In vitro and *in vivo* analysis of human cell-based immunotherapies for acute myeloid leukemia

Dissertation zur Erlangung
des Doktorgrades der Naturwissenschaften
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“The best way to have a good idea is to have a lot of ideas.”

Linus Pauling

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Zusammenfassung

Konventionelle Therapien, wie die operative Entfernung von Tumoren oder chemotherapeutische Ansätze, sind zwar bei der Beseitigung von soliden Tumoren besonders effizient, scheitern jedoch häufig daran, streuende Tumorzellen zu beseitigen ("Minimal Residual Disease"). Meist sind es aber genau diese Zellen, die Metastasen oder auch Rezidive verursachen. Sowohl die systemische Verteilung als auch die Langlebigkeit dieser Zellen in den für das Immunsystem schwer zugänglichen Nischen stellen die Krebstherapie vor eine besondere Herausforderung. Aus diesem Grunde ist die Kombination aus konventioneller Therapie und einer zusätzlichen Aktivierung des Immunsystems ein viel versprechender Ansatz in der Krebstherapie, um Tumorzellen mit metastasierendem Potenzial zu eliminieren.

Vor allem bei Patienten mit akuter myeloischer Leukämie (AML) bedeuten diese im Körper verbleibenden Tumorzellen eine lebensbedrohliche Gefahr, die nicht selten (70 % der Patienten) mit Rezidiven einhergeht. Deshalb sind gerade für die Bekämpfung von Leukämien weiterreichende Therapieformen von besonderer Bedeutung. In meiner Arbeit habe ich mich vor allem auf zwei Formen der Immmuntherapie konzentriert. Zum einem, auf die Verwendung von Dendritischen Zellen (DC) als therapeutisches Vakzin, zum anderen auf den adoptiven Transfer von T-Zell-Rezeptor (TCR)-transgenen Lymphozyten.

Eine Möglichkeit, das Immunsystem zu aktivieren, besteht in der Anwendung von ex vivo generierten DC, welche ein ausgezeichnetes immunstimulatorisches Potenzial besitzen. Für eine Anti-Tumor-Immunantwort sollten die DC sowohl IFN-γ-produzierende $CD4^+$-T-Zellen (Th1) wie auch zytotoxische T-Zellen (CTL) und natürliche Killer-Zellen (NK) induzieren können. Innerhalb dieser Doktorarbeit war es möglich, über einen Zeitraum von drei Tagen (3d), DC mit einem aktivierenden Phänotyp zu generieren. Hierfür wurden synthetische Toll-like-Rezeptor-3-,-7/8-Agonisten verwendet, welche eine Virus-abhängige Aktivierung nachahmen. Die auf diese Weise generierten DC waren in der Lage, hohe Mengen an bioaktivem IL-12 freizusetzen, und in der Folge, wirkungsvoll Effektorzellen zu aktivieren. Verglichen mit 7d-DC, die derzeit in klinischen Studien verwendet werden, konnte für die hier generierten 3d-DC eine

verbesserte Fähigkeit zur Aktivierung des angeborenen (NK Zellen) als auch des adaptiven Immunsystems (T-Zellen) nachgewiesen werden. Darüber hinaus wurde ein humanisiertes Mausmodell etabliert, welches die In-Vitro-Ergebnisse auch in vivo bestätigte. Hierfür wurden NOD/scid/IL2Rg^{null}-Mäuse mit humanen peripheren mononukleären Zellen rekonstituiert und mit reifen DC immunisiert. Im Einklang mit den in-vitro-Ergebnissen ergaben auch die in-vivo-Untersuchungen, dass die kürzer kultivierten, TLR-aktivierten DC in der Lage waren, eine potente anti-Tumor-Immunantwort zu induzieren.

Der adoptive T-Zelltransfer mit Hilfe von TCR-modifizierten Lymphozyten, stellt eine elegante und relativ leicht umzusetzende Möglichkeit für die Übertragung einer anti-Tumorantwort auf den Patienten dar. Bei diesem Ansatz werden zuvor isolierte TCR-Sequenzen, und somit auch deren Antigen-Spezifität, auf Lymphozyten des Patienten übertragen. Ein entscheidender Schritt hierbei ist die Isolierung von T-Zellen, die einen TCR mit hoher Affinität für Tumor-assoziierte Selbst-Antigene besitzen. Vor allem nicht im Thymus negativ selektionierte Lymphozyten besitzen TCR mit hoher Affinität für das jeweilige Antigen. Aus diesem Grund wurde ein fremdes MHC Molekül in Kombination mit Epitopen von Tumor-assoziierten Antigenen für die Induktion hochaffiner T-Zellen verwendet. In dieser Arbeit konnten T-Zellen mit hochaffinen TCR für die Tumor-Antigene Tyrosinase, Survivin und HMMR (hyaluronan-mediated motility receptor) gewonnen werden. TCR, die spezifisch für die beiden AML-assoziierten Antigene HMMR und Survivin sind, wurden isoliert und in Empfänger-Lymphozyten transferiert. Bei der Verwendung von Survivin-spezifischen TCR konnte ein "Brudermord" der eingesetzten Lymphozyten festgestellt werden. Eine Beobachtung, welche die Anwendung von Survivin-spezifischen TCR für den TCR-Gen-Transfer erheblich einschränkt. Weitere Analysen ergaben, dass diese Erkennung auf eine erhöhte Survivin-Expression in aktivierten Lymphozyten zurückzuführen war.

Im Gegensatz zu Survivin-spezifischen TCR zeigten Lymphozyten, welche mit HMMR-spezifischen TCR transduziert wurden, eine spezifische In-Vitro-Reaktiviät gegen AML-Zellen. Neben der Funktionalität in vitro ist das In-Vivo-Verhalten der TCR-modifizierten Lymphozyten für das therapeutische Ergebnis von entscheidender Bedeutung. Um dies zu analysieren, wurde ein Mausmodel etabliert, in welchem sowohl solide als auch leukämische humane Tumorzellen in

NOD/scid/IL2Rg^{null} Mäusen verwendet wurden. Ein signifikanter Rückgang der Tumorlast konnte nach einem Transfer von Lymphozyten, welche zuvor mit einem HMMR-spezifischen TCR modifiziert worden waren, beobachtet werden. Der Rückgang des Tumorwachstums konnte noch verstärkt werden, wenn die Empfänger-Lymphozyten einen Zytokin-induzierten Gedächtnis-Phänotyp aufwiesen. Im Einklang mit den In-Vitro-Analysen stellte sich heraus, dass ein Il-15-induzierter (Effektor-Gedächtnis) T-Zell-Subtyp das größte Anti-Tumor-Potenzial besaß.

Zusammenfassend konnte in dieser Arbeit gezeigt werden, dass sowohl die DC-basierte Vakzinierung, als auch der Transfer von spezifisch TCR-Gen-modifizierten Lymphozyten einen starken In-Vivo-Anti-Tumoreffekt zeigen und folglich vielversprechende Methoden für die Krebstherapie darstellen.

Summary

Conventional cancer therapies like surgery, radiation and chemotherapy help to eliminate primary tumor masses but often fail to eradicate disseminated tumor cells. However, it is such residual tumor cells that frequently underlie metastasis and relapse. Major obstacles for targeting such cells are wide spread dissemination and long-term persistence in niches that are difficult to reach. For example, many patients with acute myeloid leukemia (AML) show persistence of leukemia after chemotherapy – so-called minimal residual disease (MRD) – which confers a life-threatening risk for relapse in over 70% of patients. Arming the immune system to attack residual tumor cells has high therapeutic potential since immune cells can patrol the body to find and destroy residual tumor cells. Therapeutic approaches using the immune system - so-called immunotherapies - can take several forms. My project concentrated on preclinical studies of two strategies: 1) use of dendritic cells (DC) for therapeutic vaccination and 2) adoptive T cell therapy with lymphocytes expressing transgenic T cell receptors (TCR) specific for tumor-associated antigens (TAA).

In therapeutic vaccination a highly potent vaccine is needed to induce a valid immune response in patients with cancer. Effective antitumor immunity requires mobilization of IFN-γ-producing $CD4^+$ T cells (Th1 cells) and lymphocytes with cytotoxic function, including cytotoxic T lymphocytes (CTL) and natural killer (NK) cells. In my studies, high potency vaccines were developed using mature DC generated in 3 days (3d-mDC) that were stimulated with synthetic Toll-like receptor TLR3 and/or TLR7/8 agonists. This TLR stimulation mimics DC interaction with viruses and causes mDC to secrete the bioactive form of IL-12, supporting induction of effector cells. Characterization *in vitro* showed that TLR-activated 3d-mDC were superior to conventional 7d-mDC in capacity to induce Th1 cells as well as CTL. A humanized mouse model was established to verify these observations *in vivo*. NOD/scid $IL2Rg^{null}$ mice, lacking murine T, B and NK cells, were reconstituted with human peripheral mononuclear cells and vaccinated with 3d-mDC, stimulated or not with TLR agonists, and conventional 7d-mDC. Induction of CTL was quantified *ex vivo* using splenocyte populations containing human lymphocytes. The *in vivo* results were concordant with *in vitro*

observations, demonstrating the superior capacity of 3d-mDC that were stimulated with TLR agonists to induce CTL.

Adoptive T cell therapy using TCR-modified lymphocytes represents a second powerful way to provide patients with specific antitumor immunity. Here previously isolated TCR gene sequences are introduced into activated patient-derived lymphocytes, assigning them new antigen specificities. First, T cells must be isolated with TAA specificities that express high-affinity TCR which effectively recognize tumor cells. It was contended that T cell stimulation using peptide-epitopes from TAA presented on foreign MHC would allow isolation of high-affinity TCR, since these T cells had not yet undergone negative selection in the thymus. This contention was proved in individual experiments, as described in this thesis, for the antigens tyrosinase, survivin and HMMR (hyaluronan-mediated motility receptor). Since survivin and HMMR are broadly expressed in AML, TCR specific for these TAA were isolated and subsequently transferred into recipient lymphocytes. Expression of survivin-specific TCR resulted in MHC-restricted death of transduced lymphocytes due to their elevated survivin expression after activation. This precludes use of survivin-specific TCR for therapy of AML.

In contrast, transfer of an HMMR-specific TCR yielded effector lymphocytes that effectively killed AML cells *in vitro*. The behavior *in vivo* of TCR transduced lymphocytes is crucial for therapeutic outcome. To explore this capacity a xenograft mouse model was established using solid and disseminated human tumor cells injected into NOD/scid IL2Rg^{null} mice. Adoptive transfer of lymphocytes expressing an HMMR-specific TCR into tumor-bearing mice resulted in significant retardation of tumor outgrowth. Adoptive transfer of memory-like lymphocytes with higher proliferative potential and prolonged *in vivo* survival may also affect tumor growth. Analyses *in vivo* and *in vitro* showed that IL-15-induced effector memory T cells conferred the most potent antitumor immunity.

In summary, this work provides evidence for potent *in vivo* antitumor effects by either using DC-based vaccines or adoptive transfer of TCR transduced lymphocytes, opening application of both strategies for immunotherapy of cancer.

1 Introduction

1.1 The human immune system

1.1.1 Hematopoiesis and function

The human immune system is the most dynamic and flexible organ of the human body. Hematopoetic stem cells (HSC) give rise to all immune cells, display a well-characterized compartment of tissue specific stem cells and descendants of HSC maintain the steady state levels of cells within the peripheral blood (Carpenter *et al.* 2010). The self-renewal function of the immune system has been under investigation since Till and McCulloch showed that bone marrow cells give rise to spleen colonies in the 1960s (Till *et al.* 1961). Within the last decade, the hierarchical scheme of hematopoiesis was a matter of controversial debate, due to reports of leakage of cells within the conventional lineage-branches (Bell *et al.* 2008; Graf 2008; Wada *et al.* 2008). Exemplary reports show that lymphoid progenitors (e.g. pro-B-cells) lose the possibility to give rise to mature myeloid cells (Rolink *et al.* 1999; Adolfsson *et al.* 2005; Balciunaite *et al.* 2005). Nevertheless, conceptional tree models display the origin of all crucial subsets of the immune system by relying on the lymphoid-myeloid dichotomy model. The basis of all models is the decreasing self-renewal function of long term-HSC (LT-HSC), developing into short-term HSC (ST-HSC) and multi-potent progenitors (MPP) during the process of differentiation. The MPP still have the potential to evolve into all kinds of immune cell progenitors but have lost self-renewal potential. During further differentiation to common lymphoid progenitors (CLP) or common myeloid progenitors (CMP) this poly-potent differentiation capability decreases (Rosenbauer *et al.* 2007; Ceredig *et al.* 2009). CMP are determined to give rise only to myeloid cell types (megacaryocytes, erythrocytes, granulocytes, monocytes), while CLP evolve to lymphoid cell-types (natural killer (NK) cells, T lymphocytes (T cells), B lymphocytes (B cells)).

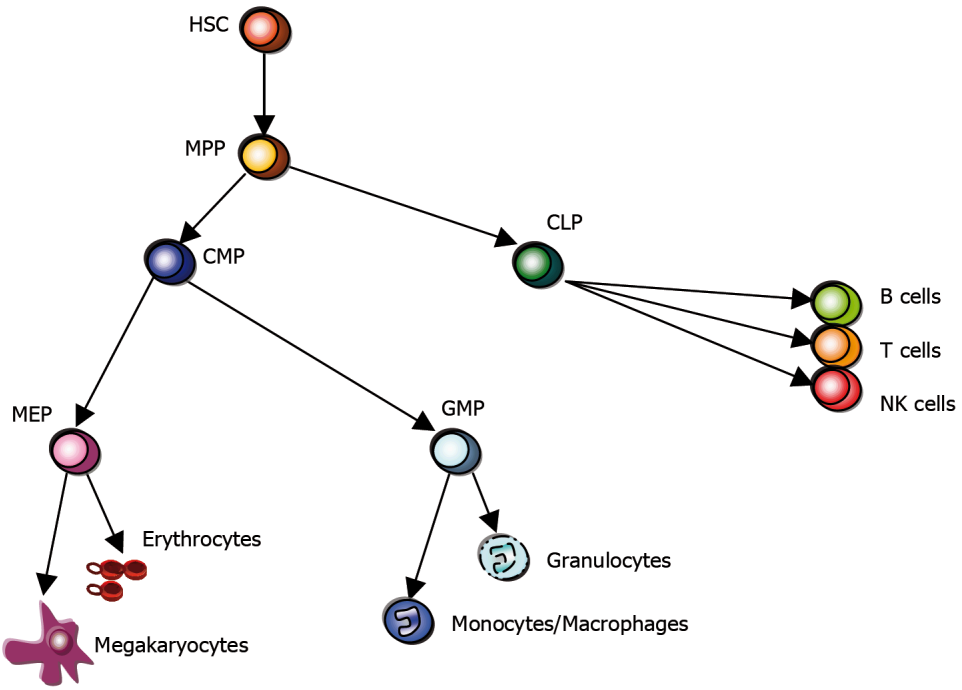

Fig. 1: Model of hematopoiesis
Lineage-based tree-scheme of hematopoiesis. The myeloid and lymphoid branches separate at the stage of multi-potent progenitors. The common lymphoid or myeloid progenitors only give rise to lymphoid or myeloid cells, respectively.
HSC (hematopoetic stem cell), MPP (multi-potent progenitor), CLP (common lymphoid progenitor), CMP (common myeloid progenitor), GMP (granulocyte/monocyte progenitor), MEP (megakaryocyt/erythrocyte progenitor) modified from (Raffegerst 2009).

The leukocytes (or white blood cells) compose the immune system, which can be subdivided into the innate and adaptive immune components. Cellular constituents of the innate system are myeloid-derived neutrophils, eosinophils, basophils, mast cells and monocytes as well as the lymphoid-derived NK cells. A common feature of “innate” cell types is their fast response to foreign particles or danger signals, without antigen-specific stimulation. In contrast, the adaptive immune system consists only of lymphoid-derived cells (T and B lymphocytes) and activation requires recognition of antigens via specific receptors (T cell receptor, TCR and B cell receptor, BCR) that are unique for each single lymphocyte. The BCR recognizes antigens directly via a membrane bound BCR or the soluble variant of the BCR, known as antibody. In contrast, TCR-mediated recognition is restricted to processed peptide sequences presented on special peptide-presenting molecules, named major-histocompartibility complexes (MHC), or human leukocyte antigen (HLA) in humans. Furthermore, for full

activation lymphocytes need costimulatory signals provided by the innate immune system. Potent activators of T cells are professional antigen-presenting cells (APC), primarily mature dendritic cells (mDC). These APC are able to ingest and process antigens and present peptide sequences to T cells on MHC-molecules. This mechanism of authorization, presenting a foreign peptide by a self-molecule, is necessary, since uncontrolled lymphocyte reactions can lead to severe damage of the body. Another control mechanism to eliminate auto-reactive T lymphocytes is founded in the thymic-selection process and described below. After thymic selection, the naïve T cell repertoire comprises cells that specifically recognize peptide-MHC (pMHC) ligands and encompass a potential diversity of at least 10^{15} different TCR (Davis *et al.* 1988).

1.1.2 Development of T lymphocytes

T lymphocytes are named after their location of development, the thymus, a primary lymphoid organ. Thymocytes evolve from CLP progenitors, which receive stimuli from thymic epithelial cells (TEC), initiating the development into thymocyte progenitors (Schlenner *et al.* 2010). Ongoing differentiation into fully mature T cells can be further separated into double-negative (DN), expressing no co-receptors, and double-positive (DP), expressing both CD4 and CD8 co-receptors. Those stages combined with a thymic selection process, are leading to single positive mature thymocytes (Carpenter *et al.* 2010; Hernandez *et al.* 2010). The stages of gradual differentiation within the DN stage can be further subdivided into the DN1 through DN4 stage (Fig. 2) (Godfrey *et al.* 1993). While DN1 cells are still able to give rise to DC, as well as NK cells, the DN2 phase is characterized by up-regulation of CD25, determining the T lymphocyte lineage. Cell survival of DN1 and DN2 is mediated by the Notch1 transcription factor, which is crucial for T cell development (Chi *et al.* 2009; Radtke *et al.* 2010). During the DN2 phase the rearrangement of TCR-β chain gene segments is initiated by fusion of D- and J-gene segments, followed by the V-segment joining within stage DN3 (Krangel 2009). These recombination-activating-gene (Rag) mediated rearrangements include additions and deletions of nucleotides within three “complementarity determining regions” (CDR) of the TCR β-chain resulting in an individual recognition pattern for each TCR chain.

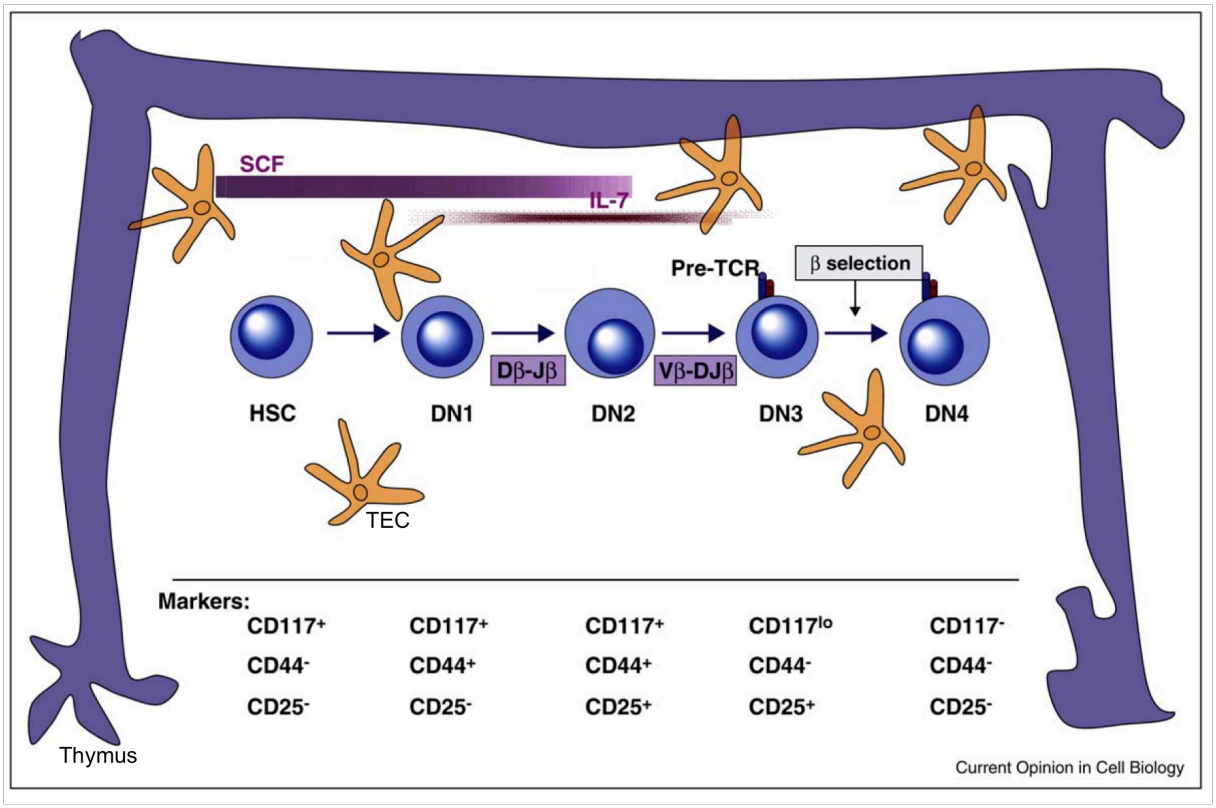

Fig. 2: Scheme of thymocyte development
Shows schematically the differentiation progress in the thymus starting with HCS through DN4 stage. Stem cell factor (SCF) and Interleukin-7 (IL-7) are shown as soluble factors maintaining the early survival. Steps of TCR rearrangements as well as TCR expression are also depicted. Cell surface markers to discriminate the different stages are listed below. (modified from (Hernandez 2010).

To prove the functionality of each β-TCR chain, β-selection occurs during DN4 stage of differentiation. Oligomerization of the β-chain with a pre-α-chain and CD3 induces a positive survival signal, initiation of the α-gene-segment rearrangement (V-J-recombination) and allelic exclusion of the non-rearranged β-chain gene. After passing the DN stages, T cell progenitors express an α- and β-chain comprising newly displayed TCR in combination with CD4 and CD8, characterizing the DP stage. DP thymocytes undergo a developmental process inducing central tolerance encompassing three stages (Starr *et al.* 2003):

I. Positive selection:
   Comprises a process in which TCR binding to pMHC ligands is verified. T cells with non-binding TCR do not receive a positive survival signal and die within the next three to four days.
II. Negative selection:
   This process reflects the core function of central tolerance, eliminating all T cells recognizing MHC ligands loaded with self-peptides by a

programmed cell-death mechanism (von Boehmer *et al.* 2010). The TEC present these self-pMHC ligands on their surface, which are than screened by developing lymphocytes. The expression of tissue-specific proteins by TEC is mediated by the transcription factor AIRE (autoimmune regulator) (Mathis *et al.* 2009).

III. Acquisition of functional competence:

The functional differentiation takes place in accordance with attainment of CD4 or CD8 co-receptor expression. The characteristics and gene expression profiles of helper T cells (Th) and cytotoxic T lymphocytes (CTL) are linked with the expression of the particular co-receptor (Corbella *et al.* 1994; Matechak *et al.* 1996). Lineage-decision is based on the TCR recognizing either MHC class I for CD8 or MHC class II for CD4 and is realized by gradual down-regulation of CD4 or CD8, in combination with ongoing MHC:TCR interactions (Brugnera *et al.* 2000; Germain 2002).

The terminal maturation of thymocytes depends on the transcription factor Klf2 (Kruppel-like factor 2), inducing L-selectin and sphingosine-1-phosphate-receptor expression, allowing the migration into the blood stream. These naïve T lymphocytes are positive for the transcription factor Fox01 (Forkhead box protein 01), which mediates homeostasis and survival in the periphery, until they receive an antigen dependent stimulation (Fabre *et al.* 2008; Kerdiles *et al.* 2009; Ouyang *et al.* 2009).

1.1.3 T lymphocyte-mediated immunity

Naïve T cells migrate via the blood stream to secondary lymphoid organs, e.g. lymph nodes or red pulp of the spleen, screening APC in the T cell area for the specific recognition of antigen. Professional APC, e.g. DC, have the ability to present endogenous and exogenous antigen on MHC class I and II molecules activating $CD8^+$ or $CD4^+$ T cells, respectively. Induction of effector T cells requires three signals delivered by the DC: (I) Interaction of pMHC ligands with TCR, (II) co-stimulatory molecules of the B7-familiy fostering the activation via CD28 or inhibition via CTLA-4 (cytotoxic T lymphocyte antigen-4) and PD-1 (programmed-death-1) of the T cell and (III) cytokines, secreted by the APC, orchestrating the immune response (Valitutti *et al.* 1995; Viola *et al.* 1999; Schuler *et al.* 2003). Only fully mature DC can deliver these signals in an

adequate fashion, while insufficient stimulation leads to T cell anergy. Over stimulation results in programmed cell death, known as "activation induced cell death" (AICD) (Marrack *et al.* 2000). Following specific antigen-recognition, a differentiation and proliferation process is initiated generating an oligoclonal effector T cell population. Among the CD4 cells, subtypes like Th1, Th2 or Th17 cells are induced with respect to the cytokine-milieu present during their priming process, conducting immune responses into distinct directions (Zheng *et al.* 1997; Szabo *et al.* 2000; Ivanov *et al.* 2006). After clearance of antigen, a "contraction" phase proceeds, leading to a reduction of the antigen-specific T cell population leaving several long-lived cells behind. These residual T cells are described as memory T cells, which are well specified for $CD8^+$ T cells, while the heterogenic phenotype of $CD4^+$ memory T cells are less well characterized. For the development of memory T cells two theories are currently under consideration: direct determination during the priming process or evolvement from the pool of effector T cells (Gerlach *et al.* 2011). There is consensus that the transit of T cells into the memory compartment depends on the antigen dose of stimulation but the required signal strength is controversially discussed (Ahmed *et al.* 1996; Lanzavecchia *et al.* 2000; Lanzavecchia *et al.* 2002). Memory cells are characterized by long-term persistence without antigen stimulation and can be subdivided into central (T_{CM}) and effector memory cells (T_{EM}) (Sallusto *et al.* 1999). Whether these memory subsets are distinct or if central memory cells give rise to disposing effector memory cells is still not completely understood (Lanzavecchia *et al.* 2002). Both memory subsets are maintained by interleukin-7 (IL-7) and IL-15, providing a lifelong immediate protection following a repeated encounter to the same antigen (Ahmed *et al.* 1996; Becker *et al.* 2002; Goldrath *et al.* 2002; Kieper *et al.* 2002)

1.2 Acute myeloid leukemia - tumorigenesis of the immune system

Leukemia is a cancer of blood or bone marrow cells and is clinically detectable by increased white blood cell counts. It was first characterized by the German physician Rudolph Virchow in 1845 ("leukos" – white; "haimia" – blood). Leukemia can be subdivided according to disease progression into acute or chronic subtypes, while the chronic variants result in a final acute state. The acute variants are characterized by rapid increase of immature (mostly bone-

marrow-derived) cells while a slower progression of more differentiated cell types characterizes chronic variants. Additionally, leukemic diseases are further divided by their lineage origin into lymphoid and myeloid types.

AML is distinguished by the clonal outgrowth of immature progenitor cells (blasts) and clinically characterized by the morphological stage of differentiation in which the transforming event took place (French-American-British system (FAB)). Furthermore, cytogenetic, molecular and immunophenotyping techniques are applied to further discriminate the stage of differentiation, recently specified by the WHO (World Health Organization) classification (Swerdlow *et al.* 2008). In 2010, the Leukemia and Lymphoma Society reported on a total of 43,050 newly diagnosed leukemic diseases in the USA (ALL 5,330; CLL 14,990; AML 12,330; CML 4,870). With respect to survival rates reported within the same time frame, AML displays the most life-threatening form of leukemia here shown as percent survival: ALL 66.4%; CLL 79.7%; AML 24.2%; CML 54.6% (www.lls.org). Leukemia ranges among the 10 most life-threatening cancer diseases, related to the death rates of other cancer types (Fig. 3, status 2007, United States). These statistical data are in consistence with observations in Germany.

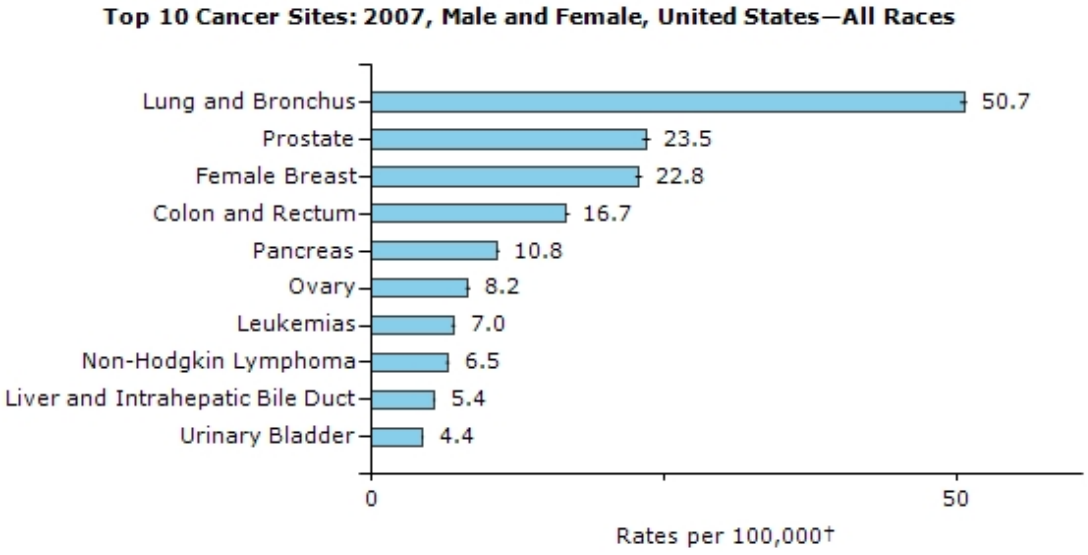

Fig. 3: Death per 100.000 U.S. citizens caused by cancer
Death rates per 100.000, age-adjusted, U.S. citizens, listing the 10 most life threatening cancer types. Statistical data of the last published evaluation in 2007. Leukemia on position 7 causing 7 deaths within 100.000 people. (http://apps.nccd.cdc.gov).

Standard therapy, applying chemotherapy (cytarabine, anthracycline) alone or in combination with bone marrow transplantation, results in 65-80% complete

remission of AML, but only in 30% of the patients, does therapy result in a long-term disease-free survival without relapse (Greiner *et al.* 2004).

AML is characterized by often-occurring abnormal karyotypes (45%) and about 15% of AML show at least 3 genetic alterations (Poppe *et al.* 2004). These chromosomal translocations, deletions, insertions or duplications generally lead to activation of “oncogenes” or inhibition of “tumor-suppression genes”, respectively. Affected genes mainly contribute to proliferation, cell survival and hematopoetic differentiation. A common theory for leukemogenesis is a two-hit model, in which the first mutation affects genes of proliferation and survival (e.g. *KIT, FLT3, NRAS/KRAS*), while the second mutation occurs at a later stage and influences the differentiation process (e.g. *CEBPA, NPM1*) (Kelly *et al.* 2002). Expression levels of proteins favoring survival and proliferation of AML cells are mostly aberrantly upregulated and required for ongoing tumor survival. Therefore, these proteins, including survivin and hyaluronan-mediated motility receptor (HMMR/Rhamm), display potential targets for immune-mediated therapeutic approaches (Greiner *et al.* 2008; Jiang *et al.* 2010).

1.3 Cancer immunosurveillance and immunoediting

As early as 1909, Paul Ehrlich claimed: “Ich bin überzeugt, dass abberrierende Keime bei dem kolossal komplizierten Verlauf der fötalen und post-fötalen Entwickung außerordentlich häufig vorkommen, dass sie aber glücklicherweise bei der überwiegenden Mehrzahl der Menschen vollkommen latent bleiben, dank der Schutzvorrichtungen des Organismus. Würden diese nicht bestehen, so könnte man vermuten, dass das Karzinom in einer gradezu ungeheuerlichen Frequenz auftreten würde.” (by P. Ehrlich (Ehrlich 1909)). Thus, he postulated that the immune system protects the organism from its own abnormal cells. Referring to this theory Thomas and Burnet described independently the theory on cancer immunosurveillance (Burnet 1964). Immunosurveillance describes the recognition and elimination of abnormal cells by the organisms’ own immune system (Dunn *et al.* 2002; Dunn *et al.* 2004a). There are several studies using immuno-incompetent mouse models proving this theory, as well as clinical data demonstrating this effect in humans (Dunn *et al.* 2004a). Direct impact on tumor cells by components of the immune system leads to drastic changes within the tumor mass. This process is described as “immunoediting”. In general, this phase

describes the protective mechanism by which the tumor is eliminated. However, this goes along with selection of tumor escape variants. Dunn and colleagues split the process of immunoediting into three major phases: (I) elimination, (II) equilibrium and (III) escape (Dunn *et al.* 2004b).

(I) Elimination:

This comprises the immune response directed against the cancer and can be further subdivided into four phases. During the first phase, the innate immune system recognizes rapid tumor growth accompanied by tissue damage. Inflammatory danger signals lead to recruitment of NK cells as well as DC. Within the second phase high amounts of secreted interferon-γ (IFN-γ) result in tumor damage, causing activation of DC to ingest tumor fragments and migrate to draining lymph nodes. During the third phase, tumor damage and inhibition of vascularization, mediated by the innate immune system, proceeds. Meanwhile, tumor-specific DC recruit and activate $CD4^+$ (Th1) and $CD8^+$ T lymphocytes, specific for TAA. Phase four describes the infiltration of those activated T cells into the tumor, leading to complete tumor cell elimination.

(II) Equilibrium:

During this phase the tumor stops growing and moves towards a long period of dormancy. This is accomplished by the immune system decreasing the cancer's capability to further grow. It eliminates some tumor cells but not enough to eradicate the tumor completely. This ongoing immune response is likely to cause escape variants of tumor cells, resulting in resistance to elimination.

(III) Escape:

This comprises a reinitiated uncontrolled tumor growth of immunologically "silent" escape variants. Cancer cells, which have escaped from the immune response, give rise to growing malignant tumors together with tumor cell spread.

With respect to AML, evidence for immunosurveillance mechanisms can be detected in patients, although it is not resolved if those mechanisms are of primary origin or induced by therapeutic approaches (Barrett *et al.* 2010). Nevertheless, increased lymphocyte counts are related to decreased and retarded relapse rates (Bruserud *et al.* 1998; Behl *et al.* 2006; Barrett *et al.*

2009). In addition to these clinical observations, several immunoescape mechanisms are described, indicating a process of immunoediting. Evidence for suppression of NK cell-mediated immunity include elevated expression levels of killer-inhibitory-receptors (KIR) on tumor cells as well as secretion of soluble suppressing factors (Panoskaltsis *et al.* 2003; Verheyden *et al.* 2004). In concordance to NK cell suppression, T cell immunity can be affected by many tumor escape mechanisms, e.g. aberrant peptide presentation or down-regulation of MHC molecules (Vago *et al.* 2009; van Luijn *et al.* 2010). Moreover, numerous observations have demonstrated that changed expression profiles of costimulatory molecules have a profound impact on T cell responses and down-regulation of B7.1 and B7.2 results in worse prognosis (Whiteway *et al.* 2003). There is a correlation of a particular CTLA-4 genotype with a higher frequency of disease (Perez-Garcia *et al.* 2009). In addition, most AML patients exhibit an immunosuppressive microenvironment, while the bone marrow comprises an ideal environment for the maintenance of leukemic stem cells (Lapidot *et al.* 2007; Zhi-Gang *et al.* 2008).

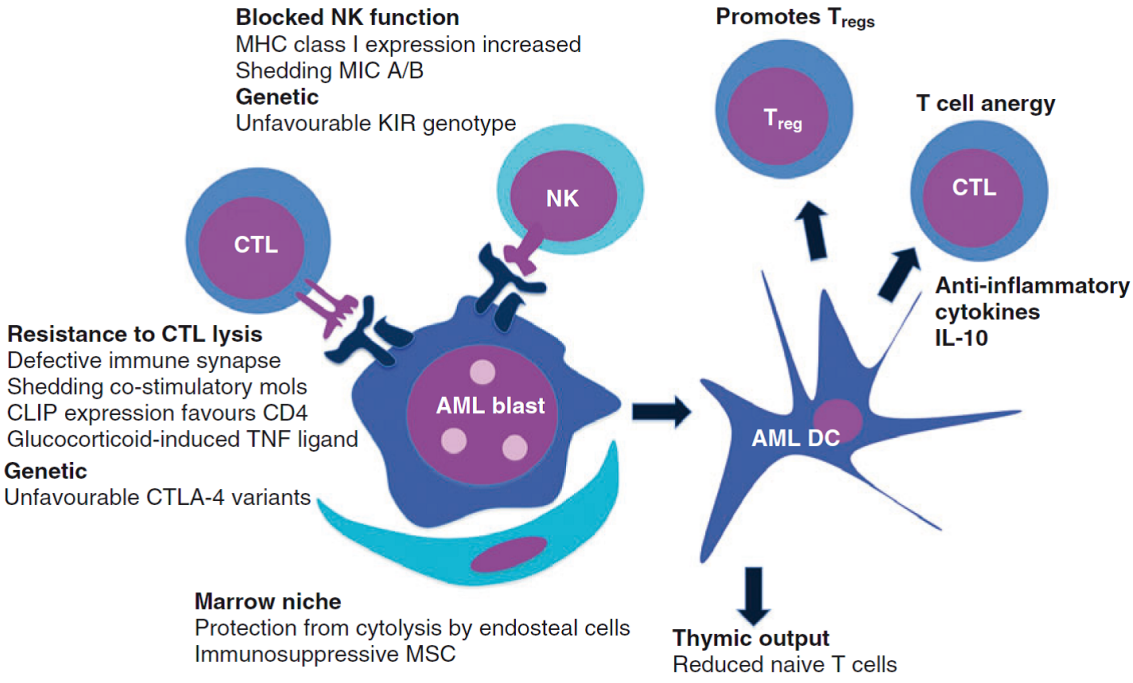

Fig. 4: Interactions of the immune system and AML cells
Escape mechanisms of AML tumor cells to conquer the immune system as well as suppressive mechanisms on T cells by tumor-derived DC (Barrett *et al.* 2010).

A unique feature of AML to suppress the immune system is based on the origin of tumor cells. These myeloid-derived tumor cells mostly share the ability

to become tolerogenic APC, comprising the dangerous potential to induce antigen-specific anergy in tumor-reactive T cells or even induce regulatory T cells (Mohty *et al.* 2001; Narita *et al.* 2001; Schui *et al.* 2002; Tong *et al.* 2008; Ge *et al.* 2009).

1.4 Immunotherapy

1.4.1 A general survey

The concept behind immunotherapy is to reinforce the patient's own immune system for recognition of tumor cells and elimination of residual cells after primary treatment, including surgery or chemotherapy. Therapeutic approaches can be subdivided along two basic principles. The first is based on boosting the immune system using classical vaccination strategies all the way through to whole cell-vaccines. The second involves transfer of antitumor immunity to the patient via the application of tumor-specific antibodies, T cells or transgenically-engineered lymphocytes.

With respect to AML, already the standard clinically applied bone marrow transplantation utilizes an approach arming components of the immune system. While transplantation of solid organs primarily uses HLA-matched donor-recipient pairs, mismatches in major histocompatibility complexes can be even beneficial for the therapy of leukemia. It has been reported that allogeneic primed lymphocytes can efficiently limit tumor outgrowth in mouse models (Bonnet *et al.* 1999; Distler *et al.* 2008). In concordance to these results, Barrett reported on an elevated Graft-versus-Leukemia (GvL) effect in patients treated with allogeneic transplants (Barrett 2008). A successful therapeutic approach of allogeneic stem cell therapy was introduced by Kolb et al. and is described as "haplo-identical" bone marrow transplantation (Kolb 2008). This therapeutic approach uses donor-recipient pairs, which differ in one HLA-haplotype (e.g. partial-mismatched siblings or parent-child combinations). After acceptance of the stem cell graft by the recipient (induced chimerism), donor lymphocytes are transferred to induce an allogeneic mediated GvL-response.

A more broadly used immunotherapeutic approach is the administration of cytokines to boost the immune system. In particular, high-dose or low-dose IL-2 was proven to prolong the survival of cancer patients by inducing antitumor immune responses (Antony *et al.* 2010). The usage of IL-2 therapy for AML is

discussed controversially due to some reports, which demonstrate beneficial effects as well as reports which show IL-2-induced relapse (Macdonald *et al.* 1991; Meloni *et al.* 1994; Stone *et al.* 2008).

Application of antibodies comprises another promising approach, which is already successfully used to treat cancer diseases. Interestingly, the treatment of Her2-positive breast cancer patients with Herceptin, an anti-Her2 antibody, is able to prolong significantly the disease-free survival (in mean 33 weeks) (Mannocci *et al.* 2010). Possible AML-associated targets for antibody-therapy are CD123, CD47, CD64 and CD33. Especially, Gemtuzumab, targeting CD33, in combination with other therapeutic strategies, showed promising clinical data (Estey *et al.* 2002; Amadori *et al.* 2005; Larson *et al.* 2005). Besides antibodies, that target TAA, blocking-antibodies are potent tools to boost or redirect immune responses (Callahan *et al.* 2010). For instance, Ipilimumab, an anti-CTLA-4 blocking-antibody, silencing an inhibitory costimulatory receptor on T cells and thereby enhancing immune responses against tumor cells showed first benefits in patients with metastatic melanoma (Hodi *et al.* 2010).

In addition to the previously described therapies, peptide vaccines in combination with strong adjuvants, such as Toll-like-receptor (TLR) agonists, like poly(I:C) or lipopolysaccarides (LPS), have been proven to be effective for the induction of potent Th1/CD8 antitumor responses. Promising antigens to target AML have been reviewed by Greiner and colleagues and include Wilms-Tumor-1 (WT-1), proteinase-3 (PR-3) and HMMR (Schmitt *et al.* 2006; Schmitt *et al.* 2009). Several studies demonstrated increased T cell responses combined with prolonged survival (Saliba *et al.* 2007; Rezvani *et al.* 2009; Greiner *et al.* 2010).

Beyond the above described modulations of the immune system, administration of whole cells, including DC and T cells, can confer antitumor immunity to patients, and will be described below.

1.4.2 Dendritic cell-based vaccination

Deduced from classical vaccination against foreign pathogens, two basic observations were made that (I) DC play a central role in inducing immune responses and (II) adjuvants used for vaccination are potent DC activators (Palucka *et al.* 2010). The main goal of establishing DC-based vaccines for cancer treatment is to prime and redirect the immune system, resulting in

reinforced recognition and elimination of tumor cells. The immune response that is needed for an effective antitumor effect can be compared to leprosy, where the indolent variant is characterized by a Th1 response, whereas the lepramatous form is characterized by a mostly lethal Th2 response. Therefore, the potent induction of a $CD4^+$ Th1-response goes along with activation of cytotoxic $CD8^+$ T cells and hence is of outstanding importance and essential for the development of antitumor vaccines (Janssen *et al.* 2003). Th1 responses or cellular immunity in general, typically are directed against intracellular pathogens, like *M. tuberculosis*, and are characterized by secretion of IFN-γ. In contrast, Th2 responses combat extracellular pathogens via secretion of IL-4 and IL-13 resulting in humoral immunity (Mosmann *et al.* 1986). DC deliver three "activator-signals" that conduct the induced immune response: signal 1 - pMHC ligands for the presentation of antigens to T lymphocytes; signal 2 - costimulatory molecules; and signal 3 - secretion of Th1 polarizing cytokines. For instance, defined members of the IL-12 family influence the type of Th response, with IL-12p70 being a potent activator of Th1 responses (Macatonia *et al.* 1995). Costimulatory signals are mostly dependent on B7-family members binding to CD28 and thereby deliver positive signals to T cells during the priming process, whereas their binding to CTLA-4 inhibits immune responses (Krummel *et al.* 1995). Depending on the signal strength and duration leading to maturation, expression profiles of these costimulatory molecules can be very diverse on mDC (Chen 2004; Greenwald *et al.* 2005).

In parallel, mDC possess an enhanced ability to interact with T cells, while immature DC (iDC) have the unique capacity to ingest antigens (Lanzavecchia *et al.* 2001). In addition, iDC contain an excellent machinery to process and present peptides on MHC molecules, but lack activating signals. The expression of high levels of costimulatory molecules as well as MHC molecules distinguishes fully mDC from iDC. Thus, it is not surprising that several clinical studies using iDC for antitumor vaccination failed, due to their tolerogenic phenotype (Schuler *et al.* 2003; Steinman *et al.* 2003). Therefore, much effort is undertaken to reveal potent adjuvants, such as microbial-derived components, to optimally induce DC maturation (Reis e Sousa 2006). Receptors that recognize such pathogen-derived structures can be sub-grouped into C-type lectins, TLR, NOD-like receptors and RIG-I-like receptors (Manicassamy *et al.* 2010; Takeuchi *et al.*

2010). In particular, TLR are known to represent potent stimulators of DC maturation. TLR downstream signaling results in activation of NFkB (nuclear factor kappa-light-chain-enhancer of activated B cells) and other immunomodulatory genes. Thereby, a modulation of the secreted cytokines as well as expression of costimulatory molecules is initiated (Gautier *et al.* 2005; Napolitani *et al.* 2005).

In contrast to conventional vaccination, where a vaccine is given preventively, antitumor vaccination is mostly applied therapeutically, with the intention to eliminate residual tumor cells. In addition, potential target antigens for vaccination are limited to overexpressed self-antigens or to antigens with rare gene mutations. Compared to over-expressed antigens, mutated epitopes provide the advantage that responses can be generated from a non-selected TCR repertoire, whereas T cells recognizing self-antigens will have undergone negative selection for high aviditiy T cells in the thymus (Parmiani *et al.* 2007; Appay *et al.* 2008). Furthermore, overcoming the tumor microenvironment that simultaneously limits potent immune responses, is one of the biggest hurdles for DC-based vaccine usage. The tolerogenic milieu in the tumor microenvironment is determined mainly by regulatory T cells as well as tolerogenic subtypes of DC, often described as myeloid-derived suppressor cells (MDSC) (Gabrilovich *et al.* 2009). These factors counteract a potent induction of antitumor immunity. Therefore, a better understanding of DC biology, resulting in robust mDC that can induce potent antitumor immunity is under intensive investigation (Steinman *et al.* 2007; Melief 2008).

With respect to AML, several attempts were made to generate DC loaded with tumor lysates or RNA-encoding TAA (Roddie *et al.* 2006; Lei *et al.* 2009; Smith *et al.* 2009; Van Driessche *et al.* 2009). So far, these attempts resulted induced antitumor responses *in vitro*, while the clinical benefits were poor.

1.4.3 Adoptive T cell transfer

Shortly after the discovery of the cellular immune response, Alexander and colleagues cured mice with murine sarcomas using syngeneic lymphocytes of previously immunized mice (Alexander *et al.* 1964). Fefer and colleagues reported on the beneficial usage of chemotherapy in combination with lymphocyte transfer into mouse models (Fefer 1969). Later on, Eberlein

described an improved effect of lymphocytes that were cultured *ex vivo* with IL-2, resulting in the cure of disseminated murine tumors (Eberlein *et al.* 1982). Likewise, Rosenberg reported on IL-2-mediated reactivation of tumor-infiltrating lymphocytes (TIL) before adoptive transfer (Rosenberg *et al.* 1986). All of these mouse models paved the way towards the usage of T lymphocytes as therapeutic agents to treat cancer. However, when compared to antibody therapy, adoptive T cell therapy still lags behind in development although several clinical applications have been very successful. In general, adoptive T cell therapy can be subdivided into the usage of primary T cells or genetically-modified lymphocytes.

Primary T cells can be of various origins and comprise patient-derived TIL, *ex vivo* primed T cell bulk cultures of autologous or allogeneic origin or even single cell-derived T cell clones. Therapeutic approaches using those T cell types were shown to be highly efficient in treating post-transplant lymphoproliferating disorders (PTLD), induced by Epstein-Barr virus (EBV) infections (Heslop *et al.* 2010). Furthermore, TIL isolated from melanoma patients, transferred into lympho-depleted patients, were demonstrated to have impressive clinical benefit (Dudley *et al.* 2002). Primary isolated lymphocytes are associated with high costs and laborious individual preparation procedures and a non-predictable efficacy.

Therefore, genetically-engineered lymphocytes provide a promising tool for adoptive cell therapy. Modifications mainly concentrate on the introduction of TCR, specific for certain TAA. In addition, provision of costimulatory molecules, beneficial cytokines or survival factors is also feasable. Several studies on isolation and transfer of high-affinity TCR into recipient lymphocytes were published and are still a subject of intense research (Cole *et al.* 1995; Morgan *et al.* 2003; Hughes *et al.* 2005; Zhao *et al.* 2005). Initial clinical studies, focusing mainly on advanced melanoma, have proven efficacy of TCR-modified lymphocytes, but also showed severe side effects due to on-target toxicity directed against normal tissues expressing the same self-proteins as the targeted tumor cell (Johnson *et al.* 2009). The clinical results of those studies suggest the combination of several different TCR to avoid selection of escape variants. Furthermore, to avoid cross-reactivity due to pairing of endogenous and transgenic TCR, modifications of the TCR genes are considered (Cohen *et al.* 2006; Sommermeyer *et al.* 2010). Such modifications concentrate mainly on the

replacement of specific amino acids or exchange of the constant regions by the murine counterparts (“murinization”) to foster pairing of the introduced TCR chains and to avoid mispairing with the endogenous chains.

An additional subject of intense research focuses on the recipient lymphocytes that are used for TCR transfer. Conflicting statements have been made regarding the T cell phenotypes that yield convincing results *in vivo* with respect to naïve, effector as well as central memory phenotypes (Berger *et al.* 2008; Hinrichs *et al.* 2009a; Heslop *et al.* 2010). Considerations to apply long-term persistent anti-viral memory cells or gamma-delta T cells are ongoing (van der Veken *et al.* 2009). Studies provide evidence that the phenotype of the recipient lymphocytes determines whether the TCR-transgenic lymphocyte will become anergic or serve as potent effector cells, thereby this topic is of outstanding interest (Dossett *et al.* 2009). In addition, since the function and importance of immunosuppression to eliminate regulatory T cells was demonstrated, pre-conditioning of therapeutic recipients is also a subject of intense research (Antony *et al.* 2005).

The main use of targeting mainly over-expressed self-antigens is a major pitfall of adoptive T cell transfer. For application of primary, patient-derived T cells, this fact limits mainly the avidity of T cells that are used for adoptive transfer. In contrast, transgenic TCR used for modification of lymphocytes can be of high affinity if they are obtained via allogeneic *in vitro* priming approaches or following genetic modifications (Morris *et al.* 2006; Kuball *et al.* 2007). Nevertheless, use of high-affinity TCR directed against self-antigens carries the risk of autoimmune reactions that affect antigen-expressing organs. It has been demonstrated by Overwijk that such autoimmune reactions accompany a potent antitumor effect (Overwijk *et al.* 2003). Therefore, the selection of target antigens has to be a carefully considered.

For the treatment of AML, haploidentical T lymphocyte transfusion into non-transplanted recipients resulted in positive clinical effects (Colvin *et al.* 2009). Additionally, several reports identifying anti-leukemic T cells in patients that would allow the application of autologous TIL were published (Zhong *et al.* 2008; Rezvani *et al.* 2009). In the meantime, attempts to isolate high-aviditiy TCR specific for AML-associated antigens and suitable for lymphocyte modification were initiated (Wolfl *et al.* 2007; Stauss *et al.* 2008).

2 Project Aims

Immunotherapeutic approaches for treatment of AML using DC-based vaccines or adoptive T cell therapy constitute promising therapeutic strategies but both still require improvement. Therefore, this thesis project was designed to focus first on *in vitro* optimization to generate such cellular agents with subsequent assessment of their efficacy *in vivo* using mouse models.

2.1 Optimization of *ex vivo*-generated DC and *in vivo* evaluation

Due to the fact that DC are potent activators of the immune system, utilization of this cell type for development of an antitumor immune response seems reasonable. Nevertheless clinical studies resulted in only poor benefit due to the marginal immune responses that were induced in patients. Several studies reported on the importance of DC-activation stimuli to induce a potent immune stimulatory phenotype in murine DC, particularly through modulation of their cytokine production (Reis e Sousa 2006). Therefore, one of the major aims of this thesis was to optimize the composition of human DC maturation cocktails by including activation signals using Toll-like receptors (TLR), which can modulate their capacity to secrete cytokines. This optimization process involved comparison of the impact of various maturation cocktails on the functional capacity of DC to activate innate and adaptive immune responses. This was assessed using a variety of immune monitoring assays including phenotypical analysis of the DC themselves as well as the activation status of NK and T cells. Further assays concentrated on the potential of those effector cells to recognize and kill tumor cells.

In addition, the duration of DC generation is an important factor, impacting on the viability and function of DC. Therefore, comparison of the most frequently used clinical form of mDC generated using a 7-day protocol and a newly established 3-day protocol was considered. These DC were compared for their potential in antigen-presentation as well as their capacity to induce antigen-specific immune responses.

Given that DC-based vaccines are usually injected intradermally or near lymph nodes, the capacity to enter secondary lymphoid organs, like lymph nodes, as well as their ability to induce potent immune responses *in vivo* are essential.

Therefore, humanized *in vivo* mouse models are needed to assess the potential of *ex vivo*-generated DC to induce immune responses. Thus, a second major aim was to establish a NOD/scid IL2Rg^{null} (NSG)-based mouse model, reconstituted with human PBMC, to allow comparison of different forms of mature DC.

2.2 *De novo*-induction of AML-specific T lymphocytes and functional analysis using xenograft mouse models

The adoptive transfer of tumor-specific T lymphocytes into patients encompasses another promising strategy to overcome insufficient antitumor immunity. Included in this approach is the use of patient-derived lymphocytes that express a pre-selected TCR as a transgenic protein, providing them with a new antigen specificity. Here it is essential to isolate high-affinity TCR, specific for broadly expressed TAA that are restricted to prevalent HLA alleles. To obtain high-affinity TCR a priming approach can be used employing DC of an HLA-A2-negative donor that are provided with *ivt*-RNA encoding a TAA of choice in combination with HLA-A2 *ivt*-RNA, in order to create new allogeneic pMHC ligands at the DC surface that can be recognized by T cells. By this means it is possible to activate T cells autologous to the DC that express high-affinity TCR since they were not negatively selected against allo-pMHC ligands in the thymus. Since HLA-A2 represents a prevalent allele within the Caucasian population use of this MHC was given first preference for development of potential therapeutic TCR. This DC priming approach was established using the melanoma associated antigen tyrosinase and consequently used to isolate T cells directed against the AML-associated antigens survivin and HMMR.

Stimulation using DC loaded with *ivt*-RNA encoding the entire antigenic protein allows the isolation of T cells directed against all intrinsic immunogenic peptides in the TAA that bind to the MHC. However, this priming approach also induces T cells that recognize the allogeneic MHC molecules expressing peptides that are not derived from the TAA, but from other proteins expressed in the DC. Therefore, methods are needed to efficiently distinguish alloreactive versus allo-restricted peptide-specific T cells of choice. Often TCR binding of MHC-peptide multimers is used to enrich specific T cell populations which are then cloned by limiting dilution and further characterized for specificity. Use of MHC-multimers for selection limits the enriched T cells to those specific for the single peptide used to construct the

MHC-multimer. Therefore, another goal of this thesis was to develop an alternative method to isolate primed T cells via the CD137 activation marker that is upregulated upon TCR stimulation. Enriched T cells are then cloned and characterized for alloreactivity versus allo-restricted peptide-specific specificity to find the TCR with the desired specificity.

Following these methodical developments, the next focus of this project was to generate TCR-modified lymphocytes and to characterize them *in vitro* for phenotype and function as well as to determine if the “new” tumor specificity was transferred via the TCR. This characterization relies on application of the same immune monitoring methods used for the characterization of effector cells induced by DC vaccines. Further investigations addressed the use of various cytokines for expansion of the TCR-transgenic recipient lymphocytes. In particular, cytokines like IL-15 and IL-7, which are linked to memory-like phenotypes, were used to improve their *in vitro* function.

A final intention of these studies was to develop a xenograft mouse model to characterize the therapeutic impact of TCR-modified lymphocytes on *in vivo* tumor growth. This consisted of establishing xenograft model systems for solid as well as disseminated human tumors based on the same immunodeficient NSG mice. NSG mice were first injected with tumor cells and then given adoptive T cell therapy with TCR-transgenic lymphocytes. To address the impact on solid tumors subcutaneous injection of either a human AML cell line (THP-1) or a human melanoma cell line (mel624.38) was performed. To assess control of disseminated tumors, as leukemia, intravenous injection of luciferase tagged THP-1 cells was performed and resulted in a leukemia-like disease. Finally, in subsequent adoptive transfer experiments, an HMMR-specific TCR expressed in different effector cell phenotypes was used to assess their role in retarding outgrowth of both solid or disseminated tumor cells.

3 Discussion

3.1 Dendritic cell-based vaccine

DC-based vaccines represent potent activators of the immune system and thereby exhibit the capacity to induce an antitumor immunity. For this purpose DC must carry optimal stimulatory properties comprising high levels of pMHC ligands, a positive costimulatory profile and a cytokine-profile conducive for antitumor immune responses. In addition, a good migratory capacity and a distinctive capacity for antigen-processing contribute to a potent induction of the immune system.

3.1.1 Improvement of *ex vivo*-generated dendritic cells

One part of this doctoral thesis comprised the optimization of *ex vivo* generated DC, to induce potent antitumor immunity. Although the commonly used DC-maturation cocktail (hereafter 4C), containing IL-1β, tumor necroses factor-α (TNF-α), prostaglandin E_2 (PGE_2) and IL-6, results in fully mDC, those DC fail to induce potent Th1-directed immune responses (Jonuleit *et al.* 1997). Several studies in mice have demonstrated that TLR agonist-induced DC activation results in Th1-polarizing DC, and recent reports have described the expression of all known TLR on human myeloid-derived DC (Toubi *et al.* 2004; Napolitani *et al.* 2005; Schreibelt *et al.* 2010). Therefore, multiple studies focusing on the activation of human DC via TLR agonists, as well as the resulting biological differences, were initiated (Jurk *et al.* 2002; Mailliard *et al.* 2004; Banchereau *et al.* 2005; Gautier *et al.* 2005; Gorden *et al.* 2005; Philbin *et al.* 2007; Zobywalski *et al.* 2007). In particular, recently published observations described that DC matured in the presence of a synthetic TLR7/8 agonist (R848) showed elevated secretion of IL-12(p70), resulting in an enhanced Th1-driven immune response (Zobywalski *et al.* 2007). The basic cocktail combination, comprising IL-1β, TNF-α, IFN-γ, PGE_2 and poly(I:C) - a TLR3 ligand mimicking double-stranded RNA (hereafter 5C) was combined with the TLR7/8 agonist R848 (hereafter 5C+R848) (Roelofs *et al.* 2005).

This work focused on the comparison of R848 and CL075, two quinoline-like molecules that interact with TLR7/8 and thereby result in a DC activation signal. The impact of different maturation cocktails were assessed using a 3-day-

generation protocol for DC, which will be discussed below. The different maturation cocktails used in this work are summarized in Table 1.

Table 1. Composition of cocktails used for DC maturation

Cocktail	Inflammatory cytokines/interferons	Other additives	TLR-ligands	Used DC protocol	Published
4C	TNF-α, IL-1β, IL-6	PGE_2		7d-DC 3d-DC	Jonuleit 1997 Bürdek 2010
5C	TNF-α, IL-1β, IFN-γ	PGE_2	poly I:C	3d-DC	Spranger 2010
5C + R848	TNF-α, IL-1β, IFN-γ	PGE_2	poly I:C, R848	7d-DC 3d-DC	Zobywalski 2005 Spranger 2010
5C + CL075	TNF-α, IL-1β, IFN-γ	PGE_2	poly I:C, CL075	3d-DC	Spranger 2010

Modified from (Spranger *et al.* 2010).

Initial analyses focused on the maturation status, assessed by typical surface markers on differently matured DC. Comparisons were made to cocktail 4C as the standard maturation protocol widely used for clinical studies. All compositions indicated in Table 1, induced equal expression of the typical DC maturation markers CD83, CD86 and HLA-DR. Furthermore, the different maturation stimuli did not alter migratory capacity, a very important feature of activated DC to foster entry into secondary lymphoid organs (Martin-Fontecha *et al.* 2009). However, DC matured with cocktails containing a TLR7/8 agonist (R848 or CL075) resulted in an elevated amount of secreted IL-12(p70), while the amount of secreted IL-10 was not affected. Cocktail 5C, which contains only poly(I:C) as a TLR3 agonist, failed to elevate levels of bioactive IL-12(p70) to a similar extent. The biological active variant of IL-12 is a key modulator for the induction of NK cells, as well as for Th1-driven T cell responses, while IL-10 is related to Th2-mediated immunity (Karimi *et al.* 2008; Pallandre *et al.* 2008).

To confirm this assumption, studies were performed focusing on innate and adaptive immunity. First, the impact of differently matured DC on the NK cell-activation status was assessed. Recent studies have revealed that DC-NK cell interactions display a central role in the induction of a potent antitumor immunity (Adam *et al.* 2005). Consistent with the elevated levels of IL-12(p70), DC matured with TLR7/8-containing cocktails showed an increased ability to activate NK cells, measured via IFN-γ secretion, CD69 expression and specific target cell lysis. In contrast, DC matured with cocktails 4C or 5C failed to activate NK cells. Second, the ability of mDC to induce $CD4^+$ Th1 cells as well as antigen-specific

$CD8^+$ CTL was determined. By implication, TLR7/8-matured DC resulted in an elevated percentage of IFN-γ positive T cells, suggesting a Th1-mediated immunity (Zhou *et al.* 2009). Furthermore, only DC matured using TLR7/8 agonists were able to induce antigen-specific CTL, resulting in specific lysis of antigen positive target cells, while 4C and 5C matured DC were not able to induce comparable responses.

Besides the cytokine profile, the composition of positive and negative costimulatory molecules, expressed by mDC, is important for T cell activation. Observations that TLR7/8-matured DC showed enhanced levels of the positive costimulatory molecule B7.1 (CD80) was consistent with an enhanced immune response, as discussed previously. Unexpectedly, B7-H1 (CD274), a molecule with primarily inhibitory characteristics, also showed slightly elevated expression levels when compared to the conventional 4C-matured DC (Keir *et al.* 2008). Several reports claim that the balance between stimulatory and inhibitory signals needs to be shifted towards a positive signal to induce potent T cell immunity (Pentcheva-Hoang *et al.* 2007). Therefore, it is very likely that TLR7/8-matured DC convey improved costimulation through this profile, compared to 4C-matured DC.

A more dramatic shift of the costimulatory profile could be observed when DC generated over different time periods were compared. Standard protocols for generation of peripheral blood-derived DC rely on a 7-day generation protocol, resulting in fully differentiated mDC (also called 7d-DC). Several studies attempted to reduce the generation time for mDC and demonstrated that these so-called “fast-DC” were superior in the induction of immune responses when compared to the 7d-matured DC (Dauer *et al.* 2003; Dauer *et al.* 2005). Minor modifications of this “fast-DC” protocol resulted in a 3-day protocol to generate so-called “young/3d-DC” ((Frankenberger *et al.* 2011) and Figure 5).

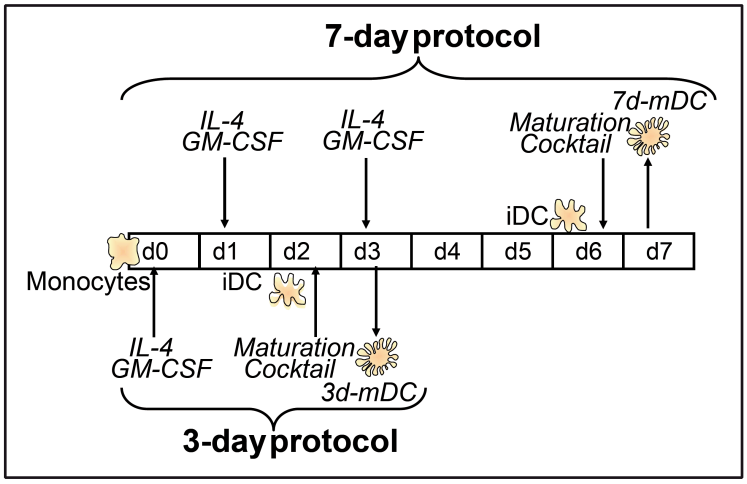

Fig. 5: *Timeschedule for generation of 7d- and 3d-DC.* (Spranger, published in (Frankenberger *et al.* 2011)).

Morphological comparisons of 7d- and 3d-DC revealed that 3d-DC were smaller in size, lower in granularity and, by trend, more robust. Analysis of surface molecules, marking the stage of maturation, did not result in significant differences between both protocols, whereas differences in the expression of the costimulatory molecules B7.1 (CD80) and B7-H1 (CD247) were striking. The observation that 7d-DC expressed higher levels of B7-H1 compared to B7.1, whereas 3d-DC showed higher expression of B7.1 than B7-H1, would suggest an enhanced costimulatory profile of 3d-DC. Indeed, functional assays demonstrated that 3d-DC generated better antigen-specific immune responses compared to 7d-DC. Improved antigen processing and presentation were determined as additional features that resulted in the enhanced immune response induced by 3d-DC.

Taken together, TLR7/8-matured 3d-DC showed superior characteristics in the induction of innate as well as antigen-specific Th1-mediated T cell responses.

3.1.2 Vaccination of humanized mice using matured dendritic cells

So far a solid *in vivo* model system for the comparison of differently matured human DC has not been reported. Therefore, a major aspect of this work was to establish an *in vivo* mouse model for validation of *ex vivo* generated human DC.

Several humanized mouse models and methods to generate humanized mice have been published (Shultz *et al.* 2007). Most models rely on NOD/scid mouse strains, which contain additional mutations in the common-gamma-chain of the IL-2 receptor gene. This mutation can either be a truncated mutation in the case of so-called NOG mice or a null mutation in NSG mice (Ito *et al.* 2002; Ishikawa

et al. 2005). Both strains are characterized by a similar phenotype, lacking T cells, B cells and NK cells. Therefore, these mouse strains are optimal hosts for xenograft-transplantation. While transplantation models using human cord blood stem cells for repopulation result in an extremely low GvHD (graft versus host disease), models relying on the transplantation of peripheral blood lymphocytes (PBL) show higher rates of meanwhile severe GvHD (Shultz *et al.* 2007). Transplantation of stem cells entails other disadvantages, including a non-human-based selection process in the thymus, which potentially result in an abnormal TCR repertoire. Thus, attempts are made to express human MHC molecules as transgenes in mice for a more appropriate selection process (Shultz *et al.* 2010).

The engraftment of PBL emerged as an adequate system for this study, due to the difficulties in obtaining cord blood stem cells in combination with monocytes for DC generation from the same healthy donor. In the first experiment, two different protocols were compared for the engraftment of human PBL in NSG mice. The more “passive” protocol used a lower cell number ($1x10^6$) in combination with a low irradiation dose of 100 cGy, while the more “aggressive” method utilized a 10-fold higher cell dose ($10x10^6$). The engraftment process lasted for 9 weeks or 4 weeks, respectively, and included two vaccinations with *ex vivo* generated human mDC of an autologous donor. While several mice treated with the “passive” 9-week protocol suffered from severe GvHD, all mice treated with the “aggressive”, 4-week protocol stayed healthy throughout the procedure of vaccination.

Consequently, the 4-week engraftment protocol was used for DC vaccination experiments to compare the *in vivo* impact of 7d-DC and 3d-DC matured with 4C cocktail to that of 3d-DC matured with a TLR7/8 agonist containing cocktail (5C+R848). Using an allogeneic model system, re-isolated splenocytes of mice vaccinated with 3d-DC resulted in an enhanced killing capacity of positive target cells. In addition, vaccination with DC matured using 5C+R848 showed increased levels of specific lysis after re-isolation. Those observations could be confirmed in an autologous setting using MART-1/Melan-A as a model antigen. Multimer-staining revealed that only 3d-DC had the capacity to induce detectable immune responses. Moreover, re-isolated human T cells of mice, vaccinated with 5C+R848 matured DC, showed elevated antigen-specific lysis of target cells,

when compared to mice immunized with 4C-matured DC. However, not all vaccinated mice showed a T cell-mediated immune response and conclusions between engraftment efficacy and percentage of $CD8^+$ T cells could not be drawn. This effect, observed for individual mice, can be explained by a presumably short survival period of DC in combination with a failure of entry into the lymph node in time. In clinical vaccination protocols, multiple applications of DC near lymph nodes may reduce this risk of failure.

Nevertheless, this *in vivo* model system encompasses the key features required for an adequate pre-clinical model system to validate and, in particular, to compare *in vivo* effects of differently generated DC-based vaccines.

3.2 Adoptive T cell transfer

While the application of DC-based vaccines represents an active strategy to induce antitumor immunity, the adoptive T cell transfer (ATT) is characterized by a passive transfer of antitumor reactive cells. Several studies have shown the efficacy of adoptively transferred T cell in mouse models as well as in clinical studies (Rosenberg *et al.* 2008). Compared to other immunotherapeutic approaches, ATT still lags in development due to a difficult and laborious preparation of the therapeutic agents in large numbers, as well as in availability of high-affinity TCR.

3.2.1 *In vitro* generation of antigen-specific T lymphocytes

The generation of T cells with high-affinity TCR, specific for TAA is up to now a critical bottleneck for the ATT approach using TCR-modified lymphocytes. Several studies, using patient-derived T cells or TIL-derived TCR resulted in poor clinical benefit, probably due to low-affinity TCR (Johnson *et al.* 2009). Reports have shown that TCR, derived from autologous cells, recognizing TAA derived from self antigens, are of low affinity to self-peptide/self-MHC ligands, while efficient tumor cell recognition involves high-affinity TCR (de Visser *et al.* 2001; Johnson *et al.* 2006). Theoretically, the induction of antigen-specific T cells, using foreign pMHC ligands, results in T cells with a presumably higher functional avidity (Rammensee *et al.* 1984). Recent studies have shown improved tumor recognition by T cells generated using allogeneic priming approaches (Morris *et al.* 2006; Schuster *et al.* 2007). Due to the excellent priming capacity of DC,

discussed above, as well as their capacity to process and present whole proteins, we established a semi-allogeneic priming approach, utilizing mDC as stimulators (Nair *et al.* 1999). Application of DC from an HLA-A2 negative donor, pulsed with *ivt*-RNA encoding the antigen of choice as well as HLA-A2, allows presentation of self-protein fragments on a foreign MHC molecules. This results in antigen-specific T cells carrying TCR with presumably higher functional avidity. Table 2 summarizes priming results for the TAA tyrosinase, survivin and HMMR. While tyrosinase is a well-described antigen to target melanoma, survivin and HMMR are potential targets for the treatment of AML (Ambrosini *et al.* 1997; Rhodes *et al.* 2004; Schmid *et al.* 2006; Slingluff *et al.* 2006; Mita *et al.* 2008). Both AML-associated antigens that are over-expressed in multiple types of cancer have been described and consequently several antitumor vaccination studies targeting these antigens, have proven their safety (Greiner *et al.* 2002; Altieri 2003; Pisarev *et al.* 2003; Greiner *et al.* 2005; Casini *et al.* 2010). Survivin is also ranked in a recently published NIH-list, which proposed suitable antigen candidates for adoptive T cell transfer, whereas HMMR was not included (Cheever *et al.* 2009).

Table 2. Composition of priming approaches tyrosinase, survivin and HMMR

			Number of clones with (%)			
Antigen	Setting	Method of sorting	No reactivity	Allo-reactivity	Antigen-specific reactivity	Published
tyrosinase	autologous	multimer	21 (55)	0 (0)	17 (45)	Wilde 2009
tyrosinase	allogeneic	multimer	8 (16)	27 (53)	16 (31)	Wilde 2009
survivin	autologous	multimer	46 (100)	0 (0)	0 (0)	Leisegang 2010
survivin	allogeneic	multimer	9 (12)	44 (60)	21 (28)	Leisegang 2010
HMMR	allogeneic	CD137	35 (23)	38 (25)	78 (52)	Spranger (manuscript)

The approaches for tyrosinase as well as for survivin mainly focused on the comparison of autologous and allogeneic priming. Supporting our contention, autologous-derived, tyrosinase-specific T cell clones resulted in lower functional avidity and poor antitumor recognition compared to allogeneically primed T cell clones. In contrast to the priming using tyrosinase, no survivin-specific T cell clones were obtained using the autologous priming approach, while the allogeneic setting repeatedly resulted in antigen-specific T cell clones with high

functional avidities. Possible reasons leading to the failure to isolate autologously primed, surivivin-specific T cell clones are discussed below. In addition to the increased functional aviditiy, T cells generated following an allogeneic setting show a higher proportion of T cell clones with a polyfunctional phenotype (unpublished observations from S. Wilde). This polyfunctional phenotype, characterized by secretion of multiple cytokines, including IFN-γ, IL-2 and TNF-α, is associated with increased recognition of tumor cells and higher functional avidity of the attendant TCR.

While the previously discussed priming approaches for the generation of tyrosinase- and survivin-specific T cells used a multimer-dependent step of isolation, the activation marker CD137 was utilized to enrich HMMR-specific lymphocytes. Several studies have proven the suitability of this $CD8^+$-restricted activation marker to eliminate allo-restricted T cells or to enrich antigen-specific T cells in an autologous priming (Wehler *et al.* 2007; Wolfl *et al.* 2007). This approach, theoretically, would allow the isolation of T cells recognizing all potential immunogenic epitopes throughout HMMR, while a multimer-dependent isolation step would have limited the specificity to a single peptide. Additionally, multimer sorting requires the validation of multimers with T cell clones that recognize the targeted peptide and are rarely at hand. Comparisons of the CD137-mediated enrichment with the multimer-sorted priming approaches resulted in a higher percentage (52%) of antigen-specific cells (see Table 2). This observation can be explained by the potentially higher number of immunogenic epitopes recognized by isolated T cell clones. This assumption is supported by a comparable absolute number of isolated, alloreactive T cell clones, indicating similar priming efficiency. In addition, analysis of the polyfunctional phenotype of HMMR-specific T cell clones revealed similar patterns compared to other priming approaches (unpublished observations). However, an evident disadvantage of this T cell-enrichment method is the laborious identification of targeted epitopes. Several approaches, including *ivt*-RNA-screening libraries or over-lapping peptides, are reasonable methods to identify targeted peptides, but require high numbers of T cells for the screening assays. Due to limitations in the expansion of primary T cell cultures, TCR-gene transfer into recipient lymphocytes is not only conceivable for adoptive transfer but also for further *in vitro* characterization of the obtained TCR.

3.2.2 Characteristics of TCR-modified lymphocytes

Since the expansion of primary T cell clones is limited, TCR gene isolation from T cell clones with strong evidence for valid tumor recognition represents an approach for further TCR characterization in the absence of sufficient T cell clones. Optional TCR transfer methods can be either transient, by electroporation of *ivt*-RNA, or stable using retroviral or lentiviral transduction approaches (Morgan *et al.* 2003; Hughes *et al.* 2005; Zhao *et al.* 2005; Morgan *et al.* 2006; Qasim *et al.* 2007; Zhou *et al.* 2009; Zhao *et al.* 2010). Since lentiviral transduction does not require proliferation of recipient cells for transgene integration, this method would allow transfer of naïve T cells without adverse *in vitro* stimulation (Morgan *et al.* 2006; Qasim *et al.* 2007). Nevertheless, integration of lentiviral transgenes can be mutagenic (Pauwels *et al.* 2009). In contrast, integration of retroviral transgenes requires potent *in vitro* stimulation to induce proliferation of lymphocytes, but is supposed to be less mutagenic (Leisegang *et al.* 2008). In addition to the TCR transfer method, isolated TCR genes can be the subject of several modifications. Improved translation of the transgenes can be achieved by codon optimization, introducing changes in the nucleodide sequence which result in enhanced translation (Scholten *et al.* 2006). In particular, modifications enhancing the likelihood of pairing of introduced TCR chains are under intensive investigation. These modifications are necessary, due to potential misspairing of the introduced chains with endogenously expressed TCR, which potentially causes severe and unpredictable side effects (van der Veken *et al.* 2009). The exchange of human constant regions by their murine counterparts resulted in a dramatic increase of pairing of the introduced TCR α- and β-chains (Cohen *et al.* 2006). While this modification can potentially result in a xenoreactive response, point mutations enhancing the chain-specific binding, have been described not to be immunogenic in humans (Cohen *et al.* 2006; Sommermeyer *et al.* 2010). Since aspects of this work comprised proliferation-mediated modulations of the recipient lymphocyte phenotypes as well as adoptive transfer into immuno-suppressed mice, usage of retrovirally-transferred TCR genes carrying the murine constant regions was adequate and non-problematic.

At first, the TCR-mediated transfer of tumor specificity was evaluated. All analyzed and successfully expressed TCR, specific for tyrosinase, survivin and HMMR, showed similar patterns of specific tumor-recognition when compared to the original T cell clones. Certainly not all TCR could be expressed on the surface of lymphocytes. This could be due to a low likelihood of TCR-chain pairing or the introduced TCR may be a “weak” competitor to endogenous TCR for expression. In particular, competition between the introduced and the endogenous TCR for expression on the cell surface is a main parameter, impacting on the expression of the introduced TCR (Heemskerk *et al.* 2007).

An unexpected observation was made when survivin-specific TCR were introduced into recipient lymphocytes. While introduction of survivin-specific TCR into lymphocytes of HLA-A2^{-} donors resulted in viable TCR-positive cells with an expected tumor recognition pattern, PBL of an HLA-A2^{+} donor showed dramatically increased numbers of apoptotic cells after TCR introduction. Further analyses, using activated and non-activated HLA-A2^{+} lymphocytes as target cells, revealed that PBL were specifically recognized and killed off by lymphocytes carrying survivin-specific TCR. While other studies report on suicide of T cells with high-affinity TCR, the percentage of dead cells detected in survivin-specific tgTCR-HLA-A2^{+} lymphocyte-populations exceeded the amount of TCR-positive cells (Molldrem *et al.* 2003). This observation indicates a fratricide-mediated death of tgTCR-negative lymphocytes. Futhermore, T cell clones with a designated non-survivin specificity were targeted by survivin-specific tgTCR-PBL, suggesting general on-target recognition of lymphocytes. Referring back to the above-mentioned observation that no autologously primed, survivin-specific T cell clones were obtained, even though multimer sorting indicated the existence of such T cells. A reasonable explanation for this discrepancy supports the contention that fratricide-mediated death occurred in emerging HLA-A2^{+} T cell clones. Altogether these results preclude the usage of survivin-specific TCR for TCR gene transfer. Also the usage of survivin for antitumor vaccination seems problematic, due to probable limitations in proliferation of high-avidity survivin-specific T cells in the draining lymph nodes.

Analyses of expression levels of other possible targets for immunotherapeutic approaches in non-activated and activated PBL on the basis of mRNA (messenger RNA) prevalence were performed. These comparisons revealed that

survivin is extreme in “x-fold” increase (non-activated vs. activated PBL) and in the amount of mRNA levels in activated PBL. Several studies speculated on fratricide-mediated limitations in the expansion of p53- and hTERT- (human telomerase reverse transcriptase) specific T cells (Kyburz *et al.* 1993; Chen *et al.* 2007). Analyzing the mRNA levels for these potentially fratricide-inducing candidate genes, revealed comparably high expression levels in activated PBL, as seen for survivin. These observations could explain why clinical approaches for induction of antitumor immunity utilizing these proteins have failed in the past (Liu *et al.* 2007; Theoret *et al.* 2008). Although the expression level of HMMR in activated PBL is comparable to that of survivin, transfer of HMMR-specific TCR into HLA-$A2^+$ PBL did not induce fratricide (unpublished observations). Feasible reasons could be founded in different protein localization or differences in protein degradation. While survivin is an anti-apoptotic protein, located in the cytosol and characterized by a fast turnover, HMMR is localized at the inner-surface of the cellular membrane. Cellular functions of this protein are still not completely resolved, though co-localization with ERK (extracellular signal-regulated kinase) as well as a transforming potential in fibroblasts have been observed (Jiang *et al.* 2010). Therefore, further analysis of the expression profile on the basis of protein localization, protein turnover as well as protein processing and presentation are needed, to make a more precise prediction of the suitability of various proteins as targets for immunotherapy. Nevertheless, a final proof will only be possible when target-specific TCR are available for specific analysis.

Since TCR150, targeting HMMR, evolved to be a suitable candidate for further analysis, expression of TCR150 in varying phenotypes of recipient lymphocytes was assessed in *in vitro* assays. Several studies are focusing on the phenotype of recipient cells that is optimal for adoptive T cell transfer (Hinrichs *et al.* 2009b; Klebanoff *et al.* 2009; Lin *et al.* 2009; Wang *et al.* 2011; Yang *et al.* 2011). While some mouse models revealed a Tc17-phenotype as potentially best suited for *in vivo* tumor recognition, consistent with published observations, Tc17-induced cells did not lyse tumor cells *in vitro* (Hinrichs *et al.* 2009b; Garcia-Hernandez Mde *et al.* 2010). In contrast to other analyzed culture conditions, tumor cell stimulation of Tc17 cells resulted in a significantly higher production of IFN-γ and TNF-α. Reports focusing on the *in vitro* characterization of killing capacity and cytokine production, comparing naïve T cells to T_{EM} and T_{CM}, proposed that a

memory-like T cell phenotype is favorable (Boyman *et al.* 2009; Lin *et al.* 2009; Neeson *et al.* 2010). Within the memory-like T cell phenotype, the capacities of T_{EM} and T_{CM} to recognize and retard tumor are still discussed controversially (Klebanoff *et al.* 2009; Mitchell *et al.* 2010; Berger *et al.* 2011; Wang *et al.* 2011; Yang *et al.* 2011). While T_{EM} are mainly characterized by a rapid antigen-specific reaction *in vitro* as well as *in vivo*, T_{CM} are distinguished by a particular long survival *in vivo*. Depending on the method of generation (isolation or induction) as well as the applied test system, T_{EM} reveal favorable properties or T_{CM} are superior in tumor retardation. The comparison of an IL-7-induced T_{CM}-phenotype to an IL-15-mediated T_{EM} phenotype, resulted in a dramatically increased *in vitro* tumor recognition by the T_{EM} phenotype. These results indicate that TCR expression in combination with an IL-15-induced T_{EM} phenotype has favorable properties for adoptive transfer of genetically-modified lymphocytes, at least in this test system.

3.2.3 Adoptive transfer of TCR-modified lymphocytes

To assess the use of TCR-modified human lymphocytes in an *in vivo* setting, xenograft-tumor transplantation models needed to be established. For this purpose, NSG mice were utilized due to their optimal immuno-suppressed status to engraft xeno-transplants, as discussed above. Initial adoptive transfer experiments targeted a solid tumor of the AML cell line THP-1 (HLA-$A2^+$, $HMMR^+$) generated using matrigel, while the final goal of this work was to validate HMMR-specific TCR150-modified lymphocytes for their benefit in targeting THP-1 as a disseminated tumor.

Adoptive transfer of a single dose of TCR150-modified lymphocytes ($2x10^5$) prepared in the presence of IL-2, resulted in a significantly reduced tumor outgrowth, when compared to mock-treated lymphocytes. Consistent with other publications, transferred lymphocytes were only detectable in mice for approximately three weeks after transfer (Wang *et al.* 2011). Additional adoptive transfer experiments using increased numbers of TCR-positive lymphocytes ($5x10^5$) did not result in further benefit. Nevertheless, T cell infiltrates were only detected in isolated tumors of mice treated with TCR150-transduced lymphocytes, while tumors of mice treated with mock-PBL or transduced with an irrelevant TCR, did not show such infiltrates. This observation indicated a TCR-

mediated antigen-specific infiltration, but further investigations are needed to resolve the abortive tumor elimination. Multiple molecular mechanisms inducing T cell anergy or exhaustion are possible. Those mechanisms could include expression of inhibitory molecules (e.g. PD-1), soluble inhibitory factors (e.g. IDO) or numerous combinations of other immunosuppressive mechanisms (Gajewski *et al.* 2006). Another obstacle in the treatment of tumor cell lines used in this study is their prominent capacity to rapidly grow in mice, compared to other xenograft systems or even syngeneic murine tumor models (Klebanoff *et al.* 2005; Distler *et al.* 2008; Carpenito *et al.* 2009; Hinrichs *et al.* 2009b; Wang *et al.* 2011).

To improve the persistence of transferred lymphocytes, adoptive transfer experiments were performed using differently induced phenotypes, described above. These experiments were performed employing a THP-1 as well as a melanoma cell line (mel624.38; HLA-A2$^+$, HMMR$^+$) as targets for TCR150-modified lymphocytes. While the persistence of lymphocytes was not altered by the different phenotypes, tumor outgrowth was dramatically retarded when T_{EM} or T_{CM} phenotypes were transferred. In particular, the T_{EM}-mediated effect was in concordance with the *in vitro* results discussed above. These observations are consistent with other studies using non-human primate or mouse model systems that revealed similar results using memory-like phenotypes for adoptive transfer (Berger *et al.* 2011; Wang *et al.* 2011). When both tumor cell lines were analyzed for growth retardation, the melanoma cell line was significantly more retarded in tumor outgrowth compared to THP-1. Possible reasons may include a different impact of the tumor microenvironment inhibiting TCR-positive lymphocytes to a different extent or due to variations in the immunogenicity of the cell lines. Further investigations are needed to resolve these discrepancies.

Significant improvement could not be observed when conventional IL-2-treated lymphocytes were compared to Tc17-cultured lymphocytes. The discrepancy with published antitumor effects may be explained by the lack of an endogenous immune system in the NSG mouse model (Hinrichs *et al.* 2009a; Hinrichs *et al.* 2009b; Garcia-Hernandez Mde *et al.* 2010). It is likely that the cytokine-producing Tc17 cells impact indirectly on the tumor cells, via activation of other compartments of the immune system. To bring these observations in line with other xenograft model systems, discrepancies may by due to shorter observation

periods, usage of higher cell doses or inadequate preincubation of effector and target cells (Klebanoff *et al.* 2005; Distler *et al.* 2008; Carpenito *et al.* 2009; Hinrichs *et al.* 2009b; Wang *et al.* 2011). Therefore, the results described here clearly demonstrate a potent antitumor effect, although all therapeutic approaches failed to eradicate solid tumors completely.

Since reports postulate that administration of IL-15 over time would prolong the persistence of human lymphocytes in NSG mice, daily administration of IL-15 was compared with two doses of DC vaccination (Wang *et al.* 2011). While vaccination with autologous DC did not result in a further deceleration of tumor outgrowth, IL-15 application resulted in a slight improvement of the rejection of the THP-1-mediated tumor. In contrast, tumor growth of mel624.38 appeared to increase. This may indicate that IL-15 is a growth factor for this cell line. Alternatively, infiltrations of TCR-modified cells may falsely appear as tumor growth (unpublished observations).

To determine the potential impact of adoptively transferred lymphocytes on disseminated tumor, we utilized a bioluminescent non-invasive *in vivo* imaging method, developed by Dr. I. Jeremias (unpublished data). This method allows a precise and sensitive detection of luciferase-tagged tumor cells in living mice. Accordingly to the results discussed above, transfer of HMMR-specific TCR150-modified T_{EM} lymphocytes ($4x10^5$/mouse) led to significant retarded tumor growth, when compared to PBL transduced with an irrelevant TCR. Administration of IL-15 under these tumor conditions resulted in a slight enhancement of growth deceleration. More dramatic results could be observed when transfer of CD8-enriched TCR-positive T cells was combined with IL-15-administrations. This combination of therapeutic agents resulted in a complete remission of the tumor, indicting that $CD8^+$ effector memory T cells in combination with IL-15 have a prominent potential to retard leukemia expansion in this model.

These results are of outstanding significance since other xenograft studies targeting disseminated human tumors, used increased cell doses for adoptive transfer (up to $1x10^7$ lymphocytes) (Zhao *et al.* 2010). Comparison of per kilogram cell doses used in the experiments discussed above ($4x10^5$ cells /mouse (à 20g); equivalent to approx. $5x10^7$ cells/kg) to doses applied in clinical

trials ($1x10^9$ to $1x10^{11}$) reveals that a minimum 2-log lesser cell dose was still sufficient to retard tumor outgrowth in this mouse model (Rosenberg *et al.* 2008).

In conclusion, TCR150, targeting HMMR, was revealed to be a potent candidate for TCR gene-transfer into lymphocytes. In particular, when introduced in recipient cells with an IL-15-induced memory-like phenotype, TCR150 demonstrated a promising antitumor effect in the NSG mouse model established in this project.

3.3 Outlook

Regarding future improvements of *in vivo* model systems for the evaluation of human DC-based vaccines, the utilization of an HLA-expressing mouse model would be of outstanding interest (Shultz *et al.* 2010). Such strains would likely enable the use of human hematopoetic stem cells for the reconstitution of mice, followed by autologous DC vaccination. Thereby, it is likely that GvHD effects can be reduced and multiple applications of DC, comparable to clinical settings, would be feasible. In addition, the NSG mouse model displays a helpful tool for resolving the importance of various administration routes for DC vaccines. Therefore, the induced immune responses following intravenous application, used in this study, could be compared to those induced after intradermal or intranodal injection, commonly used in clinical settings.

A clear challenge of the CD137-mediated enrichment of antigen-specific T cells is their epitope identification. Since identification of the targeted peptide sequences for TCR150, as well as all other HMMR-specific T cell clones, has not yet proceeded, identification of those peptides entails one crucial component of future investigations. In particular, identification of the targeted epitopes by the other HMMR-specific T cell clones would probably reveal new and predominantly immunogenic epitopes within HMMR. Besides the selection of an appropriate source of peptide (e.g. *ivt*-RNA fragments, long overlapping peptides, nonamers), the screening cells create a major hindrance in solving this problem. Up to now, no HMMR-negative cell line with suitable properties for peptide loading has been found. As an alternative method artificial APC can be applied but the missing capacity for peptide-processing would dramatically limit the number of peptides screened.

As an additional interest, the differences between survivin and HMMR on the protein-level require further studies. First experiments using confocal microscopy revealed both antigens in activated PBL, but disclosed differences in the cellular distribution as well as the distribution throughout the cell population (data not shown). Moreover, preliminary *in vivo* killing experiments have pointed out that HMMR-specific as well as survivin-specific tgTCR-lymphocytes do not eliminate activated HLA-$A2^+$ PBL *in vivo*. Additional optimization, for instance in combination with a peptide-specific vaccination strategy to induce clonal *in vivo* proliferation, is required to reinforce these observations.

An unexpected aspect of this studie was found in the differences in tumor outgrowth of THP-1 and mel624.38 after adoptive transfer of T_{EM} tgTCR150 lymphocytes. Due to the many possible factors or factor combinations impacting on the transferred lymphocytes, several experiments for revealing potential differences between the tumor cells are required to resolve the discrepancy. These analyses should be related to the tumor microenvironment, expression of inhibitory molecules or other T cell inhibitory factors. Additionally, the identification of an induced “inhibited” T cell phenotype (e.g. anergic T cells, regulatory T cells) would give insight into the inhibitory mechanisms and should therefore be investigated in more detail. Nevertheless, these observations could be of high impact for the prediction of the clinical outcome of ATT therapy.

Recent observations in clinical studies indicated a beneficial use of multiple TCR, targeting more than one antigen (Zhou *et al.* 2005). To solve this controversy the impact of either individually applied or combined therapy using HMMR- and tyrosinase-specific tgTCR-PBL can be assessed in the established xenograft melanoma mouse model.

Besides stable TCR-gene transfer, transient TCR expression via *ivt*-RNA represents a feasible technology for *in vitro* analysis of TCR functionality and avidity. In particular high-through-put screening assays, as required for the epitope screening of the HMMR-specific T cell clones, are potential *in vitro* applications. The transient TCR expression could provide a safety mechanism in the ATT approach. As a first therapeutic step of engineered lymphocytes, the transfer of transient TCR expressing lymphocytes is under consideration. This approach would allow a first insight into possible autoimmune reactions as well as intended antitumor effects, without persistence of TCR expression in patients.

Therefore, by using the NSG *in vivo* model system, comparisons of transiently TCR expressing lymphocytes to stable TCR expressing cells could be addressed. Since the use of particularly non-activated T cell phenotypes (e.g. *ex vivo* use of sorted T_{CM}) or clonal T cell populations (e.g. antiviral specific T cells) are under consideration (Stauss *et al.* 2008; Heslop *et al.* 2010). However, so far no adequate system has been able to assess these differences in detail. Transient TCR expression in T cell clones with a single specificity can limit the cross-reactivity and would allow a precise comparison of varying TCR using the same recipient cells. Furthermore, the introduction of TCR into varying phenotypes with minimal alterations of the native cells can be enabled by the usage of *ivt*-RNA for TCR gene transfer. These experiments could reveal insights into T cell biology and resolve the question of most suitable T cell phenotypes to retard tumors *in vivo*.

4 Literature

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5 Original publications and manuscripts as part of the cumulative thesis

1. **Generation of Th1-polarizing dendritic cells using TLR7/8 agonist CL075** (2010) Spranger S, Javorovic M, Bürdek M, Wilde S, Mosetter B, Tippmer S, Bigalke I, Geiger C, Schendel DJ, Frankenberger B; J Immunol. 185(1):738-47

   S. S. performed all experiments except signal-3-assays. S. S. wrote the manuscript and B. F. and D. J. S. revised it.

2. **Three-day dendritic cells for the vaccine development: antigen uptake, processing and presentation** (2010) Bürdek M, Spranger S, Wilde S, Frankenberger B, Schendel DJ, Geiger C; J Transl Med. 28;8:90

   S. S. performed experiments concerning the costimulatory profile and was involved in the development of the three-day generation protocol.

3. **NOD/scid IL-2Rg^{null} mice: a pre-clinical model system to evaluate human dendritic cell-based vaccines *in vivo*** (2011) Spranger S, Frankenberger B and Schendel DJ (submitted)

   S. S. performed all experiments and was involved in developing the experimental concept. The manuscript was written by S. S. and revised by D. J. S..

4. **Dendritic cells pulsed with RNA encoding allogeneic MHC and antigen induce T cells with superior antitumor activity and higher TCR functional avidity** (2009) Wilde S, Sommermeyer D, Frankenberger B, Schiemann M, Milosevic S, Spranger S, Pohla H, Uckert W, Busch DH, Schendel DJ; Blood 114(10):2131-9

   S. S. performed cytotoxic assays and TCR analysis.

5. **MHC-restriced fratricide of human lymphocytes expressing survivin-specific transgenic T cell receptors** (2010) Leisegang M, Wilde S, Spranger S, Milosevic S, Frankenberger B, Uckert W, Schendel DJ; J Clin Invest 120(11):3869-77.

   S. S. planned and performed cytotoxic assays and quantitative real-time analysis to assess expression-profiles of tumor-associated antigens in activated PBL. S. S. was involved in writing the manuscript.

6. **T cell receptor-transgenic lymphocytes specific for HMMR/Rhamm limit tumor outgrowth *in vivo*** (2011) Spranger S, Jeremias I, Wilde S, Mosetter B, Heemskerk MH, Schendel DJ and Frankenberger B (submitted)

   S. S. performed all experiments and was involved in developing the experimental concept. S. S. wrote the manuscript and B. F. and D. J. S. revised it.

I hereby confirm the above statements

Stefani Spranger	Dolores J. Schendel

The Journal of Immunology

Generation of Th1-Polarizing Dendritic Cells Using the TLR7/8 Agonist CL075

Stefani Spranger, Miran Javorovic, Maja Bürdek, Susanne Wilde, Barbara Mosetter, Stefanie Tippmer, Iris Bigalke, Christiane Geiger, Dolores J. Schendel and Bernhard Frankenberger

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Generation of Th1-Polarizing Dendritic Cells Using the TLR7/8 Agonist CL075

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In this paper, we describe a new method for preparation of human dendritic cells (DCs) that secrete bioactive IL-12(p70) using synthetic immunostimulatory compounds as TLR7/8 agonists. Monocyte-derived DCs were generated using a procedure that provided mature cells within 3 d. Several maturation mixtures that contained various cytokines, IFN-γ, different TLR agonists, and PGE_2 were compared for impact on cell recovery, phenotype, cytokine secretion, migration, and lymphocyte activation. Mixtures that included the TLR7/8 agonists R848 or CL075, combined with the TLR3 agonist polyinosinic:polycytidylic acid, yielded 3-d mature DCs that secreted high levels of IL-12(p70), showed strong chemotaxis to CCR7 ligands, and had a positive costimulatory potential. They also had excellent capacity to activate NK cells, effectively polarized $CD4^+$ and $CD8^+$ T cells to secrete IFN-γ and to induce T cell-mediated cytotoxic function. Thereby, mature DCs prepared within 3 d using such maturation mixtures displayed optimal functions required for vaccine development. ***The Journal of Immunology*****, 2010, 185: 000–000.**

Dendritic cells (DCs) are potent adjuvants for the induction of tumor-specific helper and killer cells in cancer patients (1, 2). DCs are the most important activators of naive cells during development of T cell-mediated immunity. Cancer immunotherapies using DC vaccination now preferably apply mature DCs (mDCs) loaded with tumor-associated Ags, which are injected near or directly into lymph nodes where they interact with naive T cells, thereby fostering activation and differentiation of $CD8^+$ CTLs. In addition, DCs can regulate cytokine polarization of T cells, and some mDCs can activate NK cells, which contribute to innate immune responses that help to orchestrate long-term Ag-specific memory T cell responses (3–6).

Various methods have been developed for preparation of mDCs starting from PBMCs or other myeloid progenitor cells. The most common method generates mDCs from monocytes over a period of 7 d (7). An alternative procedure allows mDCs to be produced within 2 d (8–10). Monocytes can be isolated either from PBMCs through plastic adherence, by isolation of $CD14^+$ cells with mAbs, or by using leukapheresis and elutriation. Isolated monocytes are cultured in vitro with GM-CSF and IL-4 or IL-13 to produce immature DCs (iDCs) (11–13). Thereafter, several alternatives can be used to obtain mDCs, each of which yields cells with somewhat different properties that must be clearly specified for DC-based vaccine development. Jonuleit et al. (7) described a maturation mixture containing TNF-α, IL-1β, IL-6, and PGE_2 (hereafter designated as the four-component mixture [4C]) that produced 7-d DCs with surface markers characteristic for mDCs, and these cells could be easily recovered in sufficient numbers for clinical application. This type of mDC has been used frequently in early-phase clinical trials of DC vaccination in patients with various forms of malignancies. However, these mDCs do not produce bioactive IL-12(p70), which is a critical cytokine for the induction of Th1 cells and activation of NK cells.

Elegant studies in mice demonstrated that activation of TLR signaling cascades could produce mDCs with Th1-polarizing capacity (14). Human DC subsets express all known human TLRs, with the most diverse expression pattern in monocyte-derived DCs and myeloid DCs (15). On the basis of these observations, a number of studies explored the use of different TLR agonists to modulate DC function, particularly with respect to cytokine secretion profiles (14, 16–25).

We previously described DCs that were matured in a 7-d protocol using a mixture that included the imidazoquinoline-like molecule R848 as a TLR7/8 agonist (26). The resultant 7-d mDCs produced substantial amounts of IL-12(p70) and displayed phenotypic and functional characteristics desired for clinical application, including high cell recoveries. However, the 7-d culture period in a good manufacturing practice facility is a strong hindrance for clinical development. Therefore, we investigated approaches to rapidly produce monocyte-derived mDCs that would display suitable characteristics for efficient use in vaccine development. The studies reported in this paper show that maturation mixtures containing the synthetic thiazoloquinoline immunostimulatory compound CL075, or R848, as a TLR7/8 agonist, when combined with polyinosinic:polycytidylic acid [poly(I:C)] as a TLR3 ligand, yielded mDCs within 3 d that showed excellent profiles with respect to recovery, phenotype, cytokine secretion, and migration, as well as capacity to activate NK cells and $CD8^+$ CTLs and to

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Abbreviations used in this paper: 4C, four-component mixture; DC, dendritic cell; iDC, immature DC; mDC, mature dendritic cell; MFI, mean fluorescence intensity; poly(I:C), polyinosinic:polycytidylic acid; pSTAT, phospho-STAT; RPMI-VLE, RPMI 1640 medium with very low endotoxin.

effectively polarize $CD4^+$ and $CD8^+$ T cells to secrete IFN-γ. Thereby, these 3-d mDCs are highly suited for development of DC-based antipathogen or antitumor vaccines.

Materials and Methods

Leukapheresis and elutriation

We used a closed system of elutriation (ELUTRA; Gambro BCT, Lakewood, CO) to obtain monocytes as progenitor cells for generation of iDCs, as described previously (26). In accordance with the Declaration of Helsinki and after approval by the Institutional Review Board of the Technical University (Munich, Germany), healthy donors underwent 180 min of leukapheresis with the COBE Spectra cell separator (Gambro BCT, Hechingen, Germany) with a modified MNC program (V6.1): separation factor was set to 700 with a collection rate of 0.8 ml/min and a target hematocrit of only 1–2%. Resulting blood cells were analyzed by automatic blood counter (ACT Dif; Beckman Coulter, Krefeld, Germany) to set up conditions for the ELUTRA system. Leukapheresis products were processed according to the manufacturer's instructions by a method of counterflow centrifugal elutriation using a fixed rotor speed (2400 rpm) and computer-controlled stepwise adjustment of media flow rate, followed by rotor-off harvesting. Five liters of running buffer consisting of RPMI 1640 medium with very low endotoxin (hereafter RPMI-VLE) (Biochrom, Berlin, Germany) with 1.5% human serum (pool of AB-positive adult males) (Institute of Transfusion Medicine, Suhl, Germany) was used for cell separation. This process resulted in five fractions with enriched monocytes present in the rotor-off fraction. The cellular composition of individual fractions was characterized by automatic cell counting and flow cytometry.

Generation of mDCs from elutriated monocytes

Cells from the rotor-off fraction, subsequently designated as fraction 5, were frozen in aliquots of 5×10^7 monocytes in freezing medium consisting of human serum albumin (20% human serum albumin solution; Octalbine, Octapharma, Langen, Germany), 20% DMSO (Merck, Darmstadt, Germany), and 10% glucose (Braun, Melsungen, Germany). Monocytes were thawed and washed with endotoxin-free PBS (Biochrom) at 1500 rpm for 10 min. Cells were resuspended and seeded at 4.5×10^7 per "nuncleon-surface" flask (80 cm^2) (Nunc, Wiesbaden, Germany) in 15 ml DC medium containing RPMI-VLE and 1.5% human serum and cultivated for 50 min at 37°C and 5% CO_2 in a humidified atmosphere. Afterward, cells were washed twice with RPMI-VLE, and 15 ml DC medium was added. On day 0, cultures were supplemented with 100 ng/ml GM-CSF (Leukine; Berlex, Richmond, VA) and 20 ng/ml recombinant human IL-4 (R&D Systems, Wiesbaden, Germany) in 3 ml fresh DC medium per flask. Full DC maturation was achieved by addition of various mixtures on day 2 (see Table I). The components for the maturation mixtures were as follows: TNF-α, IL-1β, and IL-6 (R&D Systems); poly(I:C), R848, and CL075 (InvivoGen, San Diego, CA); IFN-γ (Boehringer Ingelheim, Ingelheim, Germany); and PGE_2 (Sigma-Aldrich, Deisenhofen, Germany). For the induction of mDCs with a tolerogenic phenotype, rIL-10 (R&D Systems) was added with a final concentration of 83.33 ng/ml. After incubation of iDCs with maturation mixtures for 24 h, cells were harvested by washing twice with PBS plus 0.5% human serum with light shaking and assessed directly or cryopreserved as described above for fraction 5 monocytes.

Surface phenotyping of DCs

DCs were labeled with the following fluorescence-conjugated mAbs: CD14 (FITC, clone MΦP9), CD86 (FITC, clone 2331 FUN-1), CD80 (PE, clone L307.4), CD274 (B7-H1, FITC, clone MIH1) (all BD Biosciences, Heidelberg, Germany), HLA-DR (PE, clone B8.12.2), and CD83 (PE, clone HB15a) (Immunotech, Marseille, France). CCR7 staining was performed with rat hybridoma supernatant medium (BLR-2, clone 8E8; E. Kremmer, Helmholtz Zentrum München, Munich, Germany) and compared with a rat hybridoma isotype control (EBNA-A2, clone R3; E. Kremmer) by incubation of DCs in culture supernatant for 60 min, followed by washing and detection with secondary mouse Ab against rat IgG conjugated with cyanin-5 (Jackson ImmunoResearch Laboratories, West Grove, PA). To test viability, DCs were pelleted and resuspended for 20 min in 7-aminoactinomycin D (Sigma-Aldrich) at a final concentration of 10 μg/ml in PBS with 2% FCS. After washing, cells were analyzed by flow cytometry using FACSCalibur or LSR II instruments (BD Biosciences). Postacquisition data analysis was performed with FlowJo 8 software (Tree Star, Ashland, OR).

Signal 3 assay of cytokine secretion

mDCs were cocultured with CD40L-expressing cells as a mimic for interactions with activated T cells, as described previously (23). Briefly, mDCs were seeded in 96-well plates at concentrations of 2×10^4 cells/well and coincubated with mouse fibroblasts stably transfected with human CD40L at a concentration of 5×10^4 cells/well. DCs and CD40L fibroblasts cultured in medium alone were used to assess cytokine secretion of individual cell populations. After 24 h, plates were centrifuged, and supernatants of eight replicate wells were pooled for analysis of IL-10 and IL-12(p70).

Migration assay

After harvesting and washing, mDCs were analyzed in a Transwell migration assay, as described previously (27). In brief, the lower culture chamber of a 24-Transwell plate (Costar Corning, Corning, NY) was filled with 600 μl migration medium, consisting of RPMI-VLE, 500 U/ml GM-CSF, 250 U/ml IL-4, and 1% human serum, with or without chemokine CCL19 at 100 ng/ml (R&D Systems). mDCs were seeded in the upper chamber at 2×10^5 cells/well and incubated for 2 h at 37°C in 5% CO_2 in a humidified atmosphere. DCs from the upper and lower chambers were collected and counted using a Neubauer hemocytometer.

NK cell activation

NK cells were enriched from cryopreserved PBMCs using the Dynabeads Untouched Human NK Cells Kit (Invitrogen, Karlsruhe, Germany), according to the manufacturer's instructions. NK cells (1×10^6) were seeded in RPMI 1640 medium, supplemented with 200 mM L-glutamine, 100 mM sodium pyruvate, 10^4 U/ml penicillin and streptomycin (all Invitrogen), and 10% pooled human serum. NK cells were stimulated with 1×10^5 autologous mDCs. After 24 h, supernatant was collected and analyzed using an IFN-γ ELISA. Cocultured cells were stained afterward with CD3 (FITC, clone UCHT1; BD Biosciences), CD56 (allophycocyanin, clone N901; Immunotech), and CD69 (PE, clone TP1.55.3; Immunotech) Abs to depict the activated NK cell populations. After washing, cells were analyzed by flow cytometry using an LSR II instrument (BD Biosciences). Postacquisition data analysis was done with FlowJo 8 software (Tree Star). Aliquots of activated NK cells were also cryopreserved and later evaluated for cytotoxic function.

Activation of allogeneic T lymphocytes

Cryopreserved PBMCs isolated from $HLA\text{-}A2^-$ donors were cocultured with allogeneic mDCs prepared from $HLA\text{-}A2^+$ donors using 1×10^6 PBMCs and 1×10^5 mDCs in T cell medium (RPMI 1640 medium, 12.5 mM HEPES, 4 mM L-glutamine, and 100 U/ml penicillin and streptomycin, supplemented with 10% pooled human serum). Following 7 d of coculture, recovered lymphocytes were analyzed by flow cytometry for intracellular cytokine staining.

Activation of Ag-specific T lymphocytes

mDCs were harvested and pulsed with 1 μg/ml peptide for 120 min at 37°C and 5% CO_2 in a humidified atmosphere. For Ag-pulsing of DCs, a MART-1/Melan-A nonamer (**ELA**GIGILT; Metabion, Martinsried, Germany) as well as a peptide pool (**CEF**) comprising peptides of human CMV, EBV, and influenza A virus (PANATecs, Tuebingen, Germany) were used. Cryopreserved PBMCs isolated from $HLA\text{-}A2^+$ donors were cocultured with autologous, peptide-pulsed mDCs using 1×10^6 PBMCs and 1×10^5 mDCs in T cell medium (RPMI 1640 medium, 12.5 mM HEPES, 4 mM L-glutamine, and 100 U/ml penicillin and streptomycin, supplemented with 10% pooled human serum). Following 7 d of coculture, recovered lymphocytes were tested directly for cytotoxic activity. Recovered cells were also restimulated using the same cryopreserved batch of peptide-pulsed DCs for 24 h, at which time supernatants were collected for determination of IFN-γ content using a standard ELISA. Background activity in cytotoxicity and secretion assays was determined using non–peptide-pulsed mDCs.

Multimer staining

PBLs obtained after 7 d of coculture with 3-d peptide-pulsed mDCs were harvested, washed, and stained with MART-1/Melan-$A_{25–36}$ (**ELA**GIGILT peptide)–specific multimer. Control stainings were performed using a CMV pp65-$peptide_{495–503}$ multimer (both provided by Prof. Dr. D. Busch, Technical University of Munich). Afterward, costaining of surface molecules was performed as described above using the following Abs: CD3 (PerCP, clone SK7; BD Biosciences); CD4 (FITC, clone 13B8.2; Immunotech); and CD8 (allophycocyanin, clone SK1; BD Biosciences). Postacquisition data analysis was done using FlowJo 8 software (Tree Star).

Intracellular cytokine staining

PBLs activated for 7 d with mDCs were harvested, washed, and stimulated for 1 h at 37°C with 1 ng/ml PMA and 250 ng/ml ionomycin (Sigma-Aldrich). Afterward, brefeldin A (10 μg/ml) and monensin (50 μM) were added, and the cells were incubated for an additional 4 h. Staining of

surface molecules was performed as described above using the following Abs: CD3 (PerCP, clone SK7; BD Biosciences); CD4 (PE, clone 13B8.2; Immunotech); CD8 (allophycocyanin, clone SK1; BD Biosciences); CD16 (APC, clone 3G4; Caltag Laboratories, Buckingham, U.K.); and CD56 (PE, clone N901; Immunotech). Afterward, cells were fixed with 1% paraformaldehyde, washed twice, and permeabilized with 0.1% saponin solution (Sigma-Aldrich). Cells were incubated with IFN-γ–specific (FITC, clone 25723.11; BD Biosciences) and IL-4–specific (PE, clone 3010.211; BD Biosciences) Abs in 0.35% saponin solution in PBS for 20 min at 4°C. After washing, cells were analyzed by flow cytometry as described above. Postacquisition data analysis was performed using FlowJo 8 software (Tree Star).

Cytokine secretion measurement by ELISA and multiplex technology

Secretion of IL-12(p70) and IL-10 by mDCs in signal 3 assays as well as secretion of IL-5, IL-13, and IFN-γ by activated PBLs were detected by standard ELISA, using pretested Ab duo sets for detection of IL-5, IL-10, IL-12(p70), or IFN-γ (BD Systems, Heidelberg, Germany) and IL-13 (R&D DuoSet, Wiesbaden, Germany), according to the manufacturer's instructions. Colorimetric substrate reaction with tetramethylbenzidine and H_2O_2 was measured, after stopping the reaction with H_3PO_4, at 450 nm and wavelength correction at 620 nm and analyzed with "easy fit" software (SLT, Crailsheim, Germany). Multiple cytokine and growth factor secretion in culture supernatants of mDCs were quantified by multiplex protein arrays (Human Grp I cytokine 17-Plex Panel; Bio-Rad, Hercules, CA), according to the manufacturer's instructions. In brief, microspheres coated with cytokine-specific capture mAbs were incubated for 30 min at room temperature with 50 μl supernatant medium. After three washing steps, biotinylated detection mAbs were added and incubated for 30 min at room temperature, followed by 10 min of incubation with streptavidin-PE. A two-laser array reader (BioPlex System, Hercules, CA) simultaneously quantified 17 different cytokines, chemokines, and growth factors. Standard curves and concentrations were calculated with BioPlex Manager 4.1.1 on basis of the five-parameter logistic plot regression formula.

Cytotoxicity assay

Cytotoxic activity of NK cells and T cells that were preactivated in DC cocultures was analyzed in a standard 4-h chromium release assay. K562 cells were used as target cells in the NK assays combining 1.5×10^3 target cells and NK cells at E:T cell ratios of 10:1, 5:1, 2.5:1, and 1.25:1. Melanoma cell lines Mel624.38 (HLA-$A2^+$, MART-1/Melan-A^+) and MelA375 (HLA-$A2^+$; MART-1/Melan-A^-) (28) were used as target cells for activated T cells at E:T of 20:1, 10:1, 5:1, or 2.5:1. Specific lysis was determined as described previously (29). The percentage of specific lysis was calculated as 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Spontaneous release was assessed by incubating target cells in the absence of effector cells and was generally <15%.

Western blotting

mDCs were stimulated with the maturation mixture for an additional 2 h, harvested and washed with PBS, and lysed in lysis buffer (20 mM Tris-HCl, 150 mM NaCl [pH 7.4], 150 mM CHAPS, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, and 1× Complete). Protein concentration was detected using the BCA Protein Assay Kit (Pierce Chemical Co., Rockford, IL), according to the manufacturer's instructions. Western blotting was performed following the instructions of the NuPAGE System (Invitrogen). For specific phospho-STAT (pSTAT) staining, polyclonal anti-pSTAT Abs were used (STAT1-pTyr701 and STAT6-pTyr641; Cell Signaling Technologies, Danvers, MA), β-actin was stained using a polyclonal Ab from Sigma-Aldrich (A5060), and detection was performed using a polyclonal anti-rabbit IgG Ab (Cell Signaling Technologies).

Results

mDCs can be efficiently generated in 3 d

Monocytes were obtained by elutriation of leukapheresis products of healthy donors. These cells were used for the in vitro generation of iDCs using GM-CSF and IL-4 in a fast protocol lasting 2 d (8). DC maturation was induced on day 2, creating different populations of 3-d mDCs, using mixtures summarized in Table I. 4C represented the well-characterized 4C that did not include any TLR ligands (7). 5C+R848 was used previously to prepare 7-d mDCs (26). It included the synthetic ligand R848 (resiquimod), which is an imidazoquinoline-like molecule that interacts with TLR7/8 receptors. It also included the TLR3 agonist poly(I:C). Poly(I:C) is known also to bind to the MDA-5 receptor that belongs to the family of cytosolic helicase pattern recognition receptors (30). It is accepted that TLR3 detects extracellular viral dsRNA internalized into the endosomes, whereas RIG-I/MDA-5 detects intracellular viral dsRNA (31, 32). The synthetic thiazoloquinoline-derivative type immune response modifying compound CL075 can also induce signals via TLR7/8 in some cells, but its impact on DC maturation and function has not been reported to date (16, 17). Therefore, we also assessed this substance for its impact on DC maturation. However, a few experiments used mixture 5C alone, which did not include a TLR7/8 agonist but did contain poly(I:C) as a TLR3 agonist. Mixtures 5C, 5C+R848, and 5C+CL075 also differed from 4C in the inclusion of IFN-γ, the exclusion of IL-6, and a 4-fold reduction in the concentration of PGE_2.

Mixtures induce mature surface marker phenotypes on iDCs

mDCs were prepared from three or four healthy donors, applying the various maturation mixtures for 24 h. After a total 3-d culture period, mDCs were recovered from the culture vessels, and percentages of cell recovery were compared. The mDC recoveries ranged from 7.9 to 9%, and there were no significant differences noted among the three groups (Supplemental Table I). The three mDC populations, as well as iDCs from two donors, were then analyzed by flow cytometry for expression of surface markers that are characteristic for iDCs and mDCs. In addition to determining the percentages of positive cells, the levels of surface marker expression were also compared (Table II). The mDCs displayed the expected shift to high percentages of cells, expressing substantial levels of CD80, CD83, and CCR7. There were no substantial differences noted among the different mixtures with respect to the various surface markers that were upregulated on the mDCs.

Production of IL-12(p70) is superior by DCs matured using TLR7/8 agonists and poly(I:C)

Our goal in creating TLR-containing maturation mixtures was to generate a population of optimized DCs for clinical studies that could secrete bioactive IL-12(p70) while producing no or only low levels of IL-10 to obtain cells that would polarize Th cells in a Th1 direction (14). Furthermore, for vaccine development, it is important to assure that mDCs retain the capacity to secrete IL-12(p70) upon encounter with T cells in lymph nodes. This capacity was assessed in a so-called signal 3 assay, in which mDCs were cocultured with a murine fibroblast cell line that expresses human CD40L and thereby mimics an encounter of mDCs with $CD40L^+$ T cells. Signal 3 assays were performed with mDC populations that were prepared from multiple independent donors. After coculture of 3-d mDCs with CD40L-expressing fibroblasts for 24 h, amounts of IL-12(p70) and IL-10 released into the culture medium were determined by standard ELISA. The results are shown in Fig. 1 as mean values of IL-12(p70) and IL-10 in picograms per milliliter. The DC^{4C} populations secreted only low amounts of IL-12(p70), whereas $DC^{5C+R848}$ and $DC^{5C+CL075}$ produced very high amounts of IL-12(p70) upon stimulation via CD40, which were significantly different from those of DC^{4C}. In contrast, the mDC populations secreted only very low amounts of IL-10, with no significant differences among the three groups.

mDCs show chemotaxis to CCL19 signals

If mDCs are considered for use in vaccine development it is important to demonstrate that they have an adequate potential to migrate to lymph node sites. This is primarily governed by

Table I. *Composition of mixtures used for DC maturation*

Mixture	Population	Inflammatory Cytokines/IFNs	Other Additives	TLR Ligands
4C	DC^{4C}	TNF-α, IL-1β, IL-6	PGE_2	
5C	DC^{5C}	TNF-α, IL-1β, IFN-γ	PGE_2	Poly(I:C)
5C+R848	$DC^{5C+R848}$	TNF-α, IL-1β, IFN-γ	PGE_2	Poly(I:C), R848
5C+CL075	$DC^{5C+CL075}$	TNF-α, IL-1β, IFN-γ	PGE_2	Poly(I:C), CL075

The following concentrations were used in the individual mixtures (bold): **4C**, 10 ng/ml TNF-α, 10 ng/ml IL-1β, 1000 ng/ml PGE_2, and 15 ng/ml IL-6; **5C**, 10 ng/ml TNF-α, 10 ng/ml IL-1β, 250 ng/ml PGE_2, 5000 U/ml IFN-γ, and 20 ng/ml poly(I:C); **5C+R848**, 10 ng/ml TNF-α, 10 ng/ml IL-1β, 250 ng/ml PGE_2, 5000 U/ml IFN-γ, 20 ng/ml poly(I:C), and 1 μg/ml R848; **5C+CL075**, 10 ng/ml TNF-α, 10 ng/ml IL-1β, 250 ng/ml PGE_2, 5000 U/ml IFN-γ, 20 ng/ml poly(I:C), and 1 μg/ml CL075.

expression of the chemokine receptor CCR7, which mediates a chemotactic response to the chemokines CCL19 and CCL21 that are expressed in the lymph nodes (33, 34). On the basis of the assessment of CCR7 phenotype, it was apparent that mDCs produced with mixtures 5C+R848 and 5C+CL075 were comparable to those matured with 4C, both with respect to percentages of positive cells and levels of expression. Although substantial numbers of cells displayed this critical chemokine receptor, this does not necessarily equate directly with migratory responses to CCR7 chemokine signals. Therefore, Transwell migration assays were used to assess the spontaneous migration of the mDC populations, as well as their migratory responses to CCL19 as a chemoattractant. As illustrated in Fig. 2, low percentages of mDC populations showed spontaneous migration in the absence of chemoattractant, but substantially higher percentages of mDCs showed Transwell chemotaxis to CCL19 chemokine, with an overall significant difference to spontaneous migration in each group ($p = 0.03$). However, there were no significant differences among the three groups of mDCs.

mDCs prepared using TLR7/8 agonists strongly activate NK cells

According to phenotype, cytokine secretion, and migratory capacity, it was clear that CL075 and R848 had comparable impacts on the development of mDCs. We next addressed the capacity of $DC^{5C+R848}$ and $DC^{5C+CL075}$ to activate various lymphocyte populations as compared with DC^{4C}, which have been used most extensively in clinical studies.

It was expected that mDCs that secrete bioactive IL-12(p70) would be superior to DC^{4C} populations in the activation of NK cells. This was clearly demonstrated when enriched NK cells prepared from multiple unrelated donors were incubated for 24 h with mDCs and analyzed for secretion of IFN-γ as one parameter of NK cell activation. Only low levels of IFN-γ were secreted by NK cells stimulated with DC^{4C}, whereas NK cells released substantially more IFN-γ following contact with $DC^{5C+R848}$ or $DC^{5C+CL075}$ (Fig. 3*A*).

As a second parameter for NK cell activation, we measured upregulation of the activation marker CD69 on NK cells after 24-h activation with mDCs. Results of NK cells from one representative donor are shown in Fig. 3*B*. NK cells expressing this marker after exposure to DC^{4C} cells did not differ substantially from NK cells cultured in medium alone. Strong changes were seen for NK cells activated with $DC^{5C+R848}$ or $DC^{5C+CL075}$, showing substantially increased expression of CD69. Notably, $DC^{5C+R848}$ and $DC^{5C+CL075}$ stimulation had a strong impact on CD69 expression by both the $CD56^{dim}$ and $CD56^{bright}$ NK cells.

As a third parameter, the killing capacity of activated NK cells was determined after activation with mDCs for 24 h. Effector cells were tested in a standard 4-h chromium release assay using the K562 cell line as the target cell. NK cells cultured with DC^{4C} displayed only slightly enhanced killing activity at high E:T when compared with NK cells not exposed to DC stimulation (Fig. 3*C*). In contrast, NK cells activated by either $DC^{5C+R848}$ or $DC^{5C+CL075}$ showed greater killing at all E:T compared with NK cells activated with DC^{4C}.

mDCs prepared using TLR7/8 agonists polarize allogeneic CD4 and CD8 T cells

To analyze the effect on T cell polarization, we stimulated PBMCs containing mixtures of $CD4^+$ and $CD8^+$ T cells with allogeneic 3-d mDCs generated using either 4C, 5C+R848, or 5C+CL075 mixtures. After 1 wk of T cell-DC coculture, the allostimulated T cells were harvested, washed, and activated with PMA and ionomycin for 5 h to induce intracellular cytokine expression. The lymphocytes were then analyzed by flow cytometry for expression of CD3, CD4, and CD8 surface markers. In parallel, the fractions of cells producing IFN-γ and IL-4 were determined using intracellular cytokine staining. PBMCs cultured for the same time period in the absence of DCs served as a background control of unstimulated cells.

Table II. *Expression of typical DC surface markers*

	CD14	CD80	CD83	CD86	HLA-DR	CCR7
% Positive cells						
iDCs	25 ± 5[a]	29 ± 20	22 ± 3	99 ± 0	100 ± 0	8 ± 0
4C[b]	3 ± 0	98 ± 1	95 ± 4	100 ± 0	98 ± 4	75 ± 2
5C+R848	3 ± 1	99 ± 0	97 ± 1	98 ± 3	77 ± 24[c]	72 ± 6
5C+CL075	3 ± 1	99 ± 0	97 ± 1	98 ± 3	95 ± 8	83 ± 2
MFIs						
iDCs	19 ± 0[d]	16 ± 2	14 ± 1	144 ± 31	497 ± 124	25 ± 7
4C	20 ± 6	76 ± 12	86 ± 28	760 ± 107	732 ± 203	56 ± 8
5C+R848	14 ± 5	143 ± 33	61 ± 19	946 ± 286	542 ± 151	48 ± 10
5C+CL075	12 ± 2	144 ± 25	48 ± 5	842 ± 369	496 ± 158	58 ± 6

[a]Data represent mean percentages of positive-stained cells ± SEs for three to four independent donors.
[b]DCs matured in mixtures shown in Table I.
[c]Significant differences between 5C+R848 and 5C+CL075.
[d]Data represent MFIs ± SEs for three to four independent donors.

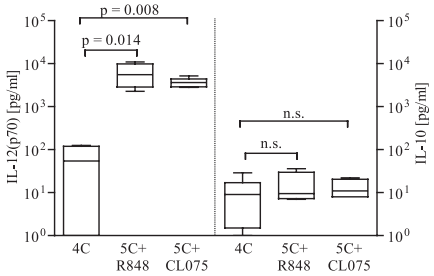

FIGURE 1. Cytokine secretion assessed by a signal 3 assay. DC populations were matured using the 3-d protocol. Independent experiments were performed with a number of n = 4–6 donors for DC^{4C}, $DC^{5C+R848}$, or $DC^{5C+CL075}$, respectively. CD40L-transfected mouse fibroblasts were used as stimulators for mDCs, mimicking the encounter with T cells that express CD40L. Supernatants were collected after 24 h of coculture. Cytokines were measured by standard ELISA. Shown are the mean values with minimum and maximum values (on a $\log_{10}$ scale) of IL-12(p70) and IL-10 detected in the supernatant. Significance was ascertained using the two-tailed Mann-Whitney U test.

In a representative example, ~6% of $CD4^+$ T cells cultured in medium alone were positive for IFN-γ, and 1.5% expressed IL-4 (Fig. 4*A*). These values increased to ~25% of $CD4^+$ T cells with IFN-γ and 6.5% with IL-4 after activation for 1 wk with DC^{4C} cells. In contrast, around twice as many $CD4^+$ T cells expressed IFN-γ after coculture with $DC^{5C+R848}$ or $DC^{5C+CL075}$, whereas $CD4^+$ T cells producing IL-4 remained at the background levels of unstimulated PBMCs. Similar effects were seen on polarization of $CD8^+$ T cells, with higher percentages of cells producing IFN-γ (Fig. 4*B*), without alterations in percentages of IL-4–stained cells (data not shown), following coculture with $DC^{5C+R848}$ or $DC^{5C+CL075}$, as compared with DC^{4C}.

IL-5 and IL-13 represent additional Th2 cytokines of importance. To determine the amount of IL-5 and IL-13 secreted by cells cultured for 7 d with allogeneic mDCs, we restimulated CD4-enriched T cells or PBLs for 24 h with allogeneic PBMCs. The levels of cytokines secreted into the supernatant medium were analyzed using standard ELISA. Neither T cells activated with DC^{4C} nor T cells stimulated with $DC^{5C+R848}$ or $DC^{5C+CL075}$ secreted appreciable amounts of IL-5 and IL-13 (data not shown).

DCs matured with TLR7/8 agonists prime better Ag-specific T cell responses

The capacity of DCs to prime naive $CD8^+$ cells to specific Ags is of particular importance for development of efficient antitumor

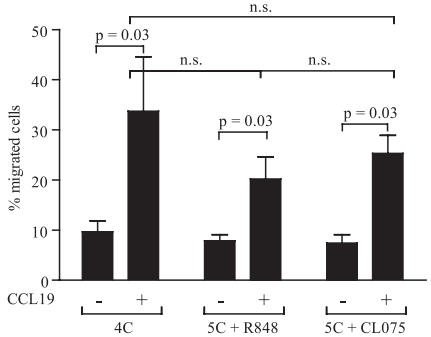

FIGURE 2. Migratory capacity of mDCs to CCR7 ligands. Depicted are data of five independent Transwell migration assays using CCL19 as a chemoattractant for DC^{4C}, $DC^{5C+R848}$, or $DC^{5C+CL075}$. Pore size of the membrane was 5 μm; chemokine concentration of CCL19 was 100 ng/ml. Shown are mean values with SEM on a $\log_{10}$ scale. Statistical analyses were performed using the Mann-Whitney U test.

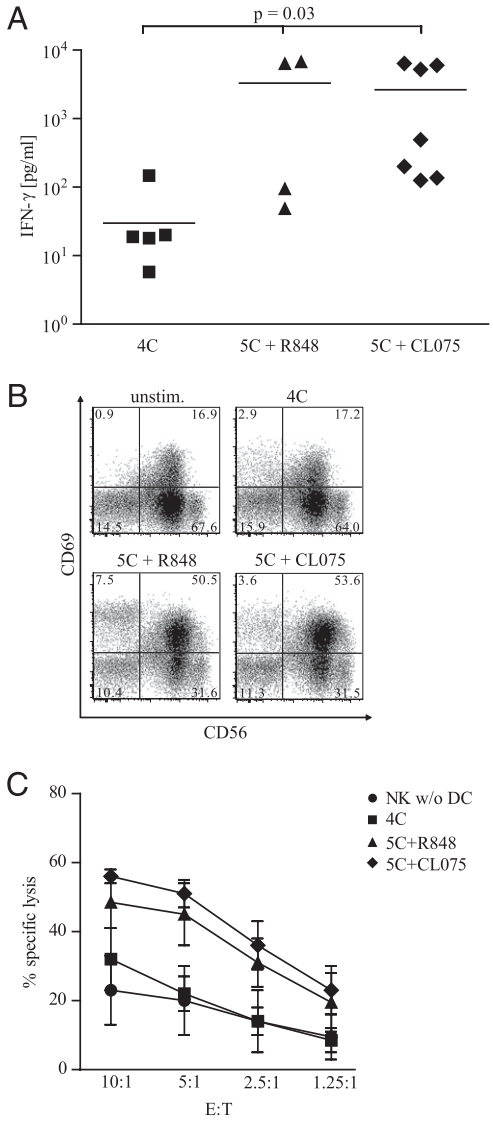

FIGURE 3. Activation of NK cells by mDCs. NK cells were prepared from several unrelated donors and incubated for 24 h with washed DCs that were matured in mixtures 4C, 5C+R848, and 5C+CL075. *A*, Secretion of IFN-γ was quantified by a standard ELISA and shown as individual values on a $\log_{10}$ scale (4C, 5C+CL075: n = 7; 5C+R848: n = 4; line represents mean value; Mann-Whitney U test was applied for statistical analyses). *B*, Cocultured cells were stained for CD3, CD56, and CD69 expression. Depicted is a representative specific staining of CD56 versus CD69 of the $CD3^-$ population (n = 4). *C*, Cytotoxic activity of NK cells assessed in a standard 4-h chromium release assay against K562 target cells at the varying effector to target cell ratios indicated on the x-axis. Data are derived using cells from two independent donors and shown as mean with SEM.

immune responses. To address this capacity of mDC populations, we cocultured PBLs from HLA-A*0201 donors for 1 wk with autologous mDCs that were pulsed with the ELA-peptide epitope of the MART-1/Melan-A Ag, which is a tumor-associated protein that is overexpressed by a large number of melanomas. In addition, the capacity of the mDCs to reactivate antiviral immune responses was assessed using the CEF pool of HLA-A2–restricted peptides from three common viruses that represent immunodominant epitopes to which many adult healthy donors show immune reactivity. After the 7-d stimulation phase, PBLs were restimulated for 24 h using peptide-pulsed DCs as well as unpulsed mDCs of the same origin to detect background

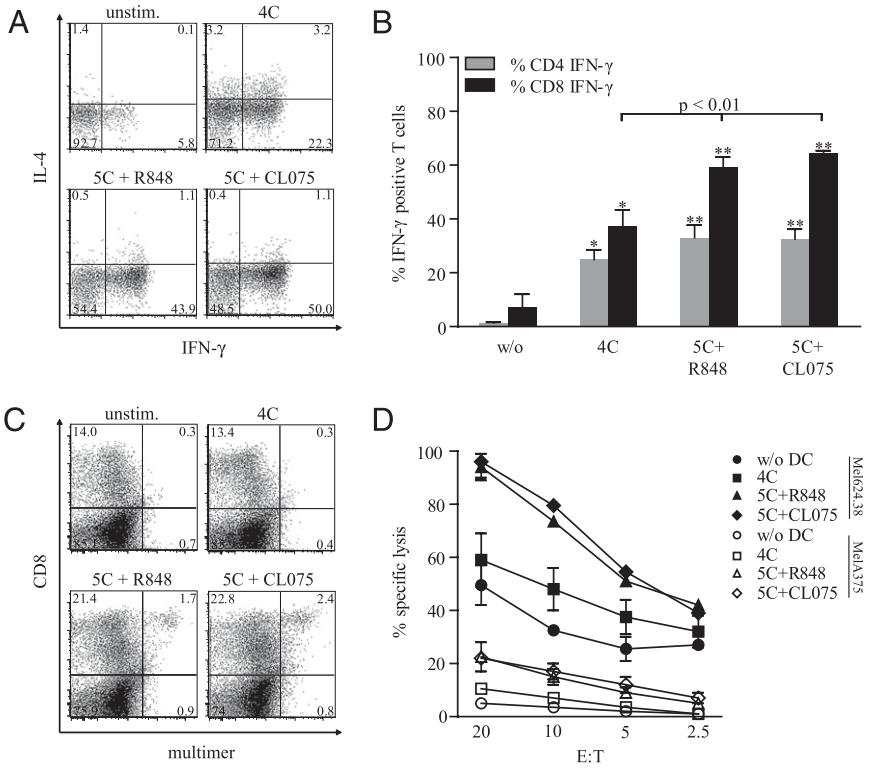

FIGURE 4. Activation of T cells by mDCs. PBMCs from HLA-A2$^-$ donors were stimulated with mDCs derived from an HLA-A2$^+$ donor for 7 d. After 5 h of stimulation with PMA and ionomycin, intracellular IFN-γ and IL-4 were analyzed using flow cytometry. *A*, Shown is a representative example of gated CD4$^+$ cells with double staining of intracellular IFN-γ and IL-4. *B*, IFN-γ–positive cells are depicted as percentages of CD4$^+$ cells (gray) and CD8$^+$ cells (black), respectively. Shown are mean values with SEM; significance was analyzed using an ANOVA test in combination with a Bonferroni posttest (*$p < 0.01$; **$p < 0.001$; each compared with unstimulated control or compared with CD8$^+$ T cells stimulated with DC^{4C}). *C*, Autologous PBLs of HLA-A2$^+$ donors were stimulated with MART-1/Melan-A (ELA-) peptide-pulsed mDCs for 7–10 d, followed by a staining with a MART-1/Melan-A multimer and CD8-specific Ab on day 10. *D*, Killing was assessed on day 7 in a standard 4 h chromium-release assay using Mel624.38 (HLA-A2$^+$, MART-1/Melan-A$^+$) and MelA375 (HLA-A2$^+$, MART-1/Melan-A$^-$) tumor cell lines as positive and negative target cells, respectively. Shown are two independent donors as mean values with SEM.

activation. PBLs stimulated with ELA- or CEF-pulsed mDC$^{5C+CL075}$ secreted noticeably higher amounts of IFN-γ compared with PBLs cocultured with peptide-pulsed mDC^{4C} (data not shown).

To assess the killing capacity of T cells primed with different mDC populations, PBLs were cocultured with ELA-pulsed mDCs for 7–10 d. Staining with a MART-1/Melan-A$_{26–35}$ multimer was performed on day 10. In a representative example, nonstimulated CD8$^+$ cells contained 0.3% multimer-positive T cells, which were not increased by coculture with DC^{4C} (Fig. 4*C*). In contrast, the multimer-positive fraction increased through coculture with DC$^{5C+R848}$ or DC$^{5C+CL075}$ to 1.7 and 2.4%, respectively, clearly demonstrating the superior capacity of DCs matured with these mixtures to induce Ag-specific CTLs.

Primed CTLs were analyzed in a standard 4-h chromium release assay using MelA624.38 (HLA-A2$^+$ and MART-1/Melan-A$^+$) and MelA375 (HLA-A2$^+$ and MART-1/Melan-A$^-$) as target cells. CTL primed with peptide-pulsed DC^{4C} showed cytotoxic activity somewhat greater than effector cells cultured without any DCs, whereas CTLs primed with peptide-pulsed DC$^{5C+R848}$ or DC$^{5C+CL075}$ showed substantially greater cytotoxic activity, which was specific for melanoma cells coexpressing MART-1/Melan-A and HLA-A2 (Fig. 4*D*).

DCs matured using 5C+CL075 display a prominent positive costimulatory profile and STAT1 activation

In addition to IL-12(p70) secretion, we observed several other differences between DC^{4C} and DC$^{5C+CL075}$ that may impact on lymphocyte activation and function. For example, differences were found with respect to expression of the positive costimulatory molecule CD80 (B7.1) versus the negative costimulatory molecule CD274 (B7-H1). A preponderance of CD80 compared with CD274 seems to be important for transmission of positive costimulatory signals to T cells. Fig. 5*A* shows mean fluorescence intensity (MFI) values of CD80 and CD274 on DCs matured using mixtures 4C or 5C+CL075, with or without addition of IL-10. Expression of CD80 was highest on DC$^{5C+CL075}$ and significantly different to DC^{4C}. DC$^{5C+CL075+IL-10}$ was generated by addition of IL-10 to the maturation mixture as a tolerogenic control. Treatment with IL-10 has been shown to reduce the stimulatory capacity of DCs, resulting in anergized T cells (35, 36). IL-10–stimulated DCs showed a reciprocal relationship of CD80 to CD274, with lower CD80 and higher CD274 expression. Similar observations were made with DC$^{5C+R848}$ (data not shown).

On a molecular level, STAT molecules are important transcription factors in the downstream signaling pathways of TLRs and IFN-γRs. DCs matured with 4C and 5C+CL075 were assessed for the activation status of the STAT proteins 1 (pTyr$_{701}$), 2 (pTyr$_{690}$), 3 (pSer$_{727}$ and pTyr$_{705}$), 5 (pTyr$_{694}$), and 6 (pTyr$_{641}$). STAT1, STAT3, and STAT6 were found to be activated in the mDCs based on their phosphorylation status (data not shown), but differences between DC^{4C} and DC$^{5C+CL075}$ were only found for STAT1 and STAT6 (Fig. 5*B*). DC$^{5C+CL075}$ displayed strongly increased STAT1 phosphorylation that was only marginal in DC^{4C}. Activation of STAT1 has been demonstrated previously for DCs matured in mixtures containing R848 (24).

DCs matured with a TLR3 agonist are inferior to those matured with TLR3 and TLR7/8 signals

Clearly mDCs prepared using a combination of TLR3 and TLR7/8 agonists were superior to DCs matured with mixture 4C that lacked TLR signaling capacity. However, the impact of the TLR3 signal alone was not clear. Therefore, comparisons of DC^{5C} and DC$^{5C+CL075}$ were made to ascertain the role of TLR3 stimulation in the absence of TLR7/8 activation (Fig. 6). Mixture 5C induced a maturation phenotype similar to 5C+CL075, albeit with somewhat lower levels of CD80, CD83, and CCR7 (Fig. 6*A*). DC^{5C} secreted high levels of bioactive IL-12(p70) in signal 3 assays that were comparable to DC$^{5C+CL075}$ and substantially greater than DC^{4C} (Fig. 6*B*). Coculture of NK cells with DC^{4C} and DC^{5C} for 24 h yielded comparable percentages of activated NK cells expressing CD69, but these were less than half the value obtained through activation by DC$^{5C+CL075}$ (Fig. 6*C*). Furthermore, secretion of

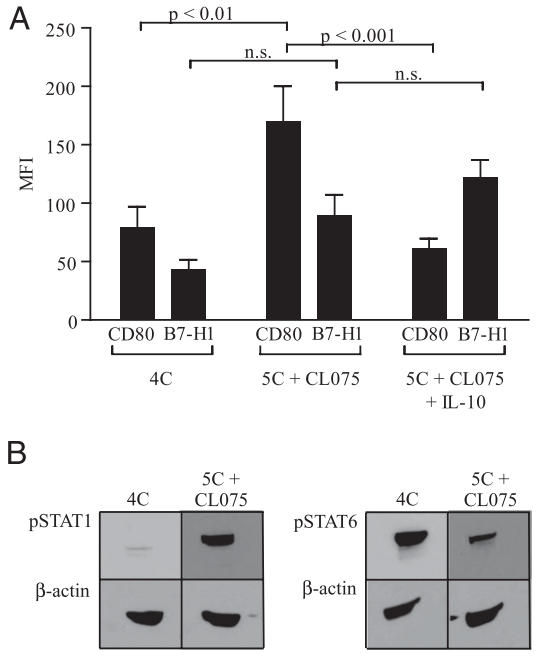

FIGURE 5. Costimulatory profile and STAT activation in mDCs. mDCs were generated with the 3-d-protocol. *A*, Shown is the expression level of the costimulatory molecules CD80 and CD274 (B7-H1) detected by flow cytometry as mean values of six individual experiments (three independent donors) with SEM. As a tolerogenic control, mDCs were prepared using 10 ng/ml IL-10 incorporated into mixture 5C+CL075. Statistical analysis was performed using an ANOVA test in combination with a Bonferroni post-test. *B*, Shown is a Western blot stained for pSTAT1 and pSTAT6; β-actin was stained as a control. Exposure times were 3 min, 30 s, and 3 s for 4C, 5C+CL075, and β-actin staining, respectively.

IFN-γ by T cells primed and restimulated with mDCs was much less for DC^{4C} and DC^{5C} compared with $DC^{5C+CL075}$ (Fig. 6*D*). Killing capacity of MART-1/Melan-A–specific CTL primed over 7 d was comparable for peptide-pulsed DC^{4C} and DC^{5C} and somewhat greater than T cells cultured in the absence of mDC. In contrast, T cells stimulated with peptide-pulsed $DC^{5C+CL075}$ displayed much higher levels of killing of tumor cells coexpressing MART-1/Melan-A and HLA-A2 (Fig. 6*E*). These results demonstrated that DCs matured using only TLR3 signals were functionally inferior in activation of both NK cells and T cells to DCs matured using the combination of TLR3 and TLR7/8 agonists.

Discussion

In DC-based vaccination, mDCs are essential to induce effective immune responses; therefore, the first generation vaccine trials that applied iDCs may not have achieved adequate in vivo maturation to allow induction of optimal immune responses. The second generation of vaccines using mDCs provided cells that were more proficient at delivering signal 1 to T cells through abundant MHC class I and class II expression and were capable of delivering signal 2 through an improved expression of costimulatory molecules, but they did not express a cytokine profile that would foster induction of optimal antitumor responses. The discovery that TLR signaling in murine DCs could activate the NF-κB pathway opened the door for inclusion of synthetic TLR agonists in maturation mixtures to modulate the cytokine profile of mDCs (37).

A variety of TLRs are expressed by human monocyte-derived DCs (38). Signaling through different TLRs, including TLR3, TLR7, and TLR8, also causes activation of the NF-κB pathway in human cells, with subsequent induction of several immunomodulatory genes (14, 24, 25). This leads to production of cytokines and chemokines, such as TNF-α, IFN-γ, IL-6, IL-10, IL-12, and MIP-1α, in various cell types (16, 17). The availability of synthetic ligands for several TLRs, including imiquimod (R837), resiquimod (R848), S-27609, CL097, CL075 (3M-002), CL087, or loxoribone, has enabled the impact of TLR activation to be assessed in various cell types (18, 22). On the basis of these observations, mixtures containing various synthetic TLR agonists have been used to prepare human mDCs. Mailliard et al. (23) described a maturation mixture composed of TNF-α, IL-1β, IFN-α, IFN-γ, and the TLR3 agonist poly(I:C), which enhanced the production of bioactive IL-12(p70) in 7-d mDCs but gave only low cell yields. This could be a limiting factor in obtaining sufficient numbers of mDCs for clinical studies if vaccination protocols do not entail direct intralymphatic injection (clinical trial ID UPCI 03-118; http://clinicaltrials.gov/ct2/show/NCT00390338) but rely instead on migration of a small proportion of mDCs to neighboring lymph nodes after s.c. or intradermal injection (39, 40). An alternative approach included only the TLR7/8 ligand resiquimod (R848) for stimulation of iDCs; however, this yielded DCs lacking several phenotypic characteristics of fully mature DCs desired for clinical use (41). Dauer et al. (10) recently incorporated TLR4 and TLR7/8 ligands in their maturation mixture to obtain 2-d mDCs secreting bioactive IL-12(p70). This study revealed that TLR ligands enhanced IL-12(p70) secretion in mDCs as well as stimulatory capacity; however, the maturation status and migratory capacity were lower than mDCs produced with mixture 4C.

We previously described DC maturation mixtures including R848 and poly(I:C) as respective agonists for TLR7/8 and TLR3, in combination with additional cytokines within a 7-d-protocol (26). CL075 is a synthetic small molecule thiazoloquinoline immunostimulatory compound that is a preferential activator of TLR8, resulting in downstream activation of NK-κB and other transcription factors. It is also involved in transcriptional activation of numerous genes encoding cytokines, chemokines, and costimulatory molecules (17). So far, the impact of CL075 on DC maturation has not been reported. We found in this study that mDCs prepared with maturation mixtures using CL075 displayed phenotypes and functions suitable for antitumor vaccine development.

Comparison of mDCs prepared without TLR agonists versus those stimulated with TLR3 [poly(I:C)], alone or in combination with TLR7/8 agonists (R848/CL075), revealed that all populations of mDCs were similar with respect to percentages of positive cells expressing costimulatory molecules. Some variations in HLA-DR and CCR7 were noted and mixture 5C+CL075 provided the highest percentages of mDCs expressing these two molecules. The good expression of CCR7 was paralleled by a strong spontaneous migratory capacity of the mDCs as well as positive chemotactic responses to CCL19 chemokine signals.

As expected, TLR signaling altered the cytokine secretion profile of mDCs, leading to high production of bioactive IL-12(p70). DCs prepared with mixtures including only a TLR3 signal or in combination with a TLR7/8 agonist induced high levels of IL-12 (p70) secretion in signal 3 assays that mimic DC encounters with T cells. These findings are supported by a study of Larangè et al. (42), which demonstrated that signaling through TLR8 had a high impact on secretion of IL-12(p70).

The modulated cytokine profile of mDCs achieved through combined TLR3 and TLR7/8 signaling had a strong impact on functional activation of NK cells, as seen by upregulation of CD69 on both $CD56^{dim}$ and $CD56^{bright}$ NK cell subpopulations, as well as on the levels of IFN-γ secretion and killing capacity by NK cells after a 24-h exposure to mDCs. A recent study also showed that IL-12(p70) secreted by DCs influences NK activation, in

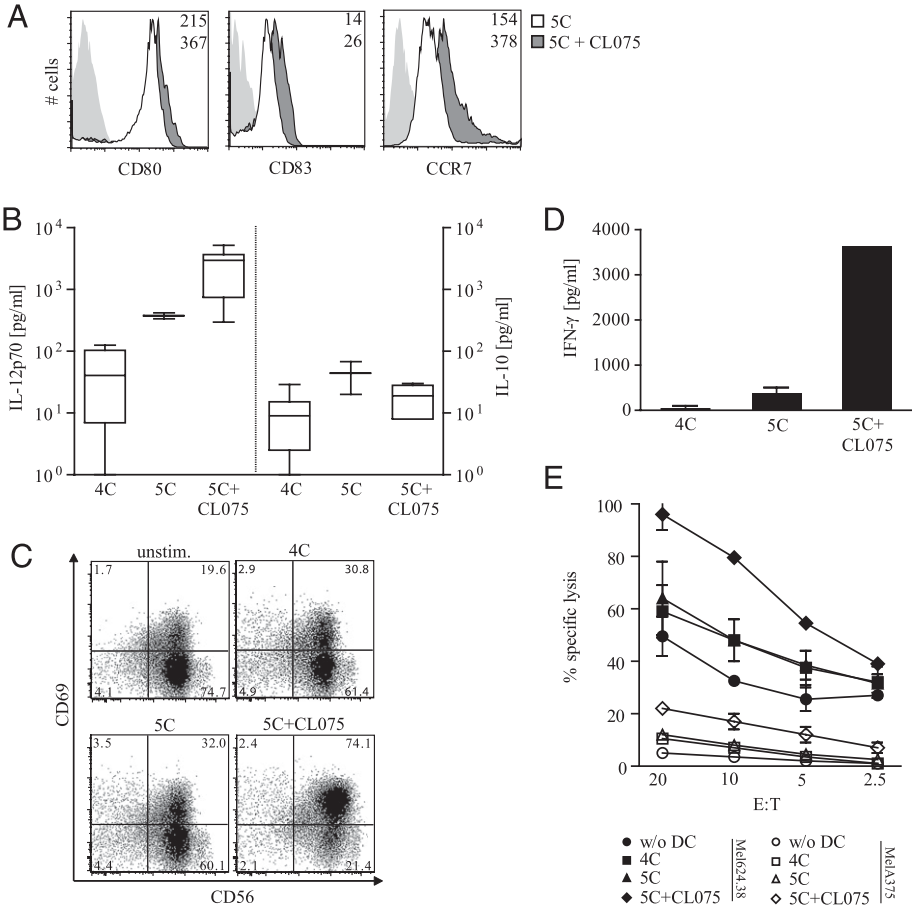

FIGURE 6. Comparison of TLR3 and TLR3 with TLR7/8 agonists for DC maturation. *A*, Depicted is a representative surface staining of CD80, CD83, and CCR7 on DCs matured with mixture 5C (open histograms) and 5C+CL075 (dark gray histograms). Unstained controls are shown in light gray. MFI values are indicated within the histograms. Data are representative for two independent donors ($n = 2$). *B*, Signal 3 assays were performed with $n = 2$–6 donors for DC^{4C}, DC^{5C}, or $DC^{5C+CL075}$. Assays were performed as described in Fig. 1, and supernatant media were collected after 24 h of coculture with CD40L-expressing fibroblasts. Cytokines were measured by standard ELISA. Shown are the mean values with minimum and maximum values (on a $\log_{10}$ scale) of IL-12(p70) and IL-10. *C*, NK cells of two individual donors were cocultured with mDCs for 24 h. Activated NK cells were stained for CD3, CD56, and CD69 expression. Depicted is specific staining of CD56 versus CD69 of the $CD3^-$ population ($n = 2$). *D*, Autologous PBLs were stimulated with MART-1/Melan-A (ELA-) peptide-pulsed mDCs for 7 d, followed by specific restimulation for 24 h using peptide-pulsed mDCs. Unpulsed mDCs were used to measure background responses. The amount of secreted IFN-γ was assessed by a standard ELISA and corrected by subtraction of background values of T cells stimulated with unpulsed mDCs. Shown are two independent donors as mean values with SEM. *E*, Autologous PBLs were stimulated with MART-1/Melan-A (ELA-) peptide-pulsed mDCs for 7 d, and killing was assessed in a standard 4-h chromium release assay using Mel624.38 ($HLA\text{-}A2^+$, MART-1/Melan-A^+) and MelA375 ($HLA\text{-}A2^+$, MART-1/Melan-A^-) tumor cell lines as positive and negative target cells, respectively. Shown are two independent donors as mean values with SEM.

particular IFN-γ secretion, in a cell-contact–dependent manner (6), as also found in our studies. Thus, DCs matured with 5C+CL075 or R848 should support innate responses that can lead to direct killing of tumor cells by activated $CD56^{dim}$ NK cells as well as through the capacity of $CD56^{bright}$ NK cells to support development of adaptive immune responses by cytokine and chemokine secretion (43).

TLR7/8 signaling with CL075 or R848 had a positive impact on the ability of $CD4^+$ and $CD8^+$ T cells to produce IFN-γ. Although some $CD4^+$ and $CD8^+$ allogeneic T cells were polarized to Th1 and Th2 cells using DC^{4C} cells, substantially higher percentages of IFN-γ–polarized T cells were obtained using TLR-activated mDCs. In addition, these mDCs were able to induce greater numbers of CTLs that displayed specific killing capacity for tumor cells. We also observed that the levels of CD80 were significantly higher on $DC^{5C+CL075}$ compared with DC^{4C}, whereas levels of CD274 were not significantly different. Therefore, it would be expected that such TLR-activated mDCs would have a superior capacity for T cell activation. This is in accordance with recent findings by Selenko-Gebauer et al. (44), who nicely showed that the expression levels of positive costimulatory molecules prevailed over low expression of inhibitory molecules in T cell activation, whereas a reverse profile resulted in poor T cell stimulation. In addition, they postulated that inhibitory molecules had a greater influence when the overall expression of costimulatory molecules was low.

The molecular mechanisms responsible for these phenotypic and functional differences in DCs matured by 4C and 5C+CL075 were indicated by the strong differences in STAT1 activation. In particular, pSTAT1-Tyr701 was strongly increased in $DC^{5C+CL075}$ compared with DC^{4C}. This activation is known to result in an altered expression profile of downstream STAT1-dependent genes, including cytokines and costimulatory molecules (42, 45). Phosphorylated STAT1 can bind to an IFN-γ–activated sequence or an IFN-stimulated response element in DNA. In both cases, this leads to increased expression of IFN-stimulated genes (46). Moreover, IFN-stimulated response element binding sites are present in the promoter regions of CD80 and CD274 (47, 48), likely explaining

the significantly enhanced expression of these molecules on DCs matured with 5C+CL075. Others found that phosphorylation of STAT1 was induced in DCs by the TLR7/8 agonist R848 and that decreased IL-12(p70), CD40, and CD83 expression was seen following inhibition of the STAT1 signaling pathway in studies using 7-d mDCs (24, 42).

Altogether, maturation mixtures including CL075 or R848 as TLR7/8 agonists, together with a TLR3 agonist, allowed mDCs to be produced in 3 d that had superior characteristics, when compared with DCs matured in the absence of TLR signals or activated by only a TLR3 signal. The production of mDCs in 3 d rather than 7 d provides a great cost reduction for DC vaccine generation, because cell cultures must not be resupplemented with cytokines during the short culture period. Furthermore, occupancy time of clean room facilities is greatly reduced. This allows rapid generation of mDCs that can be used for development of antipathogen and antitumor vaccines.

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Disclosures

S.S., M.J., D.J.S., and B.F. declare a competing interest through pending patents submitted by the Helmholtz Zentrum München, German Research Center for Environmental Health (Munich, Germany) for DC maturation mixtures.

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Three-day dendritic cells for vaccine development: Antigen uptake, processing and presentation

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Abstract

Background: Antigen-loaded dendritic cells (DC) are capable of priming naïve T cells and therefore represent an attractive adjuvant for vaccine development in anti-tumor immunotherapy. Numerous protocols have been described to date using different maturation cocktails and time periods for the induction of mature DC (mDC) *in vitro*. For clinical application, the use of mDC that can be generated in only three days saves on the costs of cytokines needed for large scale vaccine cell production and provides a method to produce cells within a standard work-week schedule in a GMP facility.

Methods: In this study, we addressed the properties of antigen uptake, processing and presentation by monocyte-derived DC prepared in three days (3d mDC) compared with conventional DC prepared in seven days (7d mDC), which represent the most common form of DC used for vaccines to date.

Results: Although they showed a reduced capacity for spontaneous antigen uptake, 3d mDC displayed higher capacity for stimulation of T cells after loading with an extended synthetic peptide that requires processing for MHC binding, indicating they were more efficient at antigen processing than 7d DC. We found, however, that 3d DC were less efficient at expressing protein after introduction of *in vitro* transcribed (*ivt*)RNA by electroporation, based on published procedures. This deficit was overcome by altering electroporation parameters, which led to improved protein expression and capacity for T cell stimulation using low amounts of *ivt*RNA.

Conclusions: This new procedure allows 3d mDC to replace 7d mDC for use in DC-based vaccines that utilize long peptides, proteins or *ivt*RNA as sources of specific antigen.

Background

The benefit of dendritic cells (DC) as adjuvants to induce tumor-specific cytotoxic T cells as well as helper T cells has been demonstrated in animal experiments and initial human trials [1,2]. In different tumor vaccines that were successfully applied in mice, mature DC (mDC) were used that were loaded with tumor antigens, supplied in various forms, including tumor extracts, short peptides or antigen-encoding RNA [3,4]. Several clinical trials using DC as tumor-vaccines have also been performed, where an increased T cell response against tumor-associated antigens could be observed [5].

DC are the most potent antigen-presenting cells for the stimulation of naïve T cells [6]. Immature DC (iDC) patrol peripheral tissues and take up antigens via macropinocytosis, phagocytosis or receptor-mediated endocytosis. After uptake of antigen, iDC process and present antigen-derived peptides on their MHC molecules. Since DC have the ability for cross-presentation, exogenous antigens can be presented on MHC-II as well as on MHC-I molecules [7]. Presentation of antigens by iDC leads to T cell anergy, deletion of T cells or the induction of IL-10-secreting T regulatory cells [8,9]. Following antigen uptake, iDC convert to a mature phenotype, characterized by the upregulation of different cell surface molecules, such as CD40, CD80 and CD83 [10]. These mDC also show higher expression of the chemokine-receptor CCR7, which plays an important role for DC homing to lymph nodes [11]. Upon arrival in the

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lymph nodes, antigen-loaded mDC are able to prime naïve T cells, which then exit the lymph nodes after antigen-encounter. The primed effector T cells can recognize and eliminate specific target cells in the periphery.

Different protocols for the generation of DC have been described to date. *In vitro*, DC can be developed from $CD34^+$ precursor cells or $CD14^+$ monocytes [10,12]. Monocytes can be enriched from peripheral blood mononuclear cells (PBMC) via plate adherence, by the use of anti-CD14 antibodies or by elutriation of leukapheresis products. iDC are usually induced by stimulation with GM-CSF and IL-4 [13,14]. It has also been shown that IL-4 could be replaced by IL-15, leading to the differentiation of monocytes into cells with properties of Langerhans cells [15-17]. Furthermore, DC can also be induced in the presence of IFN-β and IL-3 [18,19]. The induction of mDC can be initiated by several different stimuli, including microbial components (e.g. LPS as a Toll-like receptor 4 ligand), proinflammatory cytokines, viral-like stimuli [e.g. poly (I:C)] or T cell-derived molecules (e.g. CD40L) [16,18,20-24]. Depending on the composition of the maturation cocktails, mDC show different stimulatory and polarizing capacities on naïve T cells.

Most protocols for the generation of mDC require approximately one week of cell culture. As such, Jonuleit and colleagues induced mDC on day five to six of a seven-day culture period by adding a four-component maturation cocktail (hereafter 4C cocktail), containing TNF-α, IL-1β, IL-6 and PGE_2 [22], that is commonly used for the induction of DC maturation. It has been shown that mDC could also be generated within two days [25,26]. These "fast DC" were generally able to prime naïve T cells or stimulate effector cells [25,27,28]. The faster development of mDC may better reflect the situation *in vivo* [29].

In this study, we performed a systematic comparison of 3d and 7d mDC in terms of phenotype, chemokine-directed migration, antigen uptake and subsequent stimulation of cytotoxic T lymphocytes (CTL) after incubation with exogenous peptides or loading with antigen via electroporation. Because different forms of antigen are considered for use in DC-based vaccine development, it was important to demonstrate that mDC prepared in a three-day protocol would have antigen processing capacity comparable to the well known properties of 7d mDC.

Materials and methods

Peptides, antibodies and reagents

The short MART-1/Melan-A_{26-35} peptide (ELAGIGILTV) (purchased from Metabion, Martinsried, Germany) and the long MART-1/Melan-A peptide (GSGHWDFAWPWGSGLAGIGILTV) (purchased from Biosyntan, Berlin, Germany) were reconstituted in 50% DMSO containing water at a concentration of 1 mg/ml and 20 mg/ml, respectively. Further dilutions were performed in medium. Monoclonal antibodies specific for DC surface molecules were directly labelled and purchased from Becton Dickinson (Heidelberg, Germany). The unlabelled CCR7 (clone 2H4) antibody (Becton Dickinson) and the MART-1/Melan-A antibody (clone A103; Dako Cytomation, Hamburg, Germany) were detected with the additional use of secondary antibodies [Cy5-coupled $F(ab')_2$-antibody (Dianova, Hamburg, Germany) and biotinylated $F(ab')_2$-antibody (Becton Dickinson)] and streptavidin-PE (Dianova). FITC-dextran from Sigma-Aldrich (Deisenhofen, Germany) and CCL19 from R&D Systems (Wiesbaden, Germany) were used. IL-1β, IL-4, IL-6 and TNF-α were purchased from R&D Systems, IL-2 from Chiron Behring (Marburg, Germany), GM-CSF (Leukine®) from Berlex (Seattle, USA) and PGE_2 from Sigma-Aldrich.

Tumor cell lines and CTL

The melanoma cell lines Mel-93.04A12 (HLA-$A2^+$, Melan-A^+; gift from P. Schrier, Department of Immunohematology, Leiden University Hospital, Leiden, the Netherlands), Mel A375 (HLA-$A2^+$, Melan-A^-; CRL-1619; ATCC) and SK-Mel-29 (HLA-$A2^+$; gift from T. Wölfel, Third Department of Medicine, Hematology and Oncology, Johannes Gutenberg University of Mainz, Mainz, Germany) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate and non-essential amino acids. AK-EBV-B cells (gift from T. Wölfel) were cultured in RPMI 1640, containing 10% fetal calf serum. The HLA-A2-restricted, MART-1/Melan-A_{26-35} specific CTL A42 (gift from M. C. Panelli, National Institutes of Health, Bethesda, MD) were cultured in RPMI 1640 supplemented with 10% human serum (Lonza, Walkersville, USA), 2 mM L-glutamin, 1 mM sodium pyruvate, 100 IU penicillin/streptomycin, 0.5 μg/ml mycoplasma removal agent (MP Biomedicals, Eschwege, Germany) and 125 IU/ml IL-2. 5×10^5 CTL were restimulated every two weeks using 1×10^5 SK-Mel 29 and 2×10^5 AK-EBV-B (both irradiated with 100 Gy) in 1.5 ml A42 CTL medium per well of a 24-well plate. On the day of restimulation, 500 IU/ml IL-2 were added to the culture. A42 CTL were used for coculture experiments 8 days after restimulation.

Generation and culture of 3d DC and 7d DC

Monocytes were enriched from heparinized blood by Ficoll density gradient centrifugation and subsequent plate adherence or from a leukapheresis product via elutriation, as described previously [30]. For freezing of

multiple aliquots, 2-4 × 10^7 monocytes per ampule were resuspended in VLE (very low endotoxin) RPMI supplemented with 5% human serum albumin (20% Octalbin®, Octapharma, Langenfeld, Germany) and mixed 1:1 with freezing medium, containing VLE RPMI, 10% human serum albumin and 20% DMSO. After thawing, 15 × 10^6 monocytes were plated in a Nunclon™flask (80 cm^2; Nunc, Wiesbaden, Germany) in VLE RPMI medium supplemented with 1.5% human serum. For inducing the development of 2d iDC, 20 ng/ml IL-4 and 100 ng/ml GM-CSF were added to the medium immediately after plating the monocytes. On day two, 2d iDC could be harvested for study. For maturation, the 2d iDC were cultured with the four component cocktail, containing 10 ng/ml IL-1β, 15 ng/ml IL-6, 10 ng/ml TNF-α and 1000 ng/ml PGE_2 in addition to 100 ng/ml GM-CSF and 20 ng/ml IL-4 [22]. After 24 h, the 3d mDC were harvested for study. To generate 7d DC, the culture medium was supplemented with 20 ng/ml IL-4 and 100 ng/ml GM-CSF on days 1 and 3 after plating the monocytes. On day 6, the maturation cocktail (as for 3d mDC) was added to the culture of 6d iDC and 7d mDC where harvested for study after 24 h. Prior to freezing, DC were resuspended in 20% human serum albumin and mixed with equal amounts of freezing medium, containing 20% human serum albumin, 20% DMSO and 10% glucose (Braun, Melsungen, Germany).

Generation of MART-1/Melan-A *ivt*RNA

The mMESSAGEmMACHINE™Kit from Applied Biosystems (Darmstadt, Germany) was used for the production of MART-1/Melan-A *ivt*RNA. The linearized vector pcDNAI/Amp/Aa1 (gift from T. Wölfel), encoding the MART-1/Melan-A cDNA, served as a template for *in vitro* transcription. To increase the stability of the RNA, a poly-A tail was added to the *ivt*RNA with the aid of the Poly(A) Tailing Kit™(Applied Biosystems). The kits were used according to the manufacturers' instructions.

Cell surface staining of DC

The expression of cell surface molecules on DC was detected using specific monoclonal antibodies [CD14 (clone M5E2), CD83 (clone HB15e), CD209 (clone DCN46), CD40 (clone 5C3), HLA-DR (clone G46-6), CCR7 (clone 2H4), CD86 (clone 2331), CD80 (clone L307.4) and CD274 (clone M1H1), all Becton Dickinson] and measured by flow cytometry. 5 × 10^4 DC were washed with ice-cold PBS supplemented with 1% FCS and incubated for 30 min with the appropriate antibody (1:25 dilution). If the first antibody was directly linked to a fluorochrome, the cells were washed once again, as described above, and resuspended in 200 μl PBS containing 1% FCS. If use of a secondary antibody was necessary, the cells were washed and incubated with the secondary antibody for an additional 20 min, washed again and resuspended as described above. The DC were analyzed using either FACS Calibur™or LSR-II™instruments (BD Biosciences, Heidelberg, Germany). Results were evaluated using the CellQuest™(BD Biosciences) or FloJo™(Tree Star, Inc., Ashland, OR) software.

Intracellular staining of DC

For the detection of intracellular MART-1/Melan-A protein, 3 × 10^5 DC were fixed in PBS containing 1% paraformaldehyde (PFA) for 30 min on ice. After fixation, cells were washed with ice-cold PBS containing 1% FCS and resuspended in 500 μl 0.1% saponin in PBS (Sigma-Aldrich) to enable permeabilization of the cell membrane. The cells were centrifuged and the cell pellet subsequently resuspended in 0.25% saponin in PBS. The MART-1/Melan-A antibody was added to the cell suspension (dilution 1:20) and incubated for 1 h at room temperature. After incubation, the cells were washed twice in 0.1% saponin in PBS. Incubation with the secondary, Cy5-coupled antibody (dilution 1:100) was performed in 0.25% saponin in PBS for 30 min at room temperature. Before being resuspended in PBS with 1% FCS, the cells were washed in 0.1% saponin in PBS once again. The MART-1/Melan-A expression was analyzed by flow cytometry, as described for cell surface staining.

Phagocytosis assay

The phagocytosis capacity of DC was tested via uptake of FITC-dextran. 2 × 10^5 DC were resuspended in 400 μl VLE RPMI containing 1.5% human serum, supplemented with 10 μg/ml FITC-dextran for 1 h at 37°C and 5% CO_2. As controls, the same concentrations of DC were incubated in medium without FITC-dextran for 1 h at 37°C or in medium supplemented with 10 μg/ml FITC-dextran for 1 h on ice. After incubation, the cells were washed 3-4 times with ice-cold PBS containing 1% human serum and 0.1% NaN_3. The cells were resuspended in PBS containing 1% human serum and analyzed by flow cytometry.

Peptide-loading of DC

3-4 × 10^6 DC were incubated with different concentrations of the long or short MART-1/Melan-A peptides in a six-well-plate in VLE RPMI with 1.5% human serum. The incubation duration for the long peptide was 24 h and for the short peptide 2 h or 24 h. After incubation the DC were washed to remove excess peptide.

Electroporation of DC

Electroporation of DC was performed with the Gene Pulser Xcell™from Biorad (München, Germany) in 0.4 cm electroporation cuvettes (Biorad). Prior to

electroporation, DC were washed twice in ice-cold OptiMEM I medium (Invitrogen, Karlsruhe, Germany). 2-3 × 10^6 DC were resuspended in 200 μl OptiMEM I, preincubated on ice for three min and mixed with the MART-1/Melan-A *ivt*RNA (or the long MART-1/Melan-A peptide) in the electroporation cuvette. DC were pulsed with either 250 V, 150 μF or 300 V, 300 μF (exponential protocol). DC electroporated with H_2O were used as controls. Directly after pulsing, the cells were transferred into a six-well-plate, containing VLE RPMI with 1.5% human serum, and incubated at 37°C and 5% CO_2 for 24 h.

Migration assay

A standard migration assay [31] was performed to determine the migratory capacity of DC. 2 × 10^5 DC were resuspended in 100 μl migration medium (RPMI 1640 supplemented with 1% human serum, 500 U/ml GM-CSF and 250 U/ml IL-4) and incubated in the upper chamber of a 24-trans-well-plate (Costar/Corning, USA) for 2 h at 37°C and 5% CO_2. To determine chemokine-directed migration, the lower chambers contained 600 μl migration medium supplemented with 100 ng/ml CCL19 (R&D Systems). For detection of spontaneous migration and cell chemokinesis, the migration medium in the lower chamber either contained no CCL19 or CCL19 was present in both the upper and lower chambers. After 2 h of incubation the cells from the upper and lower chambers were harvested and cell counts determined with the aid of the CellTiter-Glo® Luminescent Cell Viability Assay (Promega).

Induction of antigen-specific T lymphocytes

3d and 7d mDC were harvested and pulsed with 10 μg/ml MART-1/Melan-A_{26-35} peptide (ELAGIGILTV) for 120 min at 37°C, 5% CO_2 in a humidified atmosphere. Cryopreserved autologous PBMC isolated from HLA-A2$^+$ donors were cocultured with autologous, peptide-pulsed mDC using 1 × 10^6 PBMC and 1 × 10^5 mDC in T cell medium (RPMI 1640, 12.5 mM HEPES, 4 mM L-glutamine, 100 U/ml penicillin and streptomycin, supplemented with 10% pooled human serum). After 7 days of coculture, recovered lymphocytes were restimulated using the same cryopreserved batch of peptide-pulsed DC for 24 h, at which time supernatants were collected for determination of IFN-γ content via a standard ELISA using the OptEIA™Human IFN-γ ELISA Kit from BD Biosciences (Heidelberg, Germany) according to the manufacturers' protocol.

Restimulation of effector CTL

A42 CTL were stimulated with tumor cells or antigen-loaded DC at a ratio of 2 × 10^4 CTL and 4 × 10^4 tumor cells/DC per 96-well in 200 μl A42 CTL medium. The coculture was set up 24 h after peptide-loading or pulsing of the DC with *ivt*RNA, if not otherwise indicated. The stimulation period was 24 h. Coculture supernatants were stored at -80°C for later analyses. The IFN-γ release of the stimulated A42 CTL was measured in the supernatant media by ELISA, as above.

Results

Morphology and FITC-dextran uptake of 3d mDC and 7d mDC

Immature and mature DC were generated *in vitro* using either elutriated monocytes or monocytes obtained via plate adherence of freshly isolated PBMC of healthy donors. In all experiments, the 4C described by Jonuleit and colleagues was used for DC maturation [22]. Standard 7d mDC were induced within one week, whereas 3d mDC were generated within 72 hours. The different DC types were analyzed via flow cytometry and light microscopy and compared in terms of size and morphology (Fig. 1A and 1B). It was noticeable that 3d mDC were much smaller and showed a lower granularity than 7d mDC. 3d mDC were similar in size to 2d iDC, whereas 7d mDC were clearly larger than 6d iDC (Fig. 1A and 1B). Furthermore, 3d mDC displayed a higher yield and viability compared to 7d mDC (Table 1). All four DC types displayed capacity for macropinocytosis, following incubation with 10 μg/ml FITC-dextran for 1 h at 37°C. As controls, DC were incubated without FITC-dextran under the same conditions or with FITC-dextran for 1 h at 4°C. Subsequently, FITC-dextran expression was analyzed by flow cytometry (Fig. 1C). As expected, 2d iDC and 6d iDC showed greater FITC-dextran uptake and 6d iDC achieved a higher mean fluorescence intensity compared with 2d iDC, although comparable percentages of positive cells were seen. A somewhat lower FITC-dextran uptake was usually detected in 3d mDC compared with 7d mDC. Nevertheless, immature and mature DC from both protocols displayed capacity to take up particles (e.g. antigens) from their surroundings.

Phenotype of immature and mature DC

After 2, 3, 6 and 7 days, respectively, fast DC and standard DC were stained with monoclonal antibodies specific for cell surface molecules typically expressed on iDC and mDC and subsequently analyzed via flow cytometry. 2d iDC and 6d iDC displayed no CD83 and only very low expression of CD80, which is typical for iDC. Differences between 2d iDC and 6d iDC were seen in the expression pattern of CD14, CD209 (DC-SIGN), CD86 and CCR7 in various donors (n = 3). 3d and 7d mDC showed the expected mature phenotypes, with high expression of CD83 and no expression of CD14. Both also expressed high levels of costimulatory molecules, like CD80, CD86 and CD40, as well as other cell surface

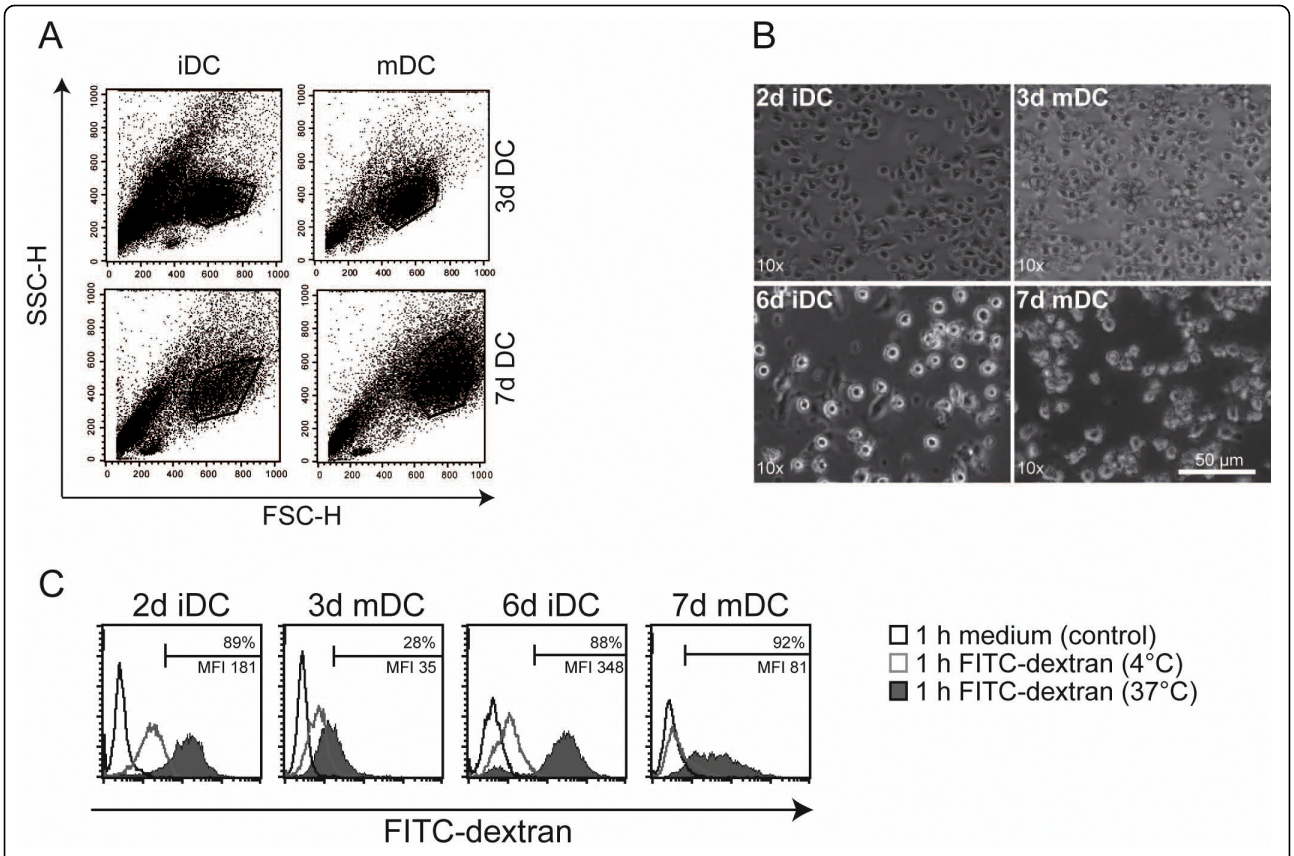

Figure 1 Morphology and FITC-dextran uptake of 3d mDC and 7d mDC. The size and the morphology of immature and mature 3d DC and 7d DC were analyzed by **(A)** flow cytometry and (**B)** light microscopy. **(C)** DC were incubated without or with 10 μg/ml FITC-dextran at 37°C or at 4°C for 1 hour. The cells were then washed three times in ice-cold PBS with 1% FCS. The uptake of FITC-dextran was analyzed by flow cytometry. Data are representative for three independent experiments. The left-most open histograms represent medium only controls, the open grey curves indicate mDC incubated with FITC-Dextran at 4°C and the filled histograms at 37°C.

molecules that are important for the function of mDC, including CD209 (DC-SIGN), HLA-DR and CCR7 (Fig. 2A and 1B). Despite the shorter culture time, 3d mDC often expressed higher levels of CD209, CD40 and HLA-DR as compared to 7d mDC. Whereas higher expression of these molecules on 3d mDC varied among different donors, HLA-DR was consistently seen to be better expressed on 3d mDC in different donors (n = 3). In contrast, expression of the inhibitory molecule CD274 (B7-H1) was consistently lower on 3d than on 7d mDC. This difference was even more striking when the expression of the positive costimulatory molecule CD80 and the inhibitory molecule CD274 was directly compared. Thus, 3d mDC displayed a stronger positive costimulatory phenotype, with higher expression of CD80 compared to CD274, whereas 7d mDC showed a lower level of CD80 compared to CD274 (Fig. 2C). Despite variability in levels of expression among different donors, these data may suggest that 3d mDC might have a slight advantage in the expression pattern of costimulatory molecules and thereby may display a higher stimulatory capacity for T cells compared to 7d mDC.

Migratory capacity of 3d mDC and 7d mDC

One of the key features of DC, besides their ability to take up antigens in the periphery, is to migrate to the lymph nodes in order to present antigenic peptides to T cells. Both 3d and 7d mDC showed a high expression of CCR7 (Fig. 2A), which is an important receptor for homing of DC to lymph nodes. To test migratory capacity, iDC and mDC were examined using a standard migration assay. DC were incubated in the upper chamber of a trans-well-plate at 37°C for 2 h. The lower chambers contained migration medium, with or without the chemokine CCL19. As an additional control for cell chemokinesis, CCL19 was placed in both the upper and lower chambers. Since CCL19 is a specific ligand for the CCR7 receptor, migration of DC towards medium containing CCL19 reveals a directed migratory capacity, whereas migration towards medium alone or in the presence of chemokine in both chambers corresponds to

Table 1 Yield, purity and viability of immature and mature DC

	2d iDC	6d iDC	3d mDC	7d mDC
Donor 1				
Yield*	n.d.[+]	n.d.[+]	8%	4%
Purity [#]	n.d.[+]	n.d.[+]	39%	34%
Viability [$]	n.d.[+]	n.d.[+]	94%	78%
Donor 3[&]				
Yield*	12%[§]	6%[§]	18%	9%
Purity[#]	57%	58%	60%	70%
Viability[$]	93%	84%	95%	86%
Donor 4[&]				
Yield*	n.d.[+]	n.d.[+]	4%	3%
Purity[#]	32%	30%	42%	60%
Viability[$]	85%	76%	86%	81%

* Yield: from PBMC with the starting population set at 100%.

[+] n.d.: not determined.

[#] Purity: SSC/FSC.

[$] Viability: propidium iodide (PI) stain.

[&]Donors 3 and 4 are identical with donors 3 and 4 in Table 2 and Table 3.

[§] These values are lower compared to 3d and 7d mDC due to cell loss from strong adherence of iDC.

spontaneous, undirected migratory capacity and a more random movement of the DC (Fig. 3). Neither 2d iDC, nor 6d iDC showed an ability to migrate, even though some expression of CCR7 was detected on these immature DC (Fig. 2A). In contrast, 3d mDC showed a higher directed migration compared with spontaneous migration. Strikingly, 7d mDC showed reduced directed migration compared to 3d mDC in all five donors tested, although the CCR7 expression on 3d mDC and 7d mDC was nearly the same (Fig. 2A). However, the differences in the directed migratory capacity between 3d and 7d mDC were not statistically significant.

MART-1/Melan-A peptide recognition on DC by A42 CTL

Next, 3d and 7d mDC were tested for their stimulatory capacity of $CD8^+$ effector T cells. Fast and standard mDC prepared from HLA-$A2^+$ donors were loaded exogenously with short MART-1/Melan-A_{26-35} peptide (ELAGIGILTV) for 2 h or 24 h. Because this peptide is only 10 amino acids long it can bind directly to HLA-A2 molecules. The peptide-loaded DC were cocultured for another 24 h with the MART-1/Melan-A-specific effector CTL A42 which recognize the MART-1/Melan-A_{26-35} peptide presented by HLA-A2 molecules. Activation of CTL A42 was measured by IFN-γ release. The MART-1/Melan-A-negative melanoma cell line Mel A375 and the MART-1/Melan-A-positive melanoma cell line Mel-93.04A12 were used as controls (Fig. 4A). A42 CTL showed IFN-γ release after stimulation with either 3d or 7d mDC. The amount of IFN-γ was higher in cocultures using DC that had an increased duration of peptide loading, indicating that 24 h of peptide loading provided DC with higher amounts of HLA-A2-peptide ligand, resulting in better stimulatory capacity. The 3d mDC were comparable to 7d mDC in their capacity to restimulate effector CTL after exogenous peptide loading for 24 h.

Uptake of long MART-1/Melan-A peptide by 3d mDC and 7d mDC

The ability of 3d and 7d mDC to take up, process and present antigen was also tested. For this purpose, a long MART-1/Melan-A peptide, consisting of 23 amino acids, was used. This peptide is too long to be exogenously loaded directly onto HLA-A2 molecules. Therefore, it has to be processed by the DC, including cleavage by the proteasome and transport to the endoplasmic reticulum for binding on MHC and export to the cell surface, where it can be recognized by CTL. 3d and 7d mDC were incubated with different amounts of long peptide for 24 h, washed and cocultured with A42 CTL for an additional 24 h. IFN-γ release by the CTL was measured via ELISA (Fig. 4B). Again, both mDC types showed capacity to stimulate CTL after incubation with the long peptide, revealing that adequate uptake of peptide occurred and both DC types were able to intracellularly process and present the correct epitope.

Electroporation of 3d mDC and 7d mDC with peptide or *ivt*RNA

To bypass the lower spontaneous uptake of antigen by mDC, it is possible to use electroporation to introduce either peptide or *ivt*RNA into DC. We compared 3d and 7d mDC that were electroporated according to optimal parameters that were previously established for 7d mDC [32]. After introduction of 1 μg, 5 μg or 10 μg of long peptide, the mDC were incubated at 37°C for 24 h and then cocultured with A42 CTL. Once again, 3d mDC showed capacity comparable to 7d mDC for stimulation of IFN-γ release by the CTL (Fig. 5A). Use of *ivt*RNA is an attractive source of antigen that can be easily and cheaply generated from any antigen-encoding cDNA. To analyze this as a source of antigen, immature and mature DC were electroporated with 24 μg of *ivt*RNA encoding MART-1/Melan-A, using the same electroporation conditions as applied with the long peptide. After 24 h of incubation at 37°C, the electroporated DC were cocultured with A42 CTL for another 24 h. Whereas 2d iDC were unable to stimulate A42 CTL, 3d mDC showed a weak but detectable stimulatory capacity. In contrast, A42 CTL responded very well to stimulation with *ivt*RNA-transfected 7d mDC (Fig. 5B).

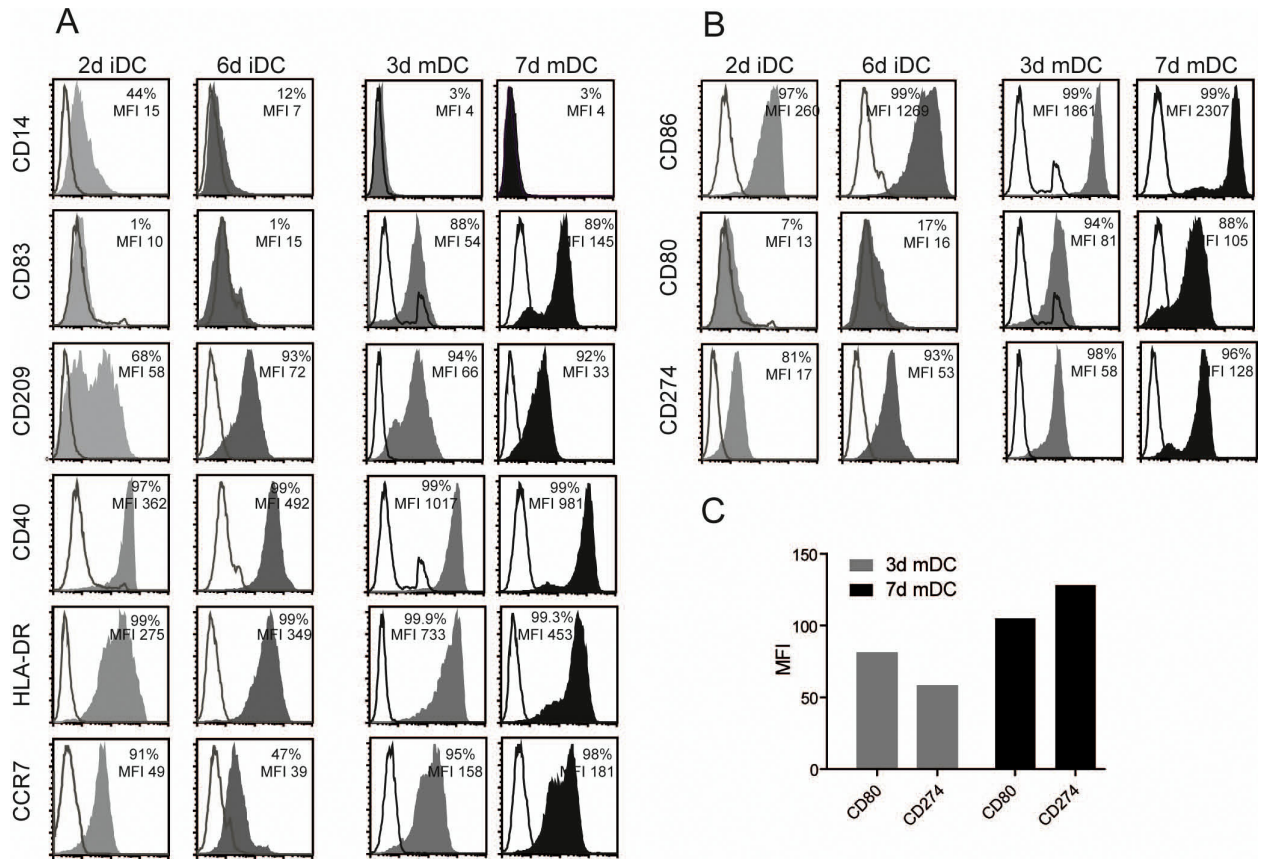

Figure 2 Phenotype of immature and mature DC. The expression of cell surface molecules on 2d iDC, 6d iDC, 3d mDC and 7d mDC was detected with specific antibodies and analyzed by flow cytometry. The open histograms correspond to the isotype controls, whereas the grey and black histograms display the specific binding of FITC- or PE-coupled antibodies. **(A)** Expression of CD14, CD83, CD209, CD40, HLA-DR and CCR7. **(B)** Expression of the B7-family-members CD86, CD80 and CD274. **(C)** Comparison of the expression of the costimulatory molecule CD80 and the inhibitory molecule CD274 on 3d mDC and 7d mDC. Data are representative for three independent experiments.

Since the electroporation conditions used in this experiment were originally established for 7d mDC, it was possible that they might be suboptimal for 3d mDC. It was seen, for example, that the stimulatory capacity of 3d mDC could be improved by using higher amounts of *ivt*RNA with these electroporation parameters (data not shown). This, however, was a poor solution for clinical application of mDC since it would increase costs for production of *ivt*RNA. Therefore, alternate electroporation conditions for 3d mDC were explored in order to improve the efficiency of *ivt*RNA transfer. After testing several variations of electroporation, modified parameters of 300 V and 300 μF (exponential protocol) were found that facilitated optimal eGFP expression in 3d mDC after transfer of *ivt*RNA (data not shown).

Based on these observations, protein expression and stimulatory capacity were again compared in 3d and 7d mDC that were loaded with MART-1/Melan-A *ivt*RNA, applying both the old and modified parameters. Hereby, 3d and 7d mDC were electroporated with 12 μg *ivt*RNA, incubated for 24 h and then cocultured with A42 CTL for an additional 24 h. Three hours after electroporation, the MART-1/Melan-A protein expression was assessed in 3d and 7d mDC via intracellular staining using a MART-1/Melan-A-specific antibody and flow cytometry (Fig. 5C). With the modified parameters (300 V, 300 μF), 3d mDC showed a higher percentage of positive cells (88% vs. 79%) and a nearly five-fold increase in MFI (361 vs. 74) compared with 3d mDC electroporated according to the older conditions. In contrast, the percentage of MART-1/Melan-A positive cells remained similar with only a slight increase in MFI (1.5-fold) in 7d mDC. Under both conditions, 7d mDC displayed a poor recovery rate 24 h after electroporation, using either the old or modified parameters (34% and 25%, respectively) compared to 3d mDC (77% and 60%, respectively). Furthermore, 3d mDC showed a higher viability after electroporation compared to 7d mDC (Table 2). The improved MART-1/Melan-A expression in 3d mDC correlated with a substantial increase in

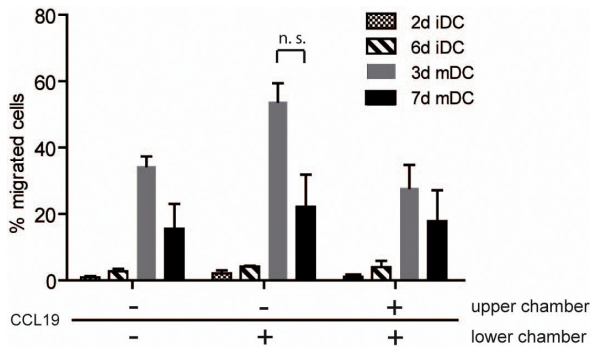

Figure 3 Migratory capacity of immature and mature DC. 2d iDC, 6d iDC, 3d mDC and 7d mDC were compared for their migratory capacity towards migration medium containing (+) or lacking (-) CCL19 in a trans-well migration assay. To measure directed migration, the medium in the lower chamber of the trans-well plate was supplied with 100 ng/ml CCL19, spontaneous migratory capacity was detected using medium that did not contain CCL19 in the lower chamber and random cell chemokinesis was determined by adding CCL19 to both the upper and the lower chambers. 2×10^5 DC were added in the upper chamber and incubated at 37°C and 5% CO_2 for 2 h. Afterwards, DC numbers in the lower chambers were determined. Shown are three independent donors as mean values with standard errors of the mean (SEM). Statistical analyses were performed using the Mann Whitney test (n.s.: not significant).

stimulatory capacity (Fig. 5D). This was detected as a three-fold higher IFN-γ release from A42 CTL. 7d mDC also showed a somewhat higher stimulatory capacity, corresponding to their higher level of protein expression.

Recoveries of 3d mDC and 7d mDC after freezing and thawing

For use in clinical application, it is important that large lots of antigen-loaded mDC can be prepared and cryopreserved in multiple aliquots for individual applications over time. To determine cell recovery after freezing and thawing, 3d and 7d mDC were frozen, without or 3 h after electroporation. After several days of storage, the DC were thawed and cell recoveries were determined (Table 2). In the absence of electroporation, the recoveries of both 3d and 7d mDC after cryopreservation and thawing were equal (68% vs. 70%, respectively). In contrast, 3d mDC displayed a greater robustness after electroporation and cryopreservation, leading to substantially higher cell recoveries compared with 7d mDC (41% vs. 18%) and to higher cell viabilities (Table 3).

Stimulation of naïve T cells

Since it is essential for DC-based vaccines to enable *de novo* priming of new T cell responses, we also

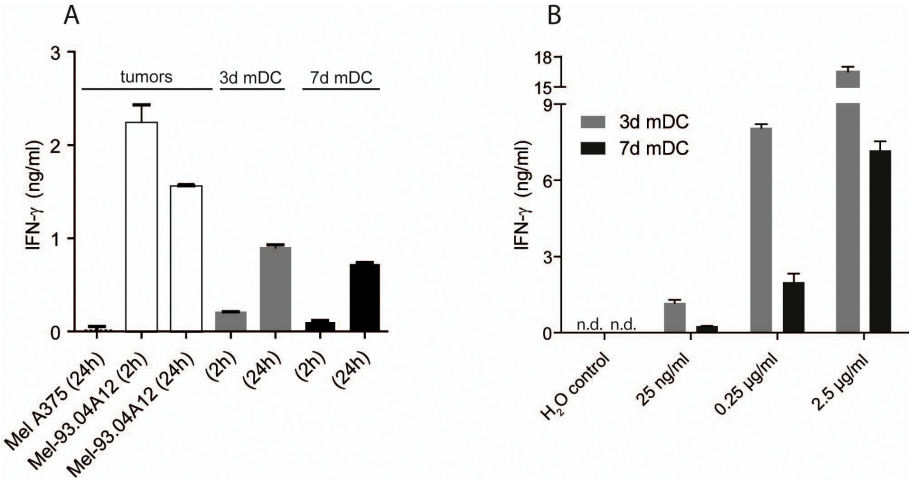

Figure 4 Recognition of MART-1/Melan-A peptide on 3d mDC and 7d mDC by MART-1/Melan-A-specific CTL. **(A)** 3d mDC and 7d mDC were exogenously loaded with 10 µg/ml short MART-1/Melan-A_{26-35} peptide for 2 h or 24 h at 37°C and 5% CO_2. After washing, the peptide-loaded DC were cocultured with MART-1/Melan-A-specific A42 CTL for 24 h at 37°C and 5% CO_2. MART-1/Melan-A-positive tumor cells (Mel-93.04A12) and MART-1/Melan-A-negative tumor cells (Mel A375) served as controls and were cocultured with A42 CTL at the same time points as the DC (2 h and 24 h). The IFN-γ release of A42 CTL was measured by IFN-γ-ELISA. The columns show mean values of triplicates with standard deviations. Data are representative for two experiments. **(B)** 3d mDC and 7d mDC were incubated with different amounts of long MART-1/Melan-A peptide for 24 h at 37°C and 5% CO_2. The DC were cocultured with A42 CTL for additional 24 h. The IFN-γ release of A42 CTL was measured by IFN-γ-ELISA. The columns show mean values of duplicates with standard deviations. The data for 2.5 µg/ml are representative for two independent experiments (n.d.: not detected).

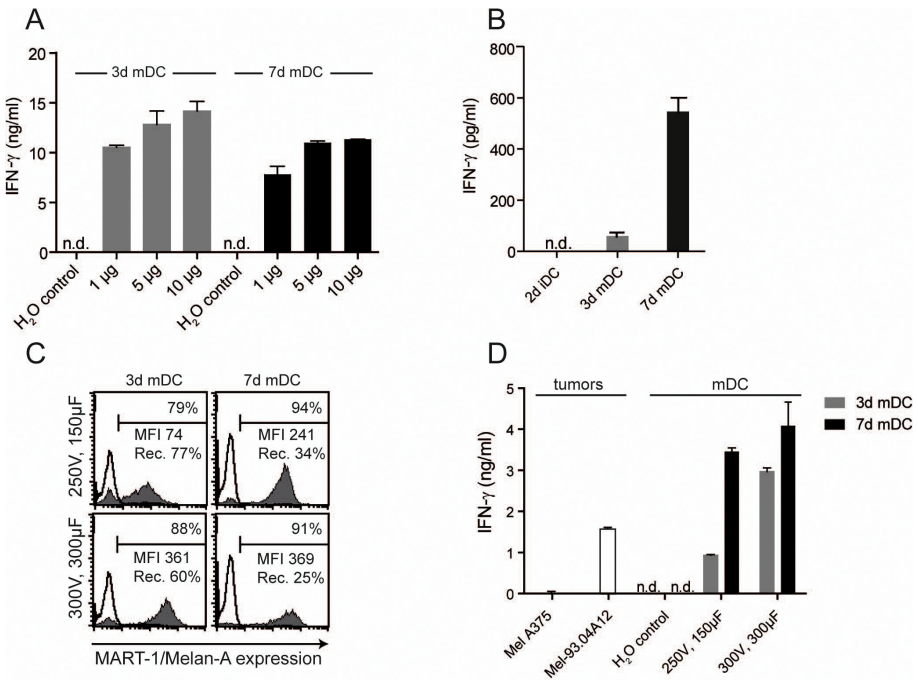

Figure 5 Electroporation of 3d mDC and 7d mDC with long MART-1/Melan-A peptide and MART-1/Melan-A-encoding *ivt*RNA. **(A)** 3d mDC and 7d mDC were electroporated (250 V, 150 µF) with 1 µg, 5 µg and 10 µg long MART-1/Melan-A peptide. After 24 h incubation at 37°C and 5% CO_2, the DC were cocultured with A42 CTL for 24 h. **(B)** 2d iDC, 3d mDC and 7d mDC were electroporated (250 V, 150 µF) with 24 µg MART-1/Melan-A *ivt*RNA, incubated at 37°C for 24 h and cocultured with A42 CTL for 24 h (n = 3). **(C)** 3d mDC and 7d mDC were electroporated with 12 µg MART-1/Melan-A *ivt*RNA at different electroporation conditions (250 V, 150 µF and 300 V, 300 µF), respectively. 3 h after electroporation, mDC were stained intracellularly with a MART-1/Melan-A-specific antibody and analyzed by flow cytometry (n = 2). **(D)** 24 h after electroporation with MART-1/Melan-A *ivt*RNA, DC were cocultured with A42 CTL for 24 h (n = 2). The IFN-γ release of the A42 CTL was measured by IFN-γ-ELISA. The bars in A, B and D show mean values of triplicates with standard deviations (Rec.: recovery; n.d.: not detected).

Table 2 Viability after electroporation, cryopreservation and thawing

		3d mDC			7d mDC		
		w/o EP	300 V 300 µF	250 V 150 µF	w/o EP	300 V 300 µF	250 V 150 µF
Donor 3	**Before freezing**						
	Cell counts (× 10^6)	1.4	0.5	0.8	0.7	0.5	0.4
	Viability*	94%	90%	91%	75%	52%	74%
	After thawing						
	Cell counts (× 10^6)	0.5	0.2	0.2	0.2	0.1	0.2
	Viability*	86%	79%	78%	51%	26%	55%
Donor 4	**Before freezing**						
	Cell counts (× 10^6)	1.1	1.4	1.3	0.7	0.7	0.6
	Viability*	89%	85%	88%	59%	58%	62%
	After thawing						
	Cell counts (× 10^6)	1.0	0.9	0.8	0.6	0.4	0.5
	Viability*	90%	90%	89%	86%	79%	84%

* Viability: PI stain.

Table 3 Recoveries of 3d mDC and 7d mDC after cryopreservation and thawing

	3d mDC - EP*		7d mDC - EP*		3d mDC + EP*		7d mDC + EP*	
	counts ($\times 10^6$)	%	counts ($\times 10^6$)	%	counts ($\times 10^6$)	%	counts ($\times 10^6$)	%
Donor 1								
Before EP*					2.0	100	2.0	100
Before freezing	1.6	100	1.2	100	1.4	70	1.3	65
After thawing	0.9	59	0.9	73	0.9	43	0.5	26
Donor 2								
Before EP*					1.5	100	1.5	100
Before freezing	1.2	100	0.5	100	1.0	65	0.3	23
After thawing	1.0	83	0.5	98	0.7	47	0.3	17
Donor 3								
Before EP*					2.0	100	2.0	100
Before freezing	1.4	100	0.7	100	0.5	25	0.5	25
After thawing	0.5	37	0.2	23	0.2	11	0.1	3
Donor 4								
Before EP*					1.5	100	1.5	100
Before freezing	1.1	100	0.7	100	1.4	93	0.7	45
After thawing	1.0	91	0.6	86	0.9	61	0.4	27
mean % after thawing ± SD+		68 ± 24		70 ± 33		41 ± 21		18 ± 11

* EP: electroporation.

+ SD: standard deviation.

analyzed the capacities of MART-1/Melan-A peptide-loaded 3d and 7d mDC to stimulate naïve T cells in autologous cocultures. PBL were primed for seven days using either MART-1/Melan-A peptide-loaded 3d or 7d mDC. At this time the primed cells were recovered and restimulated with either melanoma tumor cell lines or with the same batches of peptide-pulsed 3d and 7d mDC that were cryopreserved and thawed before use as stimulating cells. The levels of IFN-γ secretion were detected by standard ELISA. When the primed T cells were restimulated with MART-1/Melan-A-expressing tumor cells they showed low levels of cytokine release which increased substantially upon restimulation with MART-1/Melan-A peptide-pulsed 3d mDC (Fig. 6). In contrast, the IFN-γ secretion of PBL stimulated with MART-1/Melan-A peptide-pulsed 7d mDC was much weaker. As described previously for DC that were matured with 4C cocktail, we did not detect any IL-12p70 secretion after stimulation of DC using CD40-ligand expressing cells from either 3d or 7d mDC (data not shown).

Discussion

Since several different protocols for the generation of mDC using monocytes have been described to date, the aim of our study was to compare standard 7d mDC with 3d mDC in terms of phenotype, processing and presentation of antigen after transfection of either peptide or *ivt*RNA and stimulation of effector CTL. If 3d mDC display the same key characteristics as 7d mDC, they would be preferred for DC-vaccine development because of savings in time and costs.

In 2003, Dauer and colleagues published a protocol for the rapid generation of mDC *in vitro* [25,26]. These so called "fast DC" were induced from monocytes within 48 hours and showed typical phenotypic characteristics of mDC. We modified this procedure somewhat by adding the 4C maturation cocktail, designed by Jonuleit and colleagues [22], to the cultures of immature DC on the second day, thereby yielding mDC after three days of culture.

First, we observed that 3d mDC retained a smaller size and lower granularity compared to 7d mDC, as described for fast DC generated in 48 h [25,26,33]. This difference in morphology raised the issue whether 3d mDC would differ from 7d mDC in terms of antigen uptake, processing and presentation of antigen-derived peptides on the cell surface. Indeed, 3d mDC showed a lower capacity for spontaneous uptake of FITC-dextran from their surroundings, compared to 7d mDC. The

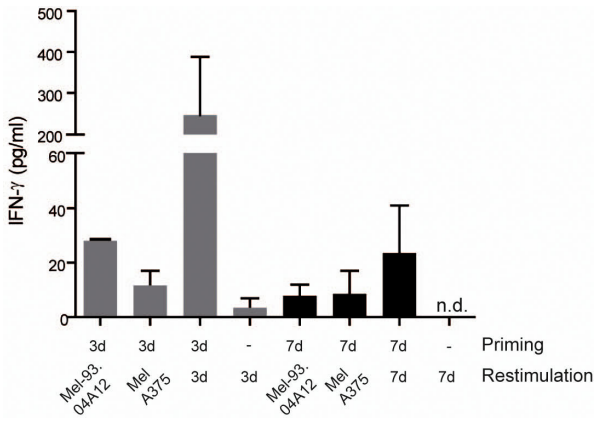

Figure 6 Stimulation of naïve T cells with MART-1/Melan-A peptide-pulsed 3d and 7d mDC. Autologous PBL were stimulated with MART-1/Melan-A peptide-pulsed 3d and 7d mDC for 7 days, followed by specific restimulation for 24 h using peptide-pulsed mDC and Melan-A-positive tumor cells. The IFN-γ release of the PBL was measured by IFN-γ-ELISA. Shown are two independent donors as mean values with SEM (n.d.: not detected).

phenotyping of 3d and 7d mDC revealed that both types of mDC expressed comparable levels of many important surface molecules. Nevertheless, some differences were observed in the levels of costimulatory molecules that play an important role in interactions with T cells. Thus, differences in the intensity of expression of the inhibitory molecule CD274 were detected, which may impact on the costimulatory capacities of 3d and 7d mDC for T cells. CD274 (B7-H1, PD-L1) on DC interacts with PD-1 on T cells and transmits an inhibitory signal [34,35]. Therefore, DC that express a preponderance of CD274 might inhibit rather than foster T cell activation. We observed that 7d mDC expressed higher levels of CD274 compared to the costimulatory molecule CD80. In contrast, 3d mDC showed a reciprocal lower expression of CD274 compared to CD80 (Fig. 2). These results indicate that 3d mDC may be more effective in activating T cells compared to 7d mDC, which would be advantageous for priming of naïve T cells. Nevertheless, when 3d and 7d mDC were loaded exogenously with short MART-1/Melan-A peptide, both DC types showed comparable capacities to stimulate A42 CTL (Fig. 4A). These findings indicated that the higher ratio of CD274 to CD80 in 7d mDC did not impair their capacity to stimulate primed effector cells (Fig. 2B and 1C). While DC loading with short peptide led to comparable stimulation of CTL, 3d and 7d mDC revealed different capacities to stimulate effector T cells after incubation with long peptide. IFN-γ release from T cells after stimulation with standard mDC loaded with long peptide was previously noted to be much higher than stimulation with standard mDC loaded with short peptide [36]. This may be due to maintenance of a persisting pool of long peptide within the DC that could be processed and presented for a longer period after uptake. In our studies 3d mDC showed equal or better stimulatory capacity for T cells after incubation with long peptide compared to 7d mDC, indicating that processing may have been more efficient in 3d mDC, since the spontaneous uptake of exogenous material was lower in 3d mDC, as evidenced by FITC-dextran uptake. After introduction of the long peptide by electroporation, comparable stimulatory capacities were found between 3d and 7d mDC. Since levels of IFN-γ release by T cells were in general lower after stimulation with DC that were electroporated with peptide compared with spontaneous peptide uptake, we speculate that electroporation might diminish somewhat the antigen processing capacity of the DC.

Next, we analyzed protein expression in 3d and 7d mDC after electroporation of *ivt*RNA, alongside their stimulatory capacity for CTL. As shown previously, antigen-loaded fast DC were clearly able to stimulate T cells [25,27]. However, we extended these observations by comparing the stimulatory capacities of 3d and 7d mDC side-by-side after electroporation of MART-1/Melan-A *ivt*RNA. Using previously established electroporation conditions, we noted that 3d mDC showed diminished stimulatory capacity compared to 7d mDC in repeated experiments with multiple donors. This was likely due to lower protein expression in 3d mDC after introduction of *ivt*RNA. The low protein expression in 3d mDC could be overcome by using greater amounts of *ivt*RNA (data not shown). However, based on the differences in morphology and size, we speculated that the more compact 3d mDC might be more robust and that more intense electroporation conditions might improve *ivt*RNA transfer, yielding better protein expression after transfer of lower amounts of *ivt*RNA. Indeed, alteration of the electroporation parameters yielded an improved protein expression in 3d mDC, which subsequently showed a much better stimulatory capacity for CTL. Thus, once 3d and 7d mDC expressed comparable levels of protein, they showed comparable stimulatory capacities for CTL.

When testing the migratory capacities of immature and mature 3d or 7d DC, we observed that immature DC displayed neither spontaneous nor chemokine-directed migration. This was also found by Dauer and colleagues for immature DC prepared in 24 h from monocytes [37]. 3d mDC appeared to have somewhat better migratory capacity than 7d mDC in multiple donors, which might be related to a less terminally-differentiated status. Since effective migration of DC is essential for the priming of naïve T cells in the lymph nodes, this characteristic supports use of 3d mDC for vaccine development.

Because 3d mDC were smaller than 7d mDC and seemed more resistant to electroporation, we speculated that they were more robust cells. Indeed, 3d mDC loading of *ivt*RNA required a stronger electroporation pulse to achieve similar protein expression to 7d mDC. Likewise, when recoveries of 3d mDC and 7d mDC were examined after cryopreservation and thawing, 3d mDC showed higher cell recoveries when the mDC were electroporated prior to cryopreservation. Furthermore, 3d mDC displayed higher cell viabilities after electroporation and cryopreservation when compared to 7d mDC. Thus, 3d mDC yielded higher cell recoveries and thereby would be superior to 7d mDC for clinical application if a DC vaccine strategy entails electroporation with antigenic proteins or antigen-encoding *ivt*RNA, followed by cryopreservation of multiple aliquots for thawing and immediate application to patients.

Since it is important that mDC are able to stimulate naïve T cells in a vaccine setting, we analyzed the stimulatory potential of MART-1/Melan-A peptide-pulsed 3d and 7d mDC on autologous PBL. Under short-term priming conditions of only seven days, we failed to detect any tumor-specific killing of MART-1/Melan-A-expressing tumor cells nor did we detect enrichment of MART-1/Melan-A-multimer-positive T cells (data not shown). Nevertheless, PBL stimulated with peptide-pulsed 3d mDC showed a higher IFN-γ release when restimulated with peptide-loaded 3d mDC compared to PBL that were primed and restimulated with 7d mDC. It has been shown previously by others, that fast DC generated in 48 h had an equal or greater capacity to stimulate naïve T cells compared to 7d mDC [28,33]. The failure to detect cytotoxic activity and detectable numbers of MART-1/Melan-A-multimer-positive T cells in our experiments is likely related to the use of 4C cocktail for DC maturation and the absence of IL-2 or IL-7 in the culture medium. It has already been described that DC matured with 4C cocktail do not produce bioactive IL-12p70, which is important for optimal polarization of naïve T cells for tumor recognition [30,38]. Improved stimulatory capacity of naïve T cells is achieved if mDC are generated using a cocktail that enables their production of IL-12p70. Recently, we showed indeed that 3d mDC, matured with TLR-ligand containing maturation cocktails display a much higher stimulatory capacity for naïve T cells than 3d mDC matured with 4C cocktail [38].

Conclusions

Here we show that 3d mDC displayed similar characteristics to 7d mDC concerning phenotype and capacity to stimulate CTL after exogenous pulsing with short peptide. We observed that 3d mDC also had good capacities to stimulate CTL after uptake and processing of long peptide and they displayed a strong chemokine-directed migration. The 3d mDC were more robust and thereby required altered conditions for introduction of RNA-encoding antigen via electroporation, however this characteristic likely accounts for higher cell recoveries after electroporation and cryopreservation compared to 7d mDC. Thus, 3d mDC offer a suitable alternative to 7d mDC for use in clinical trials, thereby saving time and costs for cell production.

Abbreviations

CCR: chemokine receptor (C-C type); CD: cluster of differentiation; CTL: cytotoxic T lymphocyte(s); DC: dendritic cell(s); EGFP: enhanced green fluorescent protein; FACS: fluorescence activated cell sorting; FCS: fetal calf serum; GM-CSF: granulocyte macrophage-colony stimulating factor; IDC: immature dendritic cell(s); IFN: interferon; IL: interleukin; *IVT*(RNA): *in vitro* transcribed RNA; MDC: mature dendritic cell(s); MHC: major histocompatibility complex; PBS: phosphate buffered saline; PGE_2: prostaglandin E_2; poly (I:C): polyriboinosinic polyribocytidylic acid; TGF: transforming growth factor; TNF: tumor necrosis factor; VLE: very low endotoxin.

Declaration of competing interests

The authors declare that they have no competing interests.

Authors' contributions

MB designed and performed the experiments and drafted the manuscript. SS and SW contributed to the initial development of 3d DC and provided scientific and technical advice. BF provided scientific advice and helped drafting the manuscript. DJS provided scientific advice, discussions of data and revised the manuscript. CG provided scientific advice, discussions of data and helped in the design of experiments. All authors read and approved the final manuscript.

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NOD/scid IL-2Rg^{null} mice: a pre-clinical model system to evaluate human dendritic cell-based vaccine strategies *in vivo*

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List of abbreviations:

4C – four component cocktail
5C+R848 – five component cocktail plus R848
ivt-RNA – *in vitro-transcribed* RNA
GvHD – Graft versus Host Disease
NSG – NOD/scid IL2Rg^{null}
TLR – Toll-like receptor

Abstract

Here we describe a new method to compare vaccination with different preparations of mature human dendritic cells (DC) in a xenograft mouse model. NOD/scid IL2Rg^{null} (NSG) mice reconstituted with human peripheral blood mononuclear cells (PBMC) were vaccinated with autologous human-derived mature DC prepared using different protocols. First, two reconstitution regimes were evaluated for engraftment rates, leading to selection of 4-week engraftment for vaccine evaluation. NSG recipients of human PBMC were vaccinated twice with DC generated *in vitro* for 3 or 7 days. DC cultured for 3 days were better at inducing antigen-specific immune responses *in vivo*. We then compared DC matured using two different maturation cocktails. Consistent with *in vitro* observations, vaccination using mature DC activated with a Toll-like receptor (TLR) 7/8 agonist resulted in enhanced immune responses. To date, appropriate *in vivo* systems for comparing human cell-based vaccines have been lacking: this humanized mouse model system provides an approach to compare characteristics of new DC-based vaccine strategies.

Introduction

Dendritic cell (DC) vaccines hold high therapeutic potential for induction of antitumor immunity in cancer patients [1,2]. Current cancer vaccines focus on mature DC (mDC) loaded with tumor-associated antigens (TAA) and injected intradermally to activate $CD8^+$ cytotoxic T lymphocytes (CTL) and natural killer (NK) cells.

Various methods have been developed for preparation of mDC for clinical studies; mostly these rely on a 6-day protocol using IL-4 and GM-CSF to induce immature DC (iDC), followed by a 24 h maturation phase [3]. Published studies demonstrated that DC generated over 2 or 3 days give comparable [4] or enhanced [5] immune responses to 7-day mDC. Most clinical trials rely on a four-component cocktail (4C) containing IL-1β, IL-6, TNF-α and PGE_2 [6]. The discovery that TLR agonists can optimally activate murine DC to secrete IL-12 [7] led to studies of the impact of TLR agonists on human mDC cytokine production [8-10]. We have described maturation cocktails using quinoline-like molecules, R848 or CL075, in 3-day and 7-day mDC [11,12]. DC matured with TLR7/8 agonists, with or without poly (I:C) as a TLR3 agonist, resulted in substantial secretion of bioactive IL-12(p70) and high potential to activate innate and adaptive immune responses [11,12].

Humanized mouse models could provide a useful tool for *in vivo* assessment of DC vaccines [13] but reports characterizing immune responses after DC-based vaccination are rare [14]. Engraftment of human peripheral blood lymphocytes (PBL) in mice has been reported using two NOD/scid strains, one with a truncated mutation (NOG) and one with a null mutation (NSG) of the IL-2-receptor γ-chain [15,16].

In this study, we used NOD/scid $IL2Rg^{null}$ (NSG) mice to engraft human PBMC and perform vaccination experiments using 3- and 7-day mDC. Additionally, we compared cocktail 4C with our maturation cocktail containing R848 as a TLR7/8 agonist (5C+R848). We demonstrate here that 3-day mDC are superior to 7-day mDC *in vivo*. Furthermore, DC matured with the 5C+R848 cocktail induced enhanced antitumor immunity compared to 4C-matured DC.

Methods

Preparation of PBMC for engraftment of NSG mice and *ex vivo* generation of mDC

Peripheral blood of healthy donors was prepared as described [12]. Afterwards PBMC were used directly for engraftment or as sources of mDC, as described [5,12]. In brief, monocytes were isolated via flask adhesion and cells were cultured for 2- or 6-days with IL-4 and GM-CSF. Afterwards, maturation cocktails were added as described [12] using 4C and 5C+R848 cocktails. See Supplementary Table 1 for cytokine concentrations. DC were harvested after 24 h, loaded with antigen and incubated for an additional 6 h before injection into human PBMC reconstituted mice.

Engraftment PBMC in NSG mice

All animal experiments were approved by the local authorities according to the legal regulations. For the 9-week reconstitution protocol, mice were irradiated with a sub-lethal dose of 100 cGy one day before intravenous injection of $1x10^6$ human PBMC, while the 4-week protocol used a single intravenous injection of $10x10^6$ PBMC, without irradiation. Mice were vaccinated on day 42 or day 14 after reconstitution, respectively (see also workflow shown in Fig. 1A and 1B).

Electroporation of mDC and vaccination of humanized mice

Specific antigen was introduced to DC in the form of *in vitro-transcribed*-RNA (*ivt*-RNA), as described [5]. Electroporation of mDC was performed using varying amounts of *ivt*-RNA (shown for each experiment), prepared from linearized T7-promotor-containing plasmids, with the mMESSAGE mMACHINE T7 kit (Ambion) following the manufacturer's instructions. We used the plasmid pCDM8HLA-A2 encoding HLA-A*0201 (gift from E. Weiß, Ludwig-Maximilians-University, Munich) and the plasmid pcDNAI containing MART-1 and WT-1 cDNA. Electroporation was performed as described in [5] and mDC were cultured 6 h following electroporation. Afterwards, mDC were injected immediately or cryopreserved for later vaccination. DC ($1x10^6$) were given twice intravenously, with a one-week interval between injections. After 7 to 14 days

mice were sacrificed and spleen cell suspensions prepared for *in vitro* characterization of immune responses.

Functional analysis and flow cytometry

Spleen cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM non-essential amino acids and 5 μM beta mercaptho-ethanol, either overnight or over a 7-day period, in the presence of 50 IU/ml IL-2 and 0.1 μg/ml OKT-3, prior to chromium-release assays. Cytotoxic assays were performed as described [12]. Tumor cell lines used as targets were cultured as described [12,17]. For flow cytometry, cells were stained using a MART-1/MelanA_{25-36}-specific multimer (peptide sequence: ELAGIGILT; PE-conjugated; kindly provided by D. Busch, Technical University of Munich) for 20 minutes, followed by washing and staining with specific CD3 (PerCP; clone SK7, BD) and CD8 (APC; clone SK1, BD) or with directly labeled CD3, CD4 (PE; clone 13B8.2, Immunotech), CD8, CD62L (FITC; clone SK11, BD) antibodies. Measurement was performed using a LRSII machine and FlowJo software for analyses.

Results

Engraftment of human PBMC in NSG mice

To analyze the ability of human mDC to induce immune responses *in vivo*, we used NSG mice xenografted with human PBMC. Initially we compared two published engraftment methods, with minor modifications [14,18]. As shown in Figure 1A and B, the protocols differed in the length of the engraftment period of 9 weeks (9-wk) (Fig. 1A) or 4 weeks (4-wk) (Fig. 1B). For the 9-wk protocol, mice were irradiated with a sub-lethal dose of 100 cGy, 24 h prior to injection of $1x10^6$ human PBMC. The mice were immunized with the DC vaccines 6 weeks later. For the 4-wk protocol non-irradiated recipients received $10x10^6$ PBMC and were immunized after two weeks. Vaccination of mice in each group consisted of two intravenous injections of $1x10^6$ mDC, with a one-week interval between them. Freshly prepared mDC from autologous donors were used for the first vaccination and cryopreserved mDC for the second.

FACS analysis of vaccinated mice showed a mean of 55.7% human $CD3^+$ T cells using the 9-wk protocol, while the 4-wk engraftment protocol resulted in a mean of 26.0% human T cells (Fig. 1C and 1D). When the CD4 and CD8 fractions were compared within the human $CD3^+$ T cells, we detected an increased mean percentage of CD8 (58.4 %; CD4 34.3 %) cells using the 9-wk protocol, while the CD8 (48.6 %, in mean) to CD4 (40.7 %, in mean) distribution after the 4-wk engraftment reflected more the normal situation in humans (Fig. 1C and 1D). Additionally, we observed that the CD62L-positive fraction of naïve cells and the level of expression, assessed as mean fluorescent intensity (MFI), were decreased using the 9-wk versus the 4-wk protocol (Fig. 1C and 1D). Furthermore, we observed signs of graft-versus-host-disease (GvHD) approximately 5 weeks after engraftment with the 9-wk protocol (not shown). Mice showed a strong loss of weight, fur loss and thin red skin. Based on these observations, we selected the 4-wk procedure for DC vaccination studies.

Induction of allo-specific responses using DC pulsed with HLA-A2-encoding RNA

To assess if mDC were able to induce immune responses *in vivo*, we used an allogeneic approach employing DC of an HLA-$A2^-$ donor. To create allo-restricted peptide ligands for T cell recognition [19], we electroporated mDC with *ivt*-RNA encoding HLA-A2 (24 μg) and MART-1/MelanA *ivt*-RNA (48 μg) as a source of antigen. In parallel, PBMC and DC from the same donor were cocultured *in vitro.* Priming efficiency of *in vitro* and *in vivo* activated PBL were compared in a chromium-release assay using THP-1 (HLA-$A2^+$, MART-1^-) and mel624.38 (HLA-$A2^+$, MART-1^+) cell lines as target cells. Only low killing activity was detected after *in vitro* priming but responses were greater against the melanoma tumor, indicating a component of antigen-specific recognition (Fig. 2A left) and lysis of both targets was enhanced when T cells were primed *in vivo* (Fig. 2A right). T cells primed with 7-day DC matured using 4C, *in vitro* and *in vivo*, resulted in only low specific recognition of tumor cells. In contrast, mDC generated using the 3-day protocol induced increased responses, in both cases. In particular we observed that T cells obtained from mice vaccinated *in vivo* with

5C+R848 matured-DC showed enhanced specific lysis of both target cells (Fig. 2A right).

MART-1/MelanA-specific immune responses in an autologous setting

To verify these findings in an autologous setting, we utilized the model antigen MART-1/MelanA for priming. For these experiments DC prepared from an HLA-$A2^+$ donor were electroporated with RNA encoding MART-1/MelanA (48 μg). Seven days after vaccination of mice, reconstituted with HLA-$A2^+$ PBMC from the same donor, splenocytes were analyzed by FACS using a MART-1-specific multimer to determine the numbers of antigen-specific cells within the human T cell fractions. 7-day cultured DC failed to induce any multimer-positive cells above the background of non-vaccinated mice, while 3-day DC derived using either 4C or 5C+R848 induced multimer-positive cells (Fig. 2B, 2C). Not all mice in a group yielded multimer-positive cells; one mouse in each group of four displayed no positive cells (not shown).

We isolated and cultured splenocytes from the vaccinated mice for one-week *in vitro* stimulation with anti-CD3 antibody and human IL-2 to enrich human T cells before assaying for function. A chromium-release assay assessed specific responses using mel624.38 cells as positive target cells and THP-1 cells as negative controls. None of the T cell fractions showed reactivity against THP-1 (not shown). As indicated by the multimer-staining results (Fig 2B), splenocytes from mice immunized with 7-day DC (4C) showed no specific lysis of tumor cells *in vitro*. In contrast, 3-day DC matured with 5C+R848 induced MART-1-specific responses in three of four mice. In mice immunized with 3-day DC matured by 4C, only one mouse showed specific lysis of the MART-1^+-cell line, although three of the four mice were positive for multimer staining. To account for variations in $CD8^+$ T cells, we calculated the percentage relative lysis, normalizing to the percentage of $CD8^+$ T cells. An additional experiment using MART-1/MelanA in combination with WT-1 as a second targeted antigen was also included into this analsyis (Fig. 2D, 2E and not shown). In both experiments 3-day DC matured with 5C+R848 were superior in capacity to induce antigen-specific $CD8^+$ CTL.

Discussion

DC vaccines *in vitro* can potently reactivate human patient T cell responses [20]. In particular, DC have the capacity to activate antigen-specific CTL-driven immune responses. Recently published studies in humans indicate that the commonly used 4C cocktail is suboptimal in its capacity to activate mDC to guide antitumor responses, whereas murine DC matured with TLR agonists resulted in more effective antitumor responses [7]. Few papers describe the use of humanized mice to assess the efficacy of human DC-vaccines [14,21]. The ability to carry out *in vivo* comparisons of new DC vaccine preparations would be of substantial interest for design of clinical trials.

We have developed a rapid and simple system to investigate various human DC-based cellular therapeutics. We were able to engraft NSG mice with human PBMC within two weeks. Although engraftment of human cord blood-derived stem cells would result in higher engraftment rates and reduced GvHD, the feasibility of obtaining stem cells and DC from the same donor is limited. In addition, testing patient-derived DC with lymphocytes of healthy HLA-matched donors is also difficult.

By adapting a 4-wk engraftment protocol of $10x10^6$ PBMC followed by two vaccinations with $1x10^6$ DC, we were able to induce and detect allo-reactive as well as antigen-specific responses in donor T cells from humanized NSG mice. In these settings, 7-day DC failed to induce potent immune responses, while DC generated in a 3-day period induced good responses using the 4C cocktail. Furthermore, DC matured with the TLR7/8 agonist R848 not only gave enhanced immune responses in individual mice, but also increased the numbers of positive responders within a cohort. It is not known whether failure to induce immune responses in some mice is due to deficiencies in vaccination or reflects variation between mice, for example, in qualitative or quantitative levels of reconstitution. In experiments reported here we did not inject DC into or near lymph nodes, a strategy commonly used in clinical protocols. Further studies will show the potential of varying the immunization route on vaccine efficacy.

In conclusion, the humanized mouse model system developed here enables investigation of therapeutic cell reagents in an *in vivo* setting, which hopefully reflects the situation in humans. In addition, this method allows to assess the

potential of human *in vitro* prepared DC to activate autologous T cells in an *in vivo* setting, using NSG mice reconstituted with responder cells. In particular, this model allows comparisons to be made among different vaccine strategies and comparison of the immunogenicity of selected tumor-associated antigens *in vivo*, prior to use in clinical studies.

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The authors declare that they have no conflict of interest.

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Figure legends

***Fig.1:* Efficacy of engraftment of human PBMC in NSG mice**

(A) Schema of 9-wk engraftment protocol. Mice were irradiated 24 h prior to i.v. injection of $1x10^6$ human PBMC. Vaccination using $1x10^6$ mDC occurred on day 42 and day 49 and spleens were isolated on day 63. (B) Schema of 4-wk engraftment protocol starting with i.v. injection of $10x10^6$ human PBMC on day 1. Injection of $1x10^6$ mDC was performed on day 14 and day 21 and spleens were isolated on day 28. (C) Representative examples for CD3 vs. SSC, CD4 vs. CD8 and CD62L expression (from left to right) of flow cytometry analysis comparing engraftment efficacy of 9-wk (left of each block) and 4-wk (right of each block). (D) Statistical analysis of engraftment efficacy comparing 9-wk and 4-wk protocol and vaccinated using 7 day- 4C (circles), 3 day- 4C (squares) or 3 day- 5C+R848 (triangles) mDC. Statistical analyses were performed using a two-tailed Mann-Whitney test and $p<0.05$ were considered significant.

***Fig. 2: In vivo* priming efficiency using DC-based vaccination**

(A) Chromium-release assay using *in vitro* (left) and *in vivo* (right) allogeneic primed T cells. DC from an HLA-$A2^-$ donor were pulsed with RNA encoding HLA-A2 and MART-1 prior to stimulation of autologous PBMC. $2x10^3$ THP-1 (HLA-$A2^+$, MART-1^-) or mel624.38 (HLA-$A2^+$, MART-1^+) target cells were tested in combination with various numbers of effectors (E:T). Data for *in vivo* primed T cells are depicted as means of four individual mice. (B) MART-1-specific multimer staining versus CD8 staining of *in vivo*-primed human T cells. Differently matured DC of an HLA-$A2^+$ donor, pulsed with MART-1 RNA, were used for vaccination. Staining was performed on day of spleen isolation. (C) Killing capacity (% specific lysis) of *ex vivo* cultured CTL populations tested individually in a chromium-release assay. $2x10^3$ mel624.38 target cells were incubated with varying numbers of splenocytes (E:T). Responses of non-immunized mice are shown as open circles while filled circles represent responses of immunized mice. Filled squares represent responses of individual mice shown in (B). (D) Relative specific lysis of individual vaccinated mice shown in (C). Values at an E:T of 20:1 were adjusted as follows: % rel. specific lysis = % spec. lysis/ (% CD8/ 100). (E) Relative lysis of isolated, *ex vivo* activated splenocytes against $2x10^3$ mel624.38 (HLA-$A2^+$, MART-1^+) or $2x10^3$ K562-A2 (HLA-$A2^+$,

WT-1^{+}) target cells at an E:T of 20:1. Mice were previously immunized twice with autologous mDC, electroporated with *ivt*-RNA encoding for MART-1 and WT-1. Given are means with SEM of 4 mice for 3d-DC (4C) and 3d-DC (5C+R848), 3 mice for 7d-DC (4C) and 1 mouse without vaccination.

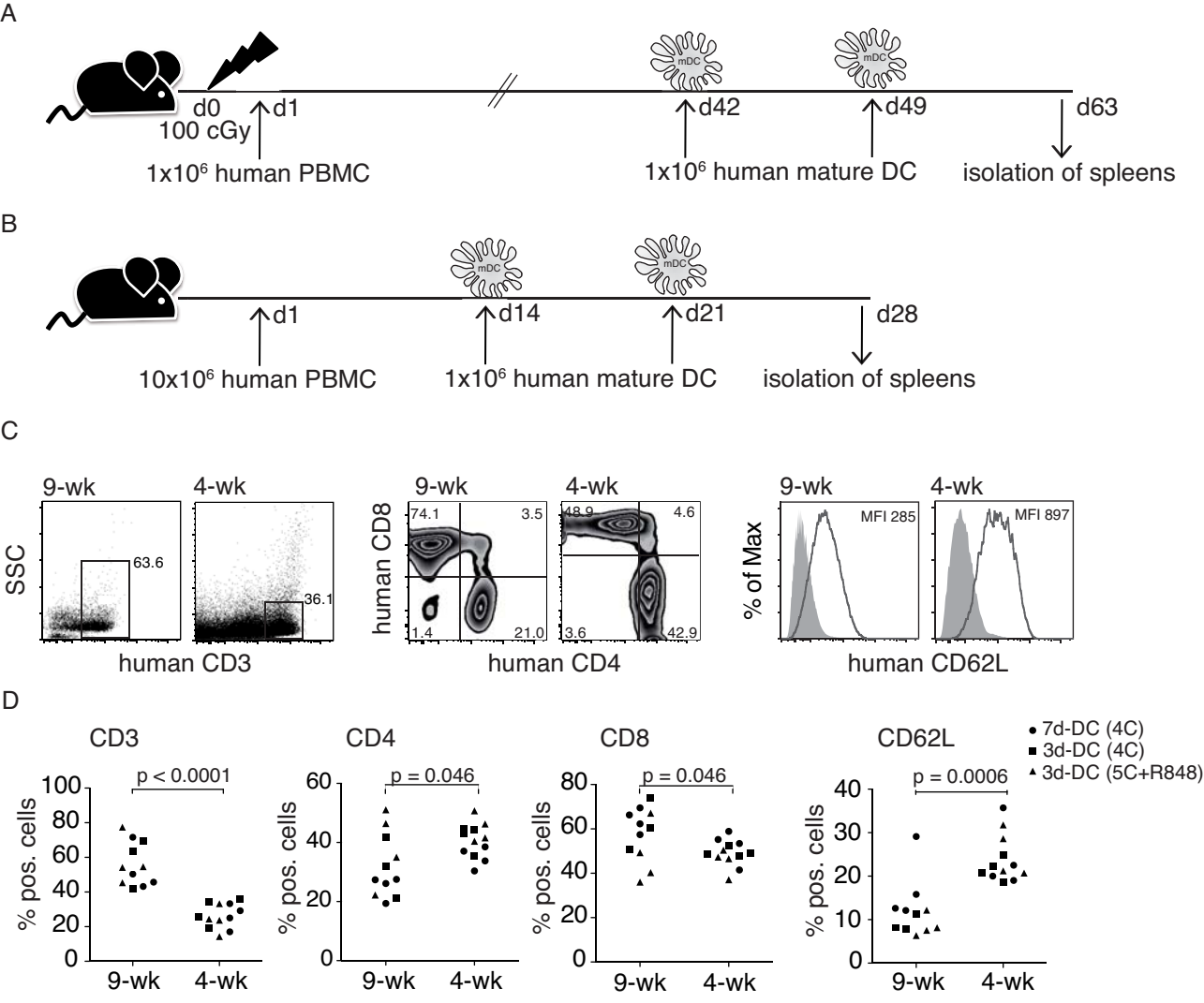
Suppl. Table 1. Composition of cocktails used for DC maturation

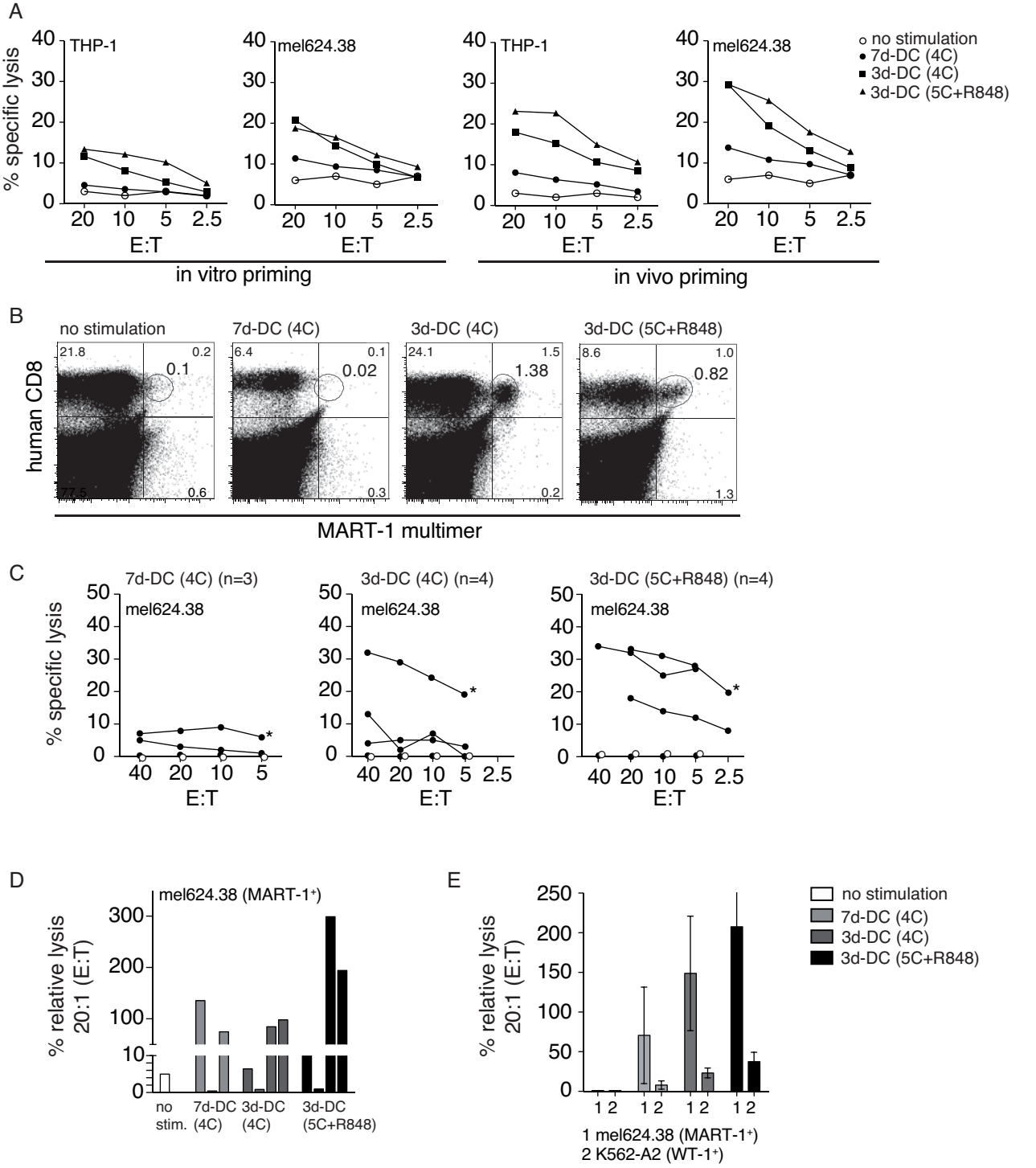
Cocktail	Population	Inflammatory cytokines/interferons	Other additives	TLR-ligands
4C	DC^{4C}	TNF-a, IL-1β, IL-6	PGE_2	
5C + R848	$DC^{5C+R848}$	TNF-a, IL-1β, IFN-γ	PGE_2	poly(I:C), R848

The following concentrations were used in the maturation cocktails:

4C: TNF-a (10 ng/ml); IL-1β (10 ng/ml); PGE_2 (1000 ng/ml); IL-6 (15 ng/ml)

5C + R848: TNF-a (10 ng/ml); IL-1β (10 ng/ml); PGE_2 (250 ng/ml); IFN-γ (5000 U/ml); poly (I:C) (20 ng/ml); R848 (1 μg/ml)


A
mDC
mDC
d0
100 cGy
d1
d42
d49
d63
1x10⁶ human PBMC
1x10⁶ human mature DC
isolation of spleens
B
mDC
mDC
d1
d14
d21
d28
10x10⁶ human PBMC
1x10⁶ human mature DC
isolation of spleens
C
9-wk
4-wk
SSC
63.6
36.1
human CD3
9-wk
4-wk
human CD8
74.1
3.5
1.4
21.0
48.9
4.6
3.6
42.9
human CD4
9-wk
4-wk
% of Max
MFI 285
MFI 897
human CD62L
D
CD3
p < 0.0001
% pos. cells
100
80
60
40
20
0
9-wk
4-wk
CD4
p = 0.046
% pos. cells
60
40
20
0
9-wk
4-wk
CD8
p = 0.046
% pos. cells
80
60
40
20
0
9-wk
4-wk
CD62L
p = 0.0006
% pos. cells
40
30
20
10
0
9-wk
4-wk
7d-DC (4C)
3d-DC (4C)
3d-DC (5C+R848)


A
% specific lysis
THP-1
mel624.38
THP-1
mel624.38
E:T
in vitro priming
in vivo priming
no stimulation
7d-DC (4C)
3d-DC (4C)
3d-DC (5C+R848)
B
no stimulation
7d-DC (4C)
3d-DC (4C)
3d-DC (5C+R848)
human CD8
MART-1 multimer
C
7d-DC (4C) (n=3)
3d-DC (4C) (n=4)
3d-DC (5C+R848) (n=4)
mel624.38
% specific lysis
E:T
D
mel624.38 (MART-1+)
% relative lysis 20:1 (E:T)
no stim.
7d-DC (4C)
3d-DC (4C)
3d-DC (5C+R848)
E
% relative lysis 20:1 (E:T)
1 mel624.38 (MART-1+)
2 K562-A2 (WT-1+)

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Dendritic cells pulsed with RNA encoding allogeneic MHC and antigen induce T cells with superior antitumor activity and higher TCR functional avidity

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Dendritic cells pulsed with RNA encoding allogeneic MHC and antigen induce T cells with superior antitumor activity and higher TCR functional avidity

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Adoptive transfer of T cells expressing transgenic T-cell receptors (TCRs) with antitumor function is a hopeful new therapy for patients with advanced tumors; however, there is a critical bottleneck in identifying high-affinity TCR specificities needed to treat different malignancies. We have developed a strategy using autologous dendritic cells cotransfected with RNA encoding an allogeneic major histocompatibility complex molecule and a tumor-associated antigen to obtain allo-restricted peptide-specific T cells having superior capacity to recognize tumor cells and higher functional avidity. This approach provides maximum flexibility because any major histocompatibility complex molecule and any tumor-associated antigen can be combined in the dendritic cells used for priming of autologous T cells. TCRs of allo-restricted T cells, when expressed as transgenes in activated peripheral blood lymphocytes, transferred superior function compared with self-restricted TCR. This approach allows high-avidity T cells and TCR specific for tumor-associated self-peptides to be easily obtained for direct adoptive T-cell therapy or for isolation of therapeutic transgenic TCR sequences. (Blood. 2009;114:2131-2139)

Introduction

T-cell responses against tumors are often directed against self-major histocompatibility complex (MHC) molecules presenting peptides derived from overexpressed self-proteins. In general, high-avidity T cells specific for self-peptide/self-MHC ligands are eliminated by negative selection to prevent autoimmunity. The T-cell receptor (TCR) affinity of remaining T cells specific for self-ligands is normally low; however, high-avidity T cells are needed to effectively eradicate tumors. Because negative selection is limited to self-MHC molecules, T cells that recognize allogeneic MHC molecules have not undergone negative selection. Thus, peptides presented by allogeneic MHC molecules can stimulate high-avidity T cells specific for tumor-associated ligands derived from overexpressed self-proteins.[1] T cells that recognize allogeneic MHC molecules irrespective of specific peptide can be distinguished from peptide-specific T cells at the clonal level and excluded.[2]

Several approaches have been used to obtain allo-restricted peptide-specific cytotoxic T lymphocytes (CTLs). For example, peptide-loaded T2 cells were used to generate HLA-A2 allo-restricted T cells.[3,4] This system is normally limited to HLA-A2 and CTLs induced with exogenous peptides often fail to effectively kill tumor cells.[5] Activated B cells coated with allogeneic peptide-MHC (pMHC) monomers were also used to prime allo-restricted $CD8^+$ T cells.[6] For broad application, this method would require development of many different pMHC monomers. Furthermore, allo-restricted peptide-specific T cells were obtained using T cells and stimulating cells derived from HLA partial-mismatched donors.[7] Here, necessary donor pairs that differ by single HLA allotypes are rare. Therefore, each of these methods has strong limitations.

We developed an approach that provides maximum flexibility to obtain allo-restricted peptide-specific T cells using RNA-transfected dendritic cells (DCs) as stimulating cells. The use of DCs as antigen-presenting cells is particularly valuable because of their superior capacity to prime naive lymphocytes. In this approach, T cells are stimulated with autologous DCs cotransfected with RNA that encode an allogeneic MHC molecule and a tumor-associated antigen (TAA), bypassing the need to search for appropriate HLA-mismatched donors for the DC source. These DCs coexpress both proteins and present allo-pMHC complexes that activate allo-restricted peptide-specific T cells. In this approach, high-avidity T cells that recognize self-peptides are more readily found because they were not subjected to negative selection in vivo.

Selected allogeneic high-avidity T cells can be used directly for adoptive T-cell therapy in HLA-haploidentical stem cell transplantation.[8] Alternatively, their TCR sequences can be used to develop designer lymphocytes.[4,9,10] Thereby, autologous T cells from tumor patients in general, or from MHC-matched donors in the case of stem cell transplantation, can be used for TCR transduction, bypassing the need for direct transfer of allogeneic T cells. Improved ability to isolate T cells with high-affinity TCR recognizing common tumor-associated ligands will allow quicker development of this therapy for more patients.

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Methods

Cell lines and effector CTLs

The human melanoma cell lines, Mel-A375 (HLA-A2$^+$, tyrosinase$^-$; CRL-1619, ATCC), Mel-93.04A12 (HLA-A2$^+$, tyrosinase$^+$; gift of P. Schrier, Department of Immunohematology, Leiden University Hospital, Leiden, The Netherlands), Mel-624.38[11] and SK-Mel-23 (HLA-A2$^+$, tyrosinase$^+$; gift of M. C. Panelli, National Institutes of Health, Bethesda, MD), SK-Mel-28 (HLA-A2$^-$, tyrosinase$^+$; MTB-72; ATCC), SK-Mel-29 (HLA-A2$^+$, tyrosinase$^+$, gift of P. Rieber, Institute of Immunology, Technical University Dresden, Dresden, Germany), WM-266-4 (HLA-A2$^+$, tyrosinase$^+$; CRL-1676; ATCC) and primary cultures of a human melanoma (passage 6-12) and MaCa1 (HLA-A2$^-$, tyrosinase$^-$, gift of Dr R. Wank, Immunotherapy Center, Munich, Germany), stable HLA-A*0201 transfectant of MaCa1 (MaCa1/A2; HLA-A2$^+$, tyrosinase$^-$, gift of E. Noessner, Institute of Molecular Immunology, Helmholtz Zentrum München, Munich, Germany), RCC-26[12] (HLA-A2$^+$, tyrosinase$^-$), PancTu1 (HLA-A2$^+$, tyrosinase$^-$, gift of P. Nelson, Department for Biological Chemistry, University Hospital LMU Munich, Munich, Germany), UTS CC 1588 (HLA-A2$^+$, tyrosinase$^-$, gift of M. Schmitz, Institute of Immunology, Technical University Dresden, Dresden, Germany) as well as the lymphoid cell line T2 (CRL-1992; ATCC) were cultured in RPMI 1640 medium supplemented with 12% fetal bovine serum, 2 mM L-glutamine, and 1 mM sodium pyruvate and nonessential amino acids.

The HLA-A2 allo-reactive CTL JB4,[13] the HLA-A*0201–restricted tyrosinase peptide-specific CTL Tyr-F8,[14] which were primed using stimulating cells pulsed with exogenous peptide (gift of P. Schrier, Department of Immunohematology, Leiden University Hospital, Leiden, The Netherlands), the HLA-A*0201–restricted tyrosinase peptide-specific, melanoma patient–derived IVS-B[15] CTLs (gift of T. Wölfel, Third Department of Medicine, Hematology, and Oncology, Johannes Gutenberg-University of Mainz, Mainz, Germany), and the HLA-A*0201–restricted melan-A peptide–specific A42[11] CTLs (gift of M. C. Panelli) were cultured as described.[11,13-15]

Production of tyrosinase, melan-A, and HLA-A2 ivt-RNA

The plasmid pCDM8-HLA-A2 with HLA-A*0201 cDNA (gift of E. Weiß, Department of Biology II, Ludwig-Maximilians-University, Munich, Germany), pZeoSV2+/huTyr with tyrosinase cDNA (gift of I. Drexler, Institute of Molecular Virology, Helmholtz Zentrum München, Munich, Germany), and pcDNAI/Amp/Aa1 with melan-A cDNA (gift of T. Wölfel) were linearized and used as in vitro transcription templates to produce RNA with the aid of the mMESSAGE mMACHINE T7 kit (Ambion) according to the manufacturer's instructions.

Fluorescence-activated cell sorter analysis

HLA-A2 molecules were stained with BB7.2 monoclonal antibody (HB82; ATCC) followed by a secondary antibody conjugated with phycoerythrin (PE; goat anti–mouse IgG; Jackson ImmunoResearch Laboratories). The intracellular tyrosinase expression in RNA-transfected DCs was detected using tyrosinase-specific primary monoclonal antibody (clone T311; Novocastra Laboratories) and Cy5-conjugated secondary antibody (rat anti–mouse IgG; Jackson ImmunoResearch Laboratories) as described.[16]

De novo priming of T cells with RNA-pulsed DCs

Blood samples from healthy donors were collected with donors' informed consent in accordance with the Declaration of Helsinki and after approval by the Institutional Review Board of the University Hospital of the Ludwig-Maximilians-University. Donor HLA types are listed in Table 1. Mature DCs were prepared from adherent monocytes and transfected with in vitro transcribed (ivt)–RNA via electroporation as previously described.[16] DCs of HLA-A2$^+$ donors were loaded with 24 μg tyrosinase ivt-RNA, and DCs of HLA-A2$^-$ donors were cotransfected with 24 μg tyrosinase ivt-RNA and 48 μg HLA-A2 ivt-RNA. On the same day, autologous CD8$^+$ T lymphocytes were enriched from peripheral blood mononuclear cells (PBMCs) via negative selection using a commercial kit according to the manufacturer's instructions (CD8$^+$ T Cell Isolation Kit II [human]; Miltenyi Biotec). Cocultures were initiated 10 hours after DC electroporation in 24-well plates (Techno Plastic Products) by adding 10^5 RNA-pulsed DCs to 10^6 CD8$^+$ T cells in RPMI 1640, supplemented with 10% heat-inactivated human serum, 4 mM L-glutamine, 12.5 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 50 μM β-mercaptoethanol, and 100 U/mL penicillin/streptomycin (T-cell medium). Interleukin-7 (IL-7, 5 ng/mL; Promokine) was added on day 0 and 50 U/mL IL-2 (Chiron Behring) after 2 days and then on every third subsequent day. Addition of IL-2 was delayed to decrease proliferation of nonspecific CD8$^+$ T cells.[17] The second stimulation of primed T cells was made after 7 days using freshly prepared RNA-pulsed DCs.

Table 1. HLA types of donors used in these studies

Donor	Antigen	HLA types
A	Tyr	HLA-A*01/*02, -B*08/*44, -Cw*05/*07
D	Tyr	HLA-A*02/*03, -B*35/*37, -Cw*04/*06
T	Tyr	HLA-A*01/*26, -B*07/*57, -Cw*06/*07
E	Tyr	HLA-A*24/*26, -B*07/*44, -Cw*07/*16

HLA multimer sorting

Seven days after the second stimulation of CD8-enriched T cells with RNA-pulsed DCs, HLA-A2–restricted tyrosinase-specific T cells were stained with a PE-labeled HLA-A2 tyrosinase$_{369\text{-}377}$ (A2-tyr) multimer,[18] CD8-specific antibody (clone RPA-T8; BD Pharmingen) and propidium iodide (2 μg/mL) for sorting. Up to 5×10^6 cells were incubated with 12 μg multimer in 100 μL phosphate-buffered saline plus 0.5% human serum. Allophycocyanin-labeled, CD8-specific antibody was then added for an additional 25 minutes. After staining, cells were washed and sorted on a FACSAria cell sorter (BD Biosciences) as described.[19]

Culture of peptide-specific CTLs

Multimer-sorted T cells were cloned by limiting dilution in round-bottom 96-well plates (TPP). IL-2 (50 IU/mL) was supplemented every 3 days with 5 ng/mL IL-7 and 10 ng/mL IL-15 (PeproTech) every 7 days. CTLs were stimulated nonspecifically with CD3-specific antibody (0.1 μg/mL; OKT-3; gift of E. Kremmer, Institute of Molecular Immunology, Helmholtz Zentrum München, Munich, Germany) and provided with 10^5 feeder cells per 96-well, consisting of irradiated (50 Gy) PBMCs derived from a pool of 5 unrelated donors and 10^4 irradiated (150 Gy) Epstein-Barr virus–transformed allogeneic B-lymphoblastoid cell line every 2 weeks. Proliferating T cells were cultured in 24-well plates (TPP) in T-cell medium plus cytokines and feeder cells. Clonality was determined by TCR–β-chain receptor analysis, as described.[20]

Peptide loading of T2 cells, PBMCs, and tumor cells

For exogenous peptide pulsing, 10^6 T2 cells were incubated at 37°C and 5% CO_2 for 2 hours with 10 μg/mL human β_2-microglobulin (Calbiochem) and titrating amounts, ranging from 10^{-5} M to 10^{-11} M, of the tyrosinase peptide YMD (tyrosinase$_{369\text{-}377}$ YMDGTMSQV; Metabion). T2 cells pulsed with 10^{-5} M influenza peptide GIL (flu: influenza matrix protein$_{58\text{-}66}$ GILGFVFTL; Metabion) served as the negative control. PBMCs were loaded with tyrosinase peptide as for T2 cells with titrating amounts ranging from 10^{-6} to 10^{-11} M. Tumor cells were loaded with either 10^{-5} M flu peptide or 10^{-5} M tyrosinase peptide YMD as described for T2 cells. After washing, peptide-loaded T2 cells, PBMCs, or tumor cells were used as target cells in cytotoxicity or as stimulating cells in interferon-γ (IFN-γ) release assays.

IFN-γ release assay

To demonstrate the stimulatory capacity of RNA-pulsed DC 10 hours after electroporation, 100 μL of JB4, Tyr-F8, IVS-B, or A42 T-cell suspensions (2×10^4 cells in 100 μL) was added to RNA-loaded DCs (4×10^4 cells in 100 μL) in round-bottom 96-well plates. T cells with mock-transfected DCs and

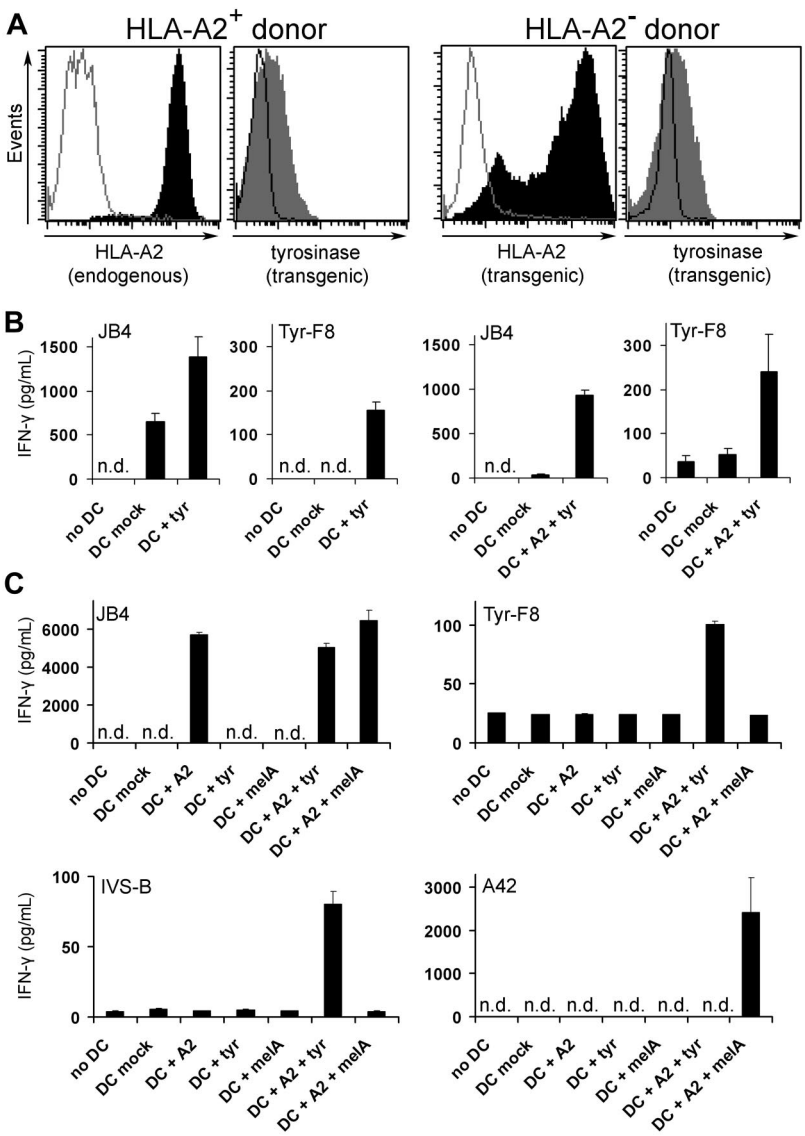

Figure 1. De novo priming of CD8+ T cells with RNA-pulsed DCs. (A) Coexpression of HLA-A2 and tyrosinase proteins was detected by flow cytometry in DCs of an HLA-A2+ donor transfected with 24 μg tyrosinase ivt-RNA (left histograms) and DCs of an HLA-A2− donor transfected with 48 μg HLA-A2 and 24 μg tyrosinase ivt-RNA (right histograms). Stained samples are represented by filled curves and corresponding controls by empty curves. Data are representative of 9 independent experiments, demonstrating that all DCs used for T-cell priming coexpressed both proteins. (B) Columns represent the amount of IFN-γ (picograms per milliliter) secreted by a tyrosinase-independent HLA-A2 allo-reactive T-cell clone (JB4) and an HLA-A2–restricted tyrosinase peptide–specific T-cell clone (Tyr-F8) after coincubation with RNA-pulsed DCs, 10 hours after electroporation. IFN-γ was quantified in culture supernatants by ELISA. Mean values and mean deviations represent duplicates. n.d. indicates not detectable. (C) DCs of an HLA-A2− donor were transfected with HLA-A2 (48 μg) and tyrosinase (24 μg) or melan-A (48 μg) ivt-RNA alone and in combination and used for coincubation with the HLA-A2 allo-reactive CTL clone (JB4), 2 HLA-A2–restricted tyrosinase peptide–specific CTL clones (Tyr-F8 and IVS-B), and an HLA-A2–restricted melan-A peptide–specific CTL clone (A42) 10 hours after electroporation. IFN-γ was quantified in culture supernatants by ELISA and presented as picograms per milliliter. Mean values and mean deviations represent duplicates. n.d. indicates not detectable.

without stimulator cells served as controls and showed negligible IFN-γ secretion.

For investigation of specificity, CTLs (2×10^3 cells in 100 μL) were incubated with various tumor cell lines (10^4 cells in 100 μL), with or without peptide pulsing, as described in "Peptide loading of T2 cells, PBMCs, and tumor cells." Culture supernatants were harvested after 24 hours of coculture and assessed by a standard enzyme-linked immunosorbent assay (ELISA) using the OptEIA Human IFN-γ Set (BD Biosciences). Data represent mean values with corresponding mean deviations calculated from duplicate determinations. For the calculation of percentage relative IFN-γ release, the maximum IFN-γ release was set to the reference value of 100% and corresponding values were calculated corresponding to this reference.

Cytotoxicity assay

Cytotoxic activity of CTLs was analyzed in a standard 4-hour chromium release assay. Melanoma cell lines and peptide-loaded T2 cells were used as target cells. T cells were cocultured with 10^3 melanoma target cells/well at effector cell to target cell (E:T) ratios of 5:1 or 10:1. For determination of functional avidity, 10^4 T cells were added to 10^3 peptide-pulsed T2 cells loaded with titrated amounts of peptide, giving a constant E:T of 10:1.

The percentage of specific lysis was calculated as: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Spontaneous release was assessed by incubating target cells in the absence of effector cells and was generally less than 15%. For the calculation of percentage relative lysis, the maximum percentage specific lysis was set to the reference value of 100%, and corresponding values were calculated corresponding to this reference. To determine half-maximum lysis, percentage relative lysis was plotted against peptide concentration. The peptide concentration at which the curve crossed 50% relative lysis was taken as the value of half-maximum lysis.[21]

Retroviral TCR gene transfer

For TCR identification of tumor-specific CTLs, regions of the TCR–α- and TCR–β-chains encoding CDR3 were amplified by polymerase chain reaction using a panel of TCRV-α and TCRV-β primers in combination with respective constant region primers as described.[22] The full TCR–α- and TCR–β-chain genes of CTL clones T58 and D115 were amplified by polymerase chain reaction using cDNA as template. Primer sequences will be provided on request. The constant regions of both TCR chains were exchanged by the murine counterparts to increase the stability of the TCR.[23] The TCR chains were linked by a 2A peptide linker (TCR-β-P2A-TCR-α),[24] codon-optimized (Geneart),[25] and cloned into the retroviral vector MP71PRE via *Not*I and *Eco*RI restriction sites.[24] Retroviral vector plasmids were cotransfected into 293T cells with expression plasmids encoding Moloney MLV gag/pol and MLV-10A1 env gene, respectively, to produce amphotropic MLV-pseudotyped retroviruses as described.[24] Ten days after

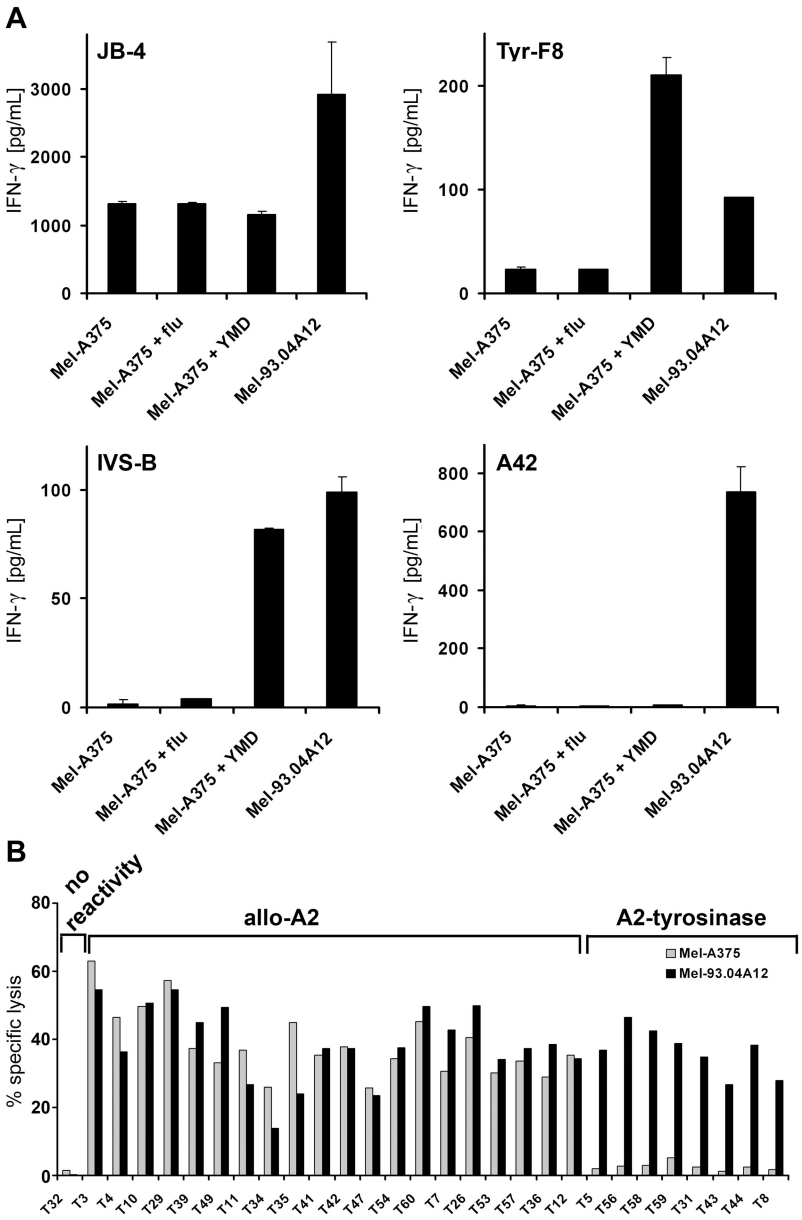

Figure 2. Recognition of peptide-loaded Mel-A375 and exemplary screening data from the DC priming. (A) HLA-A2$^+$tyrosinase$^-$ Mel-A375 were exogenously loaded with irrelevant flu peptide, the tyrosinase-peptide YMD, and compared with HLA-A2$^+$tyrosinase$^+$ Mel-93.04A12 for capacity to stimulate CTLs. IFN-γ secretion of the tyrosinase-independent HLA-A2 allo-reactive T-cell clone (JB4), 2 HLA-A2–restricted tyrosinase peptide–specific T-cell clones (Tyr-F8 and IVS-B), and an HLA-A2–restricted melan-A peptide–specific T-cell clone (A42) was measured by ELISA and given as picograms per milliliter. (B) Exemplary screening data of clones derived from a priming using DCs derived from an HLA-A2$^-$ donor loaded with HLA-A2 and tyrosinase RNA. Cytotoxic activity was assessed in a standard 4 hours chromium release assay using HLA-A2$^+$tyrosinase$^-$ Mel-A375 and HLA-A2$^+$tyrosinase$^+$ Mel-93.04A12 melanoma cells as target cells at different E:T ratios. Data are given as percentage-specific lysis.

the second transduction, peripheral blood lymphocytes (PBLs) were stained using PE-labeled A2-tyr multimer and fluorescein isothiocyanate-labeled CD8-specific antibody. Multimers presenting peptides derived from cytomegalovirus pp65 were used as controls: PE-labeled HLA-B7 pp65$_{417\text{-}427}$ (B7-pp65) multimers served as the HLA control, and HLA-A2 pp65$_{495\text{-}503}$ multimers as a peptide-specificity control. On day 15, an IFN-γ release assay was performed using T2 cells or autologous PBMCs loaded with graded amounts of tyrosinase peptide (10^{-12} M to 10^{-5} M) and the tumor cell lines MaCa1, SK-Mel-28, Mel-A375, RCC-26, PancTu 1, MaCa1/A2, UTS CC 1588, Mel-624.38, Mel-93.04A12, SK-Mel-23, SK-Mel-29, and WM-266-4 as stimulating cells at an E:T of 2:1.

Statistical analysis

The nonparametric, 2-tailed Mann-Whitney U test was used to evaluate statistical differences between datasets derived from self- and allo-restricted CTLs in cytotoxicity and IFN-γ release assays. For statistical analysis of differences between paired datasets obtained from either self- or allo-restricted CTLs in cytotoxicity and IFN-γ release assays, the nonparametric, 2-tailed Wilcoxon signed rank test was used. Values of P less than .05 were considered statistically significant. All analyses were performed using the SPSS statistical software (Version 15.0; SPSS Inc).

Results

Activation of CD8$^+$ T cells with RNA-pulsed DCs

The first step in generating CTLs was the preparation of stimulating DCs that coexpressed allogeneic HLA molecules and a selected TAA. For proof of principle, mature DCs were loaded with

Table 2. Overview of specificity of CTL from 2 HLA-A2$^+$ and 2 HLA-A2$^-$ donors

Reactivity	Self-restricted (HLA-A2$^+$ donors), no. (%)	Allo-restricted (HLA-A2$^-$ donors), no. (%)
No reactivity*	21 (55)	8 (16)
Allo-A2†	0 (0)	27 (53)
A2-tyrosinase‡	17 (45)	16 (31)
Total no.	38 (100)	51 (100)

*Clones failed to recognize HLA-A2-tyrosinase ligands.
†Clones recognized HLA-A2 without peptide.
‡Clones recognized HLA-A2-tyrosinase-peptide ligands.

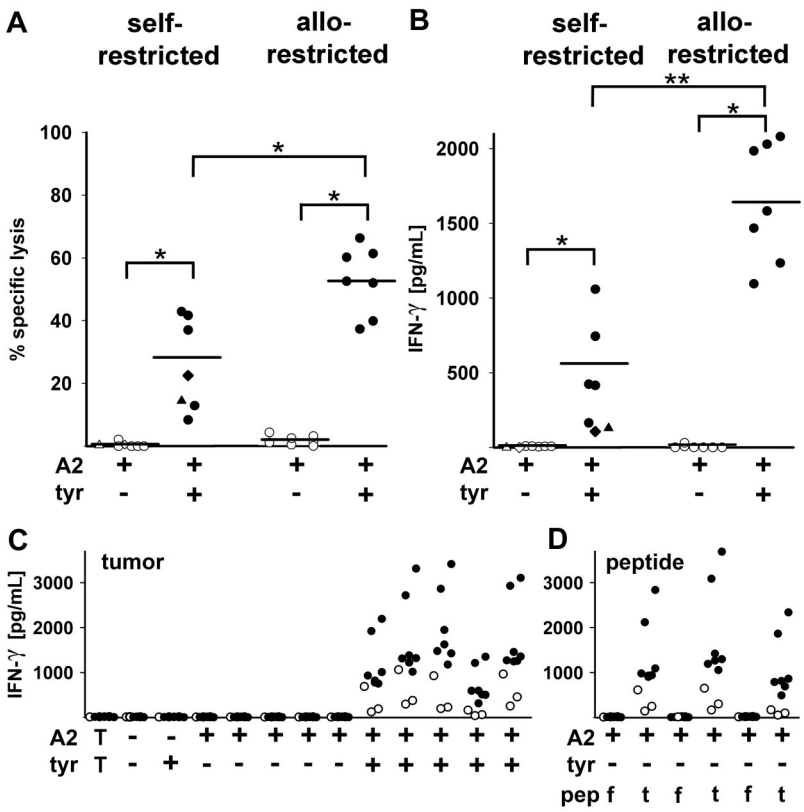

Figure 3. CTL clones from multimer-sorted cells of HLA-A2$^+$ and HLA-A2$^-$ donors primed with RNA-pulsed DCs. (A) Cytotoxic activity of 5 CTL clones from HLA-A2$^+$ and 7 CTL clones from HLA-A2$^-$ donors is shown against Mel-A375 (HLA-A2$^+$tyrosinase$^-$) and Mel-93.04A12 (HLA-A2$^+$tyrosinase$^+$) melanoma lines at E:T of 5:1. CTL clones showed significant differences in lysis of HLA-A2$^+$tyrosinase$^-$ vs HLA-A2$^+$tyrosinase$^+$ melanoma lines (nonparametric Wilcoxon signed rank test, $*P < .05$). Allo-restricted clones displayed greater mean percentage-specific lysis than self-restricted clones (52.8% vs 28.5%; nonparametric Mann-Whitney U test; $*P < .05$). The patient-derived IVS-B clone showed 22.5% specific lysis (♦), and the peptide-primed CTL clone Tyr-F8 showed 14.4% specific lysis (▲). (B) IFN-γ secretion (picograms per milliliter) by the same clones after coculture with Mel-A375 (HLA-A2$^+$tyrosinase$^-$) and Mel-93.04A12 (HLA-A2$^+$tyrosinase$^+$) lines was measured by ELISA. Allo-restricted clones showed higher mean IFN-γ secretion than self-restricted clones (1639 pg/mL vs 561 pg/mL; nonparametric Mann-Whitney U test; $**P < .005$). The IVS-B clone (♦) released 106 pg/mL IFN-γ and Tyr-F8 (▲) released 129 pg/mL. (C) IFN-γ secretion (picograms per milliliter) by 7 allo-restricted (●) and 3 self-restricted (○) T-cell clones in coculture with a panel of tumor cell lines shown from left to right: a breast carcinoma line MaCa1 (HLA-A2$^-$tyrosinase$^-$); a melanoma line SK-Mel-28 (HLA-A2$^-$tyrosinase$^+$); Mel-A375 (HLA-A2$^+$tyrosinase$^-$), a renal cell carcinoma line RCC-26 (HLA-A2$^+$tyrosinase$^-$), a pancreas carcinoma line PancTu 1 (HLA-A2$^+$tyrosinase$^-$), a stable HLA-A*0201 transfectant of MaCa1/A2 (HLA-A2$^+$tyrosinase$^-$), and a tongue carcinoma line UTS CC 1588 (HLA-A2$^+$tyrosinase$^-$); and the melanoma cell lines Mel-624.38, Mel-93.04A12, SK-Mel-23, SK-Mel-29, and WM-266-4 (all HLA-A2$^+$tyrosinase$^+$). The leftmost values designated with T show the background levels of cytokine secreted by the CTL in the absence of stimulating cells. (D) The HLA-A2$^+$tyrosinase$^-$ tumor cell lines Mel-A375, RCC-26, and MaCa1/A2 were exogenously loaded with either 10^{-5} M irrelevant flu peptide (f) or 10^{-5} M tyrosinase peptide YMD (t) and IFN-γ secretion was measured by ELISA and given as picograms per milliliter.

ivt-RNA for HLA-A2 and tyrosinase via electroporation, as described.[16] Tyrosinase ivt-RNA was transferred alone into HLA-A2$^+$ DCs or in combination with HLA-A2 ivt-RNA into HLA-A2$^-$ DCs. Protein coexpression was detected by flow cytometry with specific monoclonal antibodies (Figure 1A). Uniform surface expression of endogenous HLA-A2 molecules was detected on DCs of HLA-A2$^+$ donors, whereas transgenic HLA-A2 expression was variable on DCs of HLA-A2$^-$ donors, with some strongly positive DCs. Both DC populations coexpressed intracellular tyrosinase protein at comparable levels. Such DCs were tested for their capacity to stimulate effector CTL to secrete IFN-γ: JB4 cells recognize allogeneic HLA-A2 molecules irrespective of specific peptide,[13] whereas Tyr-F8 cells recognize tyrosinase$_{369\text{-}377}$ peptide presented by HLA-A2.[14] JB4 and Tyr-F8 cells released IFN-γ in an HLA-A2–dependent manner because mock-transfected DCs of HLA-A2$^+$ but not HLA-A2$^-$ donors activated JB4, whereas Tyr-F8 only recognized DCs expressing both HLA-A2 and tyrosinase (Figure 1B). Further analysis of CTL activation by RNA-pulsed DCs loaded with HLA-A2 and a selected TAA (tyrosinase or melan-A) is depicted in Figure 1C. JB4 recognized DCs that were loaded with HLA-A2 RNA alone and they also could respond to DC coexpressing either tyrosinase or melan-A RNA, demonstrating their allo-specificity for HLA-A2. In contrast, Tyr-F8 and IVS-B T cells, both of which recognize HLA-A2-tyrosinase$_{369\text{-}377}$ peptide complexes, only responded specifically to DCs coexpressing HLA-A2 and tyrosinase RNA. Antigen specificity was demonstrated with A42 cells, which recognize a melan-A–derived peptide presented by HLA-A2; these CTLs only responded to DCs coexpressing the 2 corresponding RNA.

The DCs shown in Figure 1A-B were used to prime purified autologous CD8$^+$ T cells using 2 rounds of stimulation with freshly prepared DCs. Peptide-specific T cells were sorted using specific HLA-A2 tyrosinase$_{369\text{-}377}$ multimer (A2-tyr multimer),[26] cloned in limiting dilution cultures and expanded using antigen-independent stimulation.[17]

Superior function of HLA-A2 allo-restricted T cells

Isolated clones were analyzed in IFN-γ release and cytotoxicity assays for function and specificity. Initial screening was done using the Mel-A375 (HLA-A2$^+$ tyrosinase$^-$) and Mel-93.04A12 (HLA-A2$^+$ tyrosinase$^+$) tumor cell lines as negative and positive controls, respectively. The capacity of these tumor lines to specifically stimulate JB4, Tyr-F8, IVS-B, and A42 is shown in Figure 2A, demonstrating that both lines can be recognized by HLA-A2 allo-specific CTL (ie, JB4 cells). However, Mel-A375 cells could not activate Tyr-F8 and IVS-B CTL unless they were pulsed with tyrosinase peptide, whereas Mel-93.04A12 cells, which expressed both endogenous tyrosinase and melan-A proteins, could activate Tyr-F8, IVS-B, and A42 directly. Screening results for exemplary clones primed against DCs derived from an HLA-A2$^-$ donor are shown in Figure 2B, depicting either tyrosinase-independent allo-reactivity for HLA-A2 or presumed specificity for HLA-A2-tyrosinase–derived ligands. In total, 38 self-restricted clones from 2 HLA-A2$^+$ donors were screened: 21 clones had no reactivity, no clones recognized HLA-A2 without tyrosinase peptide, and 17 clones recognized HLA-A2-tyrosinase-peptide ligands (Table 2). Of 51 clones isolated from 2 HLA-A2$^-$ donors, 8 were nonreactive; 27 were allo-reactive, recognizing HLA-A2 independent of tyrosinase, and 16 were specific for HLA-A2-tyrosinase ligands.

After discarding clones with tyrosinase-independent, HLA-A2 allo-reactivity, a set of strongly proliferating allo-restricted clones with presumed tyrosinase-derived peptide specificity was further analyzed compared with HLA-A2 self-restricted clones. All selected clones showed cytotoxic activity and IFN-γ release directed against Mel-93.04A12 (HLA-A2$^+$tyrosinase$^+$) but not Mel-A375

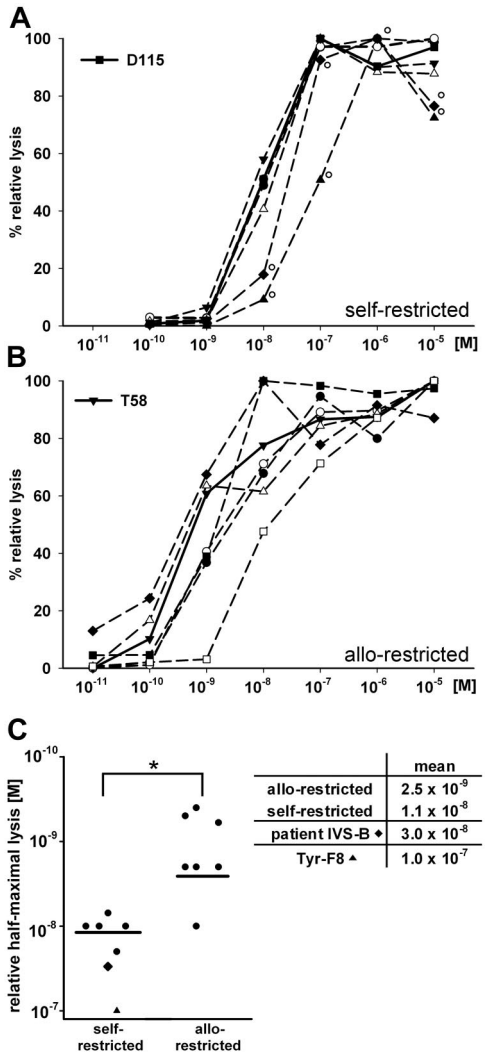

Figure 4. Comparisons of self-restricted and allo-restricted CTLs. (A-B) The cytotoxic activity of individual CTL was measured against T2 cells loaded with graded amounts of tyrosinase peptide (10^{-11} M to 10^{-5} M) at an E:T of 10:1. Peptide concentration and percentage relative lysis are displayed on the x- and y-axes, respectively. Shown are (A) patient-derived CTL IVS-B (♦°), CTL Tyr-F8 derived via T2 peptide priming (▲°), and the 5 DC-primed self-restricted CTL and (B) DC-primed allo-restricted CTL. Bold lines represent CTL D115 and T58 in panels A and B, respectively. (C) Relative values of half-maximal lysis are presented for CTL IVS-B, Tyr-F8, self-restricted CTLs (n = 5), and allo-restricted CTLs (n = 7). Means are represented as black bars. Significant differences were found between the 2 groups (nonparametric Mann-Whitney U test; $^*P < .05$).

(HLA-A2$^+$tyrosinase$^-$) melanoma cell lines (Figure 3A-B). The allo-restricted CTLs had a significantly higher mean level of specific lysis than the self-restricted CTLs (52.8% vs 28.5%) and a significantly higher mean level of IFN-γ secretion (1639 pg/mL vs 561 pg/mL), demonstrating their general superior function. The self-restricted melanoma patient-derived CTLs IVS-B and the self-restricted peptide-primed Tyr-F8 CTLs were included for comparison and showed low-intermediate killing (22.5% and 14.4%, respectively) and very low IFN-γ release (106 pg/mL and 129 pg/mL, respectively). A majority of these clones were further analyzed for IFN-γ release after coculture with a panel of tumor lines derived from several different tumor types (Figure 3C). Although all the clones failed to recognize HLA-A2$^-$ and HLA-A2$^+$tyrosinase$^-$ tumor cells, most clones secreted detectable amounts of IFN-γ after stimulation with the 5 HLA-A2$^+$tyrosinase$^+$ melanoma tumor lines. Furthermore, the allo-restricted peptide-specific CTLs showed higher levels of IFN-γ secretion compared with the self-restricted CTLs after stimulation with each of these 5 melanoma cell lines (range, 317-3417 pg/mL vs 43-1065 pg/mL). HLA-A2$^+$tyrosinase$^-$ tumor cell lines could be recognized after exogenous pulsing with specific tyrosinase YMD peptide but not with irrelevant flu peptide (Figure 3D). Again, more IFN-γ was released by allo-restricted CTL compared with self-restricted CTLs (range, 494-3693 pg/mL vs 55-655 pg/mL, respectively, Figure 3D).

Functional T-cell avidity for tyrosinase-peptide recognition was measured in a ^{51}Cr-release assay using HLA-A2$^+$ T2 cells pulsed with graded amounts of exogenous tyrosinase peptide as target cells. The peptide concentration needed for 50% relative lysis defined the value of half-maximal lysis.[21] Self-restricted clones had half-maximal values near 10^{-8} M (mean = 1.1×10^{-8} M; Figure 4A,C). Allo-restricted clones displayed a range of values and several clones responded to significantly less peptide (mean = 2.5×10^{-9} M; Figure 4B-C). This value differed significantly from self-restricted clones (Figure 4C). The patient-derived IVS-B clone and the Tyr-F8 clone required the most peptide (3.0×10^{-8} M and 1.0×10^{-7} M, respectively; Figure 4A,C).

One self-restricted T-cell clone (D115) and 1 allo-restricted T-cell clone (T58) were selected for further characterization based on their highest rank in killing of melanoma cell lines (data not shown). Responses to peptide-pulsed T2 cells showed that they localized to the middle of their respective groups with relative half-maximal lysis at lower peptide concentrations (T58: 6×10^{-10} M vs D115: 10^{-8} M; Figure 4A-B).

A side-by-side comparison of the patient-derived IVS-B CTL with self-restricted D115 and allo-restricted T58 CTL revealed that IVS-B showed the poorest response, whereas D115 showed intermediate and T58 the best recognition of HLA-A2$^+$ tumor cells that endogenously expressed tyrosinase (Figure 5A). Although all 3 CTLs failed to recognize native HLA-A2$^+$tyrosinase$^-$ tumor cell lines, these tumor lines were recognized after exogenous pulsing with specific tyrosinase YMD peptide but not with irrelevant flu peptide (Figure 5B).

To answer the question whether allo-restricted T58 CTL would also show superior recognition of primary melanoma cells, they were compared with D115 CTL and patient-derived IVS-B CTLs. Early passage tumor cells expressing tyrosinase from an HLA-A2$^-$ melanoma patient were transfected with HLA-A2 ivt-RNA to create a pair of matched cell lines, with and without the specific pMHC ligand (Figure 5C). ELISPOT assays of IFN-γ and perforin showed a hierarchy similar to that observed with established melanoma cell lines, with poor recognition by IVS-B CTLs, intermediate recognition by D115 CTLs, and the strongest IFN-γ and perforin secretion by T58 CTLs (Figure 5D-E). Recognition was HLA-A2–restricted because the primary tumor cells that were not transfected with HLA-A2 ivt-RNA were not recognized. Mel-A375 (A2$^+$tyr$^-$) were not recognized, and Mel-93.04A12 (A2$^+$tyr$^+$) were recognized by the 3 CTL clones.

Allo-restricted transgenic TCRs confer superior function

It was important to demonstrate that the superior functional avidity of allo-restricted T cells resided directly in the TCR; therefore, the TCR cDNA of D115 and T58 CTLs was isolated, modified for optimal expression, and introduced by retrovirus transfer into human PBLs of healthy donors. Multimer staining of untransduced PBLs was not seen (0.1%, data not shown). After transduction, no notable multimer staining could be detected with the irrelevant multimers B7-pp65 and A2-pp65 (0.2%-0.6%), and equal numbers

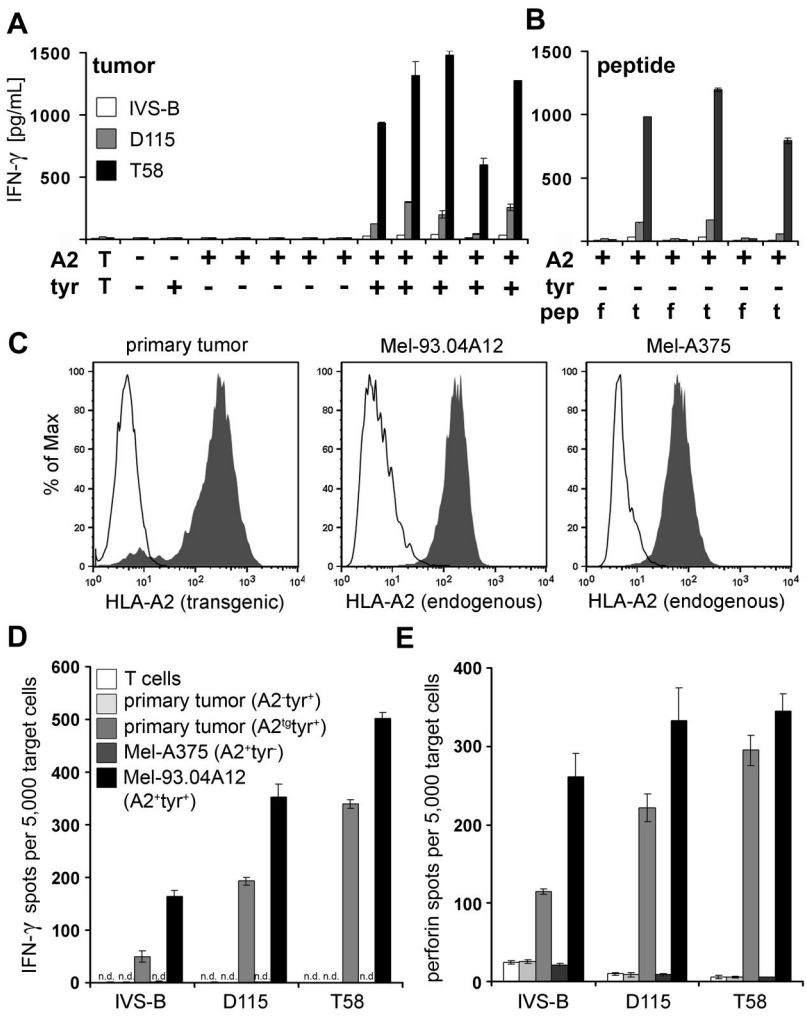

Figure 5. Tyrosinase peptide-specific CTL recognition of tumor cell lines and primary melanoma tumor cells. (A) Columns represent the amount of IFN-γ (picograms per milliliter) secreted by patient-derived IVS-B CTL, self-restricted D115 CTL, and allo-restricted T58 CTL in coculture with a panel of tumor cell lines from left to right: MaCa1 (HLA-A2$^-$tyrosinase$^-$); SK-Mel-28 (HLA-A2$^-$tyrosinase$^+$); Mel-A375, RCC-26, PancTu 1, MaCa1/A2, and UTS CC 1588 (all HLA-A2$^+$tyrosinase$^-$); Mel-624.38, Mel-93.04A12, SK-Mel-23, SK-Mel-29, and WM-266-4 (all HLA-A2$^+$tyrosinase$^+$). T indicates CTL without stimulating cells. (B) The HLA-A2$^+$tyrosinase$^-$ tumor cell lines Mel-A375, RCC-26, and MaCa1/A2 were exogenously loaded with either 10^{-5} M irrelevant flu peptide (f) or 10^{-5} M tyrosinase peptide YMD (t), and IFN-γ secretion was measured by ELISA and given as picograms per milliliter. (C) HLA-A2 expression on primary tumor cells (passage 12) of an HLA-A2$^-$ melanoma patient after transfection with 50 μg HLA-A2 ivt-RNA and on established melanoma cell lines Mel-93.04A12 (HLA-A2$^+$tyrosinase$^+$), and Mel-A375 (HLA-A2$^+$tyrosinase$^-$) was measured by flow cytometry after staining with HLA-A2–specific monoclonal antibody. Histograms represent stained samples (filled curves) and control samples (empty curves): control curves show untransfected primary tumor cells stained with HLA-A2–specific monoclonal (left histogram) or isotype control antibodies used with the melanoma cell lines (middle and right histograms). HLA-A2 expression on primary tumor cells was detected 10 hours after electroporation. (D) The capacity of the patient-derived CTLs (IVS-B), the representative self-restricted CTLs (D115), and the representative allo-restricted CTLs (T58) to secrete IFN-γ or (E) release perforin in coculture with the melanoma cells shown above was measured in ELISPOT assays. n.d. indicates not detectable.

of CD8$^+$ PBLs bound the specific A2-tyr multimer (TCR-D115, 10.9%; and TCR-T58, 10.9%; Figure 6A). The TCR-transduced PBLs were tested without enrichment for IFN-γ release after stimulation with peptide-pulsed T2 cells, whereby PBLs expressing TCR-T58 required 100-fold less peptide for half-maximal release compared with PBL expressing TCR-D115 (Figure 6B). Untransduced PBLs showed no specific IFN-γ secretion (data not shown). The PBLs with TCR-T58 also released substantially higher amounts of cytokine compared with PBLs with TCR-D115 and the concentration of peptide required to induce release of 2000 pg/mL IFN-γ differed by more than 15 000-fold (TCR-T58: 2.2×10^{-10} M vs TCR-D115: 3.5×10^{-6} M; Figure 6C). Because T2 cells have a genetic defect in peptide loading, they fail to present most endogenous peptides in their endogenous HLA-A2 molecules; thus, they express highly uniform pMHCs after exogenous loading of tyrosinase peptide. To analyze the sensitivity of TCR recognition of specific pMHC ligands within a heterogeneous pMHC setting, which occurs in cells not having defects in endogenous peptide loading, we created new stimulating cells using autologous HLA-A2$^+$ PBMCs loaded with graded amounts of tyrosinase peptide. In this case, tyrosinase peptide can only displace some endogenous peptides in HLA-A2 molecules, thereby strongly limiting these specific pMHC ligands on PBMCs. After coculture with these PBMCs, TCR-T58 PBLs secreted considerably higher amounts of IFN-γ. In addition, the lowest amount of peptide required to induce IFN-γ secretion above background levels differed substantially from TCR-D115 PBL (10^{-10} M vs 10^{-7} M; Figure 6D). Specificity was retained in the 2 TCR-transduced PBL populations, which only recognized HLA-A2$^+$tyrosinase$^-$ tumor cell lines after pulsing with specific YMD but not irrelevant flu peptide. Furthermore, HLA-A2$^+$tyrosinase$^+$ tumor cell lines, but not HLA-A2$^-$ or HLA-A2$^+$tyrosinase$^-$ tumor lines led to specific activation of both CTLs (Figure 6E-F), whereby PBL-T58 secreted significantly higher amounts of IFN-γ. Thus, by all these parameters, the allo-restricted TCR-T58 was found to be far superior to self-restricted TCR-D115 in its capacity to recognize MHC-tyrosinase-peptide complexes.

Discussion

This systematic comparison of self-restricted and allo-restricted peptide-specific CTLs clearly demonstrated that DCs that coexpressed allogeneic MHC molecules and tumor-associated antigen induced allo-restricted peptide-specific T cells with superior antitumor activity. Three features of the procedure are of particular relevance. First, we took advantage of the well-documented knowledge that allo-restricted T cells can express high-affinity TCR specific for peptide ligands of self-proteins because they were not deleted by negative selection.[1,2] Thereby, T cells that more effectively recognized tumor cells were more readily obtained. Second, we used T cells of healthy donors, taking advantage of the

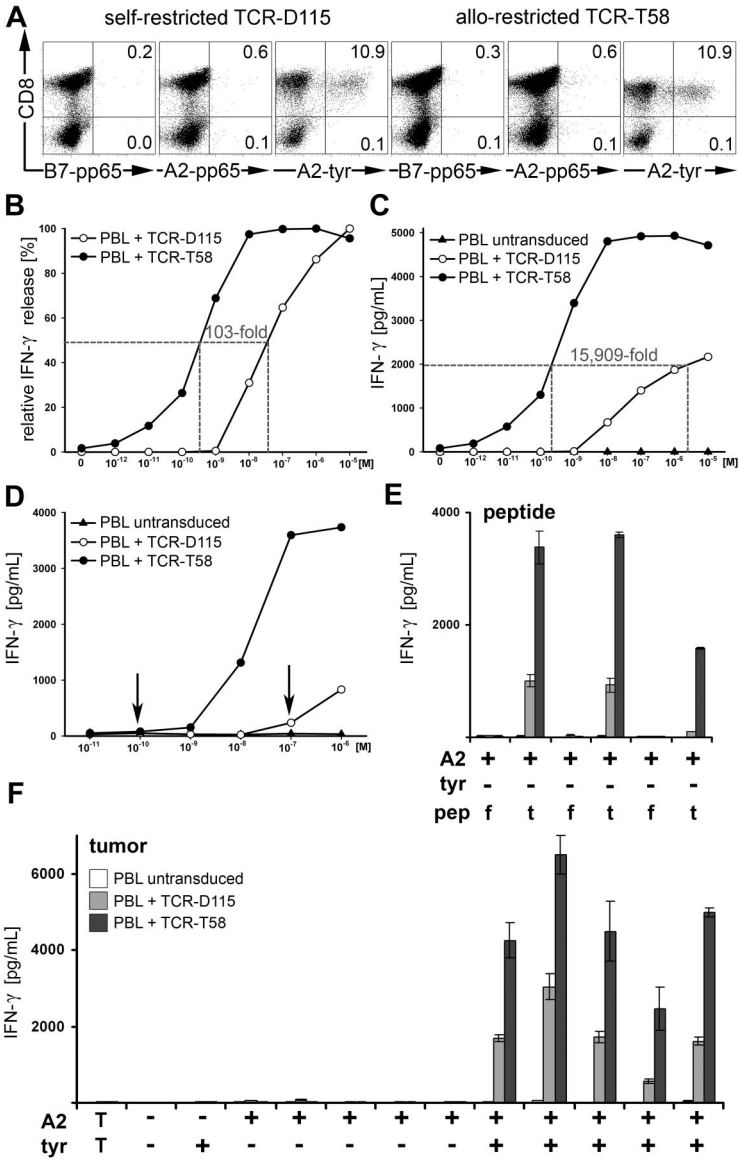

Figure 6. Transfer of antigen specificity by retroviral transfer of TCR-D115 and TCR-T58. (A) PBLs of a healthy donor were transduced with TCR-D115 or TCR-T58. Unsorted TCR-transduced PBLs were analyzed on day 10 for transgenic TCR expression using irrelevant B7-pp65 and A2-pp65 multimers and specific A2-tyr multimers. Untransduced PBLs showed no multimer binding (0.1%, data not shown). Percentages of multimer$^+$CD8$^+$ T cells are displayed in the top right quadrant. (B-C) The IFN-γ release of unsorted TCR-transduced PBLs after stimulation with T2 cells loaded with graded amounts of tyrosinase peptide (10^{-12} M to 10^{-5} M) at a ratio of 2:1. (B) The relative IFN-γ release is displayed in percentage. (C) The specific IFN-γ release is presented as picograms per milliliter. (D) Functionality of unsorted TCR-transduced PBLs was measured by IFN-γ release using autologous HLA-A2$^+$ PBMCs loaded with tyrosinase peptide (10^{-11} M to 10^{-6} M) as stimulating cells at ratio of 2:1. Untransduced PBLs (▲) showed no peptide-specific IFN-γ release. (E) The HLA-A2$^+$tyrosinase$^-$ tumor cell lines Mel-A375, RCC-26, and MaCa1/A2 were exogenously loaded with either 10^{-5} M irrelevant flu peptide (f) or 10^{-5} M tyrosinase peptide YMD (t) and IFN-γ secretion was measured by ELISA and given as picograms per milliliter. (F) Specificity of recognition was assessed by IFN-γ release after coculture with the tumor cell lines from left to right: MaCa1 (HLA-A2$^-$tyrosinase$^-$); SK-Mel-28 (HLA-A2$^-$tyrosinase$^+$); Mel-A375, RCC-26, PancTu 1, MaCa1/A2, and UTS CC 1588 (all HLA-A2$^+$tyrosinase$^-$); Mel-624.38, Mel-93.04A12, SK-Mel-23, SK-Mel-29, and WM-266-4 (all HLA$^-$A2$^+$tyrosinase$^+$). T indicates CTL without stimulating cells.

optimal priming capacity of DCs for naive cells. This allowed us to avoid use of patient lymphocytes, which often show poor proliferation and function. Third, we used ivt-RNA as the source of both allogeneic MHC molecules and TAAs. This provided maximum flexibility for creating allo-ligands specific for any MHC class I allele and any selected antigen. Furthermore, priming was not limited to known peptides because the whole TAA was available for processing and presentation within the DCs.[27] Here we used HLA multimers to isolate peptide-specific T cells, but alternative methods are available to select a broader array of T cells, such as the IFN-γ capture assay.[28] Indeed, CTLs that released high levels of IFN-γ after tumor cell stimulation often had the best killing capacity. It should also be noted that this same strategy can probably be extended to identify high-avidity allo-restricted CD4$^+$ T cells by providing DCs with ivt-RNA encoding MHC class II alleles in combination with TAAs. In this case, the ivt-RNA for TAAs can be modified to shuttle the proteins into the endosomal pathway to allow better peptide loading of class II molecules.[27]

Significant tumor regression can occur after adoptive transfer of T cells with antitumor specificity.[29] However, patient-derived T cells may have suboptimal activity, as seen with IVS-B CTLs of a melanoma patient. Furthermore, most self-restricted T cells with high-affinity TCR specific for self-peptides have undergone negative selection, and remaining T cells may be negatively controlled in the periphery. For these reasons, CTLs with appropriate specificity and function are often missing in patients with rapidly progressing tumors. Therefore, there is current interest in using precharacterized TCR genes to create designer lymphocytes for adoptive cell therapies.[4,9] Expression of TCR transgenes in activated PBLs could imbue recipient lymphocytes with antitumor activities comparable with the original CTLs,[4,9] as seen here with TCR-D115 and TCR-T58. Furthermore, some transgenic TCRs can displace endogenous TCRs, yielding lymphocytes with monoclonal TCRs.[10,30] If more TCRs with this feature can be identified, the safety of adoptive cell therapy would be improved.

Our studies comparing self-restricted and allo-restricted CTL clones specific for the same MHC-tyrosinase ligand revealed that there were inherent differences in the TCR themselves. Far superior recognition of peptide-pulsed cells was seen with PBLs expressing the allo-restricted TCR-T58 compared with the self-restricted TCR-D115. The improved capacity of TCR-T58 PBLs to respond to substantially lower amounts of peptide indicates that these T cells should be better able in vivo to effectively recognize tumor cells that express only limited amounts of

specific pMHC ligand. This is supported by the far superior recognition of melanoma tumor cell lines by PBL-T58.

The first clinical trials of adoptive transfer of TCR-transgenic T cells in melanoma patients achieved clinical responses in a number of patients with advanced disease.[31,32] These results demonstrated the therapeutic potential of this approach. It would be an advantage to provide patients with mixtures of TCR-transgenic lymphocytes that target their tumors via several different pMHC ligands to avoid immune selection of tumor cell variants that lack expression of individual TAAs. Our priming strategy should allow better access to high-avidity CTLs specific for different TAAs that are prevalent in melanomas and other tumors, which could serve as sources of therapeutic TCR sequences. Future adoptive therapy of more patients will be feasible if useful TCR sequences are available as "off-the-shelf" reagents. Our approach helps to close the technologic gap in identifying suitable TCR with specificity for common TAA ligands that have sufficient affinity to effectively recognize tumor cells.

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Authorship

Contribution: S.W. designed and performed the experiments and helped in drafting the manuscript; M.S. performed cell sorting; B.F. helped in the characterization of the TCR; D.S. and W.U. contributed retroviral transduction technology; S.S. helped in cytotoxic assays; S.M. cloned ivt-plasmids; H.P. provided ELISPOT technology; D.H.B. contributed analytical tools and data analysis; D.J.S. designed the concept and prepared the final manuscript; and all authors reviewed and edited the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests. However, a patent for specific TCRs has been submitted by the Helmholtz Zentrum München, German Research Center for Environmental Health, Munich, Germany.

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MHC-restricted fratricide of human lymphocytes expressing survivin-specific transgenic T cell receptors

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The apoptosis inhibitor protein survivin is overexpressed in many tumors, making it a candidate target molecule for various forms of immunotherapy. To explore survivin as a target antigen for adoptive T cell therapy using lymphocytes expressing survivin-specific transgenic T cell receptors (Tg-TCRs), we isolated HLA-A2–allorestricted survivin-specific T cells with high functional avidity. Lymphocytes expressing Tg-TCRs were derived from these T cells and specifically recognized HLA-A2$^+$ survivin$^+$ tumor cells. Surprisingly, HLA-A2$^+$ but not HLA-A2$^-$ lymphocytes expressing Tg-TCRs underwent extensive apoptosis over time. This demise was caused by HLA-A2–restricted fratricide that occurred due to survivin expression in lymphocytes, which created ligands for Tg-TCR recognition. Therefore, survivin-specific TCR gene therapy would be limited to application in HLA-A2–mismatched stem cell transplantation. We also noted that lymphocytes that expressed survivin-specific Tg-TCRs killed T cell clones of various specificities derived from HLA-A2$^+$ but not HLA-A2$^-$ donors. These results raise a general question regarding the development of cancer vaccines that target proteins that are also expressed in activated lymphocytes, since induction of high-avidity T cells that expand in lymph nodes following vaccination or later accumulate at tumor sites might limit themselves by self-MHC–restricted fratricide while at the same time inadvertently eliminating neighboring T cells of other specificities.

Introduction

Several recent developments have converged to improve adoptive T cell therapy of cancer. First, expression of TCRs as transgenic proteins in peripheral blood lymphocytes (PBLs) enables T cells with defined specificities to be generated in high numbers for patient-individualized therapy (TCR gene therapy), bypassing the laborious process of isolating and expanding specific T cells for individual patients (1). Second, high-affinity TCRs specific for peptides presented by different major histocompatibility complex molecules (hereafter, pMHC ligands) can be selected and employed as generic "off-the-shelf" reagents, enabling future application of small repertoires of therapeutic Tg-TCRs to achieve greater clinical efficacy (2, 3). Third, selection of recipient lymphocytes and manipulation of the microenvironment can enhance T cell survival, expansion, and long-term function after adoptive transfer in vivo (4). In addition, tumor-associated antigens (TAAs) have been elucidated that may serve as suitable target structures on tumor cells, guiding the selection of TCR specificities.

A pilot project of the National Cancer Institute (NCI) prioritized a group of TAAs for T cell therapy and vaccine development (5). These TAAs represent mutant, overexpressed, or abnormally expressed proteins in cancer cells, as well as viral proteins present in virus-associated malignancies. The role of candidate proteins in oncogenicity was an important ranking factor, based on the consideration that T cell–mediated immune selection would be limited if tumor survival was dependent on TAA expression. For example, survivin, a well-characterized inhibitor of apoptosis (6, 7), is an attractive candidate for immunotherapy, since it is not expressed in most adult tissues but is overexpressed by many tumors (8). Furthermore, survivin-specific T cells were reported by several investigators (9–18). Survivin received a top score for oncogenicity, and it was ranked 21 among the 75 prioritized TAA (5).

High-affinity TCRs that efficiently recognize tumor cells are needed for effective TCR gene therapy (19, 20). However, T cells recognizing peptides of self-proteins presented by self-MHC molecules (hereafter designated self-restricted T cells) will often be missing or display only low functional avidity due to deletional tolerance (9, 21, 22). In contrast, HLA-allorestricted T cells can be obtained with high functional avidity for self-peptides (23). Advancing upon the original concept of Stauss and coworkers (24), we recently described a versatile strategy to derive allorestricted peptide-specific T cells as sources of high-affinity TCR, using DCs that were loaded with in vitro transcribed RNA (ivt-RNA) as a source of antigen to prime naive T cells (25). For example, DCs prepared from HLA-A2$^-$ donors can be loaded with ivt-RNA encoding allogeneic HLA-A2 molecules and a self-protein, such as survivin, for use as APCs. Because tolerance is MHC restricted, the corresponding autologous T cells of HLA-A2$^-$ individuals were not subjected to negative selection by HLA-A2 and can give rise not only to HLA-A2–alloreactive T cells but also to peptide-specific T cells that recognize survivin-derived peptides

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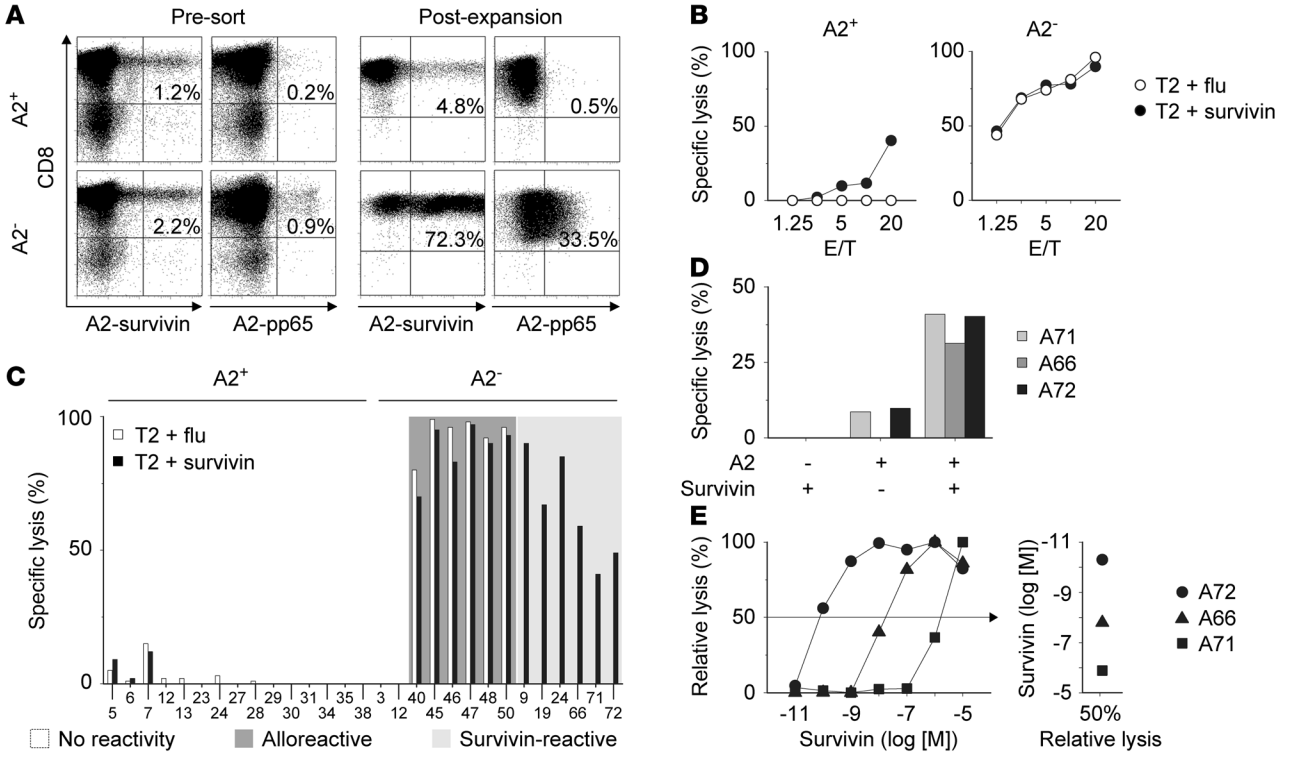

Figure 1

De novo priming of survivin-specific T cells with RNA-pulsed DCs. (**A**) Survivin-multimer staining of bulk CD8$^+$ T cell lines of HLA-A2$^+$ and HLA-A2$^-$ donors after DC priming (Pre-sort) and after 26 days of expansion following multimer sorting (Post-expansion). CMV(pp65)-multimer served as a specificity control. Percentage double-positive cells are displayed in the upper-right quadrant. (**B**) Cytotoxic activity of multimer-sorted lines measured against T2 (HLA-A2$^+$) cells pulsed with flu or survivin peptide (10^{-5} M) is presented as percent specific lysis. (**C**) Cytotoxic specificity of different T cell clones was measured against flu- and survivin-pulsed T2 cells (10^{-5} M) and presented as percent specific lysis. The x axis shows clone designation. (**D**) Cytotoxic activity of allorestricted survivin-specific T cell clones A71, A66, and A72 against Mel-1379 (HLA-A2$^-$ [A2$^-$], survivin$^+$ [S$^+$]), T2 cells loaded with 10^{-5} M flu peptide (A2$^+$, S$^-$), and Mel-624.38 tumor cells (A2$^+$, S$^+$) at an E/T ratio of 5:1. Flu-pulsed T2 cells were used as survivin-negative control, since we identified no tumor cell lines that were survivin negative. (**E**) Functional avidity of CTLs (E/T, 10:1) was measured against T2 cells pulsed with graded amounts of survivin peptide. Relative values of half-maximal killing are depicted at the right. Flu-pulsed T2 cells (10^{-5} M) were not recognized (data not shown). Cytotoxicity data represent means of duplicates measured at each E/T ratio or peptide concentration.

presented by HLA-A2. We applied this strategy to isolate high-affinity survivin-specific TCRs for use in TCR gene therapy.

Results

High-avidity allorestricted T cell clones specific for survivin are derived by DC priming. We introduced survivin ivt-RNA alone, or in combination with HLA-A2 ivt-RNA, into mature DCs prepared from HLA-A2$^+$ or HLA-A2$^-$ donors, respectively. These DCs were cocultured with autologous responding CD8$^+$ lymphocytes to induce either HLA-A2 self-restricted or allorestricted survivin-specific T cells. After two rounds of stimulation, primed cells were stained with HLA-A2–survivin$_{96\text{-}104[97L]}$ multimer (survivin-multimer) and CD8-specific antibody (Figure 1A). Double-positive cells (1.2%–2.2%) were detected in both self-restricted and allorestricted samples prior to sorting. Very few positive cells were present in self-restricted cultures that bound control HLA-A2–multimer, utilizing a peptide of cytomegalovirus pp65 protein (CMV-multimer, 0.2%). However, substantial numbers of cells from allorestricted cultures bound CMV-multimer (0.9%), most likely representing T cells that recognized HLA-A2 as an alloantigen, irrespective of survivin peptide. The survivin-multimer$^+$ T cells were isolated and cloned immediately by limiting dilution, and the remaining sorted cells were cultured as bulk T cell lines. After 26 days, the T cell lines were reanalyzed for multimer binding. Whereas fewer than 5% of self-restricted CD8$^+$ T cells bound survivin-multimer, more than 70% of allorestricted cells were survivin-multimer positive. Again, substantial numbers of cells in this T cell line bound CMV-multimer.

Both T cell lines were assessed for the capacity to kill HLA-A2$^+$ target cells that were pulsed exogenously with either survivin$_{96\text{-}104[97L]}$ (survivin) peptide or control influenza matrix protein$_{58\text{-}66}$ (flu) peptide. The self-restricted T cell line mediated a low rate of killing of survivin-pulsed T2 cells, in accordance with the low numbers of survivin-multimer$^+$ cells; it did not kill flu-pulsed target cells (Figure 1B). In contrast, the allorestricted T cell line killed both target cells. Because HLA-A2–alloreactive T cells present in the culture recognize target cells irrespective of specific peptide (25), they mask the detection of survivin-specific T cells. Therefore, HLA-A2–allorestricted survivin-specific T cells must be identified at the clonal level.

Clones derived from limiting dilution cultures were screened for cytotoxicity against the same two peptide-pulsed target cells.

Table 1
Classification of survivin-specific T cell clones after DC priming

	HLA-A2$^+$ (self-restricted)	HLA-A2$^-$ (allorestricted)
No reactivity	46 (100%)	9 (12%)
Alloreactive	0 (0%)	44 (60%)
Survivin-reactive	0 (0%)	21 (28%)
Total number	46 (100%)	74 (100%)

Clones (n = 120) were classified for specificity using 10% specific lysis of peptide-pulsed T2 cells as the positive cutoff: no reactivity designates clones recognizing neither target cell; alloreactive clones recognized survivin- and flu-pulsed T2 cells; and survivin-reactive clones recognized only survivin-pulsed T2 cells. The number and respective percentage of the total clone number are given.

Representative results of one screening assay demonstrate that survivin-specific T cell clones were not isolated from the self-restricted cultures, whereas different clones derived from the allogeneic cultures killed either both targets or only recognized survivin-pulsed cells (Figure 1C). A total of 120 T cell clones were analyzed, and no self-restricted clone with survivin specificity was isolated (Table 1). In contrast, the allogeneic cultures yielded 60% of clones that recognized both targets and 28% that recognized only survivin-pulsed target cells. The first group represented HLA-A2–alloreactive cells and was discarded.

Three clones showing potential survivin specificity were analyzed for cytotoxic activity. Target cells lacking either survivin or HLA-A2 were poorly recognized, whereas cells coexpressing HLA-A2 and survivin were efficiently killed (Figure 1D). The peptide sensitivity of killing was assessed using T2 cells pulsed with varying concentrations of survivin peptide, revealing half-maximal values ranging from 1.3×10^{-6} to 5×10^{-11} M (Figure 1E).

Survivin-specific reactivity is transferred to recipient lymphocytes by TCR gene expression. The TCR sequences of clones A66, A71, and A72 were isolated, codon optimized, and modified to express mouse TCR constant regions to improve surface expression, as described previously (26, 27). Retroviral vectors encoding both TCR chains were used to transduce activated PBLs of HLA-A2$^-$ donors. The 3 survivin-specific Tg-TCRs were expressed on comparable percentages of PBLs, as shown by binding of murine TCRβ constant region antibody (Figure 2A). The TCR-transduced PBLs killed survivin-pulsed T2 cells with different peptide sensitivities. Based on half-maximal values for cytotoxicity, a hierarchy of functional avidity was revealed that corresponded to the original T cell clones (TCR-A71 < TCR-A66 < TCR-A72) (Figure 2B).

TCR-transduced PBLs were also tested for their capacity to kill tumor cells that expressed survivin, with or without HLA-A2. Surface HLA-A2 was detected on tumor cells with specific antibody (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI43437DS1), and survivin mRNA levels were assessed by RT-PCR (Figure 2C). PBLs expressing the 3 Tg-TCRs killed UT-SCC-15, U-373, and FM-86 tumor cells, which naturally coexpressed HLA-A2 and survivin. Recognition required expression of survivin-specific Tg-TCRs, since GFP-transduced and untransduced PBLs did not mediate appreciable killing (Figure 2D). Similar results were found for 4 additional tumor lines (data not shown). KT-195 tumor cells displayed high levels of survivin mRNA, but they were HLA-A2 negative and were not recognized by TCR-transduced effector cells. Following transfection with HLA-A*0201–encoding cDNA, KT-195-A2 cells gained HLA-A2 surface expression and acquired sensitivity to effector cells modified with each of the 3 TCR-modified effector cells. In contrast, cells transfected with control vector (KT-195-VC) remained resistant to killing (Figure 2E). PBLs expressing TCR-A71, which had the lowest peptide sensitivity, recognized the FM-86 and KT-195-A2 target cells at somewhat lower levels. These two tumor cell lines expressed the lowest levels of HLA-A2 (Supplemental Table 1), indicating that T cell functional avidity impacted sensitivity of tumor cell recognition when pMHC ligand density was limited.

IFN-γ was also released by TCR-modified PBLs but not by untransduced or GFP-transduced PBLs following stimulation with tumor cells (Figure 2F). This cytokine release was pMHC specific, since it was only induced by tumor cells coexpressing survivin and HLA-A2 (data not shown).

The analysis of the KT-195 triplet of tumor cells demonstrated that Tg-TCR recognition was dependent upon HLA-A2. To demonstrate that HLA-A2 was not directly recognized in the absence of survivin peptide, we prepared artificial APCs (aAPCs), consisting of particle-bound anti-CD28 antibodies and recombinant HLA-A2-Ig molecules that were loaded exogenously with flu or survivin peptides. These aAPCs were analyzed for their capacity to induce IFN-γ secretion by PBLs expressing TCR-A72, which had the best functional avidity. Survivin-dependent recognition of this Tg-TCR was apparent, since only survivin-pulsed aAPCs led to detectable cytokine secretion. The recognition of survivin-pulsed aAPCs was also dependent upon Tg-TCR expression in the effector cells, since untransduced PBLs showed no response to the survivin-pulsed aAPCs (Figure 2G).

Survivin-specific TCR-modified cells mediate MHC-restricted fratricide. In a clinical setting, therapeutic Tg-TCRs would normally be expressed in lymphocytes of HLA-A2$^+$ patients bearing HLA-A2$^+$ survivin$^+$ tumors. Even though the survivin-specific Tg-TCRs were well expressed short-term on activated cells of both HLA-A2$^+$ and HLA-A2$^-$ donors, TCR-transgenic lymphocytes of HLA-A2$^+$ donors yielded lower recoveries after several days of culture (data not shown). Therefore, we made a closer inspection of recipient lymphocytes over a period of 2 weeks following transduction with the 3 Tg-TCRs. The percentages of PBLs that expressed Tg-TCRs ranged from 28% to 52%, and the expression profiles of each Tg-TCR in HLA-A2$^-$ and HLA-A2$^+$ recipient lymphocytes were comparable (Figure 3A). Appearance of apoptotic cells in the total population was monitored by staining with 7-aminoactomycin D (7-AAD), which intercalates into double-stranded nucleic acids of apoptotic and dead cells. While no differences in 7-AAD$^+$ cells were noted on day 1 after TCR transduction, dramatic differences in percentages of 7-AAD$^+$ cells were seen after 13 days when the HLA-A2$^-$ and HLA-A2$^+$ populations were compared (Figure 3B). Apoptosis of HLA-A2$^-$ lymphocytes ranged from 21% to 24% in TCR-modified PBLs, near the value of GFP-transduced and untransduced PBLs. In strong contrast, 72%–87% 7-AAD$^+$ cells were detected in the HLA-A2$^+$ populations containing TCR-transduced T cells. This high rate of apoptosis was dependent upon the presence of Tg-TCR–expressing T cells in the total lymphocyte population, since GFP-transduced and untransduced PBLs remained near 20%. For comparison, PBLs were transduced with a high-affinity TCR (T58) derived from an allorestricted T cell clone recognizing an epitope of tyrosinase protein presented by HLA-A2 (25) (Figure 3C). In this case, HLA-A2$^+$ recipient lymphocytes did not show any dramatic increase in apoptotic cells compared with untransduced

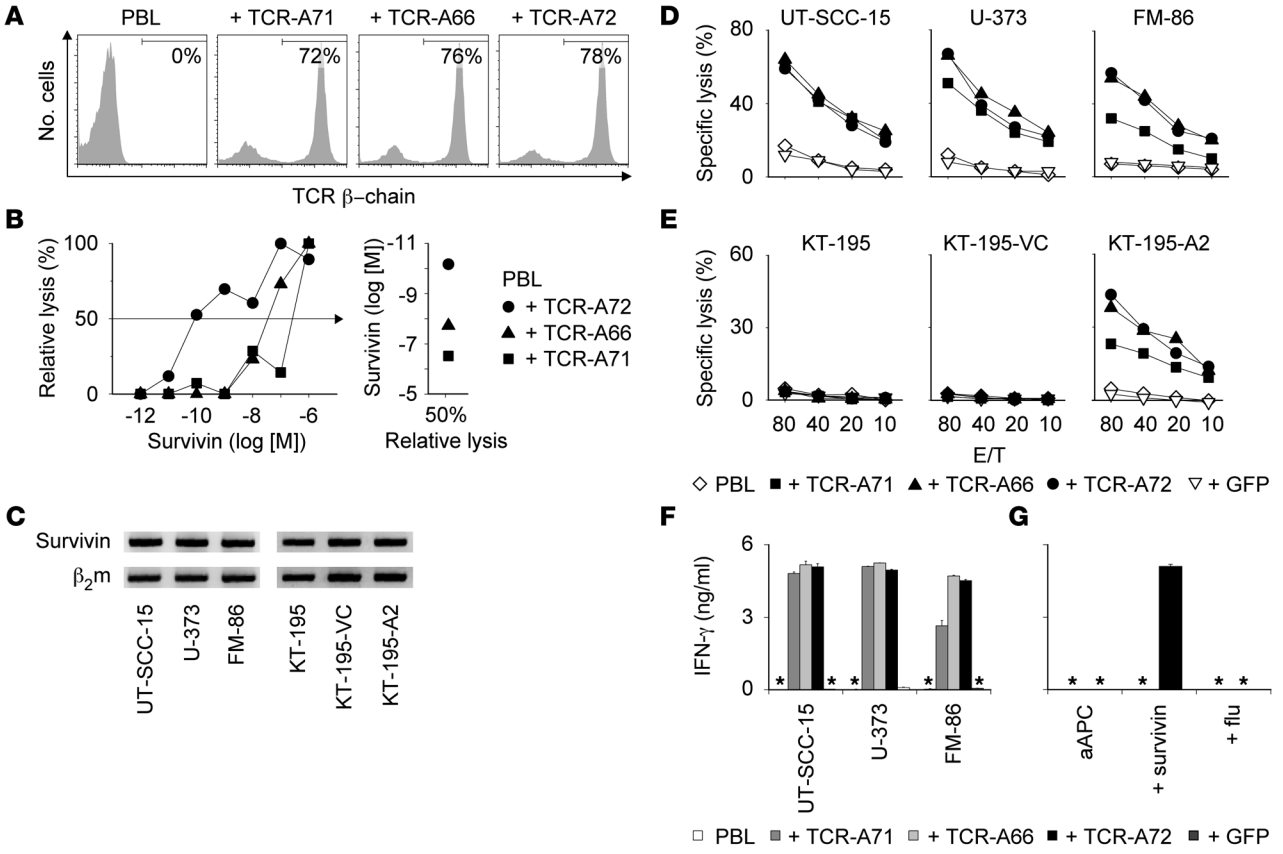

Figure 2

Redirection of antigen specificity by retroviral transfer of survivin-specific TCR genes. PBLs of a healthy HLA-A2$^-$ donors were transduced with TCR-A71, -A66, and -A72. (**A**) Unsorted TCR-transduced PBLs were analyzed on day 10 for Tg-TCR expression using murine TCRβ constant region–specific antibody. (**B**) The relative cytotoxicity is given for nonsorted TCR-transduced PBLs following stimulation with T2 cells loaded with graded amounts of survivin peptide at an E/T ratio of 20:1. Relative values of half-maximal killing are depicted at the right. (**C**) RNA expression of survivin in tumor cells was assessed by RT-PCR using survivin-specific primers and β_2-microglobulin–specific primers (β_2m) as a control. Survivin-specific cytotoxicity of TCR-modified PBLs was assessed in a standard 4-hour chromium release assay using different tumor cell lines at varying E/T ratios: (**D**) UT-SCC-15, U-373, and FM-86 (all: A2$^+$, S$^+$) and (**E**) KT-195, KT-195-VC (A2$^-$, S$^+$), KT-195-A2 (A2$^+$, S$^+$) as target cells. Untransduced PBLs and PBLs transduced with a GFP control vector served as controls. Cytotoxicity data represent means of duplicates measured at each E/T ratio or peptide concentration. (**F**) IFN-γ release at 24 hours is depicted following coculture with tumor cell lines at an E/T ratio of 2:1. (**G**) Unloaded or survivin or flu peptide–loaded aAPCs were cocultured with either untransduced PBLs or PBLs expressing TCR-A72 at an E/T ratio of 1:2. IFN-γ values for **F** and **G** are shown as mean of duplicates ± mean deviation. These experiments were done with 6 different donors, except for **B** and **G** (n = 2). Asterisk indicates values below the detection limit.

PBLs or TCR-modified PBLs from an HLA-A2$^-$ donor (Figure 3D). The accumulation of apoptotic cells was compared over time for HLA-A2$^-$ and HLA-A2$^+$ populations, containing T cells expressing survivin-specific Tg-TCRs (Figure 3E) or tyrosinase-specific Tg-TCR (Figure 3F), demonstrating that high-level apoptosis required the presence of T cells expressing survivin-specific Tg-TCRs and only occurred in HLA-A2$^+$ recipient lymphocyte populations. It should be noted that the differences between the percentages of T cells expressing a Tg-TCR and the percentages of cells undergoing apoptosis in HLA-A2$^+$ recipient PBLs revealed that death was not limited to T cells expressing survivin-specific Tg-TCRs. Thus, T cells bearing survivin-specific Tg-TCRs mediated fratricide against a substantial number of HLA-A2$^+$ lymphocytes lacking Tg-TCR expression.

Because the TCR-transgenic T cells were stimulated to achieve efficient expansion, we examined whether activated T cells could be directly killed by TCR-transduced PBLs (Figure 4A). After stimulation with either phytohemagglutinin (PHA) or a combination of CD3- and CD28-specific antibodies, activated HLA-A2$^-$ lymphocytes were not recognized by effector cells expressing Tg-TCR, even though they expressed high levels of survivin mRNA (Figure 4B). In contrast, unstimulated HLA-A2$^+$ lymphocytes were killed to a substantial degree by effector cells expressing survivin-specific Tg-TCR. Furthermore, killing increased after lymphocyte activation (Figure 4A), coinciding with increases in the basal level of survivin mRNA transcripts (Figure 4B). We also assessed whether cytotoxic T lymphocyte (CTL) clones could serve as targets for survivin-specific TCR-modified effector cells. CTLs derived from different HLA-A2$^+$ donors, with specificity for either tumor-associated peptides (A42, ref. 28; Tyr-F8, ref. 29) or an Epstein-Barr virus–derived ligand (FaLe) (D.J. Schendel, unpublished observations), were well recognized, whereas CTL clone JB4 (30), originating from an HLA-A2$^-$ donor, was not killed (Figure 4C). These CTL clones expressed survivin mRNA

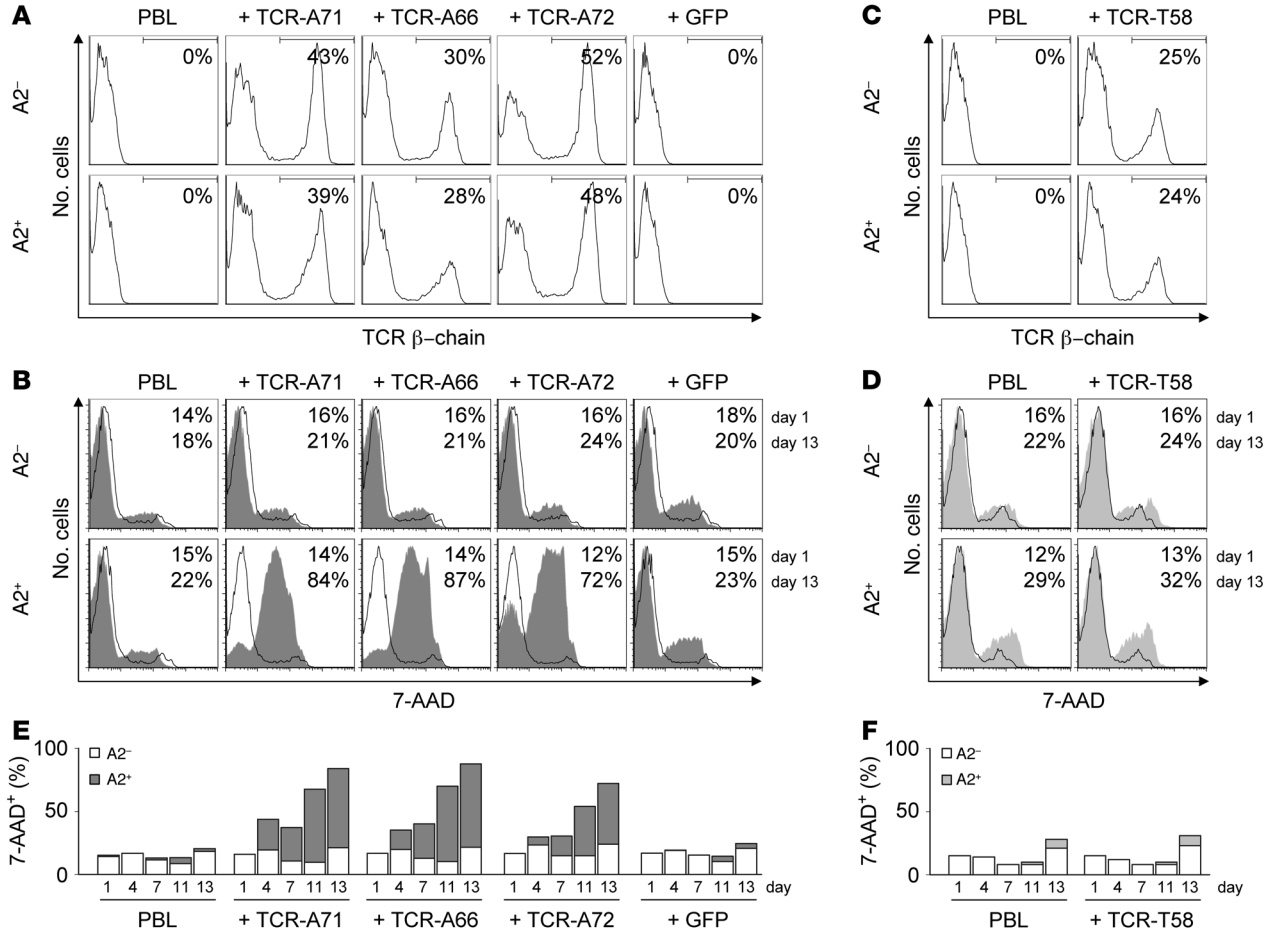

Figure 3

MHC-restricted fratricide of survivin-specific TCR-modified PBLs. HLA-A2⁻ and HLA-A2⁺ activated lymphocytes were transduced with the survivin-specific TCR-A71, -A66, and -A72. (**A**) Expression of Tg-TCRs was analyzed using murine TCRβ constant region–specific antibody at day 4 after TCR transduction. The numbers indicate the percentage of Tg-TCRβ chain expression. (**B**) TCR-modified PBLs were further cultured for 2 weeks and stained with 7-AAD to discriminate living and dead cells. The open histograms show staining 1 day after transduction; the filled gray histograms display the staining on day 13. The percentage of 7-AAD⁺ dead cells on days 1 and 13 is indicated in the upper-right corner. (**C** and **D**) The same analysis was made using PBLs transduced with a high-affinity TCR (T58) specific for a peptide derived from tyrosinase protein presented by HLA-A2 (25). Tg-TCRβ chain expression and percentage of apoptotic cells are shown as in **A** and **B**. Percentages of 7-AAD⁺ PBLs on days 1, 4, 7, 11, and 13 after TCR-transduction with (**E**) survivin- or (**F**) tyrosinase-specific TCR genes in activated lymphocytes of HLA-A2⁻ (white bars) and HLA-A2⁺ donors (gray bars). The data are representative of 2 independent experiments with 2 individual donors each.

(Figure 4D), albeit at variable levels. Two controls demonstrated the specificity of recognition. First, effector PBLs had to express a survivin-specific Tg-TCR, since GFP-transduced and untransduced PBLs did not mediate appreciable killing of target cells. Second, HLA-A2⁻ activated PBLs and HLA-A2⁻ CTLs were not killed by any effector population, demonstrating that TCR recognition was HLA-A2 restricted.

Activated T cells express several TAAs that might target them for fratricide. The wider impact of MHC-restricted fratricide was considered with respect to other TAAs, including several TAAs prioritized by the NCI Translational Research Working Group (5). Therefore, we analyzed mRNA levels in activated PBMCs and enriched CD8⁺ T cells (Figure 5) and considered two factors in this assessment. First, mRNA levels were compared in unstimulated versus stimulated T cells (CD3/CD28 activation) and expressed as x-fold increases (Figure 5, *x* axis). Second, transcript levels of each TAA in activated cells were normalized to 18S rRNA and expressed as crossing-point (CP) values, in order to demonstrate their overall prevalence with respect to each other (Figure 5, *y* axis). The CP value defined the cycle number in the logarithmic phase of the PCR, where the product was the same in all the samples that were compared; thus, low CP values revealed high levels of mRNA template, while high CP values indicated rare mRNA templates. Transcript levels of numerous TAAs increased upon activation of PBMCs and CD8⁺ T cells from around 10-fold to more than 10^7-fold when compared with unstimulated cells (Figure 5, right quadrants). As expected, TAA transcripts were expressed in activated cells at widely different levels, reflected by CP values ranging from 13 to 35 (Figure 5, *y* axis). Transcripts for several TAAs remained very low, with or without lymphocyte activation (CP, >30; Figure 5, bottom quadrants). Survivin transcripts displayed the greatest fold increase after lymphocyte stimulation (>10^7-fold) and were abundant (CP, 21). In contrast, tyrosinase transcripts did not increase upon activation and were very rare (CP, 35).

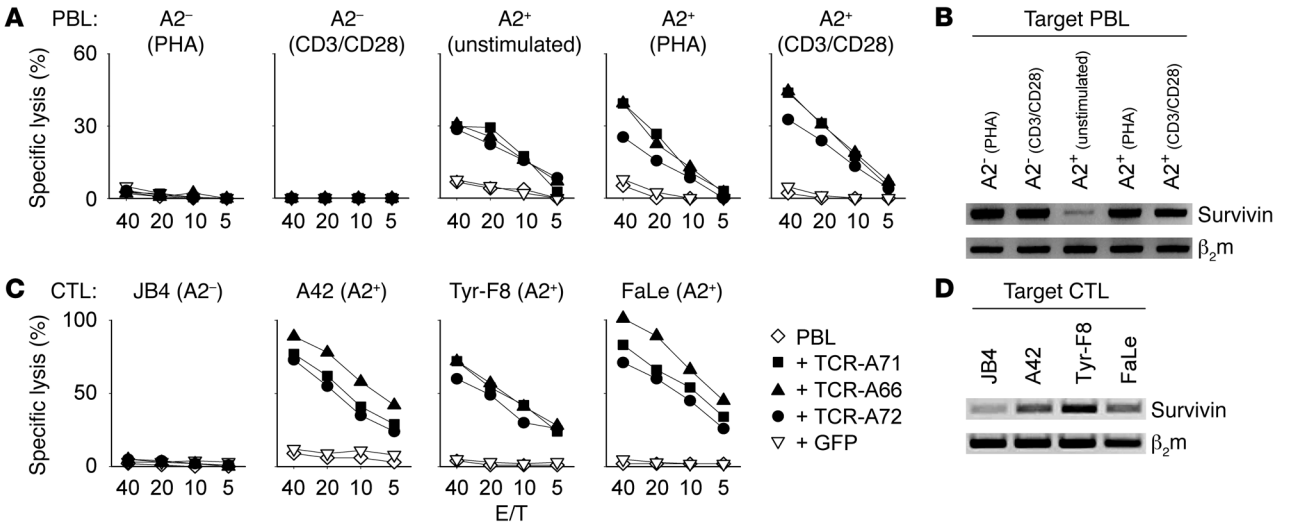

Figure 4

Cytotoxic activity of survivin-specific TCR-modified PBLs. The cytotoxicity of survivin-specific TCR-engineered PBLs using lymphocytes and T cell clones as target cells was determined in a standard 4-hour chromium release assay using varying E/T ratios, and results are presented as percent specific lysis. (**A**) Unstimulated or PHA- or CD3/CD28-activated lymphocytes of HLA-A2⁻ and HLA-A2⁺ donors were used as target cells at the given E/T ratios. (**B**) mRNA levels of survivin and β_2-microglobulin (β_2m) were analyzed by RT-PCR. (**C**) HLA-A2⁺ T cell clones with specificity for the TAA MART-1/melan-A (A42, ref. 28), tyrosinase (Tyr-F8, ref. 29), or Epstein Barr virus (FaLe) were assessed as target cells for TCR-modified effector cells. An alloreactive clone (JB4, ref. 30) derived from an HLA-A2⁻ donor served as a negative control. (**D**) Corresponding survivin mRNA levels of the T cell clones were determined and depicted as in **B**. Cytotoxicity data represent means of duplicates measured at each E/T ratio. These data are representative of 2–4 individual donors.

Therefore, the TAAs showing abundant mRNA levels (Figure 5, top quadrants) might also have the potential to generate targets for MHC-restricted fratricide.

Discussion

These studies revealed that transgenic expression of HLA-A2–restricted survivin-specific TCRs in activated PBLs led to massive apoptosis that was MHC restricted, since this only occurred in HLA-A2⁺ recipient lymphocytes. Expression of survivin in activated TCR-modified PBLs resulted in presentation of survivin-specific pMHC ligands and led to concurrent self-recognition and fratricide. MHC-restricted fratricide likely accounted primarily for our failure to expand TCR-engineered effector cells prepared using HLA-A2⁺ recipient lymphocytes, whereas expansion was readily achieved with HLA-A2⁻ recipient lymphocytes. Even though survivin-specific Tg-TCRs displayed excellent peptide sensitivities and good tumor cell recognition, which are important properties for selection of therapeutic Tg-TCRs, fratricide of HLA-A2⁺ recipient lymphocytes would preclude their use in TCR gene therapy, except in the clinical setting of HLA-A2–mismatched stem cell transplantation.

It has been reported previously that mouse T cells exposed to high levels of specific antigen can display anergy or even suicide through TCR-mediated induction of apoptosis (31, 32). Furthermore, suicide induced in human T cells by tumor cells expressing high levels of antigen altered antitumor immunity by eliminating high-affinity T cells (33). It is possible that TCR-induced suicide accounted for some of the apoptosis seen in the HLA-A2⁺ populations containing T cells expressing survivin-specific Tg-TCRs. However, the percentage of lymphocytes that underwent apoptosis was much higher than the fraction of T cells expressing a Tg-TCR; therefore, active fratricide clearly contributed to the death of Tg-TCR–negative lymphocytes. This contention was underlined by the demonstration that Tg-TCR effector cells had the capacity to directly kill both activated T cells and CTL clones of HLA-A2⁺ donors, irrespective of their particular TCR specificities.

Uncovering MHC-restricted fratricide helped to explain the loss of HLA-A2⁺ lymphocytes expressing survivin-specific Tg-TCRs over time and might also account for several other observations regarding survivin-specific T cells. After strong enrichment of T cells by survivin-multimer sorting and further culture, self-restricted T cell lines expressed only low percentages of CD8⁺ multimer⁺ T cells, whereas allorestricted lines retained high percentages of double-positive cells. Thus, it appears that expansion of survivin-specific self-restricted T cells was self-limited. We also noted early proliferation of self-restricted T cell clones in the majority of limiting dilution cultures, but T cell colonies showing robust proliferation over several weeks all proved to be nonspecific. We speculate that early clonal proliferation of survivin-specific clones may have occurred when low numbers of T cells were buffered by large numbers of feeder cells, but HLA-A2–restricted apoptosis may have hindered their later outgrowth. Because HLA-A2⁻ cells could not display the corresponding pMHC ligands, they were resistant to HLA-A2–restricted fratricide, and numerous HLA-A2–allorestricted survivin-specific T cell clones could be isolated. These results appear to be concordant with previous studies that described HLA-A2–restricted survivin-specific T cells that were propagated as T cell lines in vitro or detected in peripheral blood samples of cancer patients ex vivo, whereas survivin-specific T cell clones were difficult to obtain (9–18, 34).

Recently, one survivin-specific CTL clone that was isolated from an HLA-A2⁺ breast cancer patient recognized the same pMHC ligand as the Tg-TCR described here (18). This patient-

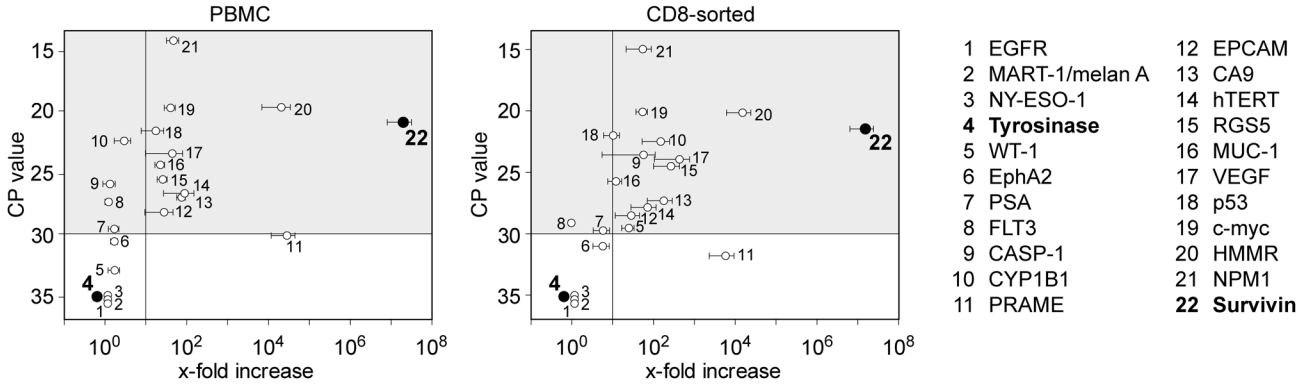

Figure 5

Analysis of mRNA levels for TAAs in activated T cells. Quantitative mRNA expression of 22 TAAs was performed using activated lymphocytes of 4 healthy donors using LightCycler technology, and the values are given as mean with SEM. For each donor, the TAA expression profile of nonactivated PBMCs and nonactivated enriched $CD8^+$ T cells was compared with that of CD3/CD28-activated PBMCs and $CD8^+$ T cells. Differences in mRNA levels detected in unstimulated versus stimulated cell populations were expressed as x-fold increases and are depicted on the *x* axis. The *y* axis represents the CP values, in order to demonstrate the overall prevalence of TAAs in activated cells. The CP value defines the cycle number in the logarithmic phase of the PCR where the product is the same in all the samples that are compared. Low CP values represent high levels of mRNA template, while high CP values indicate rare mRNA templates. An mRNA template with a CP value of less than 30 is considered rare. The housekeeping gene *18S* rRNA was processed as an internal control for normalization of samples. The data were statistically analyzed as described in Methods.

derived CTL clone was shown to recognize all $HLA\text{-}A2^+$ $survivin^+$ tumor cell lines in a small panel, with the exception of the FM-86 cell line. The authors surmised that FM-86 cells were not recognized due to disturbed pMHC ligand expression, since the tumor cells were found to have high levels of survivin mRNA. As shown here, this tumor cell line expresses relatively low levels of surface HLA-A2. We included FM-86 cells in our analyses and found that these tumor cells were recognized by effector cells transduced with each of the 3 Tg-TCRs; however, killing was less with effector cells expressing TCR-A71, the Tg-TCR that endowed the PBLs with the lowest functional avidity. The failure of the published patient CTL clone to kill FM-86 cells would be explained if it had a functional avidity decidedly lower than that of PBLs expressing TCR-A71. In addition, our Tg-TCRs were codon-optimized and modified to express murine constant regions, which imbued them with good surface expression and strong capacity to interact with tumor cells expressing low levels of pMHC ligand. Furthermore, it has been reported that TCR/CD3 expression or TCR signaling is frequently disturbed in patient-derived T cells, thereby hindering their capacity to recognize tumor cells. Such alterations may also have impacted the capacity of the patient-derived CTLs to recognize FM-86 tumor cells displaying low pMHC ligand density.

Because our survivin-specific TCRs were well expressed as transgenic proteins in activated recipient lymphocytes of $HLA\text{-}A2^-$ healthy donors, we could bypass deficits that impinge on expression, signaling, or function of patient-derived CTL clones. The 3 Tg-TCRs effected wide differences in functional avidity in recipient lymphocytes, varying by more than 4 orders of magnitude in peptide sensitivity. Based on analysis of numerous T cell clones (ref. 25 and D.J. Schendel, unpublished observations), we would classify TCR-A71 as having a relatively low affinity, whereas TCR-A72 had a very high affinity. Effector PBLs expressing TCR-A71 showed reduced recognition of FM-86 and KT-195-A2 tumor cells, which expressed the lowest levels of surface HLA-A2, indicating a relationship between T cell functional avidity and pMHC ligand density in efficacy of tumor cell recognition. It should be noted, however, that a correlation could not be drawn with respect to levels of survivin mRNA, since these tumor lines both showed high levels of survivin transcripts.

While our studies identified fratricide that was restricted by HLA-A2, it is also possible that T cells with adequate avidity could recognize additional survivin-derived peptides presented by other MHC molecules, leading to self-restricted fratricide even in $HLA\text{-}A2^-$ donors. The frequent failure to obtain self-restricted T cell clones specific for some self-peptides is often interpreted to be a consequence of deletional tolerance. Based on the results presented here, additional studies are warranted to explore the role of MHC-restricted fratricide in controlling the development of T cells specific for proteins that are well expressed in activated lymphocytes. The authors of two reports speculated that fratricide may have inhibited effective expression of a murine TCR specific for p53 in activated human lymphocytes (35) or limited expansion of T cells specific for hTERT (36), although direct experimental evidence of fratricide was not provided in these studies. On the other hand, other technical limitations could influence the expansion and isolation of such T cells.

The quantification of mRNA indicated that several other TAAs could potentially become targets for T cell–mediated fratricide, based on their high levels of expression in activated lymphocytes. In contrast, transcripts that were very rare, even upon T cell activation, would be less likely to generate pMHC ligands for self-restricted fratricide. This contention is supported by the failure of the high-affinity tyrosinase-specific TCR-T58 to induce widespread apoptosis in $HLA\text{-}A2^+$ lymphocytes. TCR-mediated fratricide specific for any TAA will be dependent on several factors, including protein expression, location, and turnover, as well as antigen processing and presentation of specific peptides by self-MHC molecules. Nevertheless, it would seem prudent to include analysis of RNA and protein expression in activated lymphocytes

as additional criteria in the selection process of candidate TAAs for development of TCR gene therapy.

MHC-restricted fratricide may also have consequences for tumor vaccine development, since this same mechanism could limit proliferation of high-avidity T cells in lymph nodes after vaccination with survivin or other TAAs that are expressed in lymphocytes (37). Some T cells might escape to the periphery, but substantial accumulation at tumor sites might again be self-limiting because of fratricide, thereby impacting clinical efficacy. Furthermore, MHC-restricted fratricide could have a local spreading effect, causing neighboring T cells with unrelated specificities to be eliminated due to presentation of target pMHC ligands, irrespective of their own TCR specificities. This contention is supported by our observation of high sensitivity of activated lymphocytes to killing by survivin-specific TCR-transduced PBLs, as well as the recognition of T cell clones of various specificities derived from HLA-A2$^+$ but not HLA-A2$^-$ donors.

Striving to attain effective antitumor immunity using TCR-transgenic effector T cells with high avidity might have the unintended consequence of causing MHC-restricted fratricide of other adaptive immune cells, if the TCR displays specificity for a pMHC ligand that is well expressed by activated lymphocytes. Our results emphasize that judicious selection of TAAs will be important for designing successful TCR gene therapies.

Methods

Cells. The cell lines Mel-624.38 (HLA-A2$^+$ [A2$^+$], survivin$^+$ [S$^+$]) (38), Mel-1379 (A2$^-$, S$^+$; M. Panelli, University of Pittsburgh, Pittsburgh, Pennsylvania, USA), UT-SCC-15 (A2$^+$, S$^+$; M. Schmitz, Technische Universität Dresden, Dresden, Germany), U-373 (A2$^+$, S$^+$; P.J. Nelson, Ludwig-Maximilians-Universität, Munich, Germany), KT-195 (A2$^-$, S$^+$; H. Gröner, Deutsche Krebsforschungszentrum, Heidelberg, Germany), and T2 (CRL-1992, ATCC) were cultured as described previously (25). KT-195-VC and KT-195-A2 are transfectants of KT-195, generated by transduction with retroviral vectors encoding GFP (MP71-iG) or HLA-A*0201 and GFP (MP71-A2iG). FM-86 cells were purchased from European Searchable Tumor Cell Bank and Database (University Tübingen, Tübingen, Germany) and cultured as described in ref. 39. The T cell clones A42 (28), FaLe (EBV-specific, generated in our facility using autologous B-LCL), JB4 (30), and Tyr-F8 (29) were cultured as described (30) and used as target cells 6 days after restimulation.

Generation of survivin peptide–specific T cells with RNA-pulsed DCs. The collection of blood and patient material was approved by the "Ethikkommission der Medizinischen Fakultät der Ludwig-Maximilians-Universität," Munich and donors gave informed consent. De novo priming of self-restricted and allorestricted peptide–specific T cells was performed using mature DCs, electroporated with 50 μg of survivin ivt-RNA, with or without 50 μg HLA-A2 ivt-RNA. The linearized plasmids pGEM4Z/survivin/A64 and pCDM8-HLA-A2 (E. Weib, Ludwig-Maximilians-Universität, Munich, Germany) were used as templates for RNA transcription. Survivin peptide–specific T cells were sorted using an HLA-A2-survivin$_{96–104[97L]}$ pentamer (survivin-multimer, ProImmune) and either expanded as bulk T cell lines or cloned in limiting dilution cultures (25).

Retroviral TCR gene transfer. TCR sequences of survivin-specific clones A71, A66, and A72 were determined, and TCRβ-2A-TCRα transgene cassettes were synthesized (GENEART) and integrated into MP71-PRE as previously described (25). To enhance surface expression, TCR constant regions were exchanged by their mouse counterparts (26) and transgene cassettes were codon optimized (27). Vector plasmids were used for production of retroviral particles and subsequent transduction of T cells (40).

FACS. Anti-CD8, anti–mouse TCRβ antibody detecting the mouse constant region of transgenic TCRβ (BD Biosciences — Pharmingen), anti–HLA-A2 antibody (AbD Serotec), survivin-multimer, and HLA-A2-CMVpp65$_{495–503}$ control multimer (CMV-multimer, D. Busch, Technische Universität, Munich, Germany) were used for analysis or sorting. Viability of TCR-modified PBLs was determined by incubation with 7-AAD (BD Biosciences — Pharmingen).

Cytotoxicity and IFN-γ release assays. Cytotoxic activities of bulk T cell lines, T cell clones, and TCR-modified PBLs were analyzed in standard 4-hour chromium release assays (25). For initial analysis at day 13 after the second unspecific stimulation, T cells were incubated with 1.5×10^3 survivin$_{96–104[97L]}$ (survivin, LMLGEFLKL) or influenza matrix protein$_{58–66}$ (flu, GILGFVFTL) peptide–loaded T2 cells (peptides: Metabion) as previously published (25). Bulk T cell lines were cocultured at various effector to target cell ratios (E/T ratios). For further analysis, allorestricted clones A71, A66, and A72 were analyzed by coculture either with 1×10^3 tumor cells (Mel-1379, Mel-624.38) or with flu peptide–loaded T2 cells (E/T, 10:1). Functional avidity of T cell clones and TCR-modified PBLs was determined by incubation with 1×10^3 survivin peptide–loaded T2 cells (10^{-12} to 10^{-5} M) at an E/T ratio of 10:1 for the T cell clones and 20:1 for TCR-modified PBLs. TCR-modified PBLs were cocultured with tumor cell lines, target PBLs or T cell clones using 2×10^3 target cells at the designated E/T. Specific, relative, and half-maximal lysis was calculated as described using duplicate samples at each E/T ratio or peptide concentration (25). Target PBL cultures were used directly after isolation or stimulated for 3 days using 100 IU/ml IL-2 (Chiron) and 5 μg/ml PHA (Roche) or anti-CD3, -CD28 antibody (40). TCR-modified PBLs (5×10^4) were incubated with given tumor cells at an E/T ratio of 2:1, and 24-hour supernatants were assessed by ELISA (BD Biosciences). aAPCs were generated as described previously (41), and 1×10^6 aAPCs were used either unloaded or loaded with 10^{-5} M survivin or flu peptide in cocultures with TCR-A72–transduced or unmodified PBLs at an E/T ratio of 1:2.

RT-PCR. Isolation of full-length RNA, cDNA synthesis, and PCR amplification of survivin and β_2-microglobulin sequences were performed as previously described (12, 40).

Real-time PCR for quantification of TAAs. To evaluate quantitative mRNA expression of TAAs, cryopreserved PBMCs of 2 donors as well as freshly drawn PBMCs of 2 donors were analyzed. For each donor, the TAA expression profile of nonactivated PBMCs and enriched CD8$^+$ T cells (CD8$^+$ T cell Isolation Kit II, Miltenyi Biotec) was compared with that of activated PBMCs and CD8$^+$ T cells. Activation of cells was performed as described above. Total RNA was extracted (TriReagent, Biozol), and equal RNA amounts were reverse transcribed using oligo(dT)$_{15}$ primer and AMV reverse transcriptase (First Strand cDNA Synthesis Kit for RT-PCR, Roche). Detection of TAA expression was performed using the LightCycler PCR Master Mix (Roche). Primers used for quantitative RT-PCR are listed in Supplemental Table 2. PCR amplification was performed with initial 10 minutes denaturation at 95°C, 35 cycles of amplification with 1 second at 95°C, 10 seconds at 56°C, and 25 seconds at 72°C except for HMMR (using 59°C as annealing temperature) and hTERT, survivin, tyrosinase (using a kit from Search-LC). Evaluation of results was done by directly plotting CP values (normalized by the housekeeping gene *18S* rRNA) as shown in Figure 5, as well as by conversion of the CPs into relative concentration of transcripts. Transcript levels obtained from nonactivated cells were set as 1 to determine an x-fold increase or decrease in transcripts in activated PBLs and CD8$^+$ T cells.

Statistics. The basis for data analysis for the different assays displayed in Figures 1–4 is provided in the individual figure legends. The values shown in Figure 5 represent mean values of transcript levels measured in cells of 4 donors, repeated in 2 independent experiments, with the exceptions that PSA was only measured in 3 donors and c-kit in only 2 donors.

The error bars represent SEM. The means and SEM were calculated using GraphPad Prism Software.

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T cell receptor-transgenic lymphocytes specific for HMMR/Rhamm limit tumor outgrowth *in vivo*

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Running Title: Adoptive T cell transfer targeting HMMR/Rhamm

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Abstract

Hyaluronan-mediated motility receptor (HMMR/Rhamm) is overexpressed in numerous tumor types, including acute myeloid leukemia (AML). Several studies have reported the existence of T cell responses directed against HMMR in AML patients, linked to better clinical outcome of disease. Therefore, we explored the use of HMMR-specific T cell receptors (TCR) for transgenic expression in lymphocytes and their *in vivo* impact on solid as well as disseminated tumors. We obtained TCR via an *in vitro* priming approach, in combination with a CD137-mediated enrichment step. Comparison of recipient lymphocytes expressing transgenic TCR (tgTCR) revealed a similar tumor recognition pattern as seen for the original T cells. Adoptive transfer experiments using a humanized xenograft mouse model resulted in significantly retarded tumor outgrowth, which could be further enhanced using IL-7- as well as IL-15-induced memory lymphocytes as tgTCR recipient cells. A combination of IL-7/IL-15-induced memory T cells showed an enhanced potency to retard the outgrowth of disseminated tumor cells, while CD8-enriched memory T cells had the ability to completely inhibit tumor growth. These findings show that transgenic expression of this TCR in memory-like $CD8^+$ recipient T cells is a potent therapeutic agent for adoptive T cell therapy of AML as well as HMMR-expressing solid tumors.

Introduction

Acute myeloid leukemia (AML) is a fast-progressing disease with an increasing incidence in elderly patients, limiting use of aggressive therapies. The most common treatments include risk-adapted polychemotherapy regimens (1, 2), 5-aza-2-deoxycytidine (3) or stem cell transplantation (SCT) (4, 5). The persistence of resistant tumor cells leads to relapse in a high percentage of patients. Immunotherapies, including adoptive T cell transfer, provide an alternative approach to eliminate residual leukemia. Consistent with adoptive transfer of tumor-infiltrating lymphocytes (TIL) (6) or genetically-modified PBL expressing chimeric antigen receptors (CAR) (7), adoptive transfer of patient-derived lymphocytes expressing transgenic antigen-specific T cell receptors (tgTCR) has the potential to target solid as well as disseminated tumors. The latter immunotherapy allows MHC-restricted, antigen-specific TCR to be isolated beforehand and later used to modify lymphocytes in a patient-specific manner, taking into account MHC allotypes and antigen profiles of the tumor. Strategies to isolate high-affinity TCR, restricted by common MHC alleles with specificity for broadly expressed tumor-associated antigens (TAA) have been described (8-11), however use of humanized mouse models to verify the quality of TCR-transgenic lymphocyte responses *in vivo* lag in development.

In addition to well-known leukemia-associated antigens, like survivin, Bcl-2 and PRAME, hyaluronan-mediated motility receptor (HMMR/Rhamm) represents a potential target for tgTCR therapy of AML. HMMR is highly expressed during embryogenesis and neural crest formation, while in adults expression is limited to testis, placenta, thymus, tonsils and bone marrow (12, 13). Moreover, it is broadly expressed in numerous types of tumors, including breast cancer, melanoma and leukemia. Several studies analyzing antigen-specific immune responses in AML

patients revealed that prolonged survival correlated with the prevalence of HMMR-specific T cells (14-17). Use of HMMR-derived peptides for antitumor vaccination resulted in a strong HMMR-specific immune response but failed to cure disease (18-20). This discrepancy between induction of peptide-specific immune responses and failure to eradicate tumor cells is partly accounted for the existence of only low-avidity T cells due to negative selection. Transfer of T cells expressing allorestricted high-affinity TCR could overcome this limitation.

In this study, we describe the development of designer lymphocytes expressing tgTCR specific for HMMR, starting with generation of allorestricted HMMR-specific T cells through to the assessment of TCR-transduced PBL *in vivo* in a humanized mouse model. We used an MHC multimer-independent method to isolate high numbers of HLA-A2 allorestricted HMMR-specific T cells using a CD137-based enrichment step (21, 22) and selected the TCR from one HMMR-specific clone to make TCR-transgenic PBL. First we characterized the function of TCR-transduced lymphocytes *in vitro* and confirmed that they had the same specificity as the original clone. Then TCR-transgenic lymphocytes were assessed for their capacity to infiltrate and retard solid tumor growth in a NOD/scid IL2Rg^{null} (NSG) mouse model. Initial studies directed us further to optimize the phenotype of TCR-transgenic lymphocytes used for adoptive transfer. By inducing an IL-7/IL-15-dependent memory phenotype within the TCR-transduced populations, we improved the impact on tumor killing *in vitro* and tumor outgrowth *in vivo*. To further characterize the role of optimized TCR-transgenic lymphocytes on disseminated human tumor cells, we successfully developed a luciferase-based tracking model to assess tumor load *in vivo*. In the settings of both solid tumor and disseminated leukemia, we showed that a single injection of TCR-transgenic HMMR-specific lymphocytes led to significant reduction

of tumor burden. These findings support the further pursuit of adoptive cell therapy using HMMR-specific TCR-transgenic lymphocytes.

Results

Induction of HMMR-specific T cells and enrichment via CD137

We used our established *in vitro* dendritic cell (DC) priming approach to generate human allorestricted HMMR-specific T cells as a source of high-affinity TCR (8, 9). Mature DC (mDC) of an HLA-A2-negative donor were simultaneously loaded with *ivt*-RNA encoding HLA-A2 and HMMR, as the MHC allotype and TAA of interest. *In vitro* priming of autologous CD8-enriched T cells was initiated on day 0 using RNA-loaded autologous mDC at a 10:1 ratio, as described previously (9). Primed cultures were restimulated once on day 7 using mDC prepared in the same manner (Figure S1A). After a 14-day resting phase, the bulk cultures were reactivated with RNA-loaded mDC that had been previously cryopreserved and T cells were subsequently stained with CD137-specific monoclonal antibody. After stimulation, 35.9% of the primed cells were CD137 positive. They were subjected to magnetic bead sorting, yielding a purity of 83.0% $CD137^+$ cells (Figure S1B). T cell clones were generated via limiting dilution directly after enrichment and restimulated every two weeks, as previously described (9).

Following a 4-week culture period, emerging T cell clones were analyzed in a standard chromium-release assay using K562-A2 cells as positive target cells ($HLA\text{-}A2^+$, $HMMR^+$) (Figure S2A, 2B) and T2 cells pulsed with an irrelevant peptide (flu) as negative controls ($HLA\text{-}A2^+$, $HMMR^-$). The isolated T cell clones could be assigned to one of three groups showing different patterns of reactivity: no reactivity for either target cell, HLA-A2 alloreactivity for both target cells and exclusive HLA-A2 allorestricted HMMR-specific killing of K562-A2 cells (Figure 1A). Of 152 clones analyzed, the HMMR-reactive clones represented the highest fraction (52%), followed by 25% HLA-A2-alloreactive and 23% non-reactive T cell clones (Figure

1C). We further analyzed the potentially HMMR-specific T cell clones for their capacity to kill an additional HLA-A2$^+$, HMMR$^+$ tumor cell (THP-1) (Figure S2A, S2B) and to secrete IFN-γ in response to tumor stimulation (Figure 1B). Additionally, we screened them for capacity to recognize one defined immunogenic epitope of HMMR (HMMR-R3; ILS), which was shown to be recognized by tumor-infiltrating T cells in AML patients (19). All of these T cell clones recognized K562-A2 and THP-1 to varying degrees. Lysis of ILS peptide-loaded T2 cells by clones 17, 61, 78 and 121 indicated potential recognition of this HMMR-nonamer, but this was not seen with IFN-γ secretion, failing to confirm that it was the particular epitope seen by these T cell clones. HMMR-reactive clones that recognized K562-A2 and THP-1 cells but not flu-pulsed T2 cells were further analyzed for secretion of several T helper-1 (Th1) cytokines (IL-2, IFN-γ, MIP-1α and TNF-α) that have been linked with poly-functionality of high-avidity T cells (Schendel, unpublished observations) (23). Based on analysis of 14 well-growing clones, 64% (9 clones) secreted all four cytokines, while 36% (5 clones) secreted three cytokines in response to both HLA-A2$^+$, HMMR$^+$ tumor lines (THP-1 and K562-A2), but not to flu-pulsed T2 cells as a negative control (Figure 1D).

Characterization of HMMR-reactive clones 67 and 150

Our further analyses concentrated on CTL67 and CTL150 which expressed different TCR sequences (data not shown). CTL150 discriminated quantitatively between THP-1 and K562-A2 tumor cells, whereas CTL67 did not. While CTL67 was less cytotoxic in recognition for K562-A2, it showed higher secretion of IFN-γ after exposure to tumor cells (Figure 1E, 1F).

In the next set of experiments, we determined whether expression of these TCR as transgenes in activated peripheral blood lymphocytes (PBL) could equip effector cells with specificity comparable to the original T cell clones. The TCR sequences of CTL67 and 150 were isolated, codon optimized and their constant regions exchanged for the murine counterparts, as described (24). Following retroviral transduction of recipient lymphocytes, we were only able to detect expression of TCR150, whereas expression of TCR67 failed in transduced lymphocytes (Figure S3A).

Tumor specificity of transgenic PBL expressing TCR150

Expression of TCR150 was demonstrated on $CD4^+$ as well as $CD8^+$ cells (Figure 2A), as detected by staining of the murine constant region of the tgTCR, as described (24). The TCR-transgenic PBL were tested for tumor recognition measuring IFN-γ secretion after stimulation with K562-A2 and THP-1 tumor cells (Figure 2B) and high levels of IFN-γ were detected. This was dependent on tgTCR expression because mock-transduced PBL and PBL transduced with a GFP control vector were not induced to secrete IFN-γ following stimulation with these tumor cells. HMMR specificity was shown by absence of IFN-γ responses by TCR150-transgenic PBL stimulated with flu-pulsed T2 cells. Interestingly, the TCR-transgenic PBL displayed the quantitative differences in recognition of THP-1 and K562-A2 seen with the original clone.

The TCR150-transgenic PBL were also analyzed for cytotoxic potential. They were compared with mock control PBL and PBL transduced with a second TCR (TCR-A72) with specificity for an epitope of the anti-apoptotic protein survivin that was also expressed by all tumor cell lines (data not shown). Both THP-1 and K562-

A2 cells were recognized by both TCR-trangenic populations, albeit only weak responses were seen against THP-1 (Figure 2C). Because the levels of cytotoxicity were low, probably due to the low percentage of $CD8^+$ cells expressing the tgTCR, we enriched TCR-transgenic cells using magnetic bead isolation of cells expressing the murine constant region of the tgTCR. Enrichment yielded more than 80% TCR-positive cells (Figure S3B and not shown) but also resulted in a significant fraction of dead cells. This may have been caused by activation-induced cell death, mediated by antibody binding to the tgTCR. Nevertheless, the cytotoxic capacity of the viable enriched TCR150- and TCR-A72-transgenic PBL was greatly increased, demonstrating the tgTCR-dependent killing of both tumor cell lines. The failure of HLA-$A2^-$, $HMMR^+$ K562 cells to be recognized by either population demonstrated the HLA-A2-restriction of both tgTCR.

To confirm that recognition by TCR150 was HMMR-specific, we used a lentiviral vector to introduce shRNA (short hairpin RNA) specific for HMMR into HMMR-expressing target cells. We first analyzed the efficacy of HMMR knock-down in $HMMR^+$ tumor lines by measuring intracellular staining for HMMR protein in comparison to the HLA-$A2^+$ human breast cancer cell line MCF-7, which is known to be $HMMR^{low}$ (Figure S2A, S2B). The $HMMR^+$ tumor cell lines, THP-1 and mel624.38, showed a strong reduction of HMMR expression (Figure S2C). Therefore this shRNA was suitable for knock-down of HMMR in target cells.

The parental tumor cell lines, THP-1 and mel624.38 (HLA-$A2^+$, $HMMR^+$), were recognized by TCR150-transgenic PBL to different degrees (Figure 2D). Following introduction of shRNA, the treated tumor cells were no longer recognized by TCR150-transgenic cells. The $HMMR^{low}$ MCF-7 cells also were not recognized by TCR150-transduced PBL. As a control we showed that all three tumor lines were

recognized by an HLA-A2 alloreactive CTL and this killing was not altered by knock-down of HMMR (data not shown). In addition we could show that the shRNA-treated and the parental melanoma cell lines were recognized to similar degrees by a MART-1-specific CTL (Figure S2).

The shRNA knock-down of HMMR with subsequent loss of recognition confirmed that TCR150 was specific for HMMR. Since none of the clones, including CTL150, recognized the HMMR-R3 epitope (ILS), we moved towards identifying the epitope using an HMMR gene transfection approach: mDC from an HLA-A2-negative donor were loaded with *ivt*-RNA encoding HLA-A2, together with *ivt*-RNA encoding full length HMMR (1-725) or with an *ivt*-RNA deletion mutant encoding the first 170 amino acids of HMMR. TCR-transgenic PBL expressing a tyrosinase-specific TCR (T58) were used as an effector control (9). As depicted in Figure 2E, TCR150-transgenic PBL recognized DC expressing full length HMMR as well as DC loaded with the deletion mutant, revealing that the targeted epitope is within the first 170 amino acids of the HMMR protein. The stimulation of TCR-transgenic PBL with T2 cells pulsed with several synthetic peptides that bind to HLA-A2 molecules did not lead to specific responses (data not shown). Further studies will be required to determine the peptide-epitope for TCR150.

Targeting of solid tumors *in vivo* using TCR150-transgenic PBL

To characterize the function of TCR150-transgenic lymphocytes *in vivo*, we utilized adoptive transfer of TCR-transgenic PBL into NSG mice, which lack murine T cells, B cells and NK cells (25). The effect of TCR150-transgenic PBL on growth of a solid tumor mass was assessed following subcutaneous injection of $1x10^6$ THP-1 cells in matrigel in the right flank. Human TCR150-transgenic PBL were injected i.v. 24 hours

later. In preliminary experiments, tumor growth in a non-treated control group was first detected around day 14 after tumor inoculation (data not shown). Therefore, in the adoptive transfer experiments, growth was measured on a day-to-day basis from this time point. The impact of injected TCR150-transgenic or mock control PBL was assessed using $2x10^5$ (Figure 3A and 3C) or $5x10^5$ (Figure 3B and 3D) TCR^+ lymphocytes, providing an E:T of 1:5 or 1:2, with the starting tumor cell number. The TCR-transduction provided 10 - 20% TCR-positive PBL in all adoptive transfer experiments and the numbers of mock-treated PBL matched the highest number of TCR150-transduced lymphocytes injected. PBL transduced with a tyrosinase-specific TCR (T58) and mock-treated control cells had no impact on THP-1 tumor outgrowth, consistent with the tumor being tyrosinase-negative. In contrast, injection of $2x10^5$ TCR150-transgenic lymphocytes reduced tumor growth to a significant extent ($p< 0.001$) (Figure 3A). Injection of the higher dose ($5x10^5$) of TCR150-transduced lymphocytes on day 1 significantly retarded tumor growth ($p<0.001$) but not more than $2x10^5$ cells (Figure 3B). Injection of a second dose of TCR150-transgenic lymphocytes ($2x10^5$) on day 14 into mice initially given the lower dose (data not shown), did not lead to further reduction in tumor size. The overall survival of mice was prolonged by 8 days in the TCR150-treated group receiving a single dose of $2x10^5$ cells, whereas the T58-treated mice showed identical mean survival times as the mock-treated control group (Figure 3C). A single injection of $5x10^5$ TCR150-transgenic lymphocytes led to longer survival of individual mice, compared to those given $2x10^5$ TCR150-PBL (Figure 3D).

The discrepancy between reduction in tumor size and survival might be explained by infiltration of lymphocytes adding to tumor volume. Immunohistochemical staining of excised tumors demonstrated the presence of

human T cells exclusively in mice treated with TCR150-transgenic lymphocytes, as evidence for TCR-mediated migration to the tumor site (data not shown). It was not possible to detect significant numbers of TCR150-expressing cells in either peripheral blood or spleens of these mice (data not shown).

Modulation of TCR-transgenic lymphocytes enhances tumor control

To prolong survival of TCR-transgenic lymphocytes *in vivo*, we attempted to induce different memory T cell phenotypes in the transduced lymphocyte populations. We initiated standard cultures with IL-2 and then changed the cytokine milieu of the PBL five days after TCR transduction, as indicated in Table 1. We determined the phenotypes of the TCR-transduced lymphocytes on day 5, before addition of new cytokines, as well as on day 13 before adoptive transfer into mice. Analysis of CD62L-expression levels was performed after pre-gating on $CD8^+/CD45RA^-$ cells, allowing discrimination between central memory (T_{CM}) ($CD62L^+$) and effector memory (T_{EM}) ($CD62L^-$) phenotypes (Figure 4A). TCR-transduced PBL showed an equal division into CM and EM phenotypes on day 5, which was preserved on day 13 when the cells were cultured under either IL-7 or Tc17-conditions. TCR-transduced PBL cultured further in the presence of only IL-2 or with IL-15 led to a decrease in the T_{CM} fraction on day 13. IL-15 culture conditions yielded cells on day 13 with an induced non-differentiated T_{EM} status ($CD28^+$, $CD27^+$) (data not shown). This phenotype was also observed for EM lymphocytes cultured under IL-7 and Tc17 conditions (data not shown).

To assess further differences in the cytokine-induced phenotypes, we performed intracellular staining (ICS) for IFN-γ and TNF-α in the four populations of TCR-transduced lymphocytes cultured for 13 days (Figure 4A). The four populations

were cultured with or without THP-1 cells for 6 h and analyzed for intracellular cytokines, in combination with lymphocyte subset surface markers. Increased amounts of intracellular cytokines were detected only in $CD8^+$ T cells after stimulation with tumor cells (Figure 4B and data not shown). This indicated that TCR150-transgenic PBL responded in a CD8-dependent fashion to antigen stimulation. Moreover, similar proportions of double-positive (IFN-γ^+, TNF-α^+) cells were found in TCR-transduced lymphocytes cultured under IL-2, IL-7 or IL-15 conditions. In contrast TCR-transduced PBL cultured with Tc17-conditions yielded at least a two-fold higher percentage of IFN-γ^+/TNF-α^+ double-positive T cells. Mock-control PBL served as a background control and showed no differences in percentages of cytokine double-positive cells after tumor stimulation.

The TCR-transgenic lymphocyte subsets were assessed for their *in vitro* cytotoxicity against THP-1 and mel624.38 target cells (Figure 4C), while K562 cells served as a negative control (data not shown). Both tumor cell lines were recognized by TCR150-transgenic PBL, whereas TCR-T58-dependent killing was restricted to the tyrosinase$^+$ melanoma cell line. TCR-transgenic PBL tested on day 5 after transduction resulted in an increased specific lysis of target cells, when compared to TCR-transgenic PBL cultured over 13 days only in the presence of IL-2. Cytotoxic capacity was not altered when TCR-transgenic PBL were cultured under Tc17 conditions and only slightly increased when TCR-transgenic PBL were exposed to IL-7 conditions. Interestingly, TCR-transgenic PBL cultured in the presence of IL-15 showed a strongly increased killing capacity for both tumor cells, compared with all other TCR-transgenic PBL populations.

To assess specific tumor recognition of the TCR-transgenic PBL cultured under different conditions in adoptive transfer experiments, $1x10^6$ THP-1 (Figure 5A)

or $4x10^5$ mel624.38 (Figure 5C) cells were injected s.c. into NSG mice. All modified PBL subsets induced a reduction in the outgrowth of both tumors when compared to mock PBL. Comparing the effect of IL7- or IL-15-treated lymphocytes with TCR-transgenic PBL cultured under IL-2 or Tc17 conditions, we were not able to detect a difference in the tumor outgrowth of THP-1, whereas that of mel624.38 was reduced to a significantly greater extent. To investigate whether this observation was due to tumor load, we injected a lower dose of $4x10^5$ THP-1 cells and treated the mice in a similar manner. While tumor outgrowth in mice treated with mock PBL or IL-2-treated TCR150-transgenic PBL (Figure 5B and data not shown) was not changed compared to the tumor curves following injection of $1x10^6$ THP-1 cells, TCR150-transgenic PBL cultured in IL-7 or IL-15 significantly reduced the outgrowth of the lower number of THP-1 cells (Figure 5B).

Reflecting the retarded tumor outgrowth, the overall survival of mice given $1x10^6$ THP-1 or $4x10^5$ mel624.38 cells followed by treatment with TCR150-transgenic PBL conditioned with IL-7 (THP-1 day 34; mel624.38 day >40) or IL-15 (THP-1 day 36; mel624.38 day > 40) was prolonged significantly for both tumors, but particularly mel624.38 cells (Figure 5F). In contrast, Tc17-conditioned PBL resulted in shorter survival (THP-1 day 28; mel624.38 day 30) compared with conventional IL-2-treated TCR-transgenic PBL (d5) (THP-1 day 31.5; mel624.38 day 33). The failure to detect human T cells in spleens or peripheral blood of sacrificed mice was not altered by the *in vitro* culture conditions (data not shown).

TCR150-transduced memory T cells limit outgrowth of disseminated tumor cells

Since we observed that IL-15- as well as IL-7-induced memory T cells expressing TCR150 showed improved ability to retard growth of solid $HMMR^+$ tumors, we tested their impact on disseminated leukemia cells. For these experiments the firefly luciferase gene was introduced into THP-1 cells by lentiviral transduction. After sorting for a purity of 100% luciferase-expressing cells, we injected $1x10^6$ $THP\text{-}1_{luc}$ cells i.v. into NSG mice. After 24 h, $2x10^5$ TCR150-transgenic PBL conditioned with IL-7 in combination with $2x10^5$ TCR150-transgenic PBL conditioned with IL-15 were adoptively transferred i.v. into mice. As a control, we used similar amounts of T58-transgenic PBL, a TCR specific for tyrosinase, since THP-1 cells are tyrosinase negative. In this control group, we detected leukemic cells in the femur of mice 14-21 days after injection (Figure 6 and Figure S4). The initial detection of leukemia was followed by a rapid spread, causing lesions in the kidneys, liver, spine and, in rare cases, in the lungs and ovaries. Approximately 10-15 days after the first detection of tumor cells, mice displayed paralysis of the hind legs, at which point they were removed from the study and sacrificed. The overall survival of the mice treated with T58-transduced PBL was 31.6 days. In contrast, a single dose of $4x10^5$ TCR150-transgenic PBL given 24 h after tumor inoculation was sufficient to dramatically retard tumor outgrowth, whereas injection of $2x10^5$ TCR150-transgenic PBL conditioned with IL-15 had no effect (Figure S4). Additionally, in one group we attempted to prolong the survival of lymphocytes *in vivo* ($4x10^5$) by exogenous IL-15 administration given i.p. daily from day 1 through day 10. This treatment resulted in no additional improvements. However, a dramatic effect was observed when exogenous IL-15-

treatment was combined with the administration of $4x10^5$ CD8-enriched TCR150-transgenic PBL. This resulted in complete control of the leukemia beyond day 50.

Discussion

Selection of a well-defined target antigen is crucial for immunotherapeutic approaches employing adoptive T cell transfer. Several studies have indicated that HMMR is over-expressed in many tumor types including prostate cancer, breast cancer, melanoma and chronic and acute leukemia (14-16, 26, 27). Over-expression of HMMR is strongly associated with a worse prognosis in solid tumors and 80% of melanoma metastasis show a dramatic over-expression of this protein (13, 14). In contrast, HMMR expression in disseminated tumors such as leukemias is related to a better clinical outcome and linked with an HMMR-specific immune response (14). In vaccination studies using an immunogenic HMMR-derived peptide, patients developed a potent immune response that correlated with clinical benefit, while no signs of toxicity were observed (20, 28). We elected to explore development of HMMR-specific T cells for TCR gene therapy in AML, since over 70% of AML show a significant over-expression of this protein, while expression in normal tissue is limited to testis, thymus, bone marrow, tonsils and placenta (15). Further characterization of the HMMR-positive cell types in these organs will show the suitability of HMMR-directed therapy using TCR-transgenic lymphocytes.

The priming of allorestricted, HMMR-specific T cells allowed us to isolate T cell clones from healthy donors (9). To overcome the limitation of T cell isolation by peptide-derived multimers, restricted to a single known epitope, we utilized T cell enrichment via the activation marker CD137. Several other studies demonstrated the use of this CD8-specific activation marker to enrich antigen-specific T cells or to deplete alloreactive T cells (21, 22). By using this approach we isolated 52% HMMR-specific T cell clones recognizing two HMMR-positive, HLA-A2-positive tumor cell lines, while the control T2 cell line (HLA-$A2^{+}$) pulsed with flu-peptide was not

recognized. A major fraction of HMMR-specific T cell clones was able to secrete the Th1-cytokines IFN-γ, IL-2, MIP-1α and TNF-α, indicating their polyfunctional phenotype (23). Selection of CTL67 and CTL150 for further studies showed that they used different TCR rearrangements. These two CTL also showed different profiles of specific cytotoxicity and varying patterns of cytokine secretion. Both clones failed to recognize the HMMR-R3 peptide (ILS) and showed negligible recognition of the HLA-$A2^+$, $HMMR^-$ target cell control. We did not identify any T cell clones recognizing the reported immunogenic peptide HMMR-R3 (ILS). This peptide appears to dominate the immune response in AML patients, whereas T cells recognizing the R3-peptide are rare in healthy individuals (19).

Using codon-optimized, murinized TCR sequences of TCR150 and TCR67 for retroviral transfer into recipient lymphocytes, we only detected surface expression of TCR150. Failure to express TCR67 could be due to inefficient TCR α- and β-chain pairing or to an overall low expression of the transferred genes. In contrast, TCR150 was expressed well on both CD4 and CD8 T cells. Tumor recognition by TCR150-transgenic PBL showed the same pattern of specificity as the original CTL. Moreover, tumor recognition by TCR150-transgenic PBL, both *in vitro* and *in vivo,* was antigen-specific. Enrichment of the TCR-transgenic fraction of PBL led to a dramatic increase in specific tumor cell lysis. Furthermore, shRNA-mediated knock-down demonstrated that recognition by TCR150-transgenic lymphocytes was dependent on HMMR expression by tumor cells. By introducing a deletion mutant of HMMR into DC, comprising only the first 170 amino acids, we were able to show that the recognized epitope is located within this fragment. Identification of the cognate epitope could not be determined by testing candidate peptides carrying known HLA-A2-binding motives, necessitating more complex approaches to identify this epitope in the future.

After confirming specificity and function *in vitro,* we determined the impact of TCR150-transduced PBL *in vivo* using as recipients the NSG mouse strain, lacking all murine T cells, B cells and NK cells (25). This strain is known to be a suitable host for engraftment of human tumors of both solid and disseminated types (25, 29). We first injected human tumor cell lines subcutaneously in matrigel to create solid tumors. In contrast to other studies using mouse model systems, we intended to explore the impact of TCR150-transduced PBL without pre-conditioning of the host mice (30), although such pre-conditioning may also impact on the tumor and contribute to better antitumor responses. Additionally, we tried to mimic the clinical relapse situation by intravenous injection of TCR-transduced PBL after tumor inoculation. In this setting, we observed that a single injection of $2x10^5$ $TCR150^+$ PBL was sufficient to retard solid tumor outgrowth to a significant extent, resulting in a prolonged overall survival. These observations were quite surprising since the ratio of $TCR150^+$ PBL to tumor cells was at a maximum of one effector cell per two tumor cells and TCR-transgenic PBL were injected only once. Nevertheless, the reduction in tumor growth using this mode of application is striking when compared with other studies performing intratumoral injection of much higher cell numbers, or pre-incubating tumor cells with effector cells *in vitro* prior to adoptive transfer (31, 32). Under these conditions, persistence of $TCR150^+$ T cells in the mice following intravenous application of $2x10^5$ or $5x10^5$ cells is probably too low to induce better control of the tumors. Further studies, injecting varying doses and regime frequencies of TCR150-transgenic PBL may lead to further improvements.

The persistence of engineered PBL in NSG mice in other studies ranged from 10-20 days (31) and could be increased by selecting central memory T cells (T_{CM}) or by administration of human IL-15 to the mice after adoptive transfer of lymphocytes

(33, 34). Other reports claimed that cells with a Tc17-phenotype were most potent for eradicating tumors in mouse models (35, 36). To further explore these options in our system, we cultured TCR150-transduced lymphocytes in the presence of different cytokines to induce cells with effector memory (T_{EM}), central memory (T_{CM}) or Tc17-phenotypes, respectively (37). In agreement with previous studies, we observed that a T_{CM}-phenotype was induced by IL-7, while IL-15 resulted in a more T_{EM}-like phenotype. *In vitro* functional analysis showed that only the IL-15 culture conditions increased the lytic capacity of TCR-transgenic PBL, while their cytokine production was altered most by Tc17 conditions. The Tc17-phenotype had no impact on tumor growth in our experiments, whereas TCR150-transgenic PBL cultured in the presence of IL-7 or IL-15 led to significant reduction of tumor outgrowth when mel624.38 cells were targeted. In contrast, outgrowth of THP-1 was not altered under these conditions. *In vitro* recognition of this tumor by the original CTL150 and by TCR150-transgenic PBL was always low, suggesting that this AML was somewhat resistant to CTL. The reasons for this remain to be explained, but may depend on antigen-processing and presentation, or on expression of inhibitory molecules or cytokines.

To address the function of TCR150-transgenic lymphocytes on disseminated human leukemia cells, we used a combination of IL-7- and IL-15-pre-conditioned PBL, due to the superior antitumor effects seen with solid tumors. TCR150-transgenic PBL given in a single dose of $4x10^5$ were sufficient to significantly retard leukemia compared to PBL transduced with an irrelevant TCR (T58). A dose dependent difference in tumor outgrowth was seen between injection of $4x10^5$ and $2x10^5$ TCR150-transgenic PBL. Further therapeutic improvements were achieved when IL-15 was administered exogenously on a daily basis, in particular when used

in combination with $4x10^5$ CD8-enriched TCR150-transgenic lymphocytes. The combination of TCR150-transgenic $CD8^+$ T cells with IL-15 resulted in a strongly prolonged control of the leukemic cells, supporting a CD8-dependent function of TCR150. Further analysis of additional HLA-$A2^+$ $HMMR^+$ leukemia and solid tumor cells, both *in vitro* and *in vivo*, may extend the potential of $TCR150^+$ IL-15-conditioned lymphocytes for use in TCR gene therapy.

Methods

Cell lines

The human acute monocytic cell line, THP-1 (HLA-A2$^+$, HMMR$^+$; TIB-202) (38) was cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum (Invitrogen). The packaging cell line 293T (human embryonal kidney, CRL-1573) (39) as well as the human breast carcinoma cell line MCF-7 (HTB-22) (40) were cultured in Dulbecco's MEM with 4 mM L-glutamine, 100 U/ml penicillin/streptomycin and 1 mM sodium pyruvate. The human chronic myeloid leukemia cell line, K562 (HLA-A2$^-$, HMMR$^+$; CCL-243) (41) and K562-A2 (HLA-A2$^+$, HMMR$^+$; medium supplemented with 1 μg/ml G418, gift from H. Pohla, Laboratory for Tumor Immunology, Ludwig-Maximilians-University, Munich, Germany), the melanoma cell line mel624.38 (HLA-A2$^+$, HMMR$^+$; gift from M.C. Panelli, National Institutes for Health, Bethesda, USA) (42) as well as the human lymphoid cell line T2 (43) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate and 1 mM non-essential amino acids. The CTL A42 was cultured and restimulated as described previously (44). Exogenous peptide pulsing of T2 cells or DC was performed using 10^{-5} M peptide (HMMR$_{166-175}$: ILSLELMKL, MART-1$_{25-36}$: ELAGIGILT and influenza matrix protein$_{58-66}$: GILGFVFTL) (Metabion) and performed as described previously (9).

Production of *in-vitro transcribed* RNA

The plasmid pReceiver-B02 with the HMMR insert (Acces. No. U29343; GeneCopoeia) and the plasmid pCDM8HLA-A2, containing the HLA-A*0201 cDNA (gift from E. Weiß, Department of Biology, Ludwig-Maximilians-University, Munich, Germany) were linearized and used as templates for *ivt*-RNA production using the

mMESSAGE mMACHINE T7 kit (Ambion). Production followed the manufacturer's instructions.

Flow cytometry analysis

For analysis of surface marker expression, cells were labeled with the following fluorescence-conjugated monoclonal antibodies: CD3 (PerCP, SK7, BD), CD4 (FITC, 13B8.2, BeckmanCoulter), CD8 (FITC, HIT8a, BD), CD8 (APC, SK1, BD), CD25 (PE-Cy7, M-A251, BD), CD27 (Alexa Fluor 700, M-T271, BD), CD28 (FITC, CD28.2, BD), CD45RA (PerCP-Cy5.5, HI100, eBioscience), CD62-L (APC, DREG-56, BD), CD137 (APC, 4B4-1, BD), CD279 (PE, MIH4, eBioscience), murine TCR constant beta-chain-region (APC, H57-597, BD). After a 60 min incubation, cells were washed and analyzed by flow cytometry using an LSRII instrument (BD). Data analyses were performed using the FlowJo 8 software (Tree Star Inc.).

Intracellular cytokine staining

Cells were cultured for 6 h, with or without tumor cells (20:1, E:T), in the presence of brefeldin A (eBioscience), with a final concentration of 15x. Before fixation, cells were washed and stained using the Live/Dead Fixable Blue Dead Stain Kit (Invitrogen), followed by staining with the following antibodies: CD4 (APC-Cy780, RPA-T4, eBioscience), CD8 (V500, RPA-T8, BD), CD69 (PerCP, FN50, BD). Fixation and permeabilization was performed using the FoxP3 Staining Buffer Set (eBioscience) according to the following steps (all performed at 4°C): fixation of cells using Fix/Perm Buffer for 30 min followed by two washing steps with Perm Buffer and staining for 30 min with the intracellular markers CD3 (PE-Cy7, SK7), IFN-γ (V450, B27), IL-2 (FITC, 5344.111), IL-4 (PE, 3010.211) and TNF-α (Alexa Fluor 700,

MAb11) (all BD). After two additional washing steps, cells were analyzed by flow cytometry (LSRII instrument; BD). Data analyses were performed using the FlowJo 8 software (Tree Star Inc.). Staining of intracellular HMMR was as for ICS starting with fixation, using the primary HMMR antibody (non conj., 2D6, Abcam) and secondary goat-anti mouse IgG2 (PE, Dianova) in sequential staining.

De novo priming of T cells with RNA-pulsed DC

Collection of blood from healthy donors was approved by the "Ethics Board of the Medical Faculty of the Ludwig-Maximilians-University" Munich, Germany and donors gave informed consent. *In vitro* priming of allorestricted T cells was performed using mDC, electroporated with 48 μg of HMMR *ivt*-RNA in combination with 24 μg HLA-A2 *ivt*-RNA. Stimulation of bulk cultures was performed as described (9) and as demonstrated in Figure S1.

CD137-mediated enrichment of HMMR-specific CTL

After a two-week resting phase, bulk culture lymphocytes were restimulated using RNA-pulsed mDC at a ratio of 10:1. Cells were adjusted to $1x10^7$/100 μl MACS buffer 9 h after stimulation and labeled with 1 μg/10^7 cells of anti-CD137-biotin antibody for 15 min at 4°C (4B4-1, Ancell). Cells were washed, resuspended and incubated with anti-biotin-microbeads containing MACS buffer, according to manufacturer's instructions (Miltenyi). After a washing step, cells were separated using an LS MACS column (Miltenyi). Flow cytometry analysis of purity was performed using a CD8-FITC antibody combined with streptavidin-PE (BD). Enriched cells were either cultured as bulk culture cell lines or were cloned immediately in limiting dilution cultures using autologous feeder cells, as published (9).

Retroviral TCR gene transfer

TCR sequences of HMMR-specific CTL67 and 150 were determined by PCR. The constant regions were exchanged using the murine counterpart (45) and TCR sequences were codon optimized (46) to enhance expression. The synthesized TCRβ-2A-TCRα transgene cassettes (GENEART) were integrated into MP71-PRE, as previously described (8). Vector plasmids were used for production of retroviral particles and subsequent transduction of T cells (24).

Lentiviral transfer of shRNA

The vectors containing shRNA targeting HMMR as well as the helper plasmids were kind gifts of M. Heemskerk. Using two pLKO.1-puro vectors with two independent shRNA sequences targeting HMMR, third generation lentiviruses were produced in 293T cells. The targeted cell lines mel624.38 and THP-1 were cultured in the mixed viral supernatant of both virus types for 48 h and selected for transduced cells using puromycin (Enzo Life Science) at a final concentration of 2 μg/ml.

Lentiviral transduction and generation of luciferase-expressing cells

The firefly luciferase construct was cloned into the pCDH vector containing a GFP-expression cassette under the control of the EF-1alpha promoter (System Biosciences). Third generation lentiviruses were produced in 293T cells. THP-1 cells were transduced overnight in the presence of polybrene and enriched by fluorescence-activated cell sorting using GFP as a marker.

Cytokine treatment of TCR-modified PBL

TCR transfer experiments using modified culture conditions were performed using X-Vivo 15 medium (Lonza) supplemented with 1.5 g/l N-acetyl-L-cystein, 50 mM HEPES, 2 mM L-glutamin and 10% human serum. TCR retroviral transduction was performed as previously published and cells were cultured with IL-2 up to day 5 (24). Cells were then washed and resuspended in culture medium supplemented with new cytokines until use in functional assays *in vitro* or adoptive transfer experiments *in vivo* (Table 1).

Chromium release and cytokine-release assays

Cytotoxic activities of T cell lines, CTL, and TCR-modified PBL were analyzed in a standard 4 h chromium-release assay (9). For initial analysis at day 13, T cells were incubated with 2×10^3 K562-A2 cells or influenza matrix protein$_{58–66}$ (flu, GILGFVFTL) peptide-loaded T2 cells, as previously described. Analysis of T cell clones at later time points was performed using the designated numbers of tumor cells and $1x10^4$ T cells for the chromium-release assay. $2x10^3$ T cells were stimulated to obtain 24 h supernatants for multiplex analysis, performed according to manufacturer's instructions using a human 17-Plex assay kit (BioRad). TCR-modified PBL, day 5 or day 13 after transduction, were cocultured with tumor cell lines using 2×10^3 target cells at the designated E:T. Specific lysis was calculated as described using duplicate samples at each E:T ratio (9). To obtain 24 h supernatants, TCR-modified PBL (4×10^4) were incubated with given numbers of tumor cells or mature, RNA-pulsed DC at an E:T ratio of 20:1, followed by a standard ELISA analysis for IFN-γ (BD).

Mice and adoptive transfer experiments

All animal experiments were approved by the local authorities according to the legal regulations for animal experimentation. For the solid tumor model, 6-8 week old NSG mice were subcutaneously injected with $4x10^5$ or $1x10^6$ tumor cells resuspended in matrigel (BD) on day 0. The indicated numbers of TCR-modified or mock-treated lymphocytes were adoptively transferred intravenously into recipient mice on day 1. The tumor size was measured every other day using a caliper and calculated as mm^2 (length x width). Mice were sacrificed when the tumor area reached a volume of 300 mm^2 or 150 mm^2 for THP-1 and mel624.38, respectively. For the disseminated leukemia model, luciferase-positive cells were intravenously injected on day 0 followed by adoptive transfer of TCR-modified on day 1. IL-15 treatment, using a daily dose of 10 μg/mouse, was started on day 1 and was injected intraperitoneally through day 10 (47). For *in vivo* imaging, mice were anesthetized by isoflurane inhalation. Luciferine (SynChem) was injected intravenously before measurement of light emission using the IVIS Lumina machine (Caliper Life Sciences).

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Figure legend

Figure 1. *In vitro* characterization of HMMR-specific T cell clones

(**A**) Clones induced by *in vitro*-priming using DC prepared from an HLA-A2-negative donor pulsed with HLA-A2 (48 μg) and HMMR (24 μg) *ivt*-RNA. Lytic capacity (% specific lysis) was assessed in a standard 4 h chromium-release assay using K562-A2 (HLA-A2$^+$, HMMR$^+$) cells as a positive target and HLA-A2$^+$ T2 cells pulsed with an irrelevant peptide (flu) as a negative control. (**B**) Screening of well-growing T cell clones was performed using K562-A2 (HLA-A2$^+$, HMMR$^+$) and THP-1 (HLA-A2$^+$, HMMR$^+$) as positive target cells, T2 cells pulsed with flu peptide (T2 flu) as a negative control, and T2 cells loaded with the HMMR-nonamer "ILS" (T2 ILS) (* not tested). The percent of specific lysis at an E:T of 5:1 is shown (upper graph) as well as IFN-γ secretion of 2x10^3 T cells stimulated with 1x10^3 tumor cells, assessed by a standard ELISA (lower graph). (**C**) Percentage of T cell clones showing no reactivity, alloreactivity or HMMR-specific reactivity in the initial screening assay. (**D**) HMMR-specific T cell clones secreting four (black) or three (grey) out of four cytokines tested in a multiplex analysis (IFN-γ, IL-2, MIP-1α, TNF-α). Supernatants were taken at 24 hours from cultures of 2x10^3 T cells stimulated with 1x10^3 target cells. (**E**) Lytic capacity shown as % specific lysis of two HMMR-specific CTL67 and CTL150, cocultured with the tumor cell lines THP-1 and K562-A2, as well as T2 cells pulsed with the ILS-peptide or the flu-peptide, respectively, at an E:T of 1:5. (**F**) Cytokine secretion profiles of CTL67 and CTL150 given in pg/ml for 2x10^3 cells at 24 h. Secretion of IFN-γ (black), IL-2 (open), and TNF-α (grey) was detected in a multiplex assay following stimulation with the four target cells described in B.

Figure 2. Characterization of TCR150-transduced lymphocytes using *in vitro* assays

(**A**) Flow cytometry staining of TCR150-transduced lymphocytes showing expression of TCR150 in CD3, CD4 and CD8 T cells (from left to right). (**B**) IFN-γ ELISA of $4x10^4$ lymphocytes stimulated with $2x10^3$ tumor cells for 24 h. THP-1 and K562-A2 cells were used as positive stimulating cells while T2 cells, pulsed with flu-peptide, were used as negative stimulating cells. PBL were transduced with TCR150 or a GFP-control vector. Mock PBL served as a background control. Data are given in pg/ml. (**C**) Specific lysis of target cells mediated by untransduced PBL (mock) (closed circles), TCR150-transduced (closed squares) or survivin-specific TCR-A72-transduced PBL (closed triangles). Open symbols represent specific lysis of targets using PBL enriched for the given TCR-expressing fraction (see Figure S3B). (**D**) Specific lysis of parental THP-1 (black circles) and mel624.38 (black squares) or HMMR-specific shRNA-treated THP-1 (gray circles) or mel624.38 (gray squares) by TCR150-modified PBL, measured in a standard chromium-release assay. In addition, the $HMMR^{low}$ cell line MCF-7 (black triangles) and mock-PBL versus THP-1 (open circles) or mel624.38 (open squares) served as controls. (**E**) IFN-γ secretion of HMMR-specific TCR150- or tyrosinase-specific T58-transduced PBL after stimulation with autologous, $HLA\text{-}A2^-$ mature DC. DC were pulsed with HLA-A2-encoding RNA alone or in combination with $HMMR_{1\text{-}725}$, representing the full length HMMR CDS, or with $HMMR_{1\text{-}170}$, representing a deletion mutant coding for amino acids 1-170.

Figure 3. Potential of TCR150-transgenic lymphocytes to retard solid tumor outgrowth *in vivo*

(**A**) Tumor outgrowth (mm^2) of $1x10^6$ THP-1 cells injected s.c. into NSG mice on day 0 followed by adoptive transfer i.v. of mock-transduced PBL (closed circles; n=6) tyrosinase-specific T58-PBL (closed triangles; n=3) or HMMR-specific TCR150-modified PBL (closed squares; n=9), on day 1 at a dose of $2x10^5$ TCR-transgenic PBL per mouse (*** $p<0.001$). For statistical analysis, a two-way ANOVA analysis was used. (**B**) Tumor outgrowth (mm^2) of $1x10^6$ THP-1 cells injected on day 0 followed by adoptive transfer of mock-PBL (closed circles; n=6) or TCR150-modified lymphocytes on day 1, at a cell-dose of $2x10^5$ (closed squares; n=9) or $5x10^5$ TCR^+ PBL (open squares; n=3). Statistics using a two-way ANOVA indicated no significant difference between the two PBL doses. (**C**) Percent survival of mice treated in the experiment described in Figure 3A. Mice injected with TCR150-modified PBL showed a significant prolonged survival of 8 days compared to mice given mock-transfected cells (Mantel-Cox test *$p=0.0022$) (**D**) Survival of mice in the experiment shown in Figure 3B. Mice injected with $5x10^5$ PBL showed no significant improved survival compared to mice given $2x10^5$ PBL (Mantel-Cox test $p=0.3312$). All experiments were performed at least twice with similar results.

Figure 4. Influence of cytokine milieu on recipient lymphocytes *in vitro*

(**A**) Surface staining of human PBL transduced with TCR150 using human CD62L antibodies on pre-gated $CD8^+$, $CD45RA^-$ cells that were cultured over a 5-day or 13-day period in the presence of different cytokines as given in Table 1. (**B**) Percentage of intracellular IFN-γ and TNF-α double-positive cells within the $CD8^+$ T cell fraction by flow cytometry analyses. Open bars represent non-stimulated PBL, while filled

bars represent PBL stimulated at an E:T of 20:1 ratio with THP-1 cells for 6 h. Mock-transfected PBL were used as a background control compared to TCR150-transduced cells, also cultured after addition of different cytokines. (**C**) Lytic capacity of T58- or TCR150-modified PBL, cultured under varying cytokine conditions, using THP-1 and mel624.38 as target cells. Specific lysis was measured in a standard 4 h chromium-release assay.

Figure 5. *In vivo* effects of conditioned TCR150-transgenic lymphocytes

(**A-C**) Tumor size in mm^2 of tumor of (**A**) $1x10^6$ THP-1 cells, (**B**) $4x10^5$ THP-1 cells or (**C**) $4x10^5$ mel624.38 cells, inoculated s.c. on day 0. Adoptive transfer of $2x10^5$ TCR-positive PBL was performed on day 1. TCR150-modified lymphocytes were pre-conditioned with IL-2- (filled circles), IL-7- (filled squares), IL-15- (filled triangles) or Tc17-medium (filled diamonds), respectively. Mock-transfected PBL served as control (open circles). A two-way ANOVA test showed a significant reduction of tumor outgrowth (* $p<0.01$; ** $p<0.001$). Results were confirmed in at least two independent experiments. (**D-F**) Percent survival of mice treated in the experiments described in Figure 5A through 5C. (**D**) Mice with THP-1 tumors ($1x10^6$) and injected with TCR150-PBL (IL-15) showed survival prolonged by 10 days (Mantel-Cox test *p=0.0011). (**E**) Mice injected with reduced numbers of tumor cells ($4x10^5$ THP-1) showed similar survival rates after injection of mock-treated PBL compared to $1x10^6$ tumor cells used, while the injection of TCR150-modified lymphocytes prolonged the survival significantly (Mantel-Cox test *p=0.0256). (**F**) mel624.38-bearing mice injected with TCR150-PBL (IL-7 or IL-15) showed a significantly improved survival compared to mice injected with TCR150-PBL (IL-2) (Mantel-Cox test *p= 0.0045 or **p=0.001). Results were confirmed in at least two independent experiments.

Experiments were performed at least twice in independent experiments with similar results.

Figure 6. Usage of IL-7- and IL-15-conditioned TCR150-transgenic PBL to target disseminated tumor cells *in vivo*

THP-1_{luc} cells were intravenously inoculated on day 0 at a dose of $1x10^6$ cells/mouse, followed by adoptive transfer of $2x10^5$ each of IL-7 and of IL-15 conditioned T58- or TCR150-modified PBL on day 1. Tyrosinase-specific T58-modified PBL served as a negative control since THP-1 cells are tyrosinase negative. In addition mice were treated with exogenous IL-15 (10 μg/mouse/day) or TCR150-modified $CD8^+$-enriched T cells ($2x10^5$ IL-7 and IL-15 cells for each conditioning) in combination with IL-15 administration. IL-15 was added daily starting from day 1 through day 10 via intraperitoneal injection. A weekly monitoring of mice started two weeks after tumor inoculation and is depicted from day 21 onwards. Additional monitoring was performed when mice showed severe signs of paralysis before they were sacrificed. Depicted are representative mice out of groups of three mice. Remaining mice are shown in Figure S4.

Figure S1. Scheme of *de novo* priming approach in combination with the CD137-mediated enrichment of antigen-specific T cells

(**A**) Workflow of the *in vitro* priming approach to induce HMMR-specific, allorestricted T cell clones. (**B**) Flow cytometry staining of restimulated bulk cultures before and after CD137-mediated magnetic bead enrichment.

Figure S2. HMMR and HLA-A2 expression in target cells

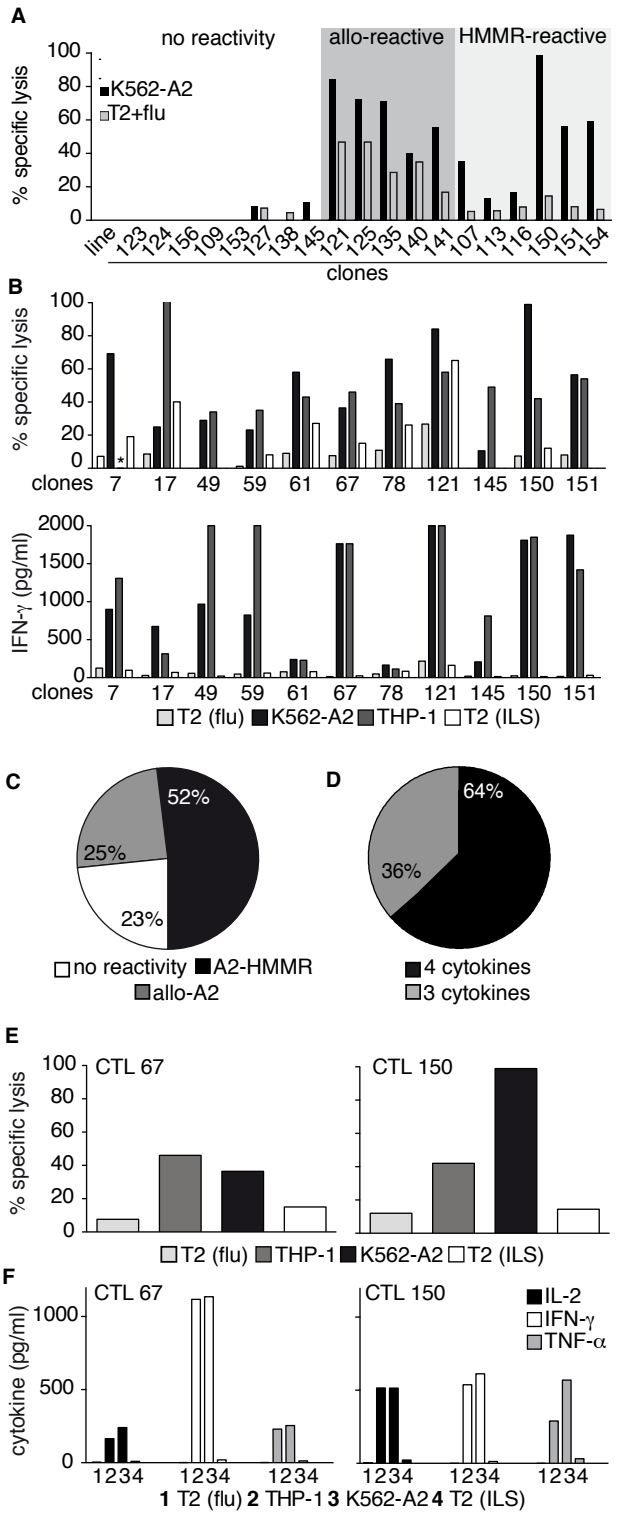
(**A**) Surface staining of HLA-A2 (open histograms) on tumor cell lines K562-A2, THP-1, mel624.38 and MCF-7. K562 was used as a negative control, shown by filled histograms. (**B**) Intracellular staining of HMMR in the parental target cell lines depicted in (A) (open histograms), with the isotype-control staining shown in filled grey histograms. (**C**) HMMR-expression in HMMR-specific shRNA-treated cell lines, THP-1 and mel624.38, is shown in filled light grey histograms, while HMMR-staining in parental cell lines is given as open histograms. (**D**) IFN-γ (pg/ml) secreted during a 24 h-coculture of $5x10^4$ MART-1/MelanA-specifc CTL A42 with $1x10^5$ mel624.38, mel624.38 treated with HMMR-specific shRNA or mDC pulsed with a MART-1_{26-35} peptide, respectively.

Figure S3. Expression levels of TCR67 and TCR150 in PBL and enrichment of TCR-positive PBL

(**A**) Expression levels of TCR67 and TCR150 in recipient lymphocytes detected by flow cytometry. Transgenic TCR expression was assessed by staining for the murine b-constant region of the TCR-construct (y-axis) versus counter-staining for human CD3, CD4 and CD8. (**B**) Enriched fractions of PBL transduced with TCR150 or TCR-A72, respectively.

Figure S4. Usage of IL-7-/ IL-15-conditioned TCR150-transgenic PBL to target disseminated tumor cells *in vivo*

In vivo imaging of additional two mice treated according to the experiment shown in Figure 6. as well as mice treated with $2x10^5$ TCR150-PBL (after IL-15 pre-conditioning conditions).


A
no reactivity
allo-reactive
HMMR-reactive
% specific lysis
K562-A2
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line 123 124 156 109 153 127 138 145 121 125 135 140 141 107 113 116 150 151 154
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clones 7 17 49 59 61 67 78 121 145 150 151
IFN-γ (pg/ml)
clones 7 17 49 59 61 67 78 121 145 150 151
T2 (flu) K562-A2 THP-1 T2 (ILS)
C
52%
25%
23%
no reactivity A2-HMMR
allo-A2
D
64%
36%
4 cytokines
3 cytokines
E
% specific lysis
CTL 67
CTL 150
T2 (flu) THP-1 K562-A2 T2 (ILS)
F
cytokine (pg/ml)
CTL 67
CTL 150
IL-2
IFN-γ
TNF-α
1234 1234 1234 1234 1234
1 T2 (flu) 2 THP-1 3 K562-A2 4 T2 (ILS)

Figure 1

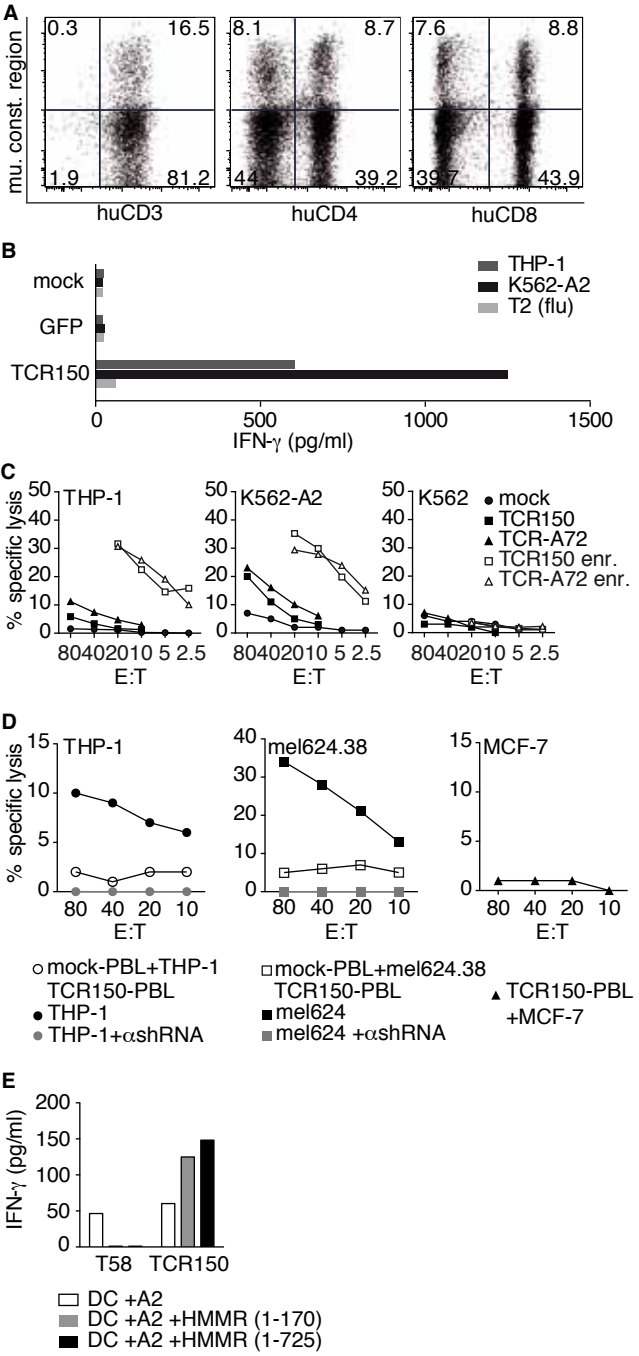

A
mu. const. region
0.3
16.5
1.9
81.2
8.1
8.7
44
39.2
7.6
8.8
39.7
43.9
huCD3
huCD4
huCD8
B
mock
GFP
TCR150
THP-1
K562-A2
T2 (flu)
0
500
1000
1500
IFN-γ (pg/ml)
C
% specific lysis
THP-1
K562-A2
K562
mock
TCR150
TCR-A72
TCR150 enr.
TCR-A72 enr.
80402010 5 2.5
E:T
D
% specific lysis
THP-1
mel624.38
MCF-7
80 40 20 10
E:T
mock-PBL+THP-1 TCR150-PBL
THP-1
THP-1+αshRNA
mock-PBL+mel624.38 TCR150-PBL
mel624
mel624 +αshRNA
TCR150-PBL +MCF-7
E
IFN-γ (pg/ml)
200
150
100
50
0
T58
TCR150
DC +A2
DC +A2 +HMMR (1-170)
DC +A2 +HMMR (1-725)

Figure 2

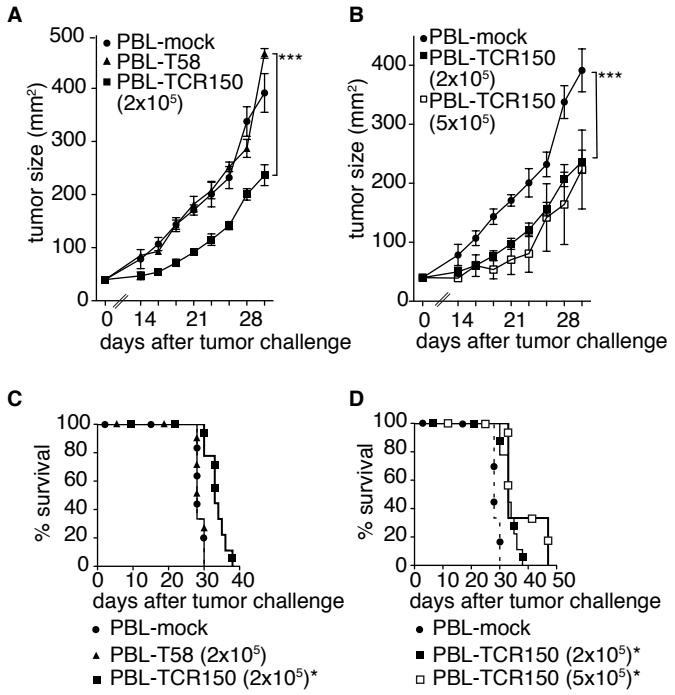

A
PBL-mock
PBL-T58
PBL-TCR150 (2x10^5)
***
tumor size (mm^2)
days after tumor challenge
B
PBL-mock
PBL-TCR150 (2x10^5)
PBL-TCR150 (5x10^5)
***
tumor size (mm^2)
days after tumor challenge
C
% survival
days after tumor challenge
PBL-mock
PBL-T58 (2x10^5)
PBL-TCR150 (2x10^5)*
D
% survival
days after tumor challenge
PBL-mock
PBL-TCR150 (2x10^5)*
PBL-TCR150 (5x10^5)*

Figure 3

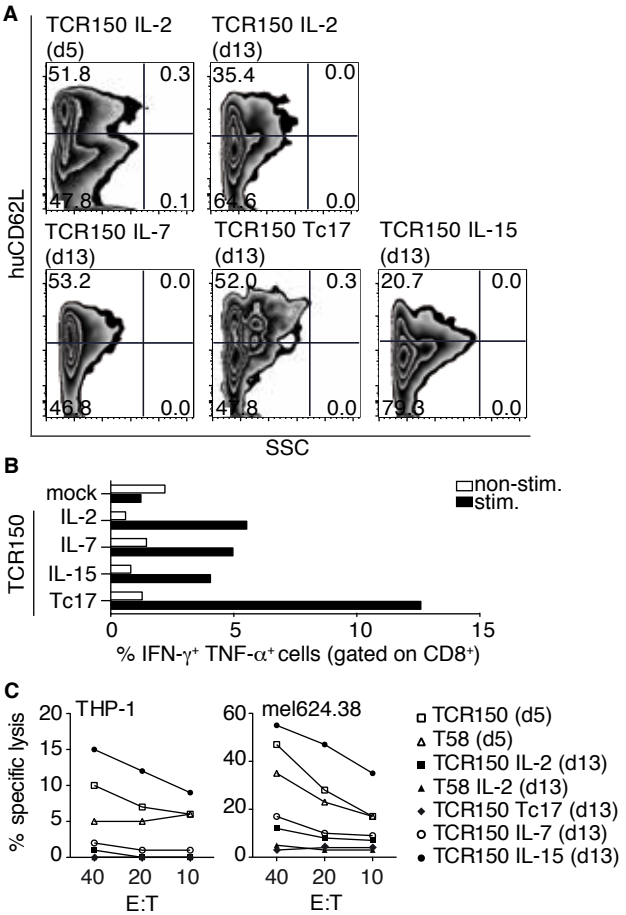

A
TCR150 IL-2 (d5)
TCR150 IL-2 (d13)
TCR150 IL-7 (d13)
TCR150 Tc17 (d13)
TCR150 IL-15 (d13)
51.8
0.3
47.8
0.1
35.4
0.0
64.6
0.0
53.2
0.0
46.8
0.0
52.0
0.3
47.8
0.0
20.7
0.0
79.3
0.0
huCD62L
SSC
B
non-stim.
stim.
TCR150
mock
IL-2
IL-7
IL-15
Tc17
0
5
10
15
% IFN-γ+ TNF-α+ cells (gated on CD8+)
C
THP-1
mel624.38
% specific lysis
E:T
TCR150 (d5)
T58 (d5)
TCR150 IL-2 (d13)
T58 IL-2 (d13)
TCR150 Tc17 (d13)
TCR150 IL-7 (d13)
TCR150 IL-15 (d13)

Figure 4

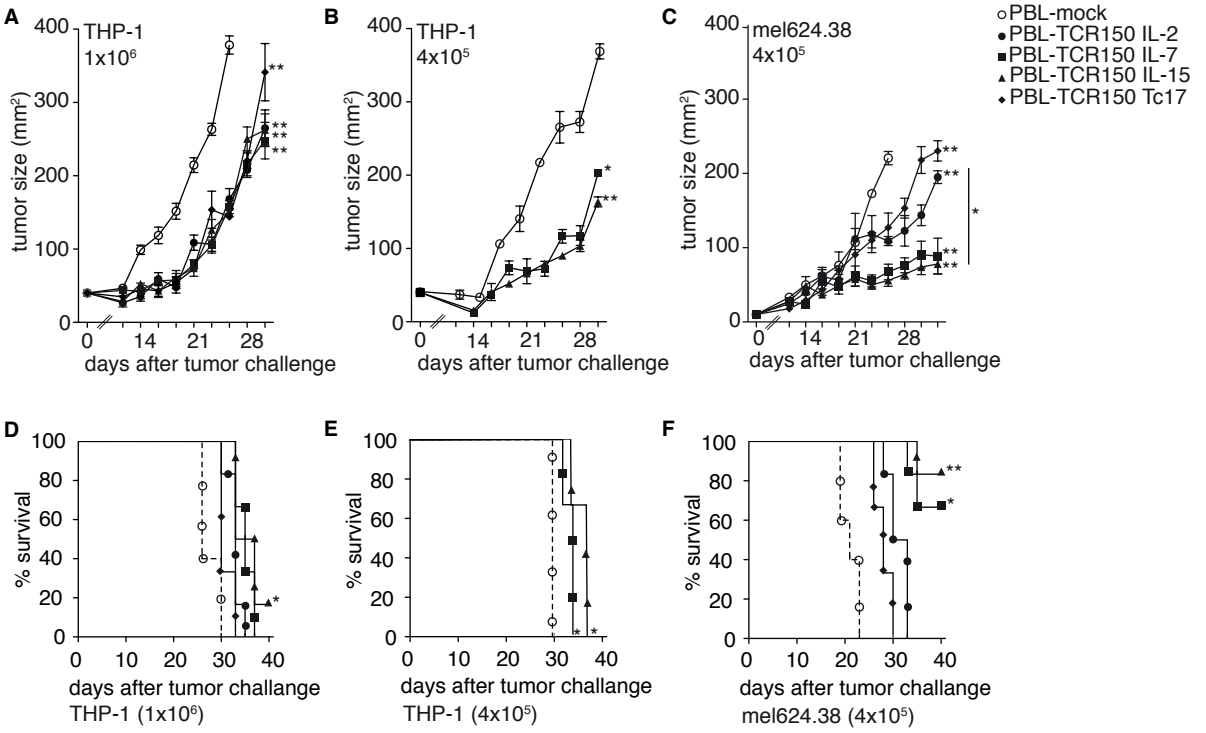

A
THP-1
1x10^6
tumor size (mm^2)
days after tumor challenge
B
THP-1
4x10^5
tumor size (mm^2)
days after tumor challenge
C
mel624.38
4x10^5
tumor size (mm^2)
days after tumor challenge
PBL-mock
PBL-TCR150 IL-2
PBL-TCR150 IL-7
PBL-TCR150 IL-15
PBL-TCR150 Tc17
D
% survival
days after tumor challange
THP-1 (1x10^6)
E
% survival
days after tumor challange
THP-1 (4x10^5)
F
% survival
days after tumor challange
mel624.38 (4x10^5)

Figure 5

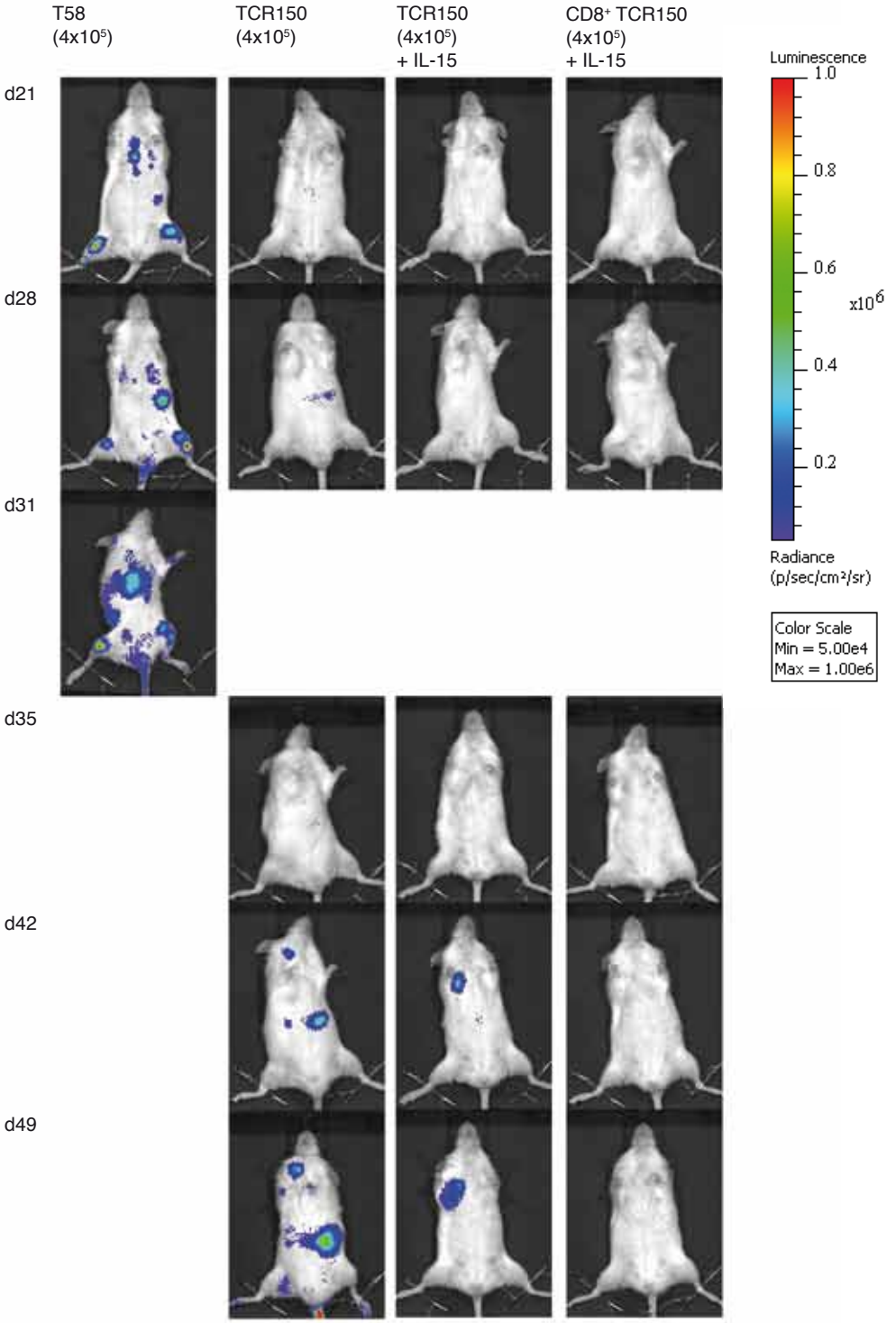

T58
(4x10⁵)
TCR150
(4x10⁵)
TCR150
(4x10⁵)
+ IL-15
CD8⁺ TCR150
(4x10⁵)
+ IL-15
d21
d28
d31
d35
d42
d49
Luminescence
1.0
0.8
0.6
0.4
0.2
x10⁶
Radiance
(p/sec/cm²/sr)
Color Scale
Min = 5.00e4
Max = 1.00e6

Figure 6

Table 1. Cytokine concentrations in conditioned medium

	cytokines				
conditions	IL-2	IL-6	IL-7	IL-15	TGF-beta
IL-2	100 U/ml	--	--	--	--
IL-7	10 U/ml	--	10 ng/ml	--	--
IL-15	10 U/ml	--	--	10 ng/ml	--
Tc17	30 U/ml	5 ng/ml	--	--	10 ng/ml

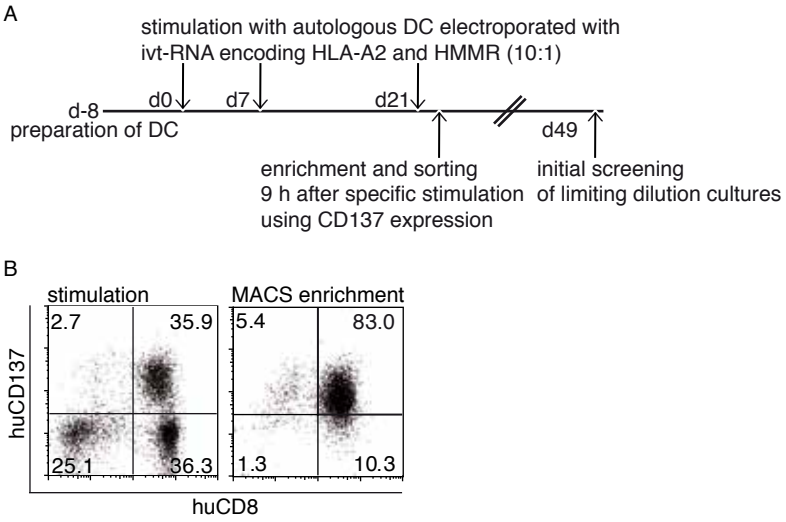

A
stimulation with autologous DC electroporated with
ivt-RNA encoding HLA-A2 and HMMR (10:1)
d-8
preparation of DC
d0
d7
d21
d49
enrichment and sorting
9 h after specific stimulation
using CD137 expression
initial screening
of limiting dilution cultures
B
stimulation
MACS enrichment
2.7
35.9
25.1
36.3
5.4
83.0
1.3
10.3
huCD137
huCD8

Figure S1

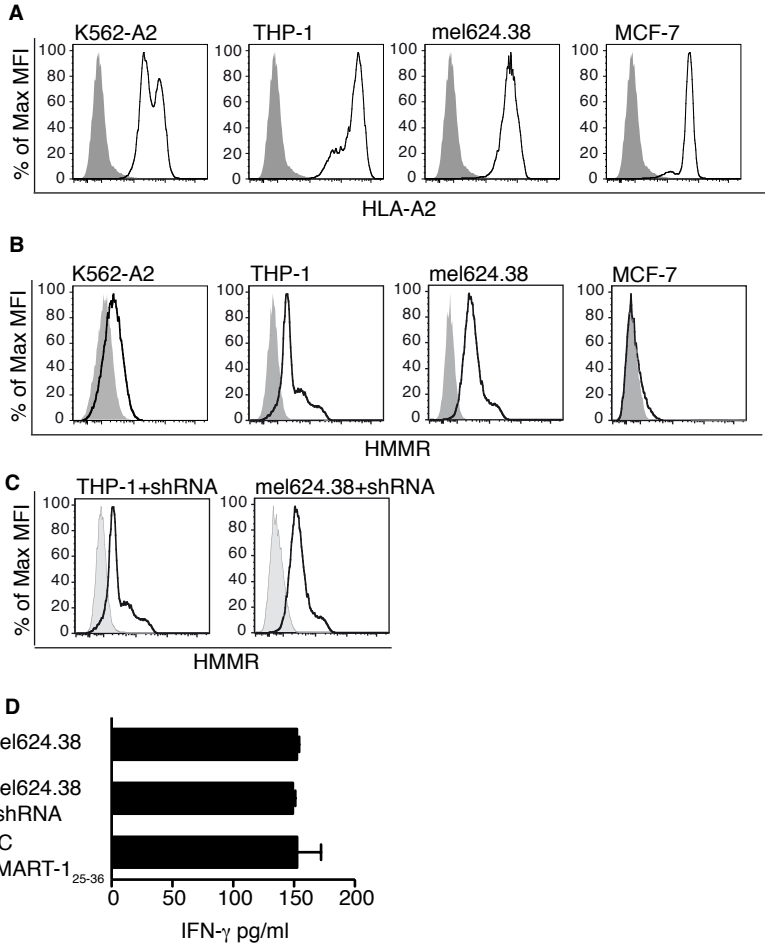
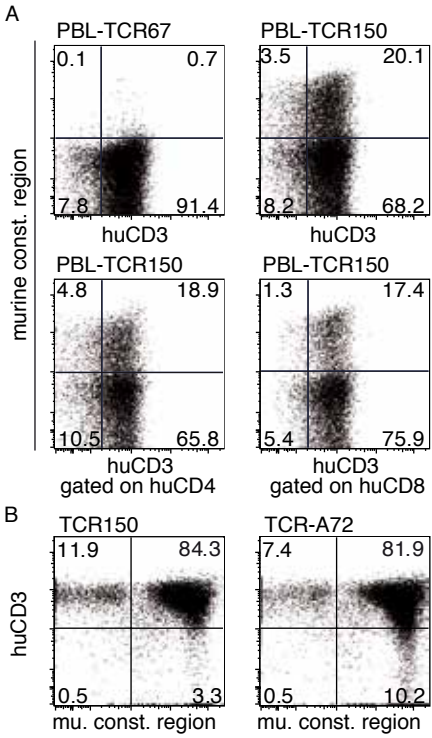

A
K562-A2
THP-1
mel624.38
MCF-7
% of Max MFI
HLA-A2
B
HMMR
C
THP-1+shRNA
mel624.38+shRNA
D
mel624.38
mel624.38 +shRNA
DC +MART-1 25-36
IFN-γ pg/ml

Figure S2


A
PBL-TCR67
PBL-TCR150
0.1
0.7
3.5
20.1
7.8
91.4
8.2
68.2
murine const. region
huCD3
huCD3
PBL-TCR150
PBL-TCR150
4.8
18.9
1.3
17.4
10.5
65.8
5.4
75.9
huCD3
gated on huCD4
huCD3
gated on huCD8

B
TCR150
TCR-A72
11.9
84.3
7.4
81.9
huCD3
0.5
3.3
0.5
10.2
mu. const. region
mu. const. region

Figure S3

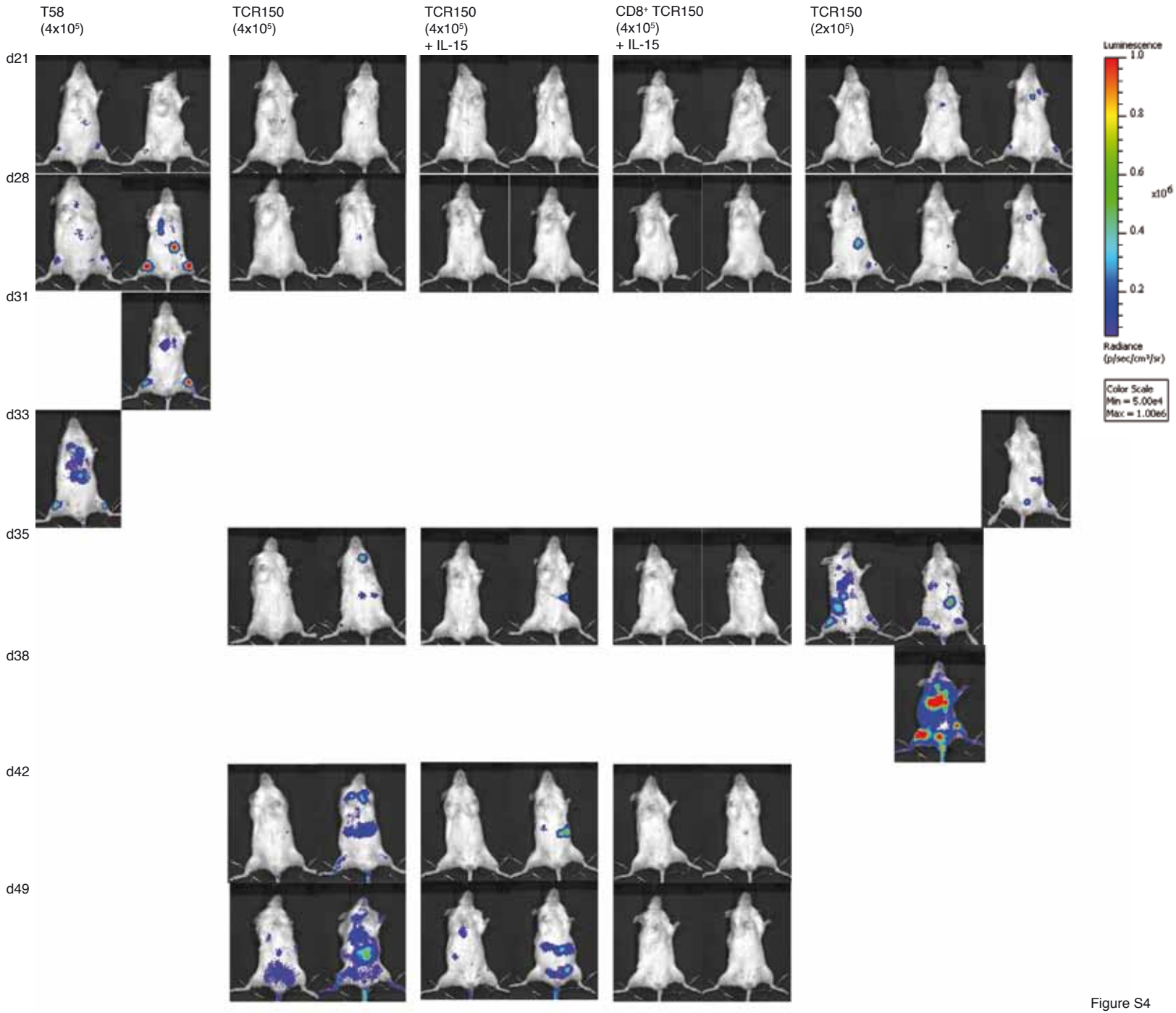

T58
(4x10⁵)
TCR150
(4x10⁵)
TCR150
(4x10⁵)
+ IL-15
CD8⁺ TCR150
(4x10⁵)
+ IL-15
TCR150
(2x10⁵)
d21
d28
d31
d33
d35
d38
d42
d49
Luminescence
1.0
0.8
0.6
x10⁶
0.4
0.2
Radiance
(p/sec/cm²/sr)
Color Scale
Min = 5.00e4
Max = 1.00e6

Figure S4

Acknowledgments

Over the last three years I had the chance to work and meet with lots of people without whom this thesis would not have been possible. Therefore, I would like to express my deepest appreciation to…

Dolores, for teaching me how to become a good scientist and for her trust in my project and all her enthusiasm.

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Bernhard, for all his support, his supervision, his answers to my questions and his trust in my ideas.

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All the other members of the Schendel-Lab for their help and support. In particular Judith for helping me with flow cytometry, Susanne for telling me the secrets of T cell priming and Sonja for introducing me into molecular cloning.

The whole IMI and the animal facility for being such a nice and helpful working environment. In particular Elfriede and Ralph for their ongoing support and their helpful discussions.

Irmela Jeremias for maintaining a very productive cooperation and in particular Volker for the informative chats in the animal facility.

Matthias Leisegang and Wolfgang Uckert, for giving me an introduction into the retroviral transfer technique and all the helpful comments.

Liz Simpson for teaching me basic mouse immunology and for giving me persistent advice on the English language.

Nadine, who once was a colleague and than became the sister I've never had.

Dennis for giving me the strength I needed and for your endless support.

Und meiner Mama für das Vertrauen in mich und die unablässige Unterstützung.

Curriculum Vitae

■ Personal and contact details

Name	**Stefani Maria Spranger**
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Nationality	German
Contact	E-mail: stefani.spranger@yahoo.de

■ Educational history

May 2008 - present
Helmholtz-Zentrum München, Munich, Germany
Anticipated degree — Dr. rer. nat. (equivalent to PhD)

May 2008
Ludwig-Maximilians-University, Munich, Germany
Degree — Diplom Biologist (equivalent to MSc)

June 2003
Theresia-Gerhardinger-Gymnasium, Munich, Germany
Degree — Abitur (equivalent to A levels)

■ Research Experience

May 2008 – present
Institute of Molecular Immunology, Helmholtz-Zentrum München, Munich, Germany
Project — Pre-clinical mouse models to evaluate immunotherapeutic approaches against AML
Position — Doctoral Student
PI — Prof. D. J. Schendel, PhD

September 2009 – Oktober 2009
Institute for Biology, Max-Delbrück Center, Berlin, Germany
Project — Acquisition of retroviral transduction of human and murine cells (PBL, splenocytes and hematopoetic stem cells)
Position — Intern
PI — Prof. Dr. W. Uckert

June 2007 – May 2008
Institute of Molecular Immunology, Helmholtz-Zentrum München, Munich, Germany
Project — Professional antigen-presenting cells: Influence of negative costimulatory molecules on the induction of innate and adaptive immunity
Position — Diploma Student
PI — Dr. B. Frankenberger

Januar 2007
Institute of Molecular Immunology, Helmholtz-Zentrum München, Munich, Germany

Project	Analyses of TCR-alpha and -beta chains of tyrosinase-specific T cell clones
Position	Research Intern
PI	Dr. B. Frankenberger

September 2006 – October 2006
Marine Biological Laboratory (MBL), Woods Hole (MA), U.S.A.

Project	Detection of differences in the expression levels of the multi-drug-resistance receptor (MDR) in strains of Schistosoma mansoni
Position	Research Intern
PI	R.M. Greenberg, PhD

■ Academic achievements

Publications (published)

Generation of Th1-polarizing dendritic cells using the TLR7/8 agonist CL075
Spranger S, Javorovic M, Bürdek M, Wilde S, Mosetter B, Tippmer S, Bigalke I, Geiger C, Schendel DJ, Frankenberger B.
J Immunol. 2010 Jul 1;185(1):738-47. Epub 2010 May 28.
http://www.ncbi.nlm.nih.gov/pubmed/20511554

The CD6 scavenger receptor is differentially expressed on a CD56^{dim} NK cell subpopulation and contributes to NK-derived cytokine and chemokine secretion
Braun M, Müller B, Ter Meer D, Raffegerst S, Simm B, Wilde S, Spranger S, Ellwart J, Mosetter B, Umansky L, Lerchl T, Schendel DJ, Falk CS.
J Innate Immun. 2010 Dec 18. [Epub ahead of print]
http://www.ncbi.nlm.nih.gov/pubmed/21178331

MHC-restricted fratricide of human lymphocytes expressing survivin-specific transgenic T cell receptors
Leisegang M, Wilde S, Spranger S, Milosevic S, Frankenberger B, Uckert W, Schendel D J.
J Clin Invest. 2010 Oct 11 [ahead of print]
http://www.jci.org/articles/view/43437?search[article_text]=&search[authors_text]=spranger

Three-day dendritic cells for vaccine development: Antigen uptake, processing and presentation
Bürdek M, Spranger S, Wilde S, Frankenberger B, Schendel DJ, Geiger C.
J Transl Med. 2010 Sep 28;8:90.PMID: 20920165 [PubMed - in process]Free PMC Article
http://www.ncbi.nlm.nih.gov/pubmed/20920165

Dendritic cells pulsed with RNA encoding allogeneic MHC and antigen induce T cells with superior antitumor activity and higher TCR functional avidity
Wilde S, Sommermeyer D, Frankenberger B, Schiemann M, Milosevic S, Spranger S, Pohla H, Uckert W, Busch DH, Schendel DJ.
Blood 2009 Sep 3; 114(10):2132-9. Epub2009 Jul 8.
http://www.ncbi.nlm.nih.gov/pubmed/19587379?ordinalpos=1&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DefaultReportPanel.Pubmed_RVDocSum

Schistosoma mansoni P-glycoprotein levels increase in response to praziquantel exposure and correlate with reduced praziquantel susceptibility
Messerli SM, Kasinathan RS, Morgan W, Spranger S, Greenberg RM.
Mol Biochem Parasitol. 2009 Sep;167(1):54-9. Epub 2009 May 3.
http://www.ncbi.nlm.nih.gov/pubmed/19406169?ordinalpos=2&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DefaultReportPanel.Pubmed_RVDocSum

Publications (submitted)

T cell receptor-transgenic lymphocytes specific for HMMR/Rhamm limit tumor outgrowth *in vivo* (2011) Spranger S, Jeremias I, Wilde S, Mosetter B, Heemskerk MH, Schendel DJ and Frankenberger B (submitted)

NOD/scid IL-2Rg^{null} mice: a pre-clinical model system to evaluate human dendritic cell-based vaccines *in vivo* (2011) Spranger S, Frankenberger B and Schendel DJ (submitted)

Conferences/Meetings attended

Symposium on Viral Offense and Immune Defense
Munich, Germany, October 6st – October 7th, 2010
Poster presentation
Generation of Th1-polarizing dendritic cells using the TLR7/8 agonist CL075

25rd International Society for Biological Therapy of Cancer
Washington DC, USA, October 1st – October 4th, 2010
Poster presentation
Fratricide of recipient lymphocytes expressing survivin-specific transgenic T cell receptors and Enrichment of AML-specific T cells with superior functional capacity and transgenic receptor expression for adoptive T cell transfer

3rd Retreat of the Helmholtz Alliance on Immunotherapy of Cancer
Allesheim, Germany June 20th – June 22nd, 2010
Talk
MHC-restricted fratricide of human lymphocytes expressing survivin-specific transgenic T cell receptors

Final Retreat of the Program Project: Viral Functions and Immune Modulation (SBF455)
Wildbad-Kreuth, Germany June 8th – June 10th, 2010
Talk
Generation of Th1-polarizing dendritic cells using the TLR7/8 agonist CL075

3rd Tegernsee Conference
Feldafing, Germany, July 2nd – July 4th, 2009
Poster presentation
A synthetic Toll-like receptor 7/8 ligand substitutes for R848 in generating mature human monocyte-derived dendritic cells with the capacity to secrete high amounts of IL-12p70 cytokine

5th International Symposium on the Clinical Use of Cellular Products – Cellular Therapy
Nürnberg, Germany, March 19th – March 20th, 2009
Poster presentation
A synthetic Toll-like receptor 7/8 ligand substitutes for R848 in generating mature human monocyte-derived dendritic cells with the capacity to secrete high amounts of IL-12p70 cytokine

5th Spring School on Immunology
Ettal, Germany, March 1st – March 6th, 2009
Poster presentation
In vivo analyses of T cells generated against AML-associated antigens

Symposium on Adoptive T cell Therapy
Berlin, Germany, May 26th – May 28th, 2008

Extracurricular activities

March 2008 – May 2011
Spokesperson of the Graduate School “Adoptive T-cell Transfer” as part of the SFB-TR36

2008 – present
Supervision of intern students in the group of Prof. D.J. Schendel

July – August 2010
Organization of a training course to generate and culture murine T cells cultures and clones for PhD students within the SFB-TR36, together with Prof Dr. E. Simpson

October 2009
Organization of the 1st PhD retreat of the SFB-TR36 in Spitzing, Germany

2009/ 2010
Organization of a guest lecture by
Prof. N. Gascoigne (Scripps Institute, LaJolla, U.S.A.)
Prof. C. Benoist (Harvard Medical School, Boston, U.S.A.)

2005 - 2008
Elected member of the Faculty Student Council at the Department for Biology (Ludwig-Maximilians-University)