on melanoma cell line Mel1300 before and after IFN-γ treatment. PD-L1 and MCSP expression was analyzed on Mel1300 before (upper panel) and after 48h IFN-γ (lower panel) exposure by flow cytometry and is shown as black solid line in histograms. Isotype controls are presented as filled histograms. Shown is one representative of three independent experiments.

After co-culture with IFN-γ treated Mel1300 cells T cells acquired both, PD-L1 and MCSP, besides membrane fragments whereas no PD-L1 could be detected on T cells after co-culture with IFN-γ–untreated Mel1300 melanoma cells (see Figure 15). Although PD-L1 can be acquired by T cells after antigen-recognition from PD-L1^{+} tumor cells, less molecules and membrane fragments are transferred from melanoma cells than from mDC. These data demonstrate that PD-L1 is not exclusively acquired from mDC but also from PD-L1 expressing melanoma cells.

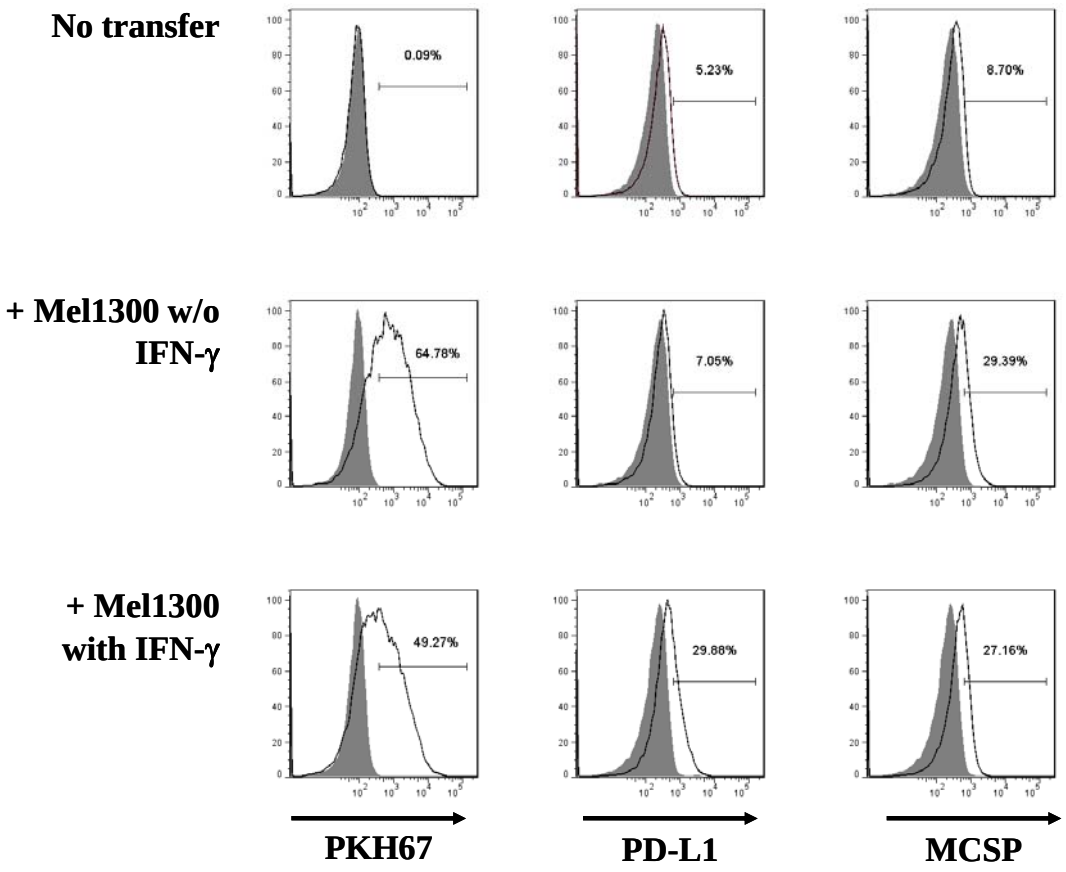


Figure 15: Acquisition of PD-L1 and MCSP from the melanoma cell line Mel1300 by $CD8^+$ T cells. PKH67 labeled, IFN-γ pre- or untreated Mel1300 melanoma cells were co-cultured with Mart-1 specific $CD8^+$ T cells. Acquisition of membrane fragments, PD-L1 and MCSP was analyzed after 2h by flow cytometry (black solid line). Negative or isotype controls are presented as filled histogram. Histograms are gated on $CD8^+PKH67^{low}$ lymphocytes. Shown is one representative of three independent experiments.

3.6. Characterization of the trogocytosis process from APC

3.6.1. PD-L1 is not up-regulated on antigen-specific T cells

PD-L1 is described to be endogenously expressed by T cells after stimulation (Keir, Butte et al. 2008). To verify that PD-L1 is not up-regulated on T cells after stimulation, Mart-1-pulsed, PKH67-labeled, PD-L1$^-$ T2 cells were used as stimulator cells for Mart-1-specific T cells (see Figure 16A). As shown in Figure 16B, the uptake of membrane fragments but not PD-L1 was detected on T cells. The transfer of PKH67 and PD-L1 from mDC onto T cells served as positive control. These data suggest that PD-L1 is rapidly acquired from PD-L1$^+$ mDC upon antigen-specific stimulation during antigen-specific interaction with mDC but not endogenously expressed on T cells within 2 hours.

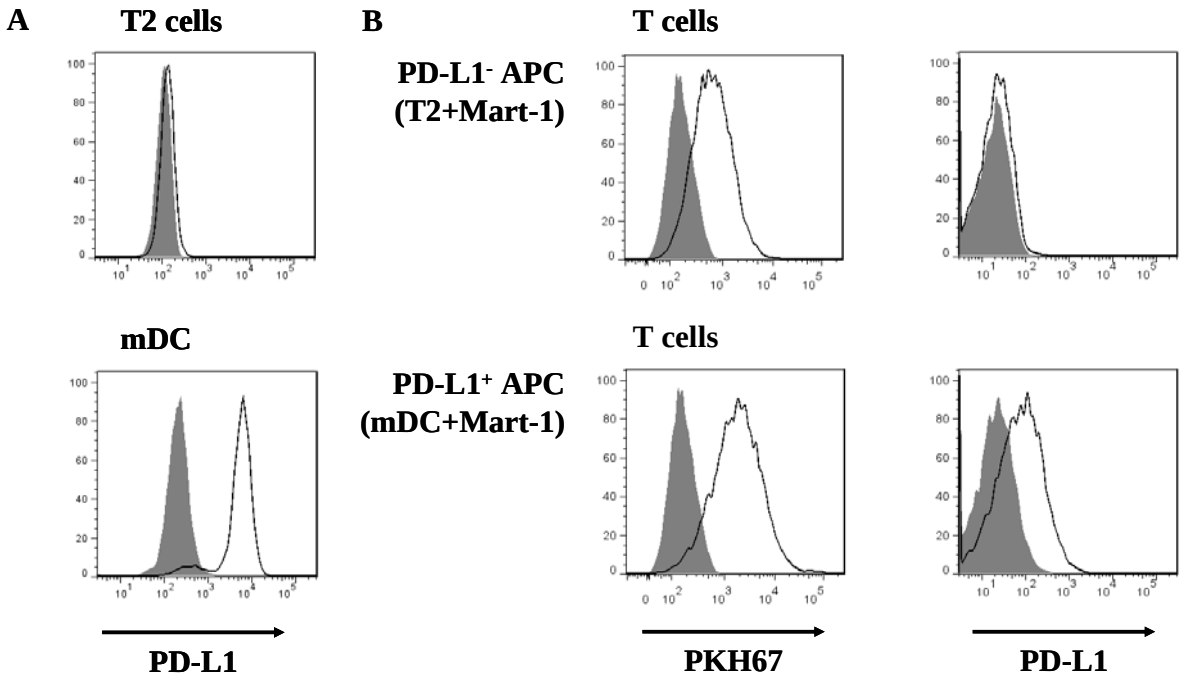
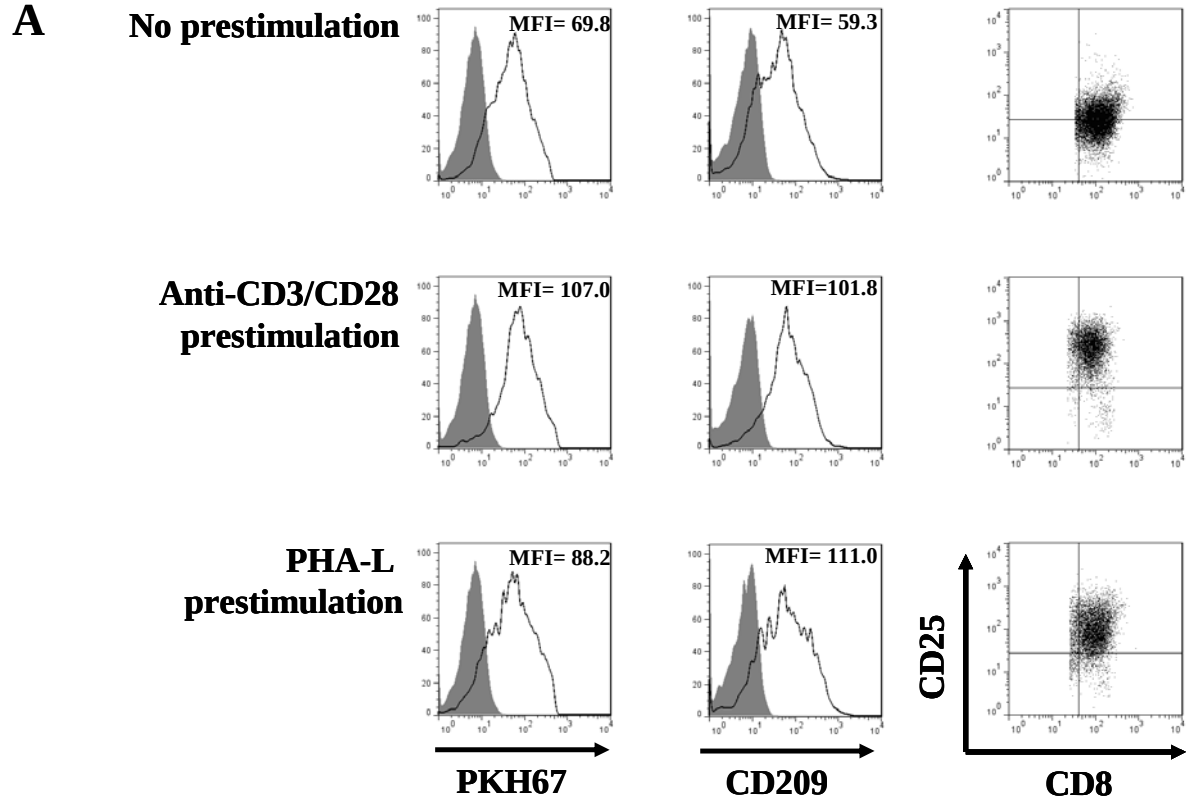


Figure 16: PD-L1 acquisition by T cells is independent on endogenous PD-L1 expression. T2 cells and mDC were pulsed with Mart-1 peptide, labeled with PKH67 and co-cultured for 2h with Mart-1-specific CD8$^+$ T cells. (A) Histograms show expression of PD-L1 on T2 cells and mDC (black solid line). (B) Histograms present the transfer of PKH67 and PD-L1 onto T cells gated on CD8$^+$PKH67^{low} lymphocytes. Filled histograms represent negative control for membrane staining and isotype control for antibody-staining. Shown is one representative of three independent experiments.

3.6.2. Acquisition of membrane fragments and surface molecules is dependent on the activation status of T cells

Next, the influence of the activation status of T cells on trogocytosis was analyzed. Antigen-specific as well as non-specific transfer was performed with $CD8^+$ T cells that were stimulated overnight with anti-CD3/CD28 beads or PHA-L. Prestimulation resulted in a slightly increased acquisition of membrane fragments and surface molecules on antigen-specific T cells in both cases (see Figure 17A). CD25 expression was up-regulated on the Mart-1-specific T-cell clone upon prestimulation with anti-CD3/CD28 beads and PHA-L. In contrast, polyclonal, unstimulated T cells did not acquire any surface molecules and membrane patches but could be triggered to acquire small amounts of membrane fragments and CD209 upon prestimulation with anti-CD3/CD28 beads as well as PHA-L (see Figure 17B). Polyclonal T cells revealed a mixed phenotype of activated and not activated cells (data not shown). After incubation in medium overnight, CD25 was down-regulated. In contrast, CD25 was highly expressed on polyclonal T cells after prestimulation with either anti-CD3/CD28 beads or PHA-L. Together, prestimulated T cells can generally acquire more membrane fragments and surface molecules than unstimulated T cells.



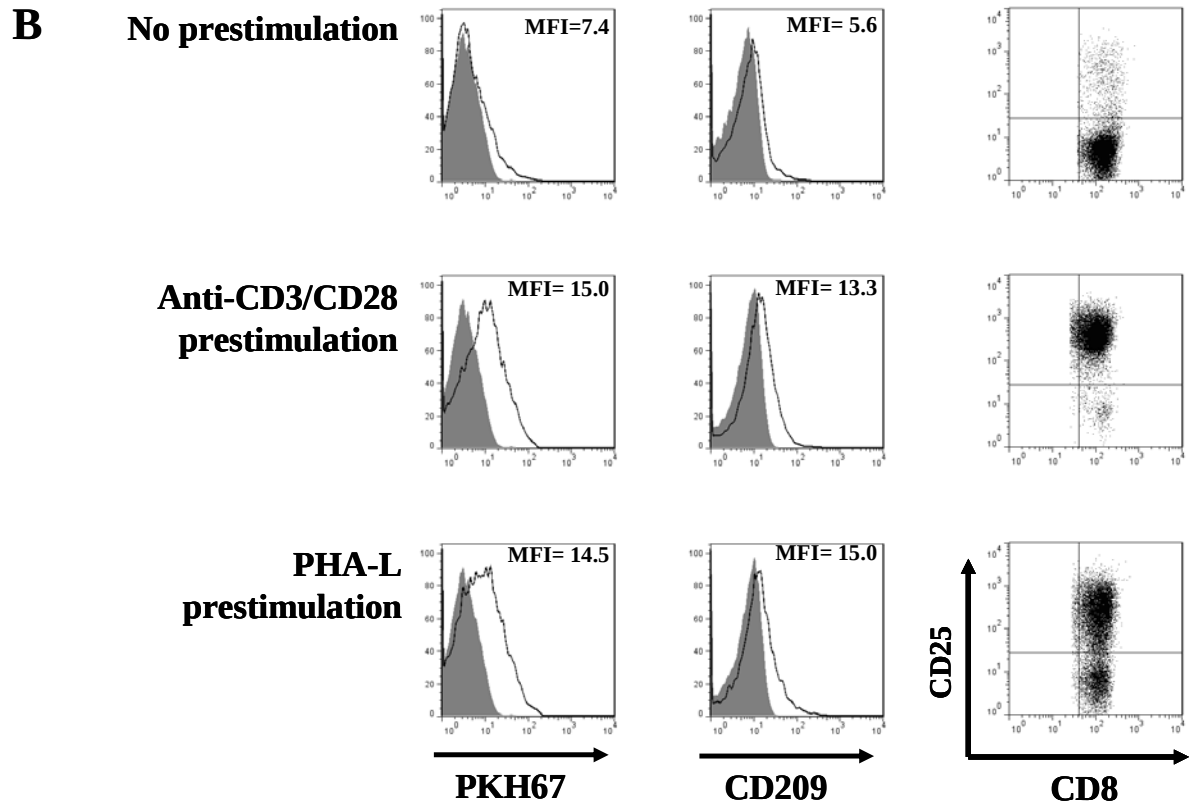


Figure 17: Transfer of membrane fragments and surface molecules of APC onto T cells is dependent on the activation status of T cells. (A) Antigen-specific $CD8^+$ or (B) polyclonal $CD8^+$ T cells were either stimulated overnight with anti-CD3/CD28 beads or PHA-L, or cultured in medium. Beads were then removed and PHA-L was washed out. T cells were subsequently co-cultured with Mart-1 peptide-pulsed, PKH67-labeled mDC. After 2h, acquisition of membrane fragments and CD209 (black solid line) as well as the expression of activation marker CD25 was measured by flow cytometry. Negative or isotype controls are presented as filled histograms. Histograms are gated on $CD8^+PKH67^{low}$ lymphocytes. Shown is one representative of three independent experiments.

3.6.3. The amount of acquired molecules on T cells is dependent on the DC to T cell ratio

To determine whether the amount of acquired molecules is higher if more APC are targeted by antigen-specific T cells, T cells were co-cultured with mDC at different ratios and analyzed for uptake of membrane fragments and PD-L1. An increase in acquired membrane fragments and PD-L1 was observed up to a T cell:DC ratio of 1:1 (see Figure 18). At a ratio of 3:1, a three-fold acquisition of membrane fragments and a two-fold transfer of PD-L1

was documented compared to a 10:1 ratio. Saturation was documented at a T cell:DC ratio of 1:3. These data demonstrate a limited capacity in the transfer of cell components.

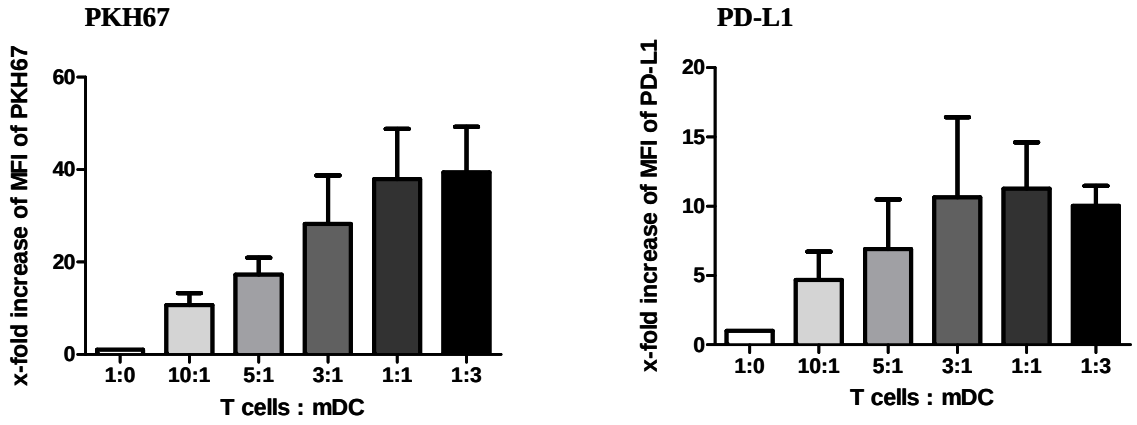


Figure 18: Influence of T cell to mDC ratio on the efficiency of uptake of membrane fragments and PD-L1. mDC were pulsed with Mart-1 peptide, labeled with PKH67 and co-cultured with Mart-1-specific T cells at different T cell:DC ratios. Data were measured by flow cytometry. The x-fold increase of the MFI of unstimulated T cells of four independent experiments (± SD) is shown for PKH67 and PD-L1 on $CD8^{+}PKH67^{low}$ T cells.

3.7. Kinetics of the transfer process

To assess the dynamics of the transfer of membrane fragments, PD-L1 and CD209, the kinetics of trogocytosis were documented at different times. As shown in Figure 19, transfer of PD-L1, CD209, and membrane patches from mDC onto T cells was evident already at 30' after co-culture. The acquisition of all three components peaked at 1.5h to 2h and rapidly decreased after 3h to 4h reaching a plateau for about 20h. Between 48h and 72h of co-culture, PD-L1, CD209 and membrane patches completely disappeared from the T-cell surface. Of interest, the acquisition of membrane fragments, PD-L1, and CD209 revealed the same kinetics indicating a similar mechanism of capture.

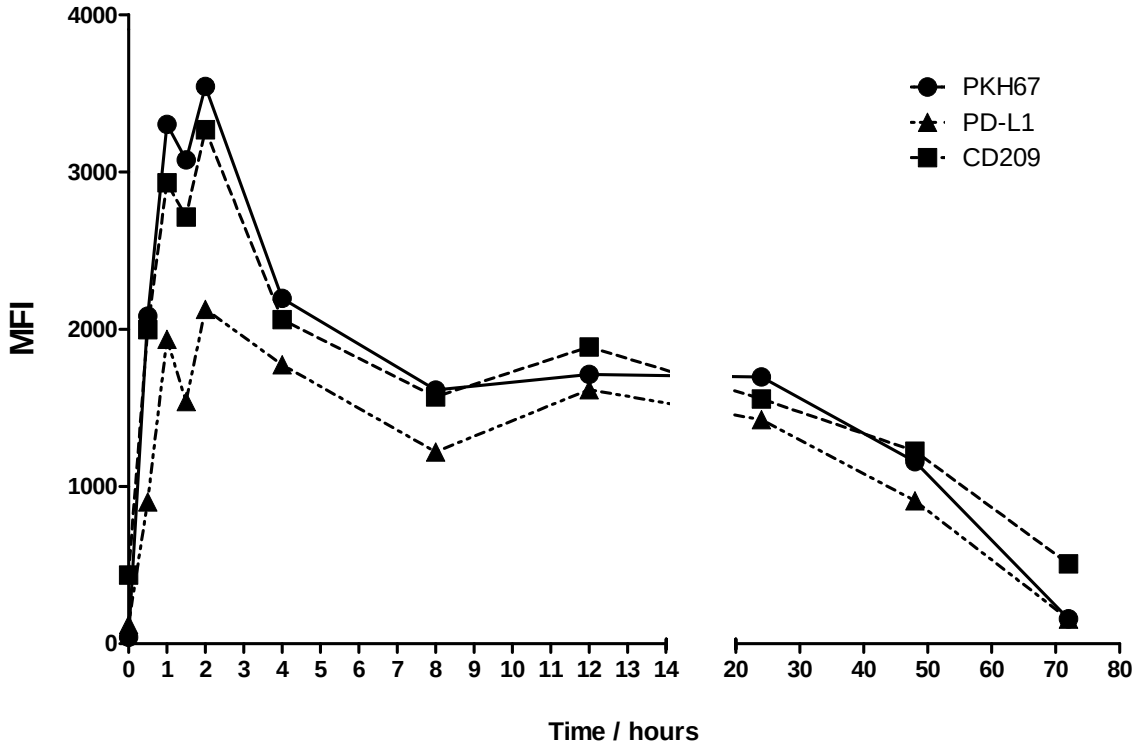


Figure 19: Kinetics of acquisition of membrane fragments, PD-L1, and CD209. Mart-1-specific T cells were co-cultured with Mart-1 peptide-pulsed, PKH67-labeled mDC. Acquisition of membrane patches, PD-L1 and CD209 was analyzed by flow cytometry at indicated times and is displayed as MFI of PKH67 ●, PD-L1: ▲, CD209: ■. Shown is one representative experiment. Kinetics were tracked three times for the acquisition of membrane fragments and two times for the acquisition of PD-L1 and CD209.

3.8. Mechanism of the transfer process

3.8.1. The transfer process from APC onto T cells requires cell-to-cell contact

Trogocytosis is defined as cell-to-cell contact dependent mechanism. To assure that both, PD-L1 and CD209 are not transferred via membrane vesicles but in a cell-to-cell contact dependent manner, peptide-pulsed DC were placed in the presence (1) or absence (2) of PKH26-labeled antigen-specific T cells in the insert well separated from T cells in the bottom well. In both settings, no transfer of PD-L1, CD209, and membrane fragments could be documented as shown in Figure 20 (PD-L1: 6.0% $\pm$ 3.8% (1) and 9.1% $\pm$ 1.0% (2) of MFI of maximal antigen-specific uptake compared to 2.5% $\pm$ 2.5% background MFI; $p<0.001$; CD209: 11% $\pm$ 15.7% (1) and 4.1% $\pm$ 0.8% (2) of MFI of maximal antigen-specific uptake compared to 5.1% $\pm$ 4.7% background MFI; membrane fragments: 5.4% $\pm$

1.5% (1) and 7.5% ± 0.9% (2) of MFI of maximal antigen-specific uptake compared to 1.0% ± 0.4 % background MFI; $p<0.001$). These experiments strongly demonstrate that trogocytosis of PD-L1 and CD209 is dependent on cell-to-cell contact.

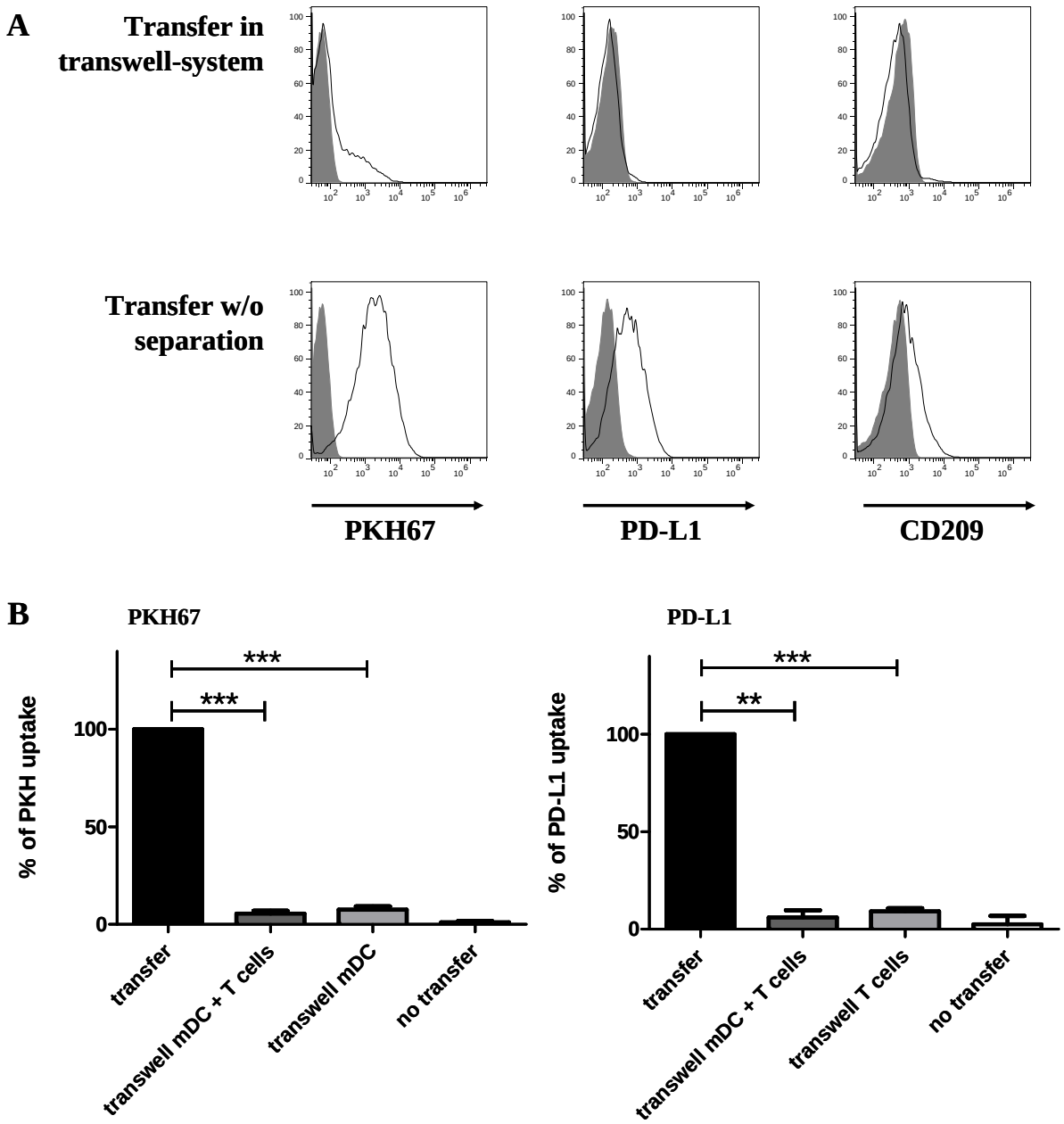
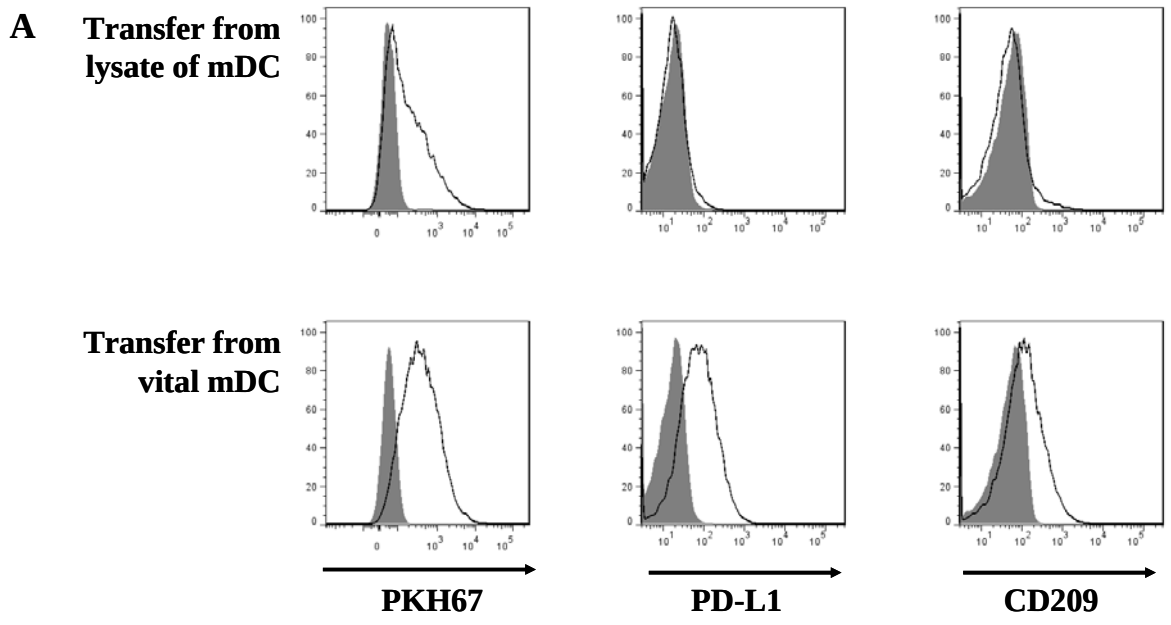


Figure 20: Acquisition of PD-L1 and CD209 by antigen-specific T cells is mediated by a cell-to-cell contact dependent mechanism. (A) Mart-1 peptide-pulsed, PKH67-labeled mDC and PKH26-labeled, Mart-1-specific T cells were set into transwell insert separated by a membrane with 5µm pores from bottom well. Mart-1-specific $CD8^+$ T cells set in bottom well were analyzed for acquisition of membrane fragments, PD-L1, and CD209 after 2h by flow cytometry (black solid line). Negative or isotype controls are presented as filled histograms. Histograms are gated on $CD8^+PKH67^{low}PKH26^-$ lymphocytes. (B) Mart-1-pulsed, PKH67-

labeled mDC were set into insert well with or without PKH26-stained T cells separated from Mart-1-specific $CD8^+$ T cells in bottom well. Data were analyzed by flow cytometry. In the bar graphs, MFI of the maximum transfer was set to 100%. Inhibited transfer is shown as percentage of maximum uptake of membrane fragments and PD-L1 of three independent experiments ± SD (*** $p<0.001$, ** $p<0.01$ paired *t* test).

3.8.2. Transfer from PD-L1 and CD209 is not mediated by soluble proteins of lysed mature dendritic cells

Next, it was analyzed whether T cells capture membrane fragments and surface molecules out of cell lysates after antigen-specific killing of APC. When Mart-1 peptide-pulsed, PKH67-labeled mDC were lysed via repeated freeze-and-thaw cycles, no transfer of PD-L1 and CD209 but low amounts of membrane fragments was documented as shown in Figure 21 (PD-L1: 8.2% ± 3.1% MFI of maximal transfer compared to 5.3% ± 2.4% background MFI, $p<0.01$; CD209: 21.4% ± 5.5% MFI of maximal transfer compared to 5.7% ± 9.9% background MFI, $p<0.01$; membrane fragments: 26.4% ± 28.4% MFI of maximal transfer compared to 5.9% ± 2.0 % background MFI, not significant). These data confirm that surface molecules are not captured after mDC lysis as consequence of the cytotoxic activity of T cells but upon close interaction with mDC.



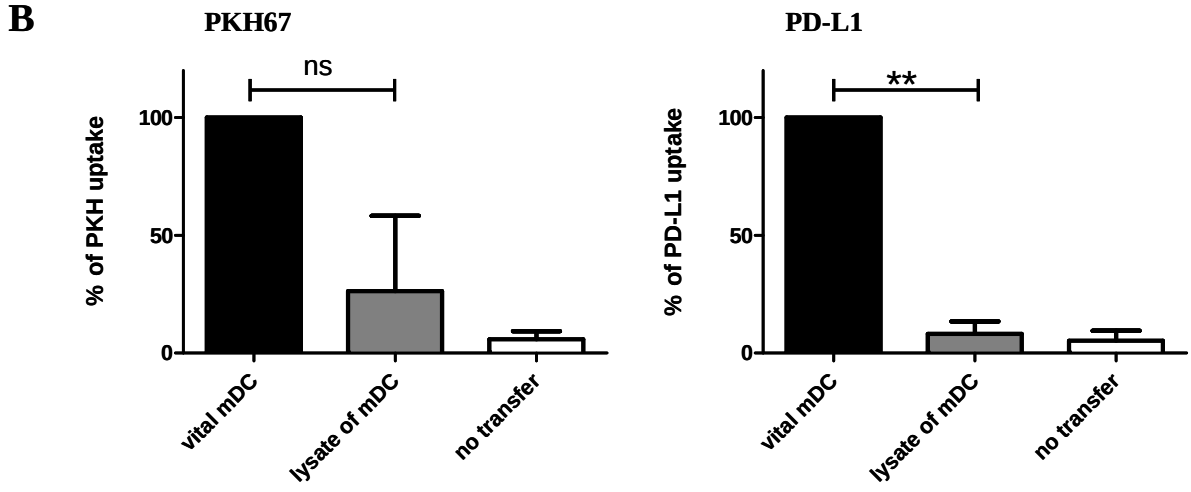


Figure 21: No impact of cell lysis of mDC on acquisition of surface molecules by T cells. Mart-1-pulsed, PKH67-labeled mDC were lysed by freeze-and-thaw cycles and incubated with Mart-1-specific $CD8^+$ T cells for 2h. As control, Mart-1-specific $CD8^+$ T cells were co-cultured with Mart-1 peptide-pulsed, PKH67-labeled mDC. Negative or isotype controls are presented as filled histograms. (A) Histograms are gated on $CD8^+PKH67^{low}$ lymphocytes. Shown is one representative of three independent experiments with similar results. (B) In bar graphs, MFI of antigen-specific transfer was set to 100% as maximum. Inhibited transfer and no transfer are shown as percentage of maximum uptake of membrane fragments and PD-L1 of three independent experiments $\pm$ SD (transfer PKH: not significant (ns) paired *t* test; PD-L1 ** $p<0.01$ paired *t* test).

3.8.3. Bystander T cells are not triggered to trogocytosis by neighboring T cells

Unspecific bystander T cells were tested for increased antigen-unspecific acquisition of membrane fragments after co-culture with Mart-1-specific T cells and Mart-1-pulsed, PKH26-labeled mDC. Of interest, bystander T cells were not triggered indirectly by cytokines or interacting cells to acquire more membrane fragments than those T cells that are non-reactive to the exposed antigen (see Figure 22). These data clearly demonstrate that the trogocytosis process is specific and can not be transferred to unspecific bystander T cells.

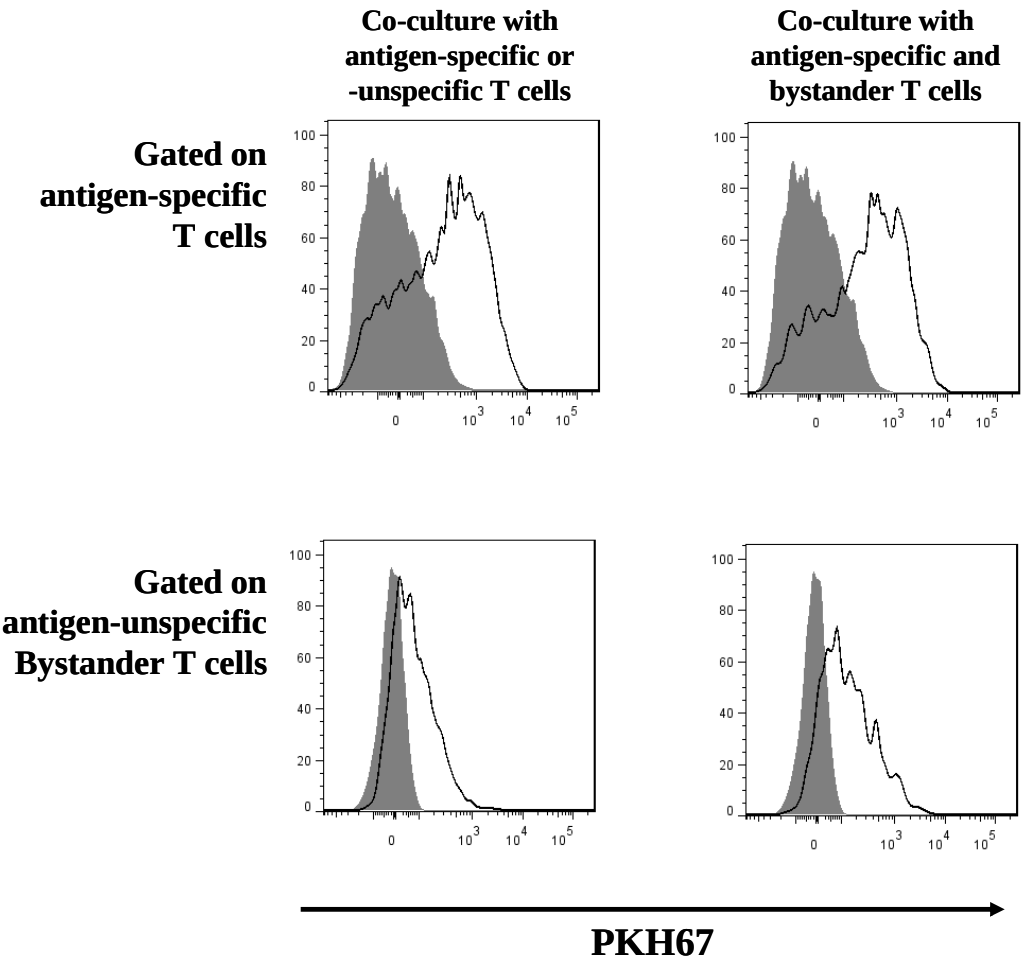


Figure 22: Trogocytosis is a direct process between antigen-specific T cells and APC and not transferred onto non-specific bystander T cells. Mart-1 peptide-pulsed and PKH67-labeled mDC were co-cultured for 2h with $CD8^+$ T cells that are either specific for the cognate Mart-1 peptide (Antigen-specific T cells in co-culture with only one T-cell population) or for the control gp100 peptide (Antigen-unspecific T cells in co-culture with only one T-cell population). On the right panel, Mart-1-pulsed, PKH67-labeled mDC were co-cultured with both Mart-1-specific and gp100-specific T cells as bystander T cells. Co-cultures were analyzed for the uptake of membrane patches on antigen-specific or –unspecific T cells by flow cytometry (black solid line). Negative controls are presented as filled histograms. Shown is one representative of four independent experiments with similar results.

3.9. Inhibition of trogocytosis

In order to explore the mechanism by which T cells acquire membrane fragments, several approaches were tested for their capacity to block trogocytosis.

3.9.1. Impairment of trogocytosis by fixation of DC

Next, the requirement for active involvement of mDC in the trogocytosis process was analyzed. To address this question, antigen-pulsed, PKH67-labeled mDC were fixed with 0.05% glutaraldehyde and co-cultured with specific $CD8^+$ T cells. As shown in Figure 23, fixation of mDC resulted in a strong inhibition of the transfer of membrane fragments, PD-L1, and CD209.

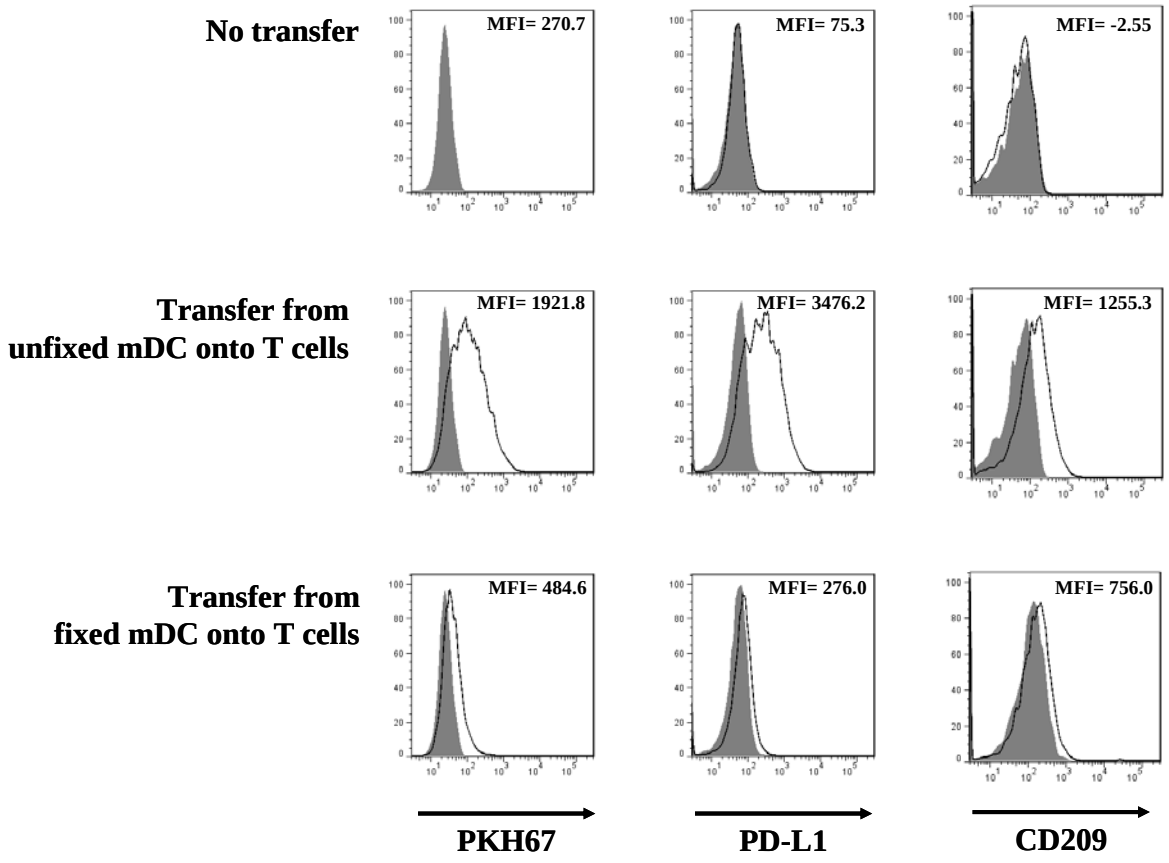


Figure 23: Impairment of trogocytosis by fixation of APC. Before co-culture, mDC were pulsed with Mart-1 peptide, labeled with PKH67 and fixed with 0.05% glutaraldehyde. After 2h, transfer of PKH67, PD-L1, and CD209 from fixed mDC onto T cells was analyzed by flow cytometry. Unfixed mDC served as

positive control. Negative or isotype controls are presented as filled histograms. Shown is one representative of three independent experiments with similar results.

Of importance, TCR-pMHC interactions between T cells and glutaraldehyde-fixed mDC must still be functional as demonstrated by the up-regulation of CD25 and CD137 on T cells after co-culture with fixed mDC (see Figure 24). These data clearly indicate that the acquisition is not caused by disruption of molecules out of the membrane of DC but depends on direct interaction of vital mDC with T cells.

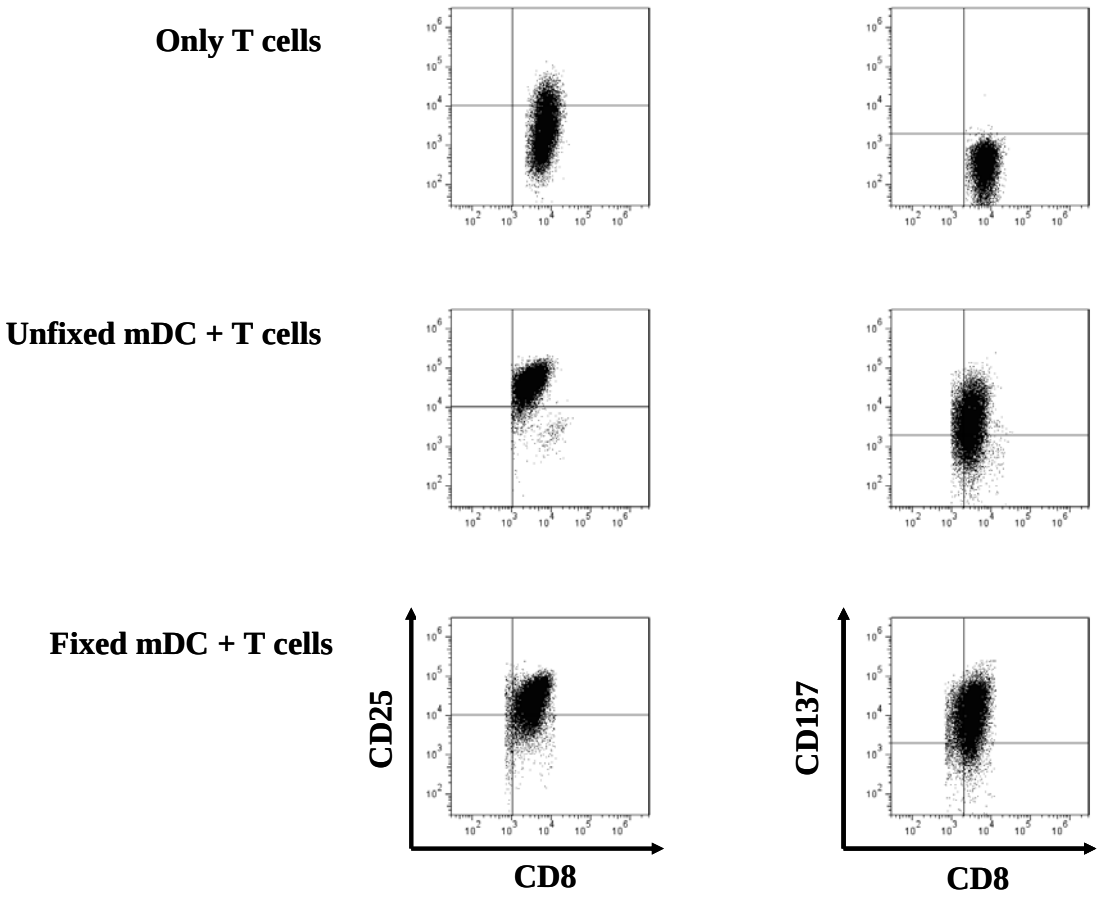


Figure 24: Activation of CD8$^+$ T cells after stimulation with fixed mDC. Mart-1-specific T cells were co-cultured with fixed or unfixed Mart-1-pulsed, PKH67-labeled mDC. After 22h, expression of activation markers CD25 and CD137 was analyzed by flow cytometry compared to unstimulated T cells. Histograms are gated on CD8$^+$PKH67^{low} lymphocytes. Shown is one representative of three independent experiments.

3.9.2. Impact of surface molecules on T cells and DC for trogocytosis

Next, surface molecules such as adhesion molecules and co-stimulatory and inhibitory molecules were blocked to analyze whether the interaction of these molecules plays an important role in triggering trogocytosis. To address this question, the co-receptor CD8, adhesion molecules ICAM-1 and ICAM-2 on antigen-specific $CD8^+$ T cells as well as co-stimulatory/-inhibitory molecules CD80 and CD86, PD-L1, and adhesion molecule CD209 on mDC were blocked by specific blocking antibodies. After co-culture, antigen-specific T cells were analyzed by flow cytometry. With the exception of CD8 (see Figure 25), blocking of ICAM-1 and ICAM-2 on T cells as well as blocking of PD-L1, CD209, CD80, and CD86 had no influence on the uptake of membrane fragments and surface molecules by T cells (Figure 26).

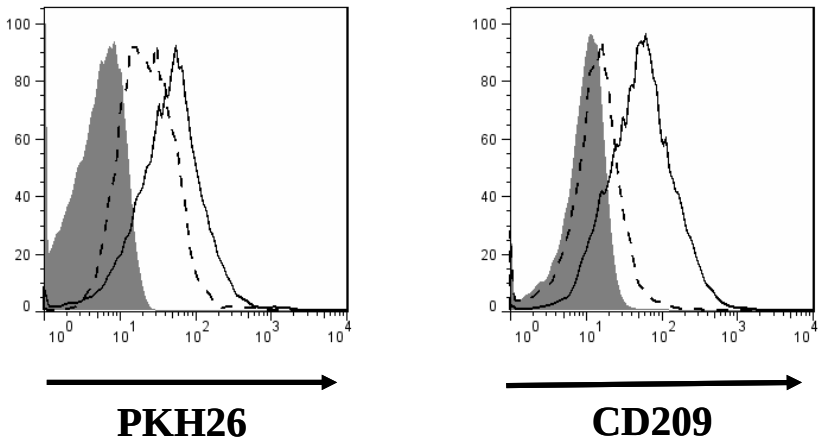


Figure 25: Blocking the CD8 co-receptor impairs trogocytosis. For blocking the CD8 co-receptor, $CD8^+$ T cells were incubated for 30' with blocking anti-CD8 antibody or with isotype control. $CD8^+$ T cells were then analyzed for acquisition of membrane fragments and CD209 after co-culture. Specific transfer is presented as black solid line, transfer with CD8 blockade as black dashed line, and negative or isotype controls are presented as filled histograms. Shown is one representative of three independent experiments.

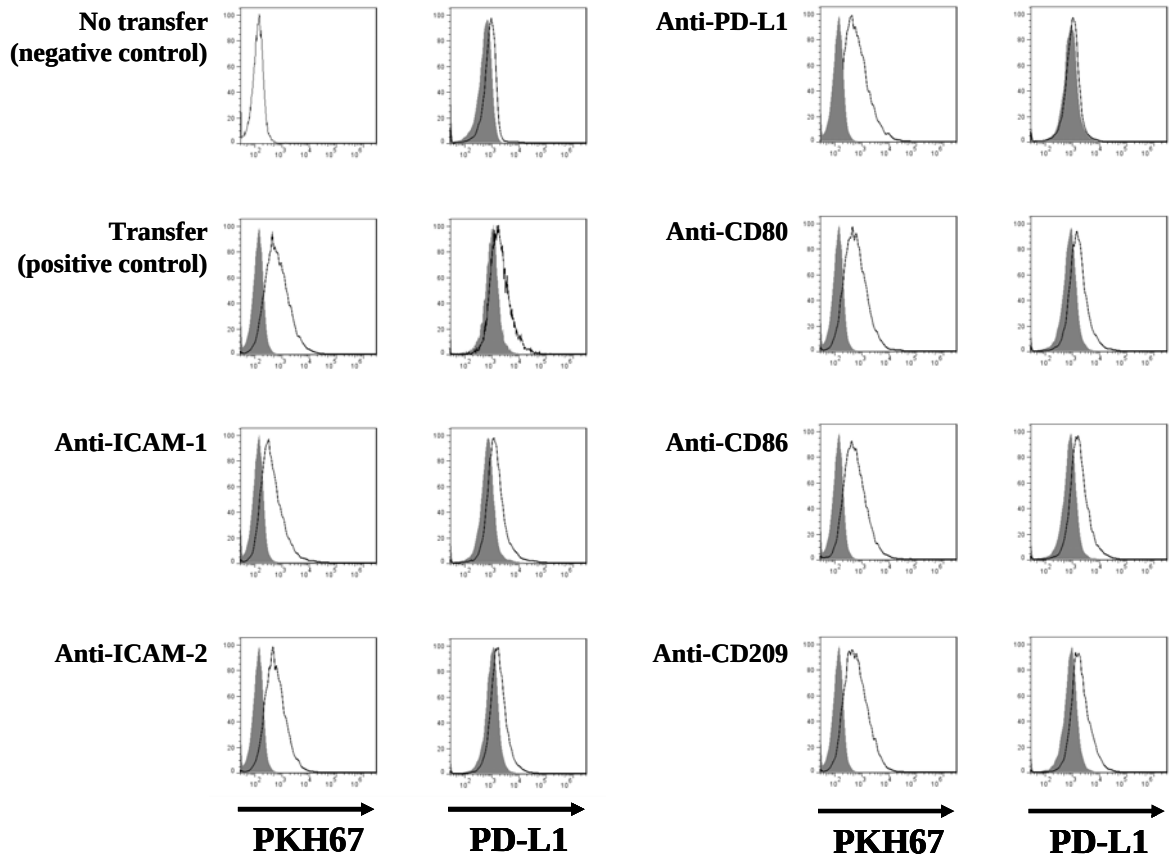


Figure 26: Impact of blocking surface molecules on trogocytosis of membrane patches and PD-L1. Antigen-specific $CD8^+$ T cells were pre-treated for 30' at 4°C with blocking anti-ICAM-1, anti–ICAM-2, or no antibody and were subsequently co-cultured with Mart-1-pulsed, PKH67-labeled mDC for 2h (left panel). Mart-1-pulsed, PKH67-labeled mDC were pretreated for 30min at 4°C with either anti-PD-L1, anti-CD80, anti-CD86, anti-CD209, or no blocking antibody and subsequently co-cultured with Mart-1-specific $CD8^+$ T cells for 2h (right panel). $CD8^+$ T cells were analyzed for acquisition of membrane fragments and PD-L1 (black solid line). Negative or isotype controls are presented as filled histograms. Histograms are gated on $CD8^+PKH67^{low}$ lymphocytes. Shown is one representative of two independent experiments.

3.9.3. Trogocytosis is not impaired by inhibitors of stability of the immunological synapse

Myosin IIA is described to play a role in the stability of the immunological synapse as well as in TCR signaling (Limouze, Straight et al. 2004; Ilani, Vasiliver-Shamis et al. 2009). The formation of cSMAC and pSMAC which can be impaired by pretreatment of T cells with blebbisatin may be important for induction of trogocytosis in human T cells. To address this point, Mart-1-specific T cells were treated with 50µM blebbistatin and co-

cultured with Mart-1-pulsed, PKH67-labeled mDC. Despite the blebbistatin treatment, trogocytosis was not impaired as shown in Figure 27A, thus organization of the immunological synapse is neither important for the transfer nor for the up-regulation of activation markers CD25 and CD137 after triggered TCR signaling (see Figure 27B).

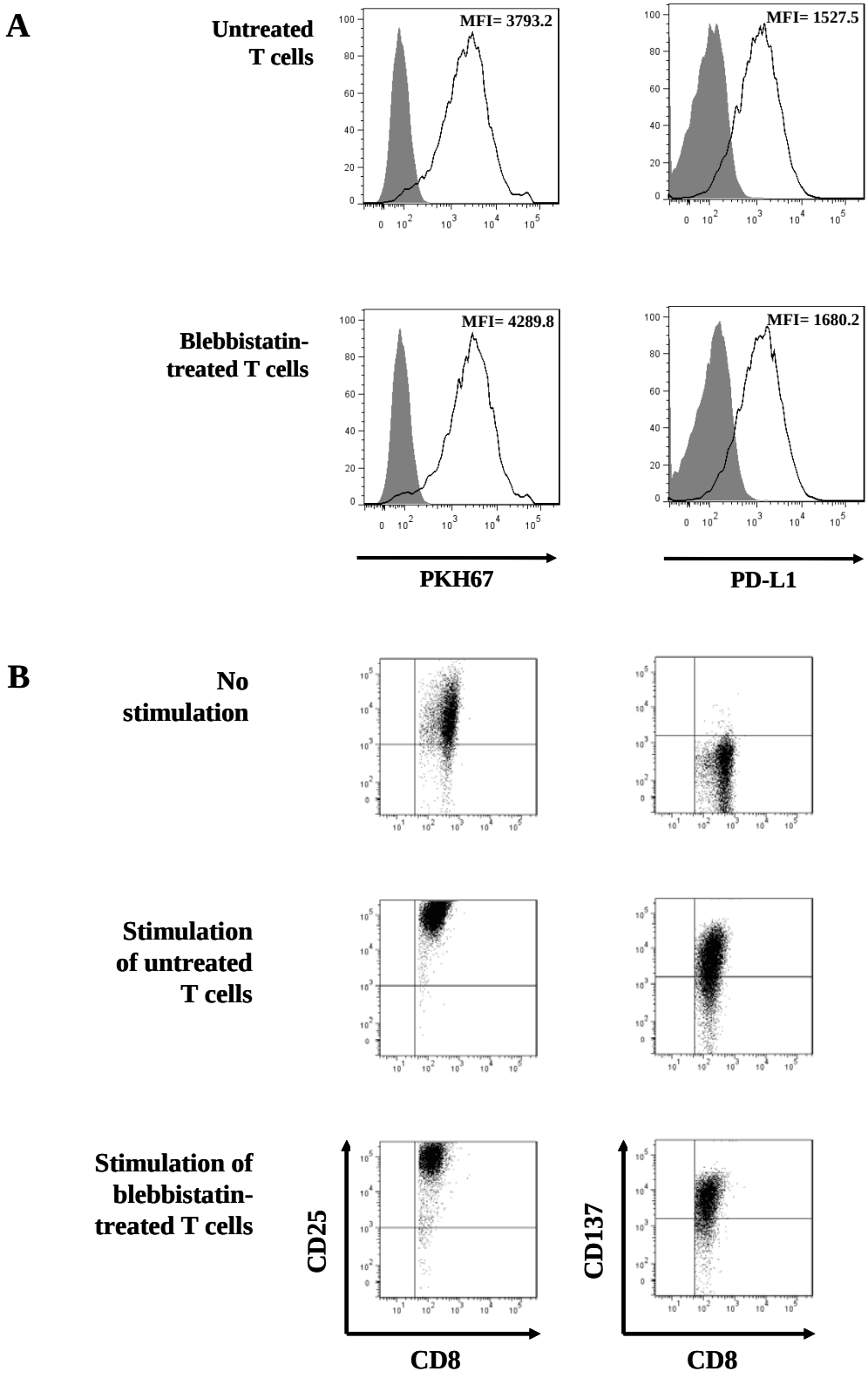


Figure 27: Trogocytosis by T cells is not impaired by prevention of cSMAC and pSMAC formation. T cells were pretreated with blebbistatin, a myosin IIA inhibitor. (A) After 2h of co-culture with antigen-pulsed, PKH67-labeled mDC, blebbistatin-treated $CD8^+$ T cells were analyzed for acquisition of membrane patches and PD-L1 compared to control T cells by flow cytometry (black solid line). Negative or isotype controls are presented as filled histograms. (B) The activation of T cells was measured by CD25 and CD137 expression after 22h of co-culture. Histograms and dot plots are gated on $CD8^+PKH67^{low}$ lymphocytes. Shown is one representative of three independent experiments with similar results.

3.9.4. TCR signaling has no influence on trogocytosis by T cells

Lck signaling is described to augment the transfer of MICA from target cells onto NK cells (McCann, Eissmann et al. 2007). Hence, $CD8^+$ T cells were pre-treated with an inhibitor of Lck, a src family kinase playing a pivotal role in TCR signaling, and analyzed for trogocytosis onto antigen-specific $CD8^+$ T cells. In contrast to the transfer of MICA, no decrease in acquisition of membrane patches, PD-L1, and CD209 was observed (see Figure 28).

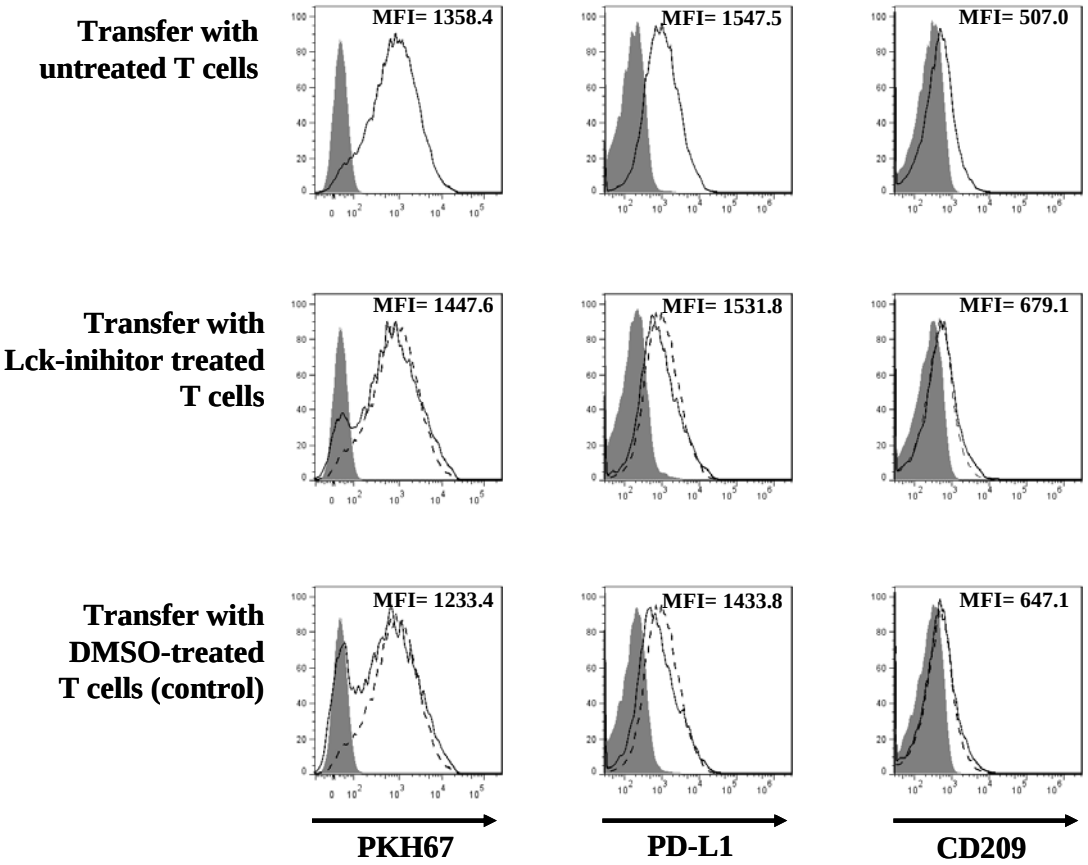


Figure 28: Trogocytosis is not impaired by inhibition of Lck. T cells were pre-incubated with 100µM Lck inhibitor or an appropriate amount of DMSO. Lck inhibitor or DMSO was washed out before trogocytosis assay with antigen-pulsed, PKH67-labeled mDC. After 2h, $CD8^+$ T cells were analyzed for the uptake of membrane fragments, PD-L1 and CD209 (black solid line). Antigen-specific transfer is shown as dashed line. Negative or isotype controls are presented as filled histograms. Histograms are gated on $CD8^+PKH67^{low}$ lymphocytes. Shown is one representative of three independent experiments.

3.9.5. vATPases in T cells play an important role in trogocytosis

vATPases were described to play a role in membrane trafficking as well as endo- and exocytosis (Marshansky and Futai 2008). It was determined whether concanamycin A (CMA), a specific inhibitor of vATPases, is able to abrogate antigen-specific trogocytosis (Huss and Wieczorek 2009). To address this question, T cells, DC, or both cell populations were exposed to CMA before co-culture and $CD8^+$ T cells were analyzed for trogocytosis. Inhibition of vATPases in mDC resulted in a slightly reduced acquisition of membrane patches, PD-L1 and CD209 by T cells. Moreover, CMA-treatment of T cells strongly inhibited the transfer of PD-L1 (13.1% + 3.8% MFI of maximal transfer compared to 4.5% + 1.2 % background MFI, $p<0.001$) and CD209 (40.0% + 30.5% MFI of maximal transfer compared to 29.0% + 19.9 % background MFI; ns) as well as the transfer of membrane fragments (28.8% + 8.0% MFI of maximal transfer compared to 6.3% + 3.3 % background MFI; $p<0.001$) (see Figure 29). Furthermore, combined exposure of T cells and DC to CMA resulted in a complete abrogation of PD-L1 and CD209 transfer. Of interest, inhibition of trogocytosis in CMA-pretreated T cells had no impact on the up-regulation of activation marker such as CD25 and CD137 (see Figure 30A). These results show that the inhibition of trogocytosis does not impair T-cell activation although pre-activation of T cells favours increased transfer capacity (see Figure 17). Moreover, degranulation marker CD107a, which is often used as cytotoxic marker for the degranulation of granzyme and perforin, was also up-regulated (Rubio, Stuge et al. 2003) showing that CD107a is not a selective marker for cytotoxicity of T cells (see Figure 30B).

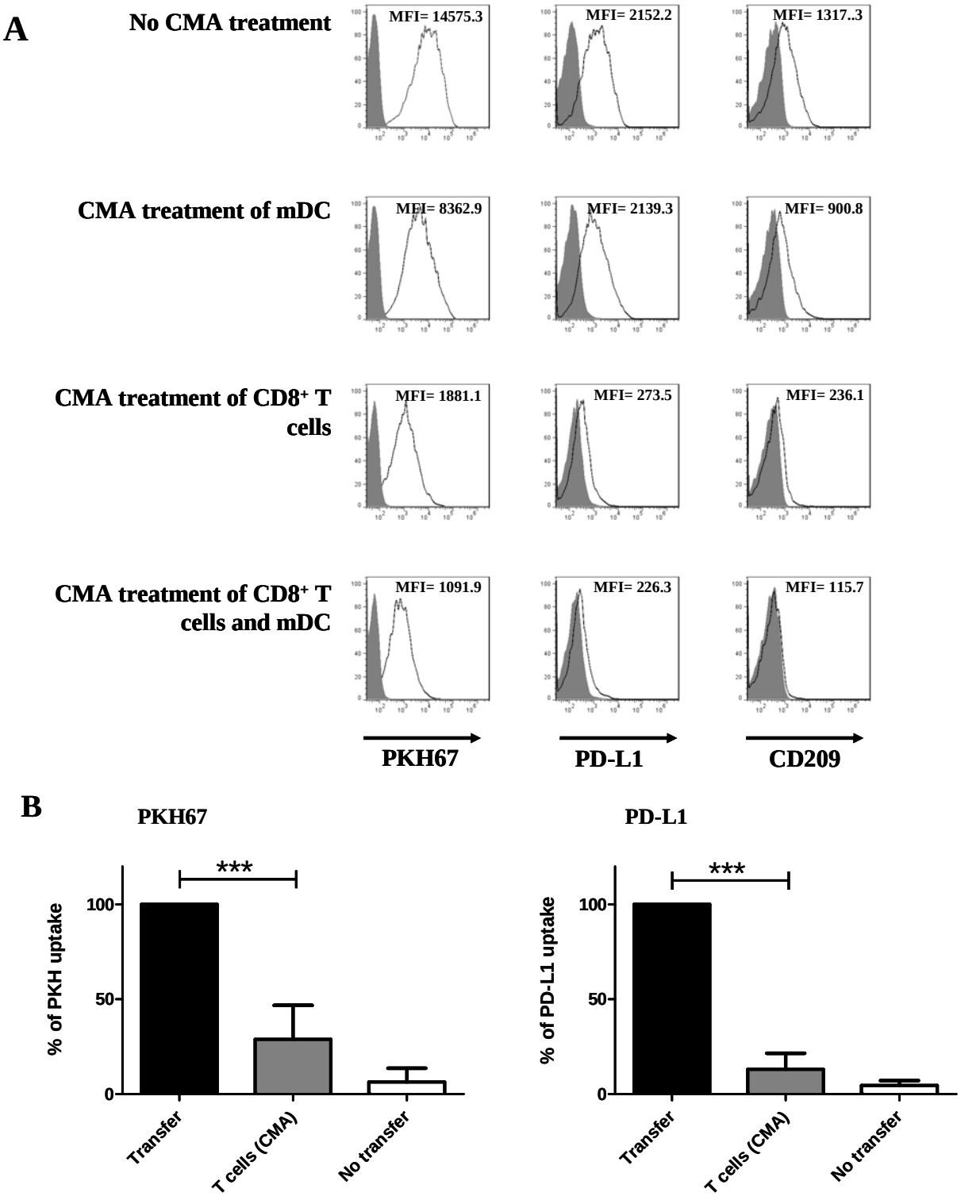


Figure 29: Impairment of trogocytosis of membrane fragments, PD-L1 and CD209 by vATPase-inhibitor Concancamycin A (CMA) pretreatment of T cells and mDC. $CD8^+$ Mart-1-specific T cells and/or mDC were pretreated overnight with CMA which was washed out before co-culture. mDC were pulsed with Mart-1 peptide and labeled with PKH67. (A) After co-culture of 2h, uptake of membrane fragments, PD-L1 and CD209 was analyzed by flow cytometry (black solid line). Negative or isotype controls are presented

as filled histograms. Histograms are gated on $CD8^{+}PKH67^{low}$ lymphocytes. Shown is one representative of three independent experiments with similar results. (B) In bar graphs, MFI data of antigen-specific transfer was set to 100% as maximum transfer. Inhibited transfer of co-culture of mDC with CMA-pretreated T cells and no transfer are shown as percentage of maximum uptake of membrane fragments and PD-L1 of four independent experiments ± SD (*** $p<0.001$, paired *t* test).

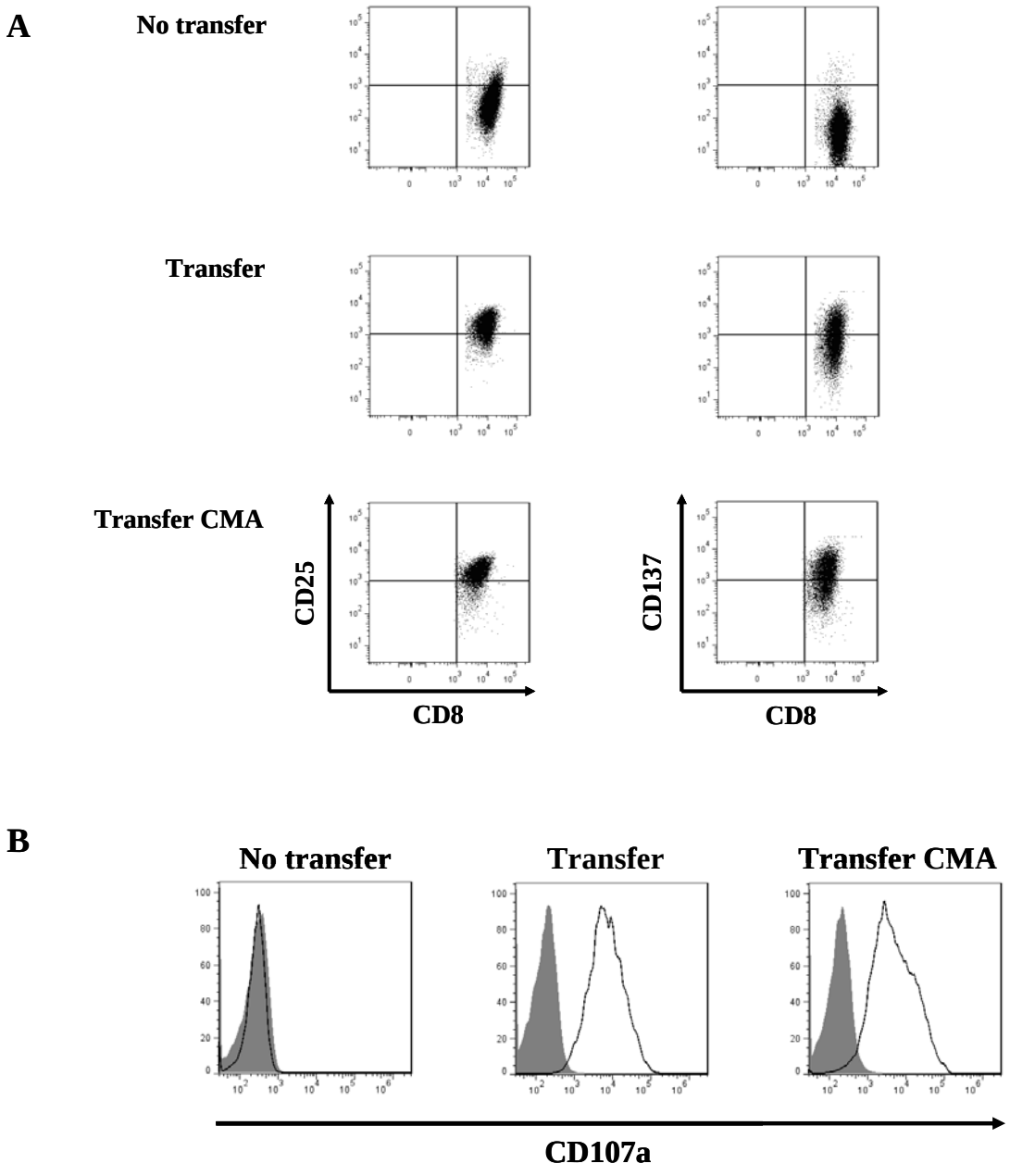


Figure 30: T cell activation despite CMA pretreatment after co-culture with mDC. Mart-1 specific T cells with or without CMA pretreatment were co-cultured with Mart-1-pulsed, PKH67-labeled mDC. Expression of activation markers CD25 and CD137 after 22h (A) as well as of degranulation marker CD107a (black solid line) after 2h (B) was analyzed by flow cytometry. Isotype controls are presented as filled

histograms Dot Plots and histograms are gated on $CD8^+PKH67^{low}$ lymphocytes. Shown is one representative of three independent experiments with similar results.

3.9.6. Inhibition of the transfer by blocking of the intracellular protein transport

Perturbation of the Golgi apparatus comes along with effects as blockade of secretion as well as disturbance of vesicular transport. Monensin is described as carboxylic ionophore binding Na^+/H^+, cations which are important in many physiologic processes (Dinter and Berger 1998). Monensin was added to the co-culture of Mart-1-specific $CD8^+$ T cells and Mart-1-pulsed, PKH67-labeled mDC. Monensin treatment was observed to cause decreased uptake of membrane patches (MFI of 40.2 compared to 100.6 without inhibition) and CD209 (MFI of 54.5 compared to 113.0 without inhibition) (see Figure 31).

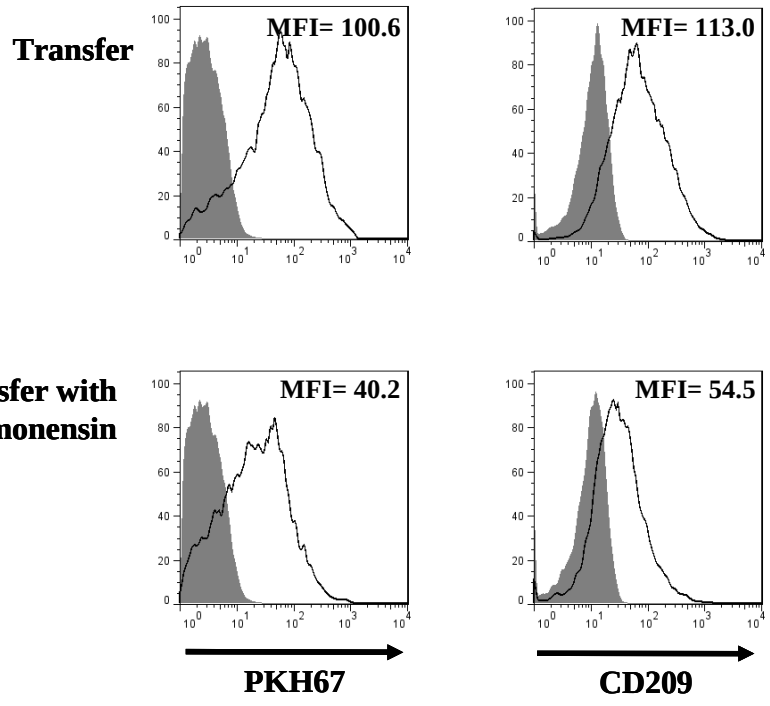


Figure 31: Blocking of intracellular protein transport by monensin impairs trogocytosis. Mart-1-specific T cells were co-cultured with Mart-1-pulsed, PKH67-labeled mDC with or without addition of Monensin, applied by 0.67µL/mL GolgiStop. After 2h, acquisition of PKH67 and CD209 was analyzed by flow cytometry (black solid line). Negative or isotype controls are presented as filled histograms. Histograms

are gated on $CD8^+PKH67^{low}$ lymphocytes. Shown is one representative of three independent experiments with similar results.

3.10. Functionality of transferred PD-L1 on T cells

In order to investigate whether acquired PD-L1 on human $CD8^+$ T cells is functionally active and inhibits PD-1 expressing neighboring T cells, the following set-up was generated as depicted in Figure 32.

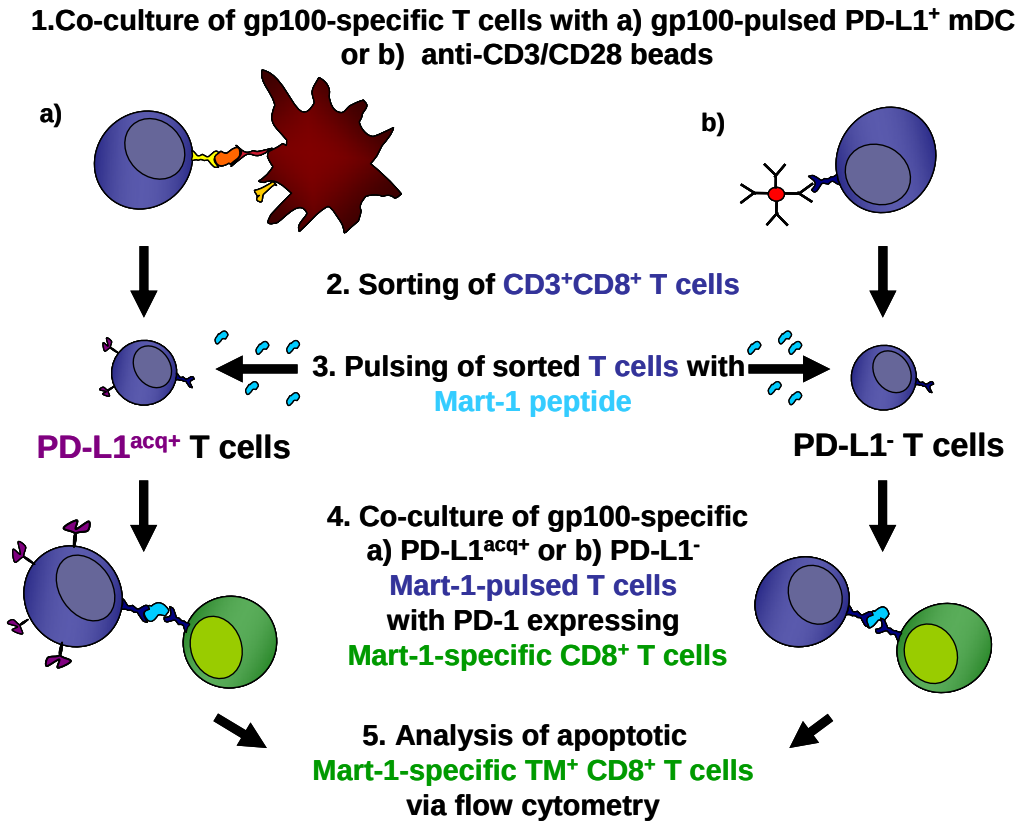


Figure 32: Experimental set-up. The graphic shows the procedure for functional analysis of acquired PD-L1: T cells of a gp100-sepcific T cell clone were co-cultured either with gp100-pulsed mDC or anti-CD3/CD28 beads for 2h. Then, $CD3^+CD8^+$ T cells were sorted out of both co-cultures, the one population showing a $PD\text{-}L1^+$ (gp100(mDC)$PD\text{-}L1^{acq+}$), the other population a $PD\text{-}L1^-$ (gp100(beads)$PD\text{-}L1^-$) phenotype. These sorted T cells were pulsed with Mart-1 peptide. Next, either gp100(mDC)$PD\text{-}L1^{acq+}$ or gp100(beads)$PD\text{-}L1^-$ sorted T cells were co-cultured with expanded, cytotoxic, Mart-1-specific T cells (CTL)

expressing PD-1. After 6h, apoptosis rate was analyzed by Annexin V and 7-AAD staining in $CD3^+CD8^+$ Mart-1 TM^+ T cells via flow cytometry.

First, a gp100-specific T-cell clone was stimulated with gp100 peptide-pulsed mDC (PD-$L1^{acq+}$) or with anti-CD3/CD28 beads (PD-$L1^-$). Next, $CD3^+CD8^+$ cells were sorted and subsequently pulsed with Mart-1 peptide to ensure interaction with Mart-1-specific neighboring T cells. Expression of acquired PD-L1 on gp100-specific T cells remained stable during the experimental procedure (see Figure 33). Of note, PD-L1 was not up-regulated on T cells after anti-CD3/CD28 bead stimulation. Next, sorted and Mart-1-pulsed gp100-specific T cells were used as APC and co-cultured with Mart-1-specific, PD-1 expressing T cells (data not shown). Of importance, Annexin V and 7-AAD staining of PD-1^+, Mart-1-specific T cells that were co-cultured with PD-L1 acquiring gp100-specific T cells revealed an increase in apoptotic cells of 28% compared to 12% in the control setting (co-culture with PD-$L1^-$ gp100-specific T cells) ($p=0.0239$) (see Figure 34). To confirm these findings, a blocking anti-PD-L1 mAb was added to the co-culture resulting in a significant reduction of apoptosis to 15.3% ($p=0.0214$). Together, these data clearly emphasize that acquired PD-L1 on antigen-specific T cells is functionally and can interact with its receptor PD-1 on neighboring T cells to induce apoptosis.

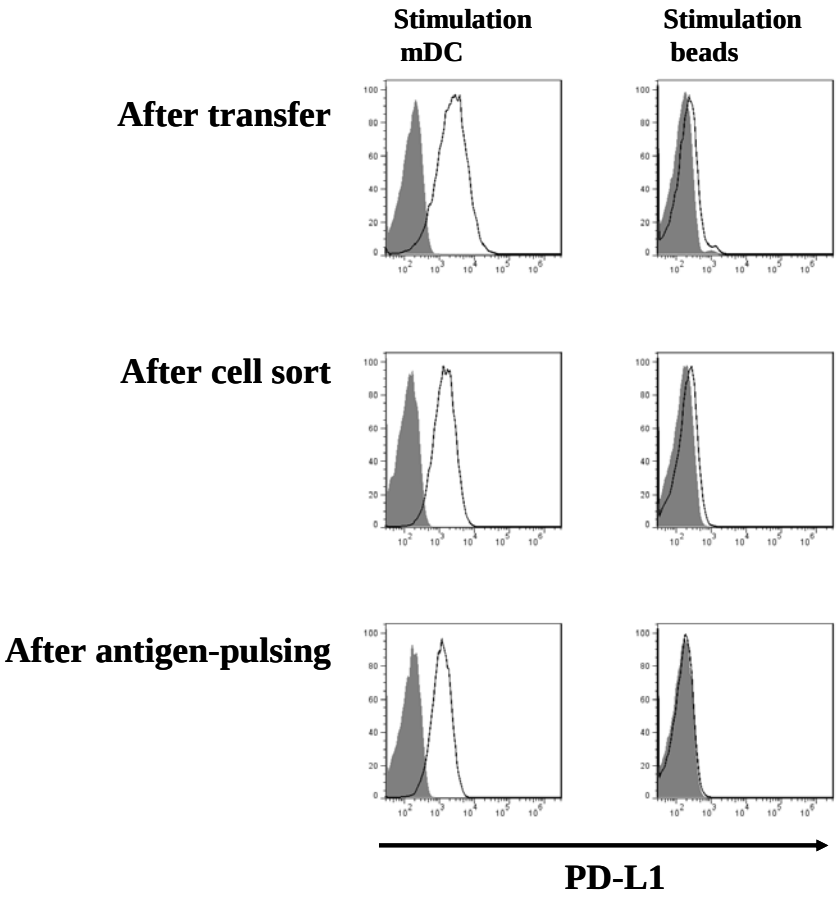


Figure 33: PD-L1 maintenance of gp100(mDC)PD-L1^{acq+} T cells during experimental procedures. Gp100-specific CD8$^+$ T-cell populations either stimulated with mDC or anti-CD3/CD28 beads were monitored for PD-L1 after stimulation, after cell sort and after antigen-pulsing (black solid line). Isotype controls are presented as filled histograms. Histograms are gated on CD8$^+$ lymphocytes. Shown is one representative of three independent experiments.

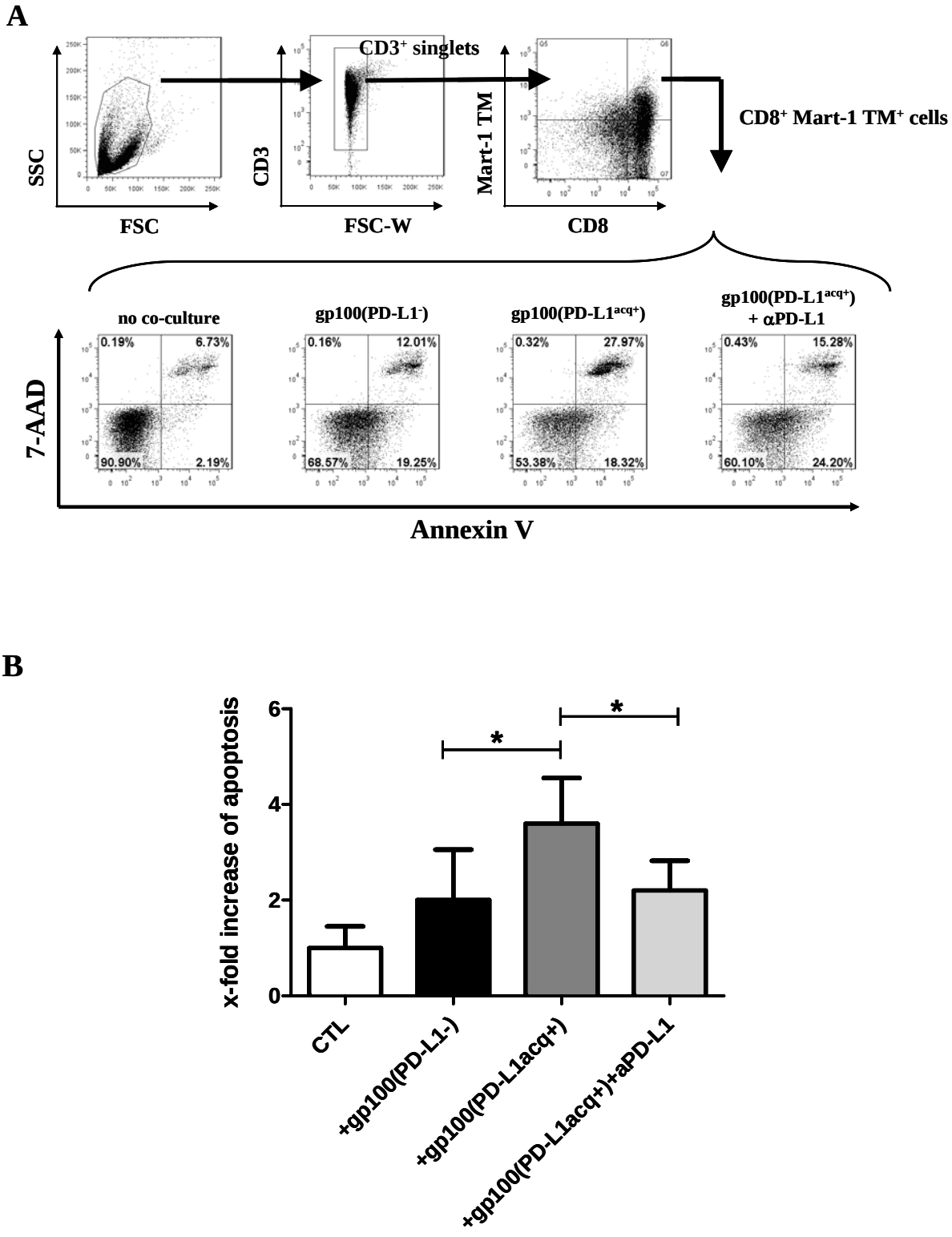


Figure 34: PD-L1 triggering apoptosis in neighboring T cells. After 6h co-culture of Mart-1-specific CTL with either gp100 PD-L1^{acq+} T cells with or without anti-PD-L1 blocking antibody (αPD-L1), or gp100 PD-L1^{-} T cells, CD3^{+}CD8^{+} Mart-1 TM^{+} CTL were analyzed for 7-AAD and Annexin V double positive cells. Shown are dot plots from one representative of three independent experiments with similar results. Bar graph shows the x-fold increase of percental apoptosis rate compared to unstimulated CD3^{+}CD8^{+} Mart-1 TM^{+} CTL with data of three independent experiments ± SD (* $p<0.05$ paired *t* test).

4. Discussion

In this study, the acquisition of PD-L1 and CD209 by human $CD8^+$ T cells from DC is demonstrated and characterized for the first time. Moreover, these data emphasize a potential new mechanism of immune regulation by acquisition of co-inhibitory molecules such as PD-L1 by $CD8^+$ T cells from both DC and melanoma cells.

Trogocytosis is described as intercellular transfer of membrane fragments. This study shows that human $CD8^+$ T cells acquire both lipid components and proteins during this process. Although many fragments are acquired out of the plasma membrane, it is not a random transfer of molecules: mDC highly express CD80, CD83, CD86, PD-L1, CD209 as well as PD-L2, but antigen-specific $CD8^+$ T cells only acquire PD-L1, CD209 as well as CD83 upon antigen-recognition on mDC. Of note, no increase of surface CD80 and CD86 can be observed in our antigen-specific human model although trogocytosis of CD80 by murine T cells has been demonstrated in other reports (Sabzevari, Kantor et al. 2001; Mostbock, Catalfamo et al. 2007). Thus, a so far unknown mechanism on DC or T cells must determine which molecules are transferred and which are not.
The structure of molecules and their anchorage in the plasma membrane may be one important feature for the exchange of molecules. Comparing the cytoplasmic parts of listed molecules, PD-L1, CD83, CD80, and CD86 are all described to have short cytoplasmic tails (Peach, Bajorath et al. 1995; Zhou and Tedder 1995; Keir, Butte et al. 2008). Just CD209 is reported to have an intracellular domain for signaling and to have internalization motifs. In the case of PD-L2, two forms are described to exist, namely one variant on the plasma surface and another located intracellular (He, Liu et al. 2004). However, the transfer of PD-L2 could not be detected at all. Despite a similar location and structure in the plasma membrane, PD-L1 is transferred very efficiently whereas no acquisition of CD80 and CD86 is documented on antigen-specific T cells. This shows that the cytoplasmic tail is not critical for the outcome in trogocytosis. In regard to CD83, a soluble form was observed which is shed from the surface of CD83 expressing cells (Hock, Kato et al. 2001; Lechmann, Berchtold et al. 2002). Thus, the detected CD83 on the surface of $CD8^+$ T cells may also result from the uptake of soluble CD83 and is not proved to be acquired by trogocytosis. In addition to these surface molecules, also cytoplasmic expressed GFP is transferred in detectable amounts in an antigen-specific manner from mDC onto T cells.

This is in accordance to the described transfer of intracellular protein H-Ras from B cells to human T and NK lymphocytes (Rechavi, Goldstein et al. 2007). Daubeuf *et al*. propose that proteins present in the internal leaflet exhibit a higher mobility than their external leaflet counter-parts and are therefore transferred more efficiently (Daubeuf, Aucher et al.). Putting the focus on the inner leaflet, it has been described that phosphatidylserine (PS) at the inner leaflet of the plasma-membrane of T cells is exposed to the outer cell upon activation showing the flexibility of the inner leaflet and its exposure to the outside (Fischer, Voelkl et al. 2006). Of note, blocking the PS exposure as well as the inhibition of trogocytosis is not accompanied by an impaired up-regulation of activation markers as shown in this study for reduced transfer of PD-L1 and CD209 by Concanamycin A or by fixation of APC. Interestingly, this PS exposure is happening very quickly providing similar kinetics as the transfer process and is probably engaged in the cell-to-cell contact formation. Thus, PS is proposed by Fischer *et al.* to contribute significantly to events at the IS. Moreover, a role of exposed PS was described in the adhesion and migration processes of murine $CD4^+$ T cells (Elliott, Surprenant et al. 2005). Furthermore, PS-receptors may be important in the up-take of microvesicles (Miyanishi, Tada et al. 2007). Regarding the common features of these two antigen-triggered processes, PS exposure may also play a role in trogocytosis.

Besides $CD8^+$ T cells, $CD4^+$ T cells as well as DN T cells acquire CD209 and membrane fragments to a similar extend after antigen-recognition. Human DN T cells have already been described by our group to acquire HLA molecules in an antigen-independent manner (Fischer, Voelkl et al. 2005). Moreover, the kinetics indicated that the mechanism of capture may be different, because of the increased amount of acquired molecules after 72 hours whereas membrane fragments, PD-L1 and CD209 in the antigen-specific setting disappeared completely from the cell surface after this period of time. In contrast, the acquisition of allogeneic MHC molecules by murine DN T cells has been demonstrated to be antigen-specific showing similar kinetics as the acquisition of HLA molecules by human DN T cells. These data suggest that different mechanisms for a cell-to-cell contact dependent transfer do exist (Ford McIntyre, Young et al. 2008). Moreover, the different T-cell subsets revealed a similar accessibility in the uptake of membrane fragments and CD209 in the case of antigen-specific acquisition whereas human DN T cells clearly acquired much more HLA molecules than $CD8^+$ T cells in the case of antigen-independent transfer of HLA-A2. It has been shown for murine $CD4^+$ and $CD8^+$ T cells that they acquire similar amounts of surface molecules after antigen-recognition as this study observes for

human trogocytosis (Daubeuf, Aucher et al.). Taken together, antigen-specific trogocytosis does occur in different T-cell subsets to a similar extent, but antigen-specific and non-specific transfer of surface molecules seem to underlie to different mechanisms.

Immune cells are interacting and networking with each other. In recent studies many cells have been described to transfer molecules between different immune and/or target cells (LeMaoult, Caumartin et al. 2007; Quah, Barlow et al. 2008; Caumartin, Favier et al. 2007; Beum, Mack et al. 2008). However, $CD8^+$ T cells acquire low amounts of membrane fragments from B cells, NK cells, and monocytes, but high amounts of membrane fragments from iDC and mDC. This is the first report comparing the efficiency of different donor cells to transfer molecules onto human T cells. This efficient trogocytosis process from DC may be due to the expression of co-stimulatory molecules. Moreover, iDC and mDC as professional APC present more pMHC complexes to T cells what also favors trogocytosis as described by Hudrisier *et al*. (Hudrisier, Riond et al. 2005). Of interest, membrane fragments and surface molecules PD-L1 and CD209 are even not equally acquired from iDC and mDC which may be connected to the higher expression of co-stimulatory molecules on mDC than on iDC mediating a stronger interaction with T cells and therefore triggering trogocytosis. Of note, CD209 is expressed at similar levels on the surface of iDC and mDC but was hardly detectable on T cells after co-culture with iDC suggesting that the molecule densitiy on the surface is not crucial for the efficiency of trogocytosis. However, Hudrisier *et al*. have shown that levels of TCR engagement correlate with the proportion of antigenic complexes amongst the plasma membrane materials captured by CTL from target cells via trogocytosis (Hudrisier, Riond et al. 2005). Thereby, DC presenting more pMHC complexes trigger trogocytosis more efficiently than monocytes, B cells and NK cells. Conform to the hypothesis that co-stimulatory molecules are important for the efficiency of the transfer, trogocytosis from melanoma cells missing co-stimulatory molecules is weaker than this from mDC. Only low amounts of highly expressed MCSP and PD-L1 are transferred from melanoma cell lines onto antigen-specific T cells.

TCR-dependency has been described as a trigger for the trogocytosis process (Huang, Yang et al. 1999; Hudrisier, Riond et al. 2001). In line with these reports, this study demonstrates that antigen-recognition by TCR is also essential for the transfer of PD-L1 and CD209 surface molecules. Thereby, the enduring strong cell-to-cell interactions as well as changes in the membrane dynamics may play a role for antigen-dependent triggering of trogocytosis. Of note, PD-L1 is also described to be expressed upon activation on human T

cells but expression of mRNA and translation into protein take much longer than PD-L1 acquisition from antigen-presenting cells via trogocytosis. Trogocytosis has been described as a phenomenon of very fast exchange of cell components compared to exosomes and nanotubes which correlates with the kinetics of the uptake of membrane patches, PD-L1, and CD209 in this study (Davis 2007). Moreover, surface-PD-L1 was neither observed after co-culture with antigen-pulsed PD-L1$^-$ T2 cells nor after stimulation with anti-CD3/CD28 beads demonstrating that PD-L1 is actually not endogenously up-regulated on T cells after antigen-specific activation with APC in this time frame. In summary, trogocytosis renders CD8$^+$ T cells immediately but just temporary to a cell with newly acquired features as kinetics show that acquired membrane patches and surface molecules completely disappear within 72 hours.

Cell-to-cell contact is one important requirement for the transfer of membrane fragments and surface molecules such as PD-L1 and CD209 onto T cells what is also reported for the uptake of HLA-G by human T cells (LeMaoult, Caumartin et al. 2007). Thus, trogocytosis is mediated by uptake of floating cell fragments after lysis of target cells but a targeted transfer triggered by antigen-recognition. Together, PD-L1 and CD209 transfer matches the characteristics of trogocytosis: the transfer is 1) very fast, 2) requires cell-to-cell contact, and 3) is triggered by antigen-recognition. Nevertheless, despite the transwell system indicating a cell-to-cell contact dependent mechanism, the involvement of membrane vesicles can not be completely excluded since vesicles have also been described to be directly transferred upon close cell-to-cell contact (Muratori, Cavallin et al. 2009). Of note, bystander T cells are not triggered by neighboring T cells to acquire surface molecules such as PD-L1 and CD209 demonstrating that trogocytosis is a targeted process that is not cross-transferred to non-reactive bystander T cells. Furthermore, these data emphasize that T cells do not capture exosomes released by activated DC. Therefore, trogocytosis can be defined as selective process to orchestrate immune responses supporting the hypothesis of HLA-matched trogocytosis by Machlenkin *et al.* (Machlenkin, Uzana et al. 2008).

The mechanisms triggering antigen-dependent trogocytosis on T cells are unknown. The immunological synapse is an important feature in T-cell activation. Candidate molecules are either adhesion molecules that are essential for close interaction between effector T cells and target cells or co-receptors as well as co-stimulatory molecules in triggering the TCR signal cascade. Blocking of the CD8 co-receptor actually impaired the transfer of membrane fragments and CD209. This observation confirms the study of Hudrisier *et al*. showing an impaired transfer of membrane fragments after blocking of CD8 on murine

$CD8^+$ T cells (Hudrisier, Riond et al. 2001). Blocking adhesion molecules as CD209, ICAM-1, and ICAM-2 as well as co-stimulatory molecules as CD80 and CD86, but also blocking of the target molecules PD-L1 and CD209 had no effect on the trogocytosis process. This may explained by the fact that one adhesion molecule or co-stimulatory molecule can be replaced by others, resulting in sustained stimulation and trogocytosis. In contrast, binding of the CD8 co-receptor is crucial for T-cell activation and for trogocytosis.

Besides this, the arrangement in and around the immunological synapse (IS) is dependent on membrane organization and may be critical for the determination which molecules are transferred or not. However, the formation of the IS endures about 30 minutes when trogocytosis is already going on and therefore the organization of the molecules in the immunological synapse does not seem to play a role for the choice of acquired molecules (Anton van der Merwe, Davis et al. 2000). As far as molecules belonging to both the cSMAC and the outer region of the IS are transferred, e.g. pMHC complexes and PD-L1, the placing in or outside the IS can also not explain which molecules are preferentially acquired by T cells. Moreover, pretreatment of T cells with blebbistatin was described to lead to a more peripheral distribution of these interactions because of an impaired transport of the molecule complexes toward the centre to arrange an IS (Ilani, Vasiliver-Shamis et al. 2009). This arrangement is also not critical to trigger trogocytosis because T cells pretreated with blebbistatin acquire similar amounts of membrane fragments and PD-L1 as untreated T cells. This demonstrates that the localization of molecules is not relevant for the acquisition from the target cell. Having a closer look onto T cell activation, the src kinases are important factors downstream the TCR signaling cascade (Salmond, Filby et al. 2009). Although the blocking of scr kinases was described by Hudrisier *et al*. to impair the uptake of membrane fragments, this could not be confirmed for the acquisition of membrane fragments, PD-L1 and CD209 by highly affine antigen-specific T cells in this study proposing that the first steps of T-cell activation as antigen-recognition and ligation of co-receptors are the more critical items in triggering trogocytosis (Hudrisier, Riond et al. 2001).

In addition, this study demonstrates that both, inhibition of vATPases and fixation of APC, strongly impair trogocytosis suggesting that the trogocytosis process is controlled by membrane dynamics as it was also shown by the dependence on an active actin cytoskeleton for trogocytosis by T cells (Hudrisier, Aucher et al. 2007). vATPases are desribed to play a role in endo-, exocytosis, and membrane trafficking (reviewed in

(Marshansky and Futai 2008)). Of interest, impairment of vATPases in mDC has only a minor impact on the acquisition of cell components from mDC by T cells whereas impairment of vATPases in T cells results in a very strong inhibition of the trogocytosis process. These data indicate that vATPases play a role in the integration of disrupted molecules from neighboring cells into the plasma-membrane. While the transfer process is abolished when vATPases are impaired, T cells became nevertheless activated measured by up-regulation of CD25 and CD137. These results clearly demonstrate that activation is not linked to trogocytosis. Furthermore, CD107a, an antigen described as T-cell degranulation marker, is up-regulated despite inhibited perforin and granzyme B secretion (Togashi, Kataoka et al. 1997; Betts, Brenchley et al. 2003). Moreover, fixation of mDC completely abrogates the transfer process suggesting that the surface of DC must not be static to enable acquisition by T cells but has to be dymamic. Up-regulation of activation markers such as CD25 and CD137 on T cells demonstrates that pMHC-complexes are still functional despite fixation of APC. Although pre-activation triggers trogocytosis as it is also described by LeMaoult *et al.*, impairment of trogocytosis does not influence the up-regulation of activation markes on T cells proposing that trogocytosis does not play a role in the cascade of events of full T-cell stimulation (LeMaoult, Caumartin et al. 2007).

Intracellular trafficking inside the cell which acquired the molecules may be a critical point in trogocytosis as the transfer of membrane fragments and surface molecules is impaired after Monensin treatment. monensin is described to inhibit vesicle trafficking by lysosomes, to disturb the golgi apparatus and to inhibit the recycling out of early endosomes (Basu, Goldstein et al. 1981; Tartakoff 1983; Muro, Mateescu et al. 2006). Of interest, molecules are recycled from early endosomes, so-called recycling endosomes, to be presented again on the plasma membrane. One possible mechanism for re-presentation on the surface again is explained via a so-called kiss-and-run mechanism (Lencer and Blumberg 2005). Eventually, acquired molecules are not integrated directly from the outside into the plasma-membrane but after recycling in early endosomes to be integrated functionally active (Jovic, Sharma et al. 2010). The kinetics of the recycling process and functionally presentation of molecules on the surface is important to link it to trogocytosis. However, the processing by recycling endosomes could explain the functionally active integration of acquired molecules into the plasma membrane.

For a broader understanding of trogocytosis, further investigations could enlighten the role of the flipping events at the plasma membrane as well as of recycling endosomes in the transfer process what may elucidate the mechanism of this phenomenon. Binding studies

could prove the functionality of acquired CD209 showing if this molecule can still enable the contact formation with other T cells after the transfer process. Furthermore, it could be tested if different effector and memory T-cell subsets show a different behavior in the transfer of surface molecules to see whether T cells acquire molecules at a special state of differentiation.

Compared to other reports on trogocytosis focusing mostly on the transfer of stimulatory molecules, this is the first report of acquisition of a widespread inhibitory molecule. Although HLA-G was described to render NK and T cells temporary to regulatory cells after acquisition of this non-classical MHC class I molecule, HLA-G is characterized by limited polymorphism and a restricted expression to immuno-privileged sites such as fetal-maternal interface (Caumartin, Favier et al. 2007; LeMaoult, Caumartin et al. 2007; Carosella, Moreau et al. 2003). This study focuses on the transfer of PD-L1 whose receptor PD-1 is expressed on a wide variety of human immune cells indicating an extensive regulatory role of PD-L1 acquisition. Thus, interaction of T cells that have acquired PD-L1, with other PD-1 expressing cells as B cells and macrophages could further modulate immune responses (Keir, Butte et al. 2008). As far as PD-L1 is not only expressed by hematopoietic cells, also the interaction of T cells with non-hematopoietic PD-L1 expressing cells should be examined, e.g. vascular endothelial cells, epithelia, and cells at immune privileged sites including trophoblasts in the placenta and retinal pigment epithelial cells or neurons in the eye. At these anatomic sites it remains unclear whether T cells can also acquire molecules such as PD-L1 and spread their function to the environment (Francisco, Sage et al.). Besides this, the parallel transfer of CD209 which is exclusively expressed on DC is also described for the first time in this study. CD209 functions as adhesion molecule initiating first interaction between DC and T cells via binding of ICAM-3 as reviewed in Zhou, Chen et al. 2006, hence acquired CD209 on T cells may temporary support the interaction and cross-talk with neighboring T cells.

Of importance, this study shows that acquired PD-L1 is able to trigger apoptosis in neighboring PD-1 expressing T cells proposing an immune-regulatory function by silencing T-cell responses after antigen-clearance. Regarding the functional outcome of surface PD-L1, the higher expression of both, PD-L1 and especially of co-stimulatory molecules on mDC, keeps the balance in favor of priming and boosting T cells. A shift causing imbalance with a predominant PD-L1 expression compared to expression of costimulatory molecules on mDC results in an overweighting inhibitory signaling of PD-L1 as described

in chronic infections (Shen, Chen et al.). In case of PD-L1 acquisition by T cells, T cells lack co-stimulatory molecules causing the outbalanced signaling of PD-L1 which results in apoptosis of neighboring T cells. Thereby, an overshooting immune reaction could be avoided. PD-1 signaling is described to result in either anergy or T-cell deletion depending on the amount of antigen-level whereat low antigen-levels cause deletion and high antigen-levels end up in anergy (Goldberg, Maris et al. 2007; Tsushima, Yao et al. 2007; Parish and Kaech 2009).

Trogocytosis of pMHC complexes was described in mice and humans (Huang, Yang et al. 1999; Tsang, Chai et al. 2003; Fischer, Voelkl et al. 2005). Thus T cells presenting acquired pMHC-complexes besides adhesion molecule CD209 and inhibitory PD-L1 to neighboring T cells trigger apoptosis because of the low antigen-amounts after clearance of antigen-presenting DC and/or infected cells and can also be killed via fratricide as it was described for T cells acquiring pMHC complexes (Huang, Yang et al. 1999). The acquisition of PD-L1 by tumor infiltrating T cells may spread the inhibitory function to the tumor environment and may therefore serve as a tumor immune escape mechanism. Many melanoma cell lines are described to up-regulate PD-L1 upon IFN-γ exposition hence in the case of an inflammatory immune response (He, Wang et al. 2005).

In chronic infections, acquired PD-L1 may contribute to exhaustion of T-cell responses because of the long lasting pathogen burden when T cells are already silenced before the pathogen is cleared. Increased PD-1 expression levels on T cells are described for chronic infections as HBV promoting the exhaustion state of T cells which may end up in deletion of T cells triggered by acquired PD-L1. However, T cells with acquired PD-L1 may also interact with PD-1 expressing B cells, macrophages, or DC as mentioned above. Of interest, PD-1 signaling can not only silence TCR signaling but also BCR signaling (Agata, Kawasaki et al. 1996). Moreover, PD-1 signaling can inhibit DC and macrophage responses to microbes and is described to be up-regulated on macrophages and monocytes in case of sepsis pointing out the diversity of possible interactions of T cells after PD-L1 acquisition (Huang, Venet et al. 2009; Yao, Wang et al. 2009).

Considering the *in vivo* relevance, human plasmacytoid DC (PDC) are described to express high levels of PD-L1 in HIV infection and generally upon activation (Francisco, Sage et al. ; Cavaleiro, Baptista et al. 2009). Moreover, accumulation of PDC in skin is shown for infection with either human papilloma virus or human herpes simplex virus where T cells can interact with those PDC and acquire PD-L1 (Sozzani, Vermi et al.). Furthermore, T_{reg}-conditioned myeloid DC (MDC) cross-talk with T cells in the periphery where T cells also

could grab PD-L1 and spread a regulatory effect (Amarnath, Costanzo et al. 2010). Thus, T cells have the chance in the periphery to acquire molecules from DC as shown in this study *in vitro* and can subsequently interact with immune cells in the environment. The relevance of trogocytosis in lymph nodes remains to be discussed as T cells have to home to the site of infection after priming. This migration probably takes longer than acquired PD-L1 is present on the surface after trogocytosis so that no T cells are silenced at the primary site of infection after activation of naïve T cells in lymph nodes. Aside from this, the co-stimulatory signals in the lymph nodes should be predominant and strong enough to overcome the inhibitory signal of acquired PD-L1.

Looking from bench to bedside, this study emphasizes that transfer of PD-L1 could have a major impact on the down-regulation of immune-responses as displayed by the induction of apoptosis and could also play a role in the failure of immune responses in chronic diseases and tumors. The acquisition of PD-L1 in *ex vivo* expanded T cells should be considered in studies for adoptive cell transfer therapies, thus the administration of T cells should be carried out at the end of a stimulation cycle to avoid the transfer of highly PD-L1$^+$ T cells as it is already described that the blockade of PD-L1 augments human tumor-specific T cell responses (Blank, Kuball et al. 2006). Zhou *et al*. recently showed that interaction between PD-1 and PD-L1 can facilitate T_{reg}-induced suppression of exogenously administered CTL propagated from non-tumor bearing mice and can dampen the anti-tumor immune responses in advanced AML disease (Zhou, Munger et al.). Not only in tumors, but also in the treatment of chronic infections as HBV where specific T cells show an increased PD-1 expression, blocking PD-L1 can cause restoration of proliferation and cytokine production *ex vivo* (Fisicaro, Valdatta et al.). Besides the adoptive cell therapy with *ex vivo* expanded T cells, administration of PD-L1 expressing DC could spread PD-L1 by trogocytosis in the tumor patients or patients with chronic infections. In conclusion, a combinatorial treatment of both, transfer of immune cells and administration of PD-1 blocking antibodie, may be an interesting strategy to avoid widespread inhibitory PD-L1 effects that can also be mediated by trogocytosis.

PD-L1 not only plays a role in cancer and chronic infections but also in the tolerance induction in autoimmunity. Thus trogocytosis of PD-L1 may act as a further regulatory mechanism for the induction or maintenance of tolerance (Francisco, Sage et al.). On the one hand, PD-L1 is described as an important factor for the induction of iT_{regs} (Francisco, Salinas et al. 2009). On the other hand, adoptive transfer studies have identified critical functions for PD-L1 on transferred T cells mediating protective effects in the recipient

animal: Latchman *et al*. has shown in an EAE model that PD-L1 on T cells could limit responses of self-reactive $CD4^+$ T cells (Latchman, Liang et al. 2004). The critical role for the maintenance of tolerance in the intestine by the PD-1/PD-L1 interaction was demonstrated in a mouse model by Reynoso *et al*. (Reynoso, Elpek et al. 2009). Missing of PD-1/PD-L1 signaling resulted in severe enteric autoimmunity. Thus, the transfer of PD-L1 may also play a role in the maintenance of tolerance and may be crucial in the depression of self-reactive T cells. Taken together, trogocytosis of PD-L1 may be involved in the balance regulation between immunity and tolerance as proposed in Figure 35.

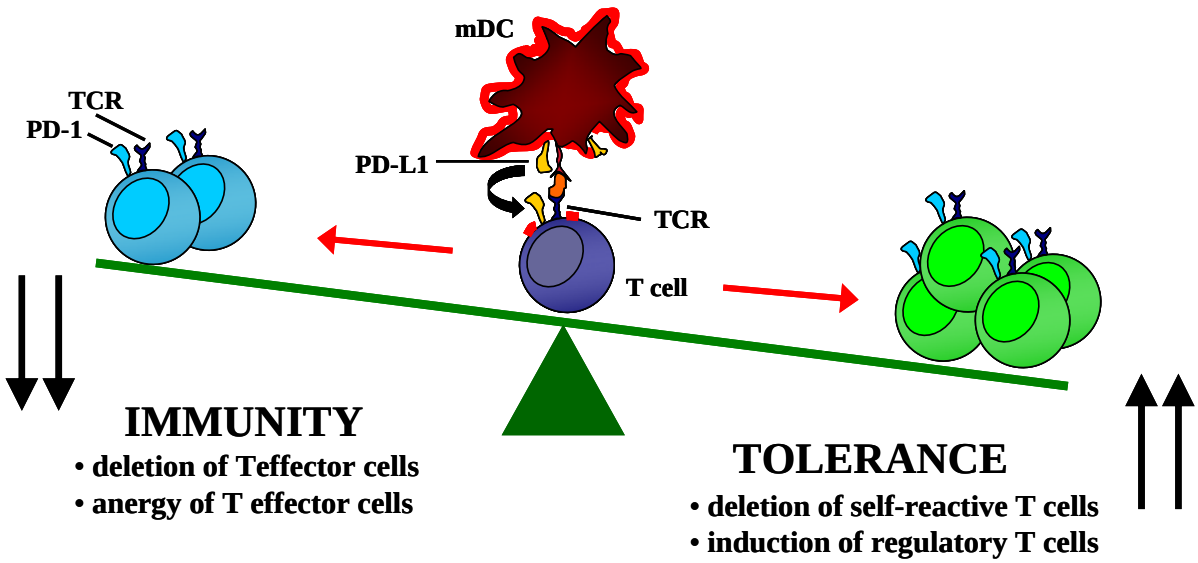


Figure 35: Potential consequences of acquired PD-L1. Acquired PD-L1 may result in inhibition of effector T-cell responses or may induce T-cell tolerance.

In summary, PD-L1 is a molecule with widespread functions in case of tolerance and immunity. It plays different roles in health, in chronic infections and tumors as well as in autoimmunity. This should emphasize the importance of this study about PD-L1 which is spread between cells via trogocytosis. To analyze the role of acquired PD-L1 *in vivo*, either an adoptive transfer mouse model in a tumor or chronic infection setting may give important insights into the physiologic role of transferred PD-L1. Tracking trogocytosis in a humanized mouse model by a GFP-linked PD-L1 construct may give further hints about the *in vivo* process. As far as PD-L1 can be both up-regulated upon stimulation and acquired by T cells via trogocytosis, PD-L1 could be blocked in the expansion of antigen-specific T cells to examine if there is a better expansion and if there are less anergic cells

resulting in better effector functions of expanded T cells as cytokine release and cytotoxicity.

Of interest, trogocytosis is described as a phenomenon carried out by many immune cells but also other kinds of cells. Such a widespread phenomenon is very probable to have an important role *in vivo* showing a high flexibility of the whole system and the possibility to confer temporary features between diverse cell populations. A profound understanding of the mechanism of trogocytosis and the physiologic relevance in immune-regulation may have important implications for novel immunotherapeutic strategies.

5. Summary

Specific T-cell responses are initiated by TCR recognition of pMHC-complexes on APC. Upon specific interaction of T cells with APC, T cells capture membrane fragments and surface molecules in a process termed trogocytosis. Exchange of membrane molecules/antigens between immune cells has been observed for a long time, but the mechanisms and functional consequences of these transfers remain uncertain. This study demonstrates that human antigen-specific $CD8^+$ T cells acquire the co-inhibitory molecule PD-L1 from mature monocyte-derived DC and tumor cells as well as the DC-specific adhesion molecule CD209 in an antigen-specific manner. Furthermore, also cytosolic proteins are transferred after antigen-recognition. Of note, not only $CD8^+$ T cells can acquire membrane fragments and surface molecules but also $CD4^+$ and DN T cells. Antigen-pulsed iDC are less effective in transferring surface molecules such as PD-L1 and CD209 onto $CD8^+$ T cells after antigen-specific recognition even though CD209 is expressed at similar levels on iDC and mDC. However, T cells interacting with monocytes, NK cells or B cells acquire only low amounts of membrane fragments. The recognition of melanoma cell lines gives rise to an efficient transfer of membrane molecules but also PD-L1 and MCSP are transferred onto T cells to a certain extend. The kinetics of the acquisition of membrane fragments, PD-L1 and CD209 reveal a maximal exposition of acquired cell components after about two hours after co-incubation with antigen-pulsed APC, being detectable up to 72 hours. Using a transwell system this study shows that the acquisition of membrane patches, PD-L1 and CD209 requires cell-to-cell contact. Furthermore, these cell components cannot be acquired by T cells from a lysate of mDC. Of importance, a parallel transfer onto antigen-unspecific bystander T cells can be excluded. Moreover, fixation of DC with glutaraldehyde completely abrogates the acquisition of PD-L1 and CD209 on T cells suggesting that an active interaction between APC and T cells is required for trogocytosis. The co-receptor CD8 seems to play an important role in this trogocytosis process since blockade of this co-receptor impairs the transfer of membrane fragments as well as of surface molecules. The formation of cSMAC and pSMAC impaired by the inhibition of Myosin IIA, is not critical for triggering trogocytosis. Furthermore, blocking of the src kinase lck has no impact on the transfer of membrane fragments, PD-L1, and CD209 excluding a crucial role of the downstream TCR

signal cascade in trogocytosis. The transfer process can be blocked after pretreatment of T cells with concanamycin A, a specific inhibitor of vacuolar ATPases, playing an important role in endo- and exocytosis, in the acidification of intracellular vesicles and in membrane trafficking. Monensin which is described to inhibit intracellular vesicle trafficking, to disturb the golgi apparatus as well as to inhibit the recycling out of early endosomes, is also capable of impairing trogocytosis, suggesting that intracellular vesicles may be of relevance for the transfer process. Moreover, an endogenous up-regulation of PD-L1 can be excluded as T-cell stimulation by antigen-pulsed PD-L1$^-$ T2 cells or by anti-CD3/CD28 beads does not result in surface expression of PD-L1. Of importance, CD8$^+$ T cells which acquired PD-L1 complexes are able to induce apoptosis in neighboring PD-1 expressing CD8$^+$ T cells that can be completely blocked by an anti-PD-L1 antibody. In summary this study demonstrates for the first time new aspects in the mechanistic regulation of trogocytosis and shows that human antigen-specific CD8$^+$ T cells take up functionally active PD-L1 from APC in an antigen-specific manner triggering apoptosis of PD-1 expressing T cells. The transfer of functionally active co-inhibitory molecules from APC onto human CD8$^+$ T cells may serve to limit clonal expansion of antigen-specific T-cell responses and to maintain tolerance by elimination of self-reactive T cells, but may also play a major role for T-cell exhaustion in chronic infection and tumor immunosurveillance.

Zusammenfassung

Spezifische T-Zell-Antworten werden über die Erkennung von Peptid-MHC-Komplexen auf Antigen-präsentierenden Zellen (APC) durch T-Zell-Rezeptoren (TCR) initiiert. Nach antigen-spezifischer Interaktion von T Zellen mit APC können T Zellen sowohl Membranfragmente als auch Oberflächenmoleküle von APC akquirieren, ein Vorgang, der als Trogozytose definiert wird. Der Austausch von Membranmolekülen und –antigenen wurde schon vor langer Zeit beschrieben. Die zugrunde liegenden Mechanismen sowie funktionelle Konsequenzen dieser Transfervorgänge bleiben bisher jedoch ungeklärt. Die Untersuchungen der vorliegenden Arbeit zeigen, dass humane antigen-spezifische $CD8^+$ T Zellen sowohl koinhibitorische PD-L1 Moleküle von reifen dendritischen Zellen (mDC) und auch Tumorzellen akquirieren können, aber auch dass das DC-spezifische Molekül CD209 bei der antigen-spezifischen Interaktion von T Zellen aufgenommen wird. Des Weiteren werden auch zytosolisch vorliegende Proteine nach Antigenerkennung auf die T Zelle übertragen. Bemerkenswerter Weise können neben den $CD8^+$ T Zellen auch $CD4^+$ und DN T Zellen Membranfragmente und Oberflächenmoleküle akquirieren. Antigen-beladene unreife DC (iDC) sind allerdings weniger effizient bei der Übertragung von den Oberflächenmolekülen PD-L1 und CD209 nach Antigen-Erkennung auf $CD8^+$ T Zellen als mDC, obwohl auf iDC und mDC eine vergleichbare CD209 Expression vorliegt. T Zellen, die dagegen mit Monozyten, NK Zellen oder B Zellen interagieren, akquirieren nur geringe Mengen an Membranfragmenten. Die Erkennung von Melanomzelllinien führt ebenfalls zu einer effizienten, antigen-spezifischen Übertragung von Membranfragmenten, aber auch PD-L1 und das Melanomantigen MCSP werden in detektierbareren Mengen auf T Zellen übertragen. Als ein limitierender Faktor ist die begrenzte Aufnahme von Zellkomponenten bei einer überwiegenden Anzahl an APC im Vergleich zu T Zellen zu nennen: ab einem gewissen Verhältnis von Zielzellen zu Effektorzellen erreicht der Transfervorgang eine Sättigung und es können nicht noch mehr Membranfragmente oder Oberflächenmoleküle aufgenommen werden. Die Kinetik der Übertragung von Membranfragmenten, PD-L1 und CD209 zeigt, dass nach einer zweistündigen Kokultur von T Zellen mit APC die maximale Menge an transferierten Zellkomponenten auf der T Zelle vorliegt. Diese auf der Zelloberfläche aufgenommenen Membranfragmente und –moleküle können jedoch bis zu 72 Stunden detektiert werden. In dieser Arbeit konnte außerdem mittels eines sogenannten Transwell-Systems gezeigt werden, dass die Übertragung von Membranfragmenten, PD-L1

und CD209 ein Zell-Zell-Kontakt abhängiger Prozess ist. Außerdem können diese Zellkomponenten nicht aus einem Pool von lysierten, antigen-beladenen mDC von T Zellen aufgenommen werden. Des Weiteren kann eine antigen-unspezifische Übertragung auf Bystander T Zellen ausgeschlossen werden. Die Fixierung der mDC mit einer Glutaraldehydlösung kann außerdem die Übertragung von PD-L1 und CD209 komplett unterbinden, was darauf hinweist, dass eine dynamische Interaktion zwischen T Zellen und APC beim Trogozytoseprozess vorausgesetzt werden muss. Der CD8 Korezeptor scheint eine wichtige Rolle bei der Trogozytose zu spielen, da dessen Blockade eine Beeinträchtigung der Übertragung von Membranfragmenten und Oberflächenmolekülen zur Folge hat. Es wurde beschrieben, dass die Inhibition von Myosin IIA die Bildung von cSMAC und pSMAC negativ beeinflusst, was jedoch nicht entscheidend für den Verlauf der Trogozytose zu sein scheint. Außerdem übt auch die Blockade der Src-Kinase Lck, die eine Rolle in der TCR-Signalkaskade spielt, keinen inhibitorischen Effekt auf die Übertragung von Membranefragmenten, PD-L1 und CD209 aus, was eine entscheidende Rolle in der TCR Signalkaskade im Ablauf der Trogozytose ausschließt. Dahingegen hat die Inhibierung von vATPasen, die eine wichtige Rolle bei sowohl bei Endo- und Exozytosevorgängen, bei der Ansäuerung von intrazellulären Vesikeln als auch in Membrandynamik spielen, durch die Vorbehandlung der T Zellen mit Concanamycin A einen stark beeinträchtigenden Effekt auf die Übertragung von Zellkomponenten per Trogozytose. Auch Monensin, welches zur Beeinträchtigung des intrazellulären Vesikelverkehrs führt, die Prozesse am Golgi Apparat stört als auch das Recycling aus frühen Endosomen inhibiert, reduziert den Transfer von Membranfragmenten und Oberflächenmolekülen. Eine endogene Hochregulation und Expression von PD-L1 kann ausgeschlossen werden, da T Zellen sowohl nach Stimulation mit antigen-beladenen, PD-L1$^-$ T2 Zellen als auch mit anti-CD3/CD28 Beads kein PD-L1 auf der T-Zelloberfläche exprimieren. Bemerkenswerterweise können CD8$^+$ T Zellen, welche das PD-L1 akquiriert haben, in benachbarten PD-1 exprimierenden T Zellen Apoptose auslösen. Dieser Effekt kann komplett durch Zugabe eines PD-L1 blockierenden Antikörpers verhindert werden. Zusammengefasst beschreibt die vorliegende Arbeit neue mechanistische Aspekte der Trogozytose beschreibt sowie die Übertragung von funktionell aktivem PD-L1 von APC auf humane CD8$^+$ T Zellen, welches in PD-1 exprimierenden T Zellen Apoptose auslösen kann. Der Transfer von funktionell aktiven koinhibitorischen Molekülen von APC auf humane CD8$^+$ T Zellen könnte dazu dienen, die klonale Expansion von antigen-spezifischen T-Zellantworten zu limitieren sowie durch Eliminierung von selbst-reaktiven

T Zellen Toleranz aufrecht zu erhalten, könnte aber auch eine tragende Rolle bei der funktionalen Erschöpfung von T Zellen bei chronischen Infektionen und bei der Tumor-Immunüberwachung spielen.

6. Abbreviations

7-AAD	7-Aminoactinomycin
ADCC	Antibody dependent cell cytotoxicity
AICD	Activation induced cell death
APC	Allophycocyanin
APC	Antigen presenting cells
ATP	Adenosine triphosphate
β2m	β-2 microglubulin
BCR	B cell receptor
CD	Cluster of differentiation
CLL	Chronic lymphocytic leukemia
CMA	Concanamycin A
CMV	Cytomegalo-Virus
cSMAC	Central supramolecular activation cluster
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4; CD154
DC	Dendritic cells
DC-SIGN	DC-specific ICAM-3 grabbing non-integrin; CD209
DMSO	Dimethyl sulfoxide
DN	$CD4^-$ $CD8^-$ double-negative
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr Virus
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence activated cell sorter
FasL	Fas-Ligand
FcR	Fc receptor
FCS	Fetal calf serum
FITC	Fluorescein-Isothiocyanat
Foxp3	Forkhead box P3
FSC	Forward scatter
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
GVHD	Graft-versus-host disease;
h	Hours
HBV	Hepatitis B Virus
HIV	Human immunedeficiency virus
HLA	Human leukocyte antigen
HSA	Human serum albumin
ICAM	Intercellular adhesion molecule
ICOS	Inducible co-stimulator
IFN-γ	Interferon-γ
IgG	Immunglobulin G
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motifs

ITIM	Immunoreceptor tyrosine-based inhibition motifs
LAG-3	Lymphocyte activation gene-3
LFA-1	Leukocyte function-associated antigen-1
MCSP	Melanoma-associated chondroitin sulfate proteoglycan
MDR	Multi-drug resistance
MHC	Major histocompabitility complex
MHC I	MHC-class I complex
MFI	Mean fluorescence intensity
MLR	mixed-lymphocyte-reaction
Melan-A	MART-1; self-antigen on melanocytes which is overexpressed on melanoma cells
MNC	Mononuclear cells
MS	Multiple sclerosis
NK cells	Natural killer cells
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood-mononuclear cells
PBS	Phosphate buffered saline
PD-1	Programmed-death 1; member of the B7-CD28 family
PD-L1	Programmed death-ligand 1
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-proteine complex
PFA	Paraformaldehyde
PGE_2	Prostaglandin E2
PHA	Phytohemagglutinin
pMHC	Peptide-MHC complex
PPR	Pathogen pattern receptor
PS	Phosphatidylserine
pSMAC	Peripheral supramolecular activation cluster
RNA	Ribonucleic acid
RT	Room temperature
RTX	Rituximab
SD	Standard deviation
SSC	Side scatter
TAA	Tumor associated antigen
TAP	transporter associated with antigen-processing
TCGF	T-cell growth factor
TCR	T-cell receptor
Teff	Effector T cells
TGF-β	Transforming-growth-factor-β
Th	Helper T cells
TIL	Tumor infiltrating lymphocytes
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNT	Tunneling nanotubes
TM	MHC-Tetramer
TRAIL	TNF-related apoptosis inducing ligand
Treg	Regulatory T cells
vATPases	Vacuolar ATPases
VEGF	Vascular endothelial growth factor

7. Literature

Abbas, A. K. and C. A. Janeway, Jr. (2000). "Immunology: improving on nature in the twenty-first century." Cell **100**(1): 129-138.

Acuto, O. and F. Michel (2003). "CD28-mediated co-stimulation: a quantitative support for TCR signalling." Nat Rev Immunol **3**(12): 939-951.

Admyre, C., S. M. Johansson, et al. (2006). "Direct exosome stimulation of peripheral human T cells detected by ELISPOT." Eur J Immunol **36**(7): 1772-1781.

Agata, Y., A. Kawasaki, et al. (1996). "Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes." Int Immunol **8**(5): 765-772.

Agrawal, M., R. J. Garg, et al. (2010). "Tyrosine kinase inhibitors: the first decade." Curr Hematol Malig Rep **5**(2): 70-80.

Ahmed, K. A., M. A. Munegowda, et al. (2008). "Intercellular trogocytosis plays an important role in modulation of immune responses." Cell Mol Immunol **5**(4): 261-269.

Almand, B., J. R. Resser, et al. (2000). "Clinical significance of defective dendritic cell differentiation in cancer." Clin Cancer Res **6**(5): 1755-1766.

Alonso, R., M. C. Rodriguez, et al. (2005). "Diacylglycerol kinase alpha regulates the secretion of lethal exosomes bearing Fas ligand during activation-induced cell death of T lymphocytes." J Biol Chem **280**(31): 28439-28450.

Amarnath, S., C. M. Costanzo, et al. (2010). "Regulatory T cells and human myeloid dendritic cells promote tolerance via programmed death ligand-1." PLoS Biol **8**(2): e1000302.

Andre, F., N. Chaput, et al. (2004). "Exosomes as potent cell-free peptide-based vaccine. I. Dendritic cell-derived exosomes transfer functional MHC class I/peptide complexes to dendritic cells." J Immunol **172**(4): 2126-2136.

Andre, F., N. E. Schartz, et al. (2002). "Malignant effusions and immunogenic tumour-derived exosomes." Lancet **360**(9329): 295-305.

Andreola, G., L. Rivoltini, et al. (2002). "Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles." J Exp Med **195**(10): 1303-1316.

Anton van der Merwe, P., S. J. Davis, et al. (2000). "Cytoskeletal polarization and redistribution of cell-surface molecules during T cell antigen recognition." Semin Immunol **12**(1): 5-21.

Arita, S., E. Baba, et al. (2008). "B cell activation regulates exosomal HLA production." Eur J Immunol **38**(5): 1423-1434.

Arnold, R., D. Brenner, et al. (2006). "How T lymphocytes switch between life and death." Eur J Immunol **36**(7): 1654-1658.

Attia, P., G. Q. Phan, et al. (2005). "Autoimmunity correlates with tumor regression in patients with metastatic melanoma treated with anti-cytotoxic T-lymphocyte antigen-4." J Clin Oncol **23**(25): 6043-6053.

Baj-Krzyworzeka, M., M. Majka, et al. (2002). "Platelet-derived microparticles stimulate proliferation, survival, adhesion, and chemotaxis of hematopoietic cells." Exp Hematol **30**(5): 450-459.

Baj-Krzyworzeka, M., R. Szatanek, et al. (2007). "Tumour-derived microvesicles modulate biological activity of human monocytes." Immunol Lett **113**(2): 76-82.

Barber, D. L., E. J. Wherry, et al. (2006). "Restoring function in exhausted CD8 T cells during chronic viral infection." Nature **439**(7077): 682-687.

Basu, S. K., J. L. Goldstein, et al. (1981). "Monensin interrupts the recycling of low density lipoprotein receptors in human fibroblasts." Cell **24**(2): 493-502.

Betts, M. R., J. M. Brenchley, et al. (2003). "Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation." J Immunol Methods **281**(1-2): 65-78.

Beum, P. V., D. A. Mack, et al. (2008). "Binding of rituximab, trastuzumab, cetuximab, or mAb T101 to cancer cells promotes trogocytosis mediated by THP-1 cells and monocytes." J Immunol **181**(11): 8120-8132.

Bhatnagar, S. and J. S. Schorey (2007). "Exosomes released from infected macrophages contain Mycobacterium avium glycopeptidolipids and are proinflammatory." J Biol Chem **282**(35): 25779-25789.

Bhatnagar, S., K. Shinagawa, et al. (2007). "Exosomes released from macrophages infected with intracellular pathogens stimulate a proinflammatory response in vitro and in vivo." Blood **110**(9): 3234-3244.

Blackburn, S. D., H. Shin, et al. (2009). "Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection." Nat Immunol **10**(1): 29-37.

Blanchard, N., D. Lankar, et al. (2002). "TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex." J Immunol **168**(7): 3235-3241.

Blank, C., J. Kuball, et al. (2006). "Blockade of PD-L1 (B7-H1) augments human tumor-specific T cell responses in vitro." Int J Cancer **119**(2): 317-327.

Blank, C. and A. Mackensen (2007). "Contribution of the PD-L1/PD-1 pathway to T-cell exhaustion: an update on implications for chronic infections and tumor evasion." Cancer Immunol Immunother **56**(5): 739-745.

Bleijs, D. A., T. B. Geijtenbeek, et al. (2001). "DC-SIGN and LFA-1: a battle for ligand." Trends Immunol **22**(8): 457-463.

Bona, C., R. Robineaux, et al. (1973). "Transfer of antigen from macrophages to lymphocytes. II. Immunological significance of the transfer of lipopolysaccharide." Immunology **24**(5): 831-840.

Bopp, T., C. Becker, et al. (2007). "Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression." J Exp Med **204**(6): 1303-1310.

Brezinschek, R. I., N. Oppenheimer-Marks, et al. (1999). "Activated T cells acquire endothelial cell surface determinants during transendothelial migration." J Immunol **162**(3): 1677-1684.

Bromley, S. K., W. R. Burack, et al. (2001). "The immunological synapse." Annu Rev Immunol **19**: 375-396.

Bronte, V., T. Kasic, et al. (2005). "Boosting antitumor responses of T lymphocytes infiltrating human prostate cancers." J Exp Med **201**(8): 1257-1268.

Busch, A., T. Quast, et al. (2008). "Transfer of T cell surface molecules to dendritic cells upon CD4+ T cell priming involves two distinct mechanisms." J Immunol **181**(6): 3965-3973.

Buschow, S. I., E. N. Nolte-'t Hoen, et al. (2009). "MHC II in dendritic cells is targeted to lysosomes or T cell-induced exosomes via distinct multivesicular body pathways." Traffic **10**(10): 1528-1542.

Campbell, D. J., C. H. Kim, et al. (2001). "Separable effector T cell populations specialized for B cell help or tissue inflammation." Nat Immunol **2**(9): 876-881.

Carosella, E. D., P. Moreau, et al. (2003). "HLA-G molecules: from maternal-fetal tolerance to tissue acceptance." Adv Immunol **81**: 199-252.

Carreno, B. M., F. Bennett, et al. (2000). "CTLA-4 (CD152) can inhibit T cell activation by two different mechanisms depending on its level of cell surface expression." J Immunol **165**(3): 1352-1356.

Carter, L., L. A. Fouser, et al. (2002). "PD-1:PD-L inhibitory pathway affects both CD4(+) and CD8(+) T cells and is overcome by IL-2." Eur J Immunol **32**(3): 634-643.

Caumartin, J., B. Favier, et al. (2007). "Trogocytosis-based generation of suppressive NK cells." Embo J **26**(5): 1423-1433.

Cavaleiro, R., A. P. Baptista, et al. (2009). "Major depletion of plasmacytoid dendritic cells in HIV-2 infection, an attenuated form of HIV disease." PLoS Pathog **5**(11): e1000667.

Chauveau, A., A. Aucher, et al. "Membrane nanotubes facilitate long-distance interactions between natural killer cells and target cells." Proc Natl Acad Sci U S A **107**(12): 5545-5550.

Clayton, A., J. P. Mitchell, et al. (2007). "Human tumor-derived exosomes selectively impair lymphocyte responses to interleukin-2." Cancer Res **67**(15): 7458-7466.

Coulie, P. G., V. Brichard, et al. (1994). "A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas." J Exp Med **180**(1): 35-42.

Cousens, L. P., R. Peterson, et al. (1999). "Two roads diverged: interferon alpha/beta- and interleukin 12-mediated pathways in promoting T cell interferon gamma responses during viral infection." J Exp Med **189**(8): 1315-1328.

Coussens, L. M. and Z. Werb (2002). "Inflammation and cancer." Nature **420**(6917): 860-867.

Cox, A. L., J. Skipper, et al. (1994). "Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines." Science **264**(5159): 716-719.

Coyle, A. J. and J. C. Gutierrez-Ramos (2001). "The expanding B7 superfamily: increasing complexity in costimulatory signals regulating T cell function." Nat Immunol **2**(3): 203-209.

Coyle, A. J., S. Lehar, et al. (2000). "The CD28-related molecule ICOS is required for effective T cell-dependent immune responses." Immunity **13**(1): 95-105.

Curiel, T. J., G. Coukos, et al. (2004). "Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival." Nat Med **10**(9): 942-949.

Daubeuf, S., A. Aucher, et al. "Preferential transfer of certain plasma membrane proteins onto T and B cells by trogocytosis." PLoS One **5**(1): e8716.

Davis, D. M. (2007). "Intercellular transfer of cell-surface proteins is common and can affect many stages of an immune response." Nat Rev Immunol **7**(3): 238-243.

Denzer, K., M. van Eijk, et al. (2000). "Follicular dendritic cells carry MHC class II-expressing microvesicles at their surface." J Immunol **165**(3): 1259-1265.

Dinter, A. and E. G. Berger (1998). "Golgi-disturbing agents." Histochem Cell Biol **109**(5-6): 571-590.

Domaica, C. I., M. B. Fuertes, et al. (2009). "Tumour-experienced T cells promote NK cell activity through trogocytosis of NKG2D and NKp46 ligands." EMBO Rep **10**(8): 908-915.

Dong, H., S. E. Strome, et al. (2002). "Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion." Nat Med **8**(8): 793-800.

Drose, S. and K. Altendorf (1997). "Bafilomycins and concanamycins as inhibitors of V-ATPases and P-ATPases." J Exp Biol **200**(Pt 1): 1-8.

Eder, J. P., P. W. Kantoff, et al. (2000). "A phase I trial of a recombinant vaccinia virus expressing prostate-specific antigen in advanced prostate cancer." Clin Cancer Res **6**(5): 1632-1638.

Elliott, J. I., A. Surprenant, et al. (2005). "Membrane phosphatidylserine distribution as a non-apoptotic signalling mechanism in lymphocytes." Nat Cell Biol **7**(8): 808-816.

Engering, A., T. B. Geijtenbeek, et al. (2002). "The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells." J Immunol **168**(5): 2118-2126.

Escudier, B., T. Dorval, et al. (2005). "Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of thefirst phase I clinical trial." J Transl Med **3**(1): 10.

Fischer, K., S. Voelkl, et al. (2006). "Antigen recognition induces phosphatidylserine exposure on the cell surface of human CD8+ T cells." Blood **108**(13): 4094-4101.

Fischer, K., S. Voelkl, et al. (2005). "Isolation and characterization of human antigen-specific TCR alpha beta+ CD4(-)CD8- double-negative regulatory T cells." Blood **105**(7): 2828-2835.

Fisicaro, P., C. Valdatta, et al. "Antiviral intrahepatic T-cell responses can be restored by blocking programmed death-1 pathway in chronic hepatitis B." Gastroenterology **138**(2): 682-693, 693 e681-684.

Ford McIntyre, M. S., K. J. Young, et al. (2008). "Cutting edge: in vivo trogocytosis as a mechanism of double negative regulatory T cell-mediated antigen-specific suppression." J Immunol **181**(4): 2271-2275.

Fortin, J. F., R. Cantin, et al. (1998). "T cells expressing activated LFA-1 are more susceptible to infection with human immunodeficiency virus type 1 particles bearing host-encoded ICAM-1." J Virol **72**(3): 2105-2112.

Fortis, C., M. Foppoli, et al. (1996). "Increased interleukin-10 serum levels in patients with solid tumours." Cancer Lett **104**(1): 1-5.

Francisco, L. M., P. T. Sage, et al. "The PD-1 pathway in tolerance and autoimmunity." Immunol Rev **236**: 219-242.

Francisco, L. M., V. H. Salinas, et al. (2009). "PD-L1 regulates the development, maintenance, and function of induced regulatory T cells." J Exp Med **206**(13): 3015-3029.

Freeman, G. J., A. J. Long, et al. (2000). "Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation." J Exp Med **192**(7): 1027-1034.

Fritzsching, B., B. Schwer, et al. (2002). "Release and intercellular transfer of cell surface CD81 via microparticles." J Immunol **169**(10): 5531-5537.

Gabrilovich, D., T. Ishida, et al. (1998). "Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo." Blood **92**(11): 4150-4166.

Game, D. S., N. J. Rogers, et al. (2005). "Acquisition of HLA-DR and costimulatory molecules by T cells from allogeneic antigen presenting cells." Am J Transplant **5**(7): 1614-1625.

Gattinoni, L., C. A. Klebanoff, et al. (2005). "Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells." J Clin Invest **115**(6): 1616-1626.

Geijtenbeek, T. B., D. J. Krooshoop, et al. (2000). "DC-SIGN-ICAM-2 interaction mediates dendritic cell trafficking." Nat Immunol **1**(4): 353-357.

Giri, P. K. and J. S. Schorey (2008). "Exosomes derived from M. Bovis BCG infected macrophages activate antigen-specific CD4+ and CD8+ T cells in vitro and in vivo." PLoS One **3**(6): e2461.

Goldberg, M. V., C. H. Maris, et al. (2007). "Role of PD-1 and its ligand, B7-H1, in early fate decisions of CD8 T cells." Blood **110**(1): 186-192.

Gonzalez, S., V. Groh, et al. (2006). "Immunobiology of human NKG2D and its ligands." Curr Top Microbiol Immunol **298**: 121-138.

Greenwald, R. J., G. J. Freeman, et al. (2005). "The B7 family revisited." Annu Rev Immunol **23**: 515-548.

Griffith, T. S., T. Brunner, et al. (1995). "Fas ligand-induced apoptosis as a mechanism of immune privilege." Science **270**(5239): 1189-1192.

Guicciardi, M. E. and G. J. Gores (2009). "Life and death by death receptors." Faseb J **23**(6): 1625-1637.

Gurke, S., J. F. Barroso, et al. (2008). "The art of cellular communication: tunneling nanotubes bridge the divide." Histochem Cell Biol **129**(5): 539-550.

He, X. H., Y. Liu, et al. (2004). "Cloning and identification of two novel splice variants of human PD-L2." Acta Biochim Biophys Sin (Shanghai) **36**(4): 284-289.

He, Y. F., X. H. Wang, et al. (2005). "Sustained low-level expression of interferon-gamma promotes tumor development: potential insights in tumor prevention and tumor immunotherapy." Cancer Immunol Immunother **54**(9): 891-897.

Held, G., V. Poschel, et al. (2006). "Rituximab for the treatment of diffuse large B-cell lymphomas." Expert Rev Anticancer Ther **6**(8): 1175-1186.

Hernandez, J., S. Aung, et al. (2001). "Phenotypic and functional analysis of CD8(+) T cells undergoing peripheral deletion in response to cross-presentation of self-antigen." J Exp Med **194**(6): 707-717.

Hildeman, D. A., Y. Zhu, et al. (2002). "Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim." Immunity **16**(6): 759-767.

Hock, B. D., M. Kato, et al. (2001). "A soluble form of CD83 is released from activated dendritic cells and B lymphocytes, and is detectable in normal human sera." Int Immunol **13**(7): 959-967.

HoWangYin, K. Y., E. Alegre, et al. "Different functional outcomes of intercellular membrane transfers to monocytes and T cells." Cell Mol Life Sci **67**(7): 1133-1145.

Huang, J. F., Y. Yang, et al. (1999). "TCR-Mediated internalization of peptide-MHC complexes acquired by T cells." Science **286**(5441): 952-954.

Huang, X., F. Venet, et al. (2009). "PD-1 expression by macrophages plays a pathologic role in altering microbial clearance and the innate inflammatory response to sepsis." Proc Natl Acad Sci U S A **106**(15): 6303-6308.

Huber, V., S. Fais, et al. (2005). "Human colorectal cancer cells induce T-cell death through release of proapoptotic microvesicles: role in immune escape." Gastroenterology **128**(7): 1796-1804.

Huber, V., P. Filipazzi, et al. (2008). "More insights into the immunosuppressive potential of tumor exosomes." J Transl Med **6**: 63.

Hudrisier, D., A. Aucher, et al. (2007). "Capture of target cell membrane components via trogocytosis is triggered by a selected set of surface molecules on T or B cells." J Immunol **178**(6): 3637-3647.

Hudrisier, D. and P. Bongrand (2002). "Intercellular transfer of antigen-presenting cell determinants onto T cells: molecular mechanisms and biological significance." Faseb J **16**(6): 477-486.

Hudrisier, D., J. Riond, et al. (2005). "T cell activation correlates with an increased proportion of antigen among the materials acquired from target cells." Eur J Immunol **35**(8): 2284-2294.

Hudrisier, D., J. Riond, et al. (2001). "Cutting edge: CTLs rapidly capture membrane fragments from target cells in a TCR signaling-dependent manner." J Immunol **166**(6): 3645-3649.

Hunt, J. S. and H. T. Orr (1992). "HLA and maternal-fetal recognition." FASEB J **6**(6): 2344-2348.

Huss, M. and H. Wieczorek (2009). "Inhibitors of V-ATPases: old and new players." J Exp Biol **212**(Pt 3): 341-346.

Hutloff, A., A. M. Dittrich, et al. (1999). "ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28." Nature **397**(6716): 263-266.

Ilani, T., G. Vasiliver-Shamis, et al. (2009). "T cell antigen receptor signaling and immunological synapse stability require myosin IIA." Nat Immunol **10**(5): 531-539.

Irving, B. A. and A. Weiss (1991). "The cytoplasmic domain of the T cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways." Cell **64**(5): 891-901.

Islam, A., X. Shen, et al. (2007). "The brefeldin A-inhibited guanine nucleotide-exchange protein, BIG2, regulates the constitutive release of TNFR1 exosome-like vesicles." J Biol Chem **282**(13): 9591-9599.

Joly, E. and D. Hudrisier (2003). "What is trogocytosis and what is its purpose?" Nat Immunol **4**(9): 815.

Josefowicz, S. Z. and A. Rudensky (2009). "Control of regulatory T cell lineage commitment and maintenance." Immunity **30**(5): 616-625.

Jovic, M., M. Sharma, et al. (2010). "The early endosome: a busy sorting station for proteins at the crossroads." Histol Histopathol **25**(1): 99-112.

Kawakami, Y., S. Eliyahu, et al. (1994). "Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection." Proc Natl Acad Sci U S A **91**(14): 6458-6462.

Kawakami, Y., S. Eliyahu, et al. (1995). "Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression." J Immunol **154**(8): 3961-3968.

Keir, M. E., M. J. Butte, et al. (2008). "PD-1 and its ligands in tolerance and immunity." Annu Rev Immunol **26**: 677-704.

Klibi, J., T. Niki, et al. (2009). "Blood diffusion and Th1-suppressive effects of galectin-9-containing exosomes released by Epstein-Barr virus-infected nasopharyngeal carcinoma cells." Blood **113**(9): 1957-1966.

Knauf, W. U., B. Ehlers, et al. (1995). "Serum levels of interleukin-10 in B-cell chronic lymphocytic leukemia." Blood **86**(11): 4382-4383.

La Boissiere, S., A. Izeta, et al. (2004). "Compartmentalization of VP16 in cells infected with recombinant herpes simplex virus expressing VP16-green fluorescent protein fusion proteins." J Virol **78**(15): 8002-8014.

Langrish, C. L., Y. Chen, et al. (2005). "IL-23 drives a pathogenic T cell population that induces autoimmune inflammation." J Exp Med **201**(2): 233-240.

Latchman, Y. E., S. C. Liang, et al. (2004). "PD-L1-deficient mice show that PD-L1 on T cells, antigen-presenting cells, and host tissues negatively regulates T cells." Proc Natl Acad Sci U S A **101**(29): 10691-10696.

Lechmann, M., S. Berchtold, et al. (2002). "CD83 on dendritic cells: more than just a marker for maturation." Trends Immunol **23**(6): 273-275.

Lehmann, B. D., M. S. Paine, et al. (2008). "Senescence-associated exosome release from human prostate cancer cells." Cancer Res **68**(19): 7864-7871.

Leitner, J., K. Grabmeier-Pfistershammer, et al. "Receptors and ligands implicated in human T cell costimulatory processes." Immunol Lett **128**(2): 89-97.

LeMaoult, J., J. Caumartin, et al. (2007). "Exchanges of membrane patches (trogocytosis) split theoretical and actual functions of immune cells." Hum Immunol **68**(4): 240-243.

LeMaoult, J., J. Caumartin, et al. (2007). "Immune regulation by pretenders: cell-to-cell transfers of HLA-G make effector T cells act as regulatory cells." Blood **109**(5): 2040-2048.

Lencer, W. I. and R. S. Blumberg (2005). "A passionate kiss, then run: exocytosis and recycling of IgG by FcRn." Trends Cell Biol **15**(1): 5-9.

Limouze, J., A. F. Straight, et al. (2004). "Specificity of blebbistatin, an inhibitor of myosin II." J Muscle Res Cell Motil **25**(4-5): 337-341.

Machlenkin, A., R. Uzana, et al. (2008). "Capture of tumor cell membranes by trogocytosis facilitates detection and isolation of tumor-specific functional CTLs." Cancer Res **68**(6): 2006-2013.

Mackensen, A., G. Carcelain, et al. (1994). "Direct evidence to support the immunosurveillance concept in a human regressive melanoma." J Clin Invest **93**(4): 1397-1402.

Makarenkova, V. P., V. Bansal, et al. (2006). "CD11b+/Gr-1+ myeloid suppressor cells cause T cell dysfunction after traumatic stress." J Immunol **176**(4): 2085-2094.

Mangan, P. R., L. E. Harrington, et al. (2006). "Transforming growth factor-beta induces development of the T(H)17 lineage." Nature **441**(7090): 231-234.

Marincola, F. M., Y. M. Hijazi, et al. (1996). "Analysis of expression of the melanoma-associated antigens MART-1 and gp100 in metastatic melanoma cell lines and in in situ lesions." J Immunother Emphasis Tumor Immunol **19**(3): 192-205.

Marshansky, V. and M. Futai (2008). "The V-type H+-ATPase in vesicular trafficking: targeting, regulation and function." Curr Opin Cell Biol **20**(4): 415-426.

McCann, F. E., P. Eissmann, et al. (2007). "The activating NKG2D ligand MHC class I-related chain A transfers from target cells to NK cells in a manner that allows functional consequences." J Immunol **178**(6): 3418-3426.

McDowall, A., B. Leitinger, et al. (1998). "The I domain of integrin leukocyte function-associated antigen-1 is involved in a conformational change leading to high affinity binding to ligand intercellular adhesion molecule 1 (ICAM-1)." J Biol Chem **273**(42): 27396-27403.

Medzhitov, R. (2007). "Recognition of microorganisms and activation of the immune response." Nature **449**(7164): 819-826.

Mescher, M. F., J. M. Curtsinger, et al. (2006). "Signals required for programming effector and memory development by CD8+ T cells." Immunol Rev **211**: 81-92.

Minami, Y., T. Kono, et al. (1993). "The IL-2 receptor complex: its structure, function, and target genes." Annu Rev Immunol **11**: 245-268.

Miyanishi, M., K. Tada, et al. (2007). "Identification of Tim4 as a phosphatidylserine receptor." Nature **450**(7168): 435-439.

Monks, C. R., B. A. Freiberg, et al. (1998). "Three-dimensional segregation of supramolecular activation clusters in T cells." Nature **395**(6697): 82-86.

Monleon, I., M. J. Martinez-Lorenzo, et al. (2001). "Differential secretion of Fas ligand- or APO2 ligand/TNF-related apoptosis-inducing ligand-carrying microvesicles during activation-induced death of human T cells." J Immunol **167**(12): 6736-6744.

Morelli, A. E., A. T. Larregina, et al. (2004). "Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells." Blood **104**(10): 3257-3266.

Morse, M. A., J. Garst, et al. (2005). "A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer." J Transl Med **3**(1): 9.

Mostbock, S., M. Catalfamo, et al. (2007). "Acquisition of antigen presentasome (APS), an MHC/costimulatory complex, is a checkpoint of memory T-cell homeostasis." Blood **109**(6): 2488-2495.

Munn, D. H., M. D. Sharma, et al. (2004). "Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes." J Clin Invest **114**(2): 280-290.

Muntasell, A., A. C. Berger, et al. (2007). "T cell-induced secretion of MHC class II-peptide complexes on B cell exosomes." Embo J **26**(19): 4263-4272.

Muratori, C., L. E. Cavallin, et al. (2009). "Massive secretion by T cells is caused by HIV Nef in infected cells and by Nef transfer to bystander cells." Cell Host Microbe **6**(3): 218-230.

Muro, S., M. Mateescu, et al. (2006). "Control of intracellular trafficking of ICAM-1-targeted nanocarriers by endothelial Na+/H+ exchanger proteins." Am J Physiol Lung Cell Mol Physiol **290**(5): L809-817.

Nishimura, H., Y. Agata, et al. (1996). "Developmentally regulated expression of the PD-1 protein on the surface of double-negative (CD4-CD8-) thymocytes." Int Immunol **8**(5): 773-780.

Nolte-'t Hoen, E. N., S. I. Buschow, et al. (2009). "Activated T cells recruit exosomes secreted by dendritic cells via LFA-1." Blood **113**(9): 1977-1981.

Onfelt, B., S. Nedvetzki, et al. (2006). "Structurally distinct membrane nanotubes between human macrophages support long-distance vesicular traffic or surfing of bacteria." J Immunol **177**(12): 8476-8483.

Onfelt, B., S. Nedvetzki, et al. (2004). "Cutting edge: Membrane nanotubes connect immune cells." J Immunol **173**(3): 1511-1513.

Parish, I. A. and S. M. Kaech (2009). "Diversity in CD8(+) T cell differentiation." Curr Opin Immunol **21**(3): 291-297.

Peach, R. J., J. Bajorath, et al. (1995). "Both extracellular immunoglobin-like domains of CD80 contain residues critical for binding T cell surface receptors CTLA-4 and CD28." J Biol Chem **270**(36): 21181-21187.

Perkins, D., Z. Wang, et al. (1996). "Regulation of CTLA-4 expression during T cell activation." J Immunol **156**(11): 4154-4159.

Peters, P. J., H. J. Geuze, et al. (1989). "Molecules relevant for T cell-target cell interaction are present in cytolytic granules of human T lymphocytes." Eur J Immunol **19**(8): 1469-1475.

Peters, P. J., G. Raposo, et al. (1995). "Major histocompatibility complex class II compartments in human B lymphoblastoid cells are distinct from early endosomes." J Exp Med **182**(2): 325-334.

Powell, D. J., Jr., M. E. Dudley, et al. (2005). "Transition of late-stage effector T cells to CD27+ CD28+ tumor-reactive effector memory T cells in humans after adoptive cell transfer therapy." Blood **105**(1): 241-250.

Quah, B. J., V. P. Barlow, et al. (2008). "Bystander B cells rapidly acquire antigen receptors from activated B cells by membrane transfer." Proc Natl Acad Sci U S A **105**(11): 4259-4264.

Quah, B. J. and H. C. O'Neill (2007). "Mycoplasma contaminants present in exosome preparations induce polyclonal B cell responses." J Leukoc Biol **82**(5): 1070-1082.

Rafii, A., P. Mirshahi, et al. (2008). "Oncologic trogocytosis of an original stromal cells induces chemoresistance of ovarian tumours." PLoS One **3**(12): e3894.

Raposo, G., H. W. Nijman, et al. (1996). "B lymphocytes secrete antigen-presenting vesicles." J Exp Med **183**(3): 1161-1172.

Rechavi, O., I. Goldstein, et al. (2009). "Intercellular exchange of proteins: the immune cell habit of sharing." FEBS Lett **583**(11): 1792-1799.

Rechavi, O., I. Goldstein, et al. (2007). "Intercellular transfer of oncogenic H-Ras at the immunological synapse." PLoS One **2**(11): e1204.

Redmond, W. L., B. C. Marincek, et al. (2005). "Distinct requirements for deletion versus anergy during CD8 T cell peripheral tolerance in vivo." J Immunol **174**(4): 2046-2053.

Restifo, N. P., F. Esquivel, et al. (1993). "Identification of human cancers deficient in antigen processing." J Exp Med **177**(2): 265-272.

Reynoso, E. D., K. G. Elpek, et al. (2009). "Intestinal tolerance is converted to autoimmune enteritis upon PD-1 ligand blockade." J Immunol **182**(4): 2102-2112.

Riond, J., J. Elhmouzi, et al. (2007). "Capture of membrane components via trogocytosis occurs in vivo during both dendritic cells and target cells encounter by CD8(+) T cells." Scand J Immunol **66**(4): 441-450.

Rippo, M. R., S. Moretti, et al. (2004). "FLIP overexpression inhibits death receptor-induced apoptosis in malignant mesothelial cells." Oncogene **23**(47): 7753-7760.

Romero, P., P. R. Dunbar, et al. (1998). "Ex vivo staining of metastatic lymph nodes by class I major histocompatibility complex tetramers reveals high numbers of antigen-experienced tumor-specific cytolytic T lymphocytes." J Exp Med **188**(9): 1641-1650.

Rosenberg, S. A., J. C. Yang, et al. (1994). "Treatment of 283 consecutive patients with metastatic melanoma or renal cell cancer using high-dose bolus interleukin 2." Jama **271**(12): 907-913.

Rubio, V., T. B. Stuge, et al. (2003). "Ex vivo identification, isolation and analysis of tumor-cytolytic T cells." Nat Med **9**(11): 1377-1382.

Rudd, C. E. (1990). "CD4, CD8 and the TCR-CD3 complex: a novel class of protein-tyrosine kinase receptor." Immunol Today **11**(11): 400-406.

Rustom, A., R. Saffrich, et al. (2004). "Nanotubular highways for intercellular organelle transport." Science **303**(5660): 1007-1010.

Sabzevari, H., J. Kantor, et al. (2001). "Acquisition of CD80 (B7-1) by T cells." J Immunol **166**(4): 2505-2513.

Salmond, R. J., A. Filby, et al. (2009). "T-cell receptor proximal signaling via the Src-family kinases, Lck and Fyn, influences T-cell activation, differentiation, and tolerance." Immunol Rev **228**(1): 9-22.

Scholer, A., S. Hugues, et al. (2008). "Intercellular adhesion molecule-1-dependent stable interactions between T cells and dendritic cells determine CD8+ T cell memory." Immunity **28**(2): 258-270.

Schwartz, R. H. (2003). "T cell anergy." Annu Rev Immunol **21**: 305-334.

Segura, E., C. Guerin, et al. (2007). "CD8+ dendritic cells use LFA-1 to capture MHC-peptide complexes from exosomes in vivo." J Immunol **179**(3): 1489-1496.

Segura, E., C. Nicco, et al. (2005). "ICAM-1 on exosomes from mature dendritic cells is critical for efficient naive T-cell priming." Blood **106**(1): 216-223.

Sharpe, A. H. and G. J. Freeman (2002). "The B7-CD28 superfamily." Nat Rev Immunol **2**(2): 116-126.

Shen, T., X. Chen, et al. "Increased PD-L1 expression and PD-L1/CD86 ratio on dendritic cells were associated with impaired dendritic cells function in HCV infection." J Med Virol **82**(7): 1152-1159.

Shevach, E. M. (2008). "Immunology. Regulating suppression." Science **322**(5899): 202-203.

Skipper, J. C., P. H. Gulden, et al. (1999). "Mass-spectrometric evaluation of HLA-A*0201-associated peptides identifies dominant naturally processed forms of CTL epitopes from MART-1 and gp100." Int J Cancer **82**(5): 669-677.

Slingluff, C. L., Jr., G. Yamshchikov, et al. (2001). "Phase I trial of a melanoma vaccine with gp100(280-288) peptide and tetanus helper peptide in adjuvant: immunologic and clinical outcomes." Clin Cancer Res **7**(10): 3012-3024.

Sowinski, S., C. Jolly, et al. (2008). "Membrane nanotubes physically connect T cells over long distances presenting a novel route for HIV-1 transmission." Nat Cell Biol **10**(2): 211-219.

Sozzani, S., W. Vermi, et al. "Trafficking properties of plasmacytoid dendritic cells in health and disease." Trends Immunol **31**(7): 270-277.

Steinman, R. M. and H. Hemmi (2006). "Dendritic cells: translating innate to adaptive immunity." Curr Top Microbiol Immunol **311**: 17-58.

Stinchcombe, J. C., G. Bossi, et al. (2001). "The immunological synapse of CTL contains a secretory domain and membrane bridges." Immunity **15**(5): 751-761.

Szabo, S. J., B. M. Sullivan, et al. (2003). "Molecular mechanisms regulating Th1 immune responses." Annu Rev Immunol **21**: 713-758.

Tabiasco, J., A. Vercellone, et al. (2003). "Acquisition of viral receptor by NK cells through immunological synapse." J Immunol **170**(12): 5993-5998.

Tamai, K., N. Tanaka, et al. (2010). "Exosome secretion of dendritic cells is regulated by Hrs, an ESCRT-0 protein." Biochem Biophys Res Commun **399**(3): 384-390.

Tartakoff, A. M. (1983). "Perturbation of vesicular traffic with the carboxylic ionophore monensin." Cell **32**(4): 1026-1028.

Tatari-Calderone, Z., R. T. Semnani, et al. (2002). "Acquisition of CD80 by human T cells at early stages of activation: functional involvement of CD80 acquisition in T cell to T cell interaction." J Immunol **169**(11): 6162-6169.

Taylor, D. D. and C. Gercel-Taylor (2005). "Tumour-derived exosomes and their role in cancer-associated T-cell signalling defects." Br J Cancer **92**(2): 305-311.

Terabe, M., S. Matsui, et al. (2000). "NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway." Nat Immunol **1**(6): 515-520.

Thakur, A. and L. G. Lum (2010). "Cancer therapy with bispecific antibodies: Clinical experience." Curr Opin Mol Ther **12**(3): 340-349.

Thery, C., L. Duban, et al. (2002). "Indirect activation of naive CD4+ T cells by dendritic cell-derived exosomes." Nat Immunol **3**(12): 1156-1162.

Thery, C., M. Ostrowski, et al. (2009). "Membrane vesicles as conveyors of immune responses." Nat Rev Immunol **9**(8): 581-593.

Togashi, K., T. Kataoka, et al. (1997). "Characterization of a series of vacuolar type H(+)-ATPase inhibitors on CTL-mediated cytotoxicity." Immunol Lett **55**(3): 139-144.

Tonks, N. K., C. D. Diltz, et al. (1991). "Purification and assay of CD45: an integral membrane protein-tyrosine phosphatase." Methods Enzymol **201**: 442-451.

Trowbridge, I. S. and M. L. Thomas (1994). "CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development." Annu Rev Immunol **12**: 85-116.

Tsang, J. Y., J. G. Chai, et al. (2003). "Antigen presentation by mouse CD4+ T cells involving acquired MHC class II:peptide complexes: another mechanism to limit clonal expansion?" Blood **101**(7): 2704-2710.

Tsushima, F., S. Yao, et al. (2007). "Interaction between B7-H1 and PD-1 determines initiation and reversal of T-cell anergy." Blood **110**(1): 180-185.

Valmori, D., V. Dutoit, et al. (2003). "Simultaneous CD8+ T cell responses to multiple tumor antigen epitopes in a multipeptide melanoma vaccine." Cancer Immun **3**: 15.

van Gisbergen, K. P., L. C. Paessens, et al. (2005). "Molecular mechanisms that set the stage for DC-T cell engagement." Immunol Lett **97**(2): 199-208.

van Kooyk, Y. and T. B. Geijtenbeek (2002). "A novel adhesion pathway that regulates dendritic cell trafficking and T cell interactions." Immunol Rev **186**: 47-56.

Vignali, D. A., L. W. Collison, et al. (2008). "How regulatory T cells work." Nat Rev Immunol **8**(7): 523-532.

Voehringer, D., T. A. Reese, et al. (2006). "Type 2 immunity is controlled by IL-4/IL-13 expression in hematopoietic non-eosinophil cells of the innate immune system." J Exp Med **203**(6): 1435-1446.

Wahl, S. M., J. Wen, et al. (2006). "TGF-beta: a mobile purveyor of immune privilege." Immunol Rev **213**: 213-227.

Walker, J. D., C. L. Maier, et al. (2009). "Cytomegalovirus-infected human endothelial cells can stimulate allogeneic CD4+ memory T cells by releasing antigenic exosomes." J Immunol **182**(3): 1548-1559.

Watkins, S. C. and R. D. Salter (2005). "Functional connectivity between immune cells mediated by tunneling nanotubules." Immunity **23**(3): 309-318.

Wegener, A. M., F. Letourneur, et al. (1992). "The T cell receptor/CD3 complex is composed of at least two autonomous transduction modules." Cell **68**(1): 83-95.

Williams, M. A. and M. J. Bevan (2007). "Effector and memory CTL differentiation." Annu Rev Immunol **25**: 171-192.

Wolfers, J., A. Lozier, et al. (2001). "Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming." Nat Med **7**(3): 297-303.

Yao, S., S. Wang, et al. (2009). "PD-1 on dendritic cells impedes innate immunity against bacterial infection." Blood **113**(23): 5811-5818.

Yoshinaga, S. K., J. S. Whoriskey, et al. (1999). "T-cell co-stimulation through B7RP-1 and ICOS." Nature **402**(6763): 827-832.

Yu, X., S. L. Harris, et al. (2006). "The regulation of exosome secretion: a novel function of the p53 protein." Cancer Res **66**(9): 4795-4801.

Zeelenberg, I. S., M. Ostrowski, et al. (2008). "Targeting tumor antigens to secreted membrane vesicles in vivo induces efficient antitumor immune responses." Cancer Res **68**(4): 1228-1235.

Zhang, X., J. C. Schwartz, et al. (2004). "Structural and functional analysis of the costimulatory receptor programmed death-1." Immunity **20**(3): 337-347.

Zhang, Z. X., L. Yang, et al. (2000). "Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression." Nat Med **6**(7): 782-789.

Zhou, L. J. and T. F. Tedder (1995). "Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily." J Immunol **154**(8): 3821-3835.

Zhou, Q., M. E. Munger, et al. "Program death-1 (PD-1) signaling and regulatory T cells (Tregs) collaborate to resist the function of adoptively transferred cytotoxic T lymphocytes (CTLs) in advanced acute myeloid leukemia (AML)." Blood.

Zhou, T., Y. Chen, et al. (2006). "DC-SIGN and immunoregulation." Cell Mol Immunol **3**(4): 279-283.

Zinkernagel, R. M. and P. C. Doherty (1974). "Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system." Nature **248**(450): 701-702.

Zinkernagel, R. M. and P. C. Doherty (1997). "The discovery of MHC restriction." Immunol Today **18**(1): 14-17.

Zippelius, A., P. Batard, et al. (2004). "Effector function of human tumor-specific CD8 T cells in melanoma lesions: a state of local functional tolerance." Cancer Res **64**(8): 2865-2873.

Zitvogel, L., A. Regnault, et al. (1998). "Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes." Nat Med **4**(5): 594-600.

8. Appendix

8.1. Curriculum Vitae

PERSONAL DATA

Name	Gary, Regina Anna-Maria
Date of birth	18th of July 1979
Place of birth	Pfaffenhofen a. d. Ilm
Nationality	german
Email	regina.gary@uk-erlangen.de

EDUCATION

12 / 2006 – present	**PhD Student of Biology**, **University of Regensburg / University of Erlangen-Nuremberg** **"Characterization and functional analysis of the transfer of cell components from human antigen-presenting cells onto T cells via antigen-specific trogocytosis "** • **Supervison**: Prof. Dr. Andreas Mackensen and Prof. Dr. Susanne Modrow
03 / 2008 – present	• **Institute**: Department of Hematology and Oncology, University of Erlangen-Nuremberg
12 / 2006 – 02 / 2008	• I**nstitute**: Department of Hematology and Oncology, University of Regensburg
05 / 2006- 10 / 2006	**Internship at the Queensland Institute of Medical Research, Brisbane, Australia** **"Investigations in the differentiation of mouse $CD8^+$ T cells and their contribution to tumor immunity"** • Supervision: Dr. Norbert Kienzle and Prof. Anne Kelso

10 / 2000 – 01 / 2006	**Diploma studies in Biology, University of Regensburg**
	• Grade: 1.1
	• Major subjects: (Medical) Microbiology Genetics Organic Chemistry
	• Diploma thesis 03 / 2005 – 01 / 2006 in the Institute for Medical Microbiology and Hygiene, University of Regensburg
	"Investigations in the translocation-mechanism of urea-adjuvated BZLF1 protein of the Epstein-Barr Virus: basis for the establishment of a novel technology for the identification of new T-cell epitopes"
	• Supervison: Dr. Ludwig Deml
1999 – 2000	**Apprenticeship** as Sculptor (not finished)
1990 – 1999	**Schyren-Gymnasium Pfaffenhofen**: Grade 1.6

COURSES AND CERTIFICATES

2010	Scientific Writing Workshop, Harald Köster, University of Erlangen, Germany
	Introduction in GCP, Center for Clinical Studies Erlangen, Germany
2009	Presenting Professionally Workshop in English, ICCON, Andrea Roos, Germany
	Flow Cytometry Seminar, Erlangen, Germany
	Course in Statistical Analysis, University of Erlangen, Germany
2008	Education seminar for project leaders and authorised persons for Biologic Security (BBS), University of Regensburg, Germany
	Spring School on Immunology of the DGfI, Ettal, Germany
2006	Research Animal Workshop, University of Queensland, Australia

CONFERENCES AND MEETINGS

12 / 2010	52nd ASH Meeting in Orlando, Florida, USA – abstract chosen for oral presentation
10 / 2010	3rd International GK Symposium on Regulators of Adaptive Immunity in Erlangen, Germany – Poster
10 / 2010	3rd Symposium of the Graduate School of SFB 643 in Veilbronn, Germany - Talk
08 / 2010	14th International Congress of Immunology, Kobe, Japan – Talk and Poster
11 / 2009	2nd Symposium of the Graduate School of SFB 643 in Effeltrich, Germany - Talk
09 / 2009	European Congress of Immunology Satellite-Symposium Erlangen, Germany - Talk
03 / 2009	5th Cellular Therapy Meeting 2009 Nuremberg, Germany – Poster
09 / 2008	Joint Annual Meeting of Immunology, Vienna, Austria – Poster Presentation
03 / 2008	Spring School on Immunology of the DGfI, Ettal, Germany – Poster Presentation

GRANTS

08 / 2010	EFIS travel grant for the ici2010

LANGUAGES

German	Mother tongue
English	Fluent
French	Basic conversation

PUBLIC RELATIONS

10 / 2009	"Lange Nacht der Wissenschaften" (Night of Sciences), Graduiertenkolleg SFB 643, Erlangen, Germany

PUBLICATIONS

- Barabas S, **Gary R**, Bauer T, Lindner J, Lindner P, Weinberger B, Jilg W, Wolf H, Deml L. Urea-mediated cross-presentation of soluble Epstein-Barr Virus BZLF1 protein. PLoS Pathogens (2008)

- Voelkl S, **Gary R**, Mackensen A. Characterization of the immunoregulatory function of human TCR$\alpha\beta^+$ $CD4^-CD8^-$ double negative T-cells. *Under review at the European Journal of Immunology*

- Zaiss MM, Kurowska-Stolarska M, Böhm C, **Gary R**, Scholtysek C, Stolarski B, Kerr S, Millar NL, Kamradt T, McInnes IB, David J-P, Liew FY, Schett G. Interleukin (IL)-33 is a negative regulator of osteoclast formation and an inhibitor of bone resorption. *Submitted*

- **Gary R**, Voelkl S, Palmisano R, Mackensen A. Transfer of functional PD-L1 from human antigen-presenting cells onto T cells via trogocytosis. *Manuscript in preparation*

8.2. Danksagung

Mein besonderer Dank gilt meinem Betreuer Prof. Dr. Andreas Mackensen für die Aufnahme in seine Laborfamilie und das in mich gesetzte Vertrauen, für seine stetige fachliche Begleitung und für seine ständige Ansprechbarkeit in allen Phasen der Entstehung dieser Arbeit trotz des Umzugs nach Erlangen und dem Berg an sonstiger Arbeit, die sich bei ihm am Schreibtisch stapelt. Bedanken möchte ich mich zudem für sein Verständnis, seine freundschaftliche Art und die großen Freiräume bei dieser Arbeit.

Des Weiteren möchte ich mich herzlich bei Frau Prof. Dr. Susanne Modrow für die Betreuung und Begutachtung meiner Dissertation sowie für die Vertretung vor der Naturwissenschaftlichen Fakultät III der Universität Regensburg bedanken.

Prof. Dr. Reinhard Andreesen möchte ich danken, dass ich die ersten 15 Monate die Arbeiten für meine Promotion in seiner Abteilung in der Uniklinik in Regensburg durchführen konnte.

Prof. Dr. Evelyn Ullrich danke ich herzlich, dass sie die Rolle des 2. Mentors neben Prof. Dr. Susanne Modrow in meiner Betreuungskommission des Graduiertenkollegs SFB643 übernommen hat und stets zur Diskussion meiner Daten bereit war.

Dr. Ralf Palmisano aus dem Institut für klinische Mikrobiologie und Hygiene, Universität Erlangen-Nürnberg möchte ich sehr für die Hilfe am konfokalen Mikroskop danken sowie Dr. Niels Schaft und Dr. Jan Dörrie aus dem RNA Labor, Dermatologie, Universität Erlangen-Nürnberg für die Unterstützung bei der RNA-Transfektion.

Dem Graduiertenkolleg des SFB643 möchte ich u.a. für die Möglichkeit zur Teilnahme an zahlreichen Kursen, zur Interaktion mit vielen anderen Forschern und für die finanzielle Unterstützung meiner Reise zum internationalen Immunologiekongress nach Japan danken.

Bei Dr. Co Bosch möchte ich mich für das Interesse und für die rege Diskussion meiner Daten bedanken, sowie dafür dass er beim Umzug rechtzeitig da war, um den allerletzten Schrank ins Häuschen zu tragen ☺.

Des Weiteren möchte ich mich bei den Erlanger TAs bedanken, hierbei vor allem bei Thea für die stets helfende Hand, sowie auch bei Steff, Kerstin, Barbara und Steffi. Steff und ihrem Walter danke ich außerdem für die kräftige Hilfe beim Umzug. Außerdem möchte ich mich zusätzlich bei Kathrin, Irena, Alex, Stefan, Sabine, Jonas und Heiko bedanken dass wir rundherum eine familiäre Truppe sind und man einiges hier zu lachen hat.

Ein besonderes Dankeschön gilt den Regensburger TAs Annegret, Jana, Monika und Sandra, für die Hilfe am Anfang im Labor, die schönen gemeinsamen morgendlichen Kaffeepausen sowie für ihre Freundschaft – schön dass der Kontakt bis heute nicht abgebrochen ist und wir uns jährlich auf das Grillevent in Annegrets Schrebergarten freuen können.

Bei Kathrin und Sabine möchte ich mich außerdem bedanken, dass man mit ihnen stets rechnen kann, wenn es darum geht zum Mittagessen zu gehen oder sich Schokolade reinzuhauen – was wäre man ohne Essensweggefährten – ein gesunder Geist wohnt in einem wohlgesättigten Körper, oder so ähnlich.

Bei Sam und Mike möchte ich mich sehr für ihre Hilfe bedanken: für die Mühe und Zeit beim Korrekturlesen der Dissertation, für die Hilfe bei PC-Problemen, für kritische Kommentare und Fragen bei Vorträgen, und besonders für ihre stets „aufbauenden“ Worte, die man von ihnen bekommt ob man will oder nicht ☺. Mike danke ich außerdem für seine stete Hilfsbereitschaft wie beim Umzug und für’s gelegentliche Retten vor dem Hungertod (Burger, Schnitzel).

Meinen Med3 Freunden Mario und Charlotte sowie Kathrin und Stefan möchte ich sehr für die Integration in Erlangen danken. Bei Mario möchte ich mich für jede zusätzliche Lachfalte bedanken ☺.

Mein größter Dank gilt der guten Katrin, die mit mir sämtliche Phasen der Doktorarbeit durchlebt hat, mit der ich zahlreiche Stunden um Stunden im Zug und an Bahnhöfen verbracht habe und die immer ein höchst willkommener Gast in meinem Häuschen war – ich hoffe sehr, dass wir auch ein Leben nach der Promotion haben und natürlich weiterhin

hin und wieder gemeinsam auf ein gutes Konzert gehen ☺. Außerdem hoffe ich mal wieder zu Ferien auf dem Land in Habelsbach eingeladen zu werden. Danke für alles!

Meiner Mama und Albert danke ich herzlichst für ihre stetige Unterstützung, vor allem für die Hilfe, dass nach dem Umzug nach Erlangen mein Häuschen so schnell wie möglich wohnlich und 1a ausgestattet war, sowie für ihr Verständnis, wenn „das Kind“ mal wieder arg im Stress ist und so gar keine Zeit hat.

Besonders danke ich meiner „Meierfamilie“ in Regensburg, wo die regelmäßige Nahrungsversorgung gesichert war, und die für mich ein steter Anlaufspunkt über Jahre hinweg war. Vor allem danke ich Torsten, der immer für mich da ist, auf den ich mich immer verlassen kann und der mich bei meinem Umzug nach Erlangen vor dem Nervenzusammenbruch gerettet hat und den für-kleine-Reginas-viel-zu-großen schweren LKW gefahren hat. Meiner „Schwanenfamilie“ in Erlangen danke ich für die herzliche Aufnahme, so dass ich meine „Meierfamilie“ nicht so sehr vermissen musste, ebenfalls für das leckere Essen, das stetige Interesse an den T Zellen und für die familiäre Atmosphäre.

Zu guter letzt möchte ich mich noch bei meinen „5 besten Mädels“ bedanken: bei meinen Martini-Biologinnen Marianne und Lisi, die immer an mich glauben und mit denen ich bestimmt mit 70 noch Martini trinken werde ☺, bei Nadine, die mir seit dem Studium eine sehr gute Freundin ist, sowie bei Sonja und Vroni, mit denen man durch Dick und Dünn gehen kann und auf die immer Verlass ist schon seit unzähligen Jahren. 1000 Dank dass ihr fünf immer für mich da seid!

8.3. Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet.
Die in der Danksagung aufgeführten Personen haben mir in der jeweils beschriebenen Weise unentgeltlich geholfen.
Weitere Personen waren an der inhaltlich-materiellen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht entgeltliche Hilfe eines Promotionsberaters oder anderer Personen in Anspruch genommen. Niemand hat von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.
Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Regensburg, 09.11.2010

_____
(Regina Gary)