DISSERTATION

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Local Low Dose Irradiation Triggers Tumor Infiltration by Adoptively Transferred and Host T Lymphocytes and Enhances Immunotherapy in Mice

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Abstract

The use of immunotherapeutic approaches for the treatment of cancer is limited because of the intrinsic resistance of tumors to T cell infiltration and effector function. Enhanced infiltration of T cells can be achieved by inducing an activated tumor microenvironment utilizing whole body irradiation in mice. However, radiotherapy of human cancer with high doses is not applicable in some patients due to complications associated with organ damage. We hypothesized that locally applied low dose irradiation is sufficient to create a niche favoring immune effector cell entry to the tumor.

The RIP1-Tag5 (RT5) transgenic mouse model expressing the simian virus 40 derived T antigen (Tag) as a model tumor antigen was employed for this study. Following *in vitro* activation, Tag specific T cells derived from donor mice were injected into RT5 mice previously irradiated with doses ranging from 0.5 to 6 Gray. Histological examination demonstrated that transfer of activated tumor-specific CD4 or CD8 positive T cells alone resulted in low T cell frequencies in the tumor tissue, whereas a combination treatment including locally applied low dose irradiation and adoptive transfer of Tag specific T cells boosted tumor infiltration. Reduced tumor hemorrhaging was associated only with the latter treatment and indicated a treatment response. Local enrichment of adoptively transferred activated tumor-specific T cells was found to modulate the tumor microenvironment providing endogenous T cell subsets access to the tumor tissue. The observed effects correlated with the presence of innate immune cells in the tumor micromilieu which mediated tumor infiltration of T cells by production of nitric oxide (NO). Depletion of this cell population or suppression of NO synthase prevented the treatment effect as indicated by tumor regrowth and increase in mortality.

This is the first demonstration of enhanced influx of immune effector cells triggered by a combination treatment with local low dose irradiation and adoptive T cell transfer that relies on activation of the tumor microenvironment mediated by NO producing innate immune cells. We believe this treatment approach can be a foundation for the development of a novel and promising cancer therapy that utilizes an activated tumor microenvironment to selectively enrich immune effector cells facilitating immune-mediated tumor destruction.

Zusammenfassung

Das Anwendungsspektrum immunotherapeutischer Maßnahmen für die Behandlung von Krebserkrankungen ist, aufgrund der intrinsischen Resistenz von Tumoren gegenüber der Infiltrierung durch T-Zellen und deren Effektorfunktionen begrenzt. In Mäusen kann eine Steigerung der Infiltration durch T-Zellen durch das Induzieren einer aktivierten Tumor-Mikroumgebung durch Ganzkörperbestrahlung erreicht werden. Jedoch ist der Einsatz einer Hochdosis-Strahlentherapie für Krebserkrankungen beim Menschen für manche Patienten nicht geeignet, da Komplikationen aufgrund von Organschäden auftreten können. Wir vermuten daher, dass eine lokal applizierte Niedrigdosis-Bestrahlung genügt, um eine Nische zu schaffen, die den Eintritt von Immuneffektorzellen in den Tumor begünstigt.

Das transgene RIP1-Tag5 (RT5) Mausmodell, in welchem Mäuse das vom Simian-Virus 40 stammende „T Antigen“ (Tag) als Modell-Tumorantigen exprimieren, wurde für diese Studie verwendet. Im Anschluss an *in vitro*-Aktivierung wurden Tag-spezifische T-Zellen in RT5-Mäuse injiziert, die mit Dosen von 0,5 bis 6 Gray bestrahlt worden sind. Anhand histologischer Auswertung konnte gezeigt werden, dass der Transfer von aktivierten Tumor-spezifischen CD4 oder CD8 positiven T-Zellen zu niedrigen T-Zell-Frequenzen im Tumorgewebe führte, während eine Kombinationsbehandlung aus lokal applizierter Niedrigdosis-Bestrahlung und adoptivem T-Zell-Transfer die Tumorinfiltration wesentlich verstärkte. Eine Reduktion von Tumor-Hämorrhagien war nur mit letzterer Behandlung assoziiert und indizierte ein Ansprechen der Therapie. Des Weiteren wurde gezeigt, dass die lokale Anreicherung adoptiv transferierter, aktivierter Tumor-spezifischer T-Zellen die Tumor-Mikroumgebung veränderte, wodurch endogenen T-Zell-Subpopulationen Eintritt in das Tumorgewebe ermöglicht wurde. Die beobachteten Effekte korrelierten mit der Präsenz von Zellen der angeborenen Immunität im Tumor-Mikromilieu, welche die Tumor-Infiltration durch T-Zellen mit der Produktion von Stickoxid (NO) vermittelten. Depletion dieser Zellpopulation bzw. Suppression von NO-Synthase verhinderte ein Ansprechen der Therapie, wie durch erneutes Tumorwachstum und Anstieg der Mortalität gezeigt werden konnte.

Diese Studie zeigt zum ersten Mal, dass ein erhöhter Influx von Immuneffektorzellen, der durch eine Kombinationsbehandlung aus lokal applizierter Niedrigdosis-Bestrahlung und adoptivem Transfer von T-Zellen hervorgerufen wurde, auf einer Aktivierung der Tumor-Mikroumgebung beruht, welche durch NO-produzierende Zellen der angeborenen Immunität vermittelt wird. Wir sind der Ansicht, dass dieser Behandlungsansatz eine Basis für die Entwicklung einer neuen und vielversprechenden Krebstherapie sein kann, welche eine aktivierte Tumor-Mikroumgebung nutzt, um durch selektive Anreicherung von Immuneffektorzellen eine Tumordestruktion einzuleiten.

Meinen Eltern

Ne dubita, cum magna petes, impendere parva.

[M. Porcius Cato major, *Distichs*]

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Abbreviation index

℃	Degree Celsius
aa	Amino acids
A	Alanin
Ab	Antibody
ADI	Adoptive Immunotherapy
Ag	Antigen
ALCAM	Activated leukocyte cell adhesion molecule
APC	Antigen presenting cell
B	Belgium
BM	Bone marrow
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
CD	Cluster of differentiation
CLIP	Clodronate loaded liposomes
CpG	Cytosine-phosphorothioate-guanine
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen 4
D	Germany
d	Day
DC	Dendritic cell(s)
ddH_2O	Double distilled water
DKFZ	Deutsches Krebsforschungszentrum (German Cancer Research Center)
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
et al.	Et alii
FACS	Fluorescence-activated cell sorter (flow cytometry)
FCS	Fetal calf serum
Fc	Fragment crystallisable

FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box protein 3
FSC	Forward scatter
g	Gram(s)
g	9,81m/s
G	Glycin
GM-CSF	Granulocyte and monocyte colony stimulating factor
GVHD	Graft versus host desease
Gy	Gray
H	Histidin
hr	Hour(s)
HA	Hemagglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen
HPMEC	Human primary microvascular endothelial cells
HUVEC	Human umbilical vein endothelial cells
I	Isoleucin
ICAM	intercellular adhesion molecule
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
int	Intermediate
IU	International units
K	Lysin
l	Liter(s)
L	Leucin
L	Ligand
LD	Low-dose
LPS	Lipopolysaccharid
m	Months
M	Molar (mol/l)
mAb	Monoclonal antibody(ies)

MACS	Magnetic-activated cell sorting
MAdCAM	Mucosal vascular addressin cell adhesion molecule
ME	Mercaptoethanol
MHC	Major histocompatibility complex
min	Minute(s)
ml	Mililiter
µl	Microliter
N	Asparagin
NaCl	Sodium chloride
NF	Nuclear factor
NK	Natural killer
NL	The Netherlands
NOD/Scid	Non-obese diabetic/severe combined immunodeficiency
n.s.	Not significant
n.t.	Not tested
ODN	Oligodeoxynucleotide(s)
o/n	Over night
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PGE2	Prostaglandin E2
PLIP	PBS loaded liposomes
PI	Propidium iodide
Q	Glutamin
R	Arginin
rb	Retinoblastoma
RIP	Rat insulin promoter
rpm	Rounds per minute
RPMI	Roosvelt Park Memorial Institute
RT	Room temperature
RT	Radiotherapy
RT5	RIP1-Tag5
S	Serin
SD	Standard deviation

SEM	Standard error of the mean
SSC	Side scatter
SV40	Simian virus 40
T	Threonin
TA	Tumor antigen
Tag	T antigen
TAA	Tumor-associated antigen
TC	T cell(s)
TCR	T cell receptor
TCRtg	TCR transgenic
TGF	Transforming growth factor
Th	Helper T cell
TIL	Tumor infiltrating lymphocyte(s)
™	Trademark
TNF	Tumor necrosis factor
Treg	Regulatory T cell(s)
trp	Transformation related protein
U	Units
USA	United States of America
V	Valin
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
vs.	Versus
v/v	Volume per volume
W	Tryptophan
W	Watt
WHO	World Health Organization
w/o	Without
w/v	Weight per volume
µg	Microgram(s)
µl	Microliter(s)
Y	Tyrosin

1 Introduction

Until the end of last century it was questioned whether the human immune system was capable of recognizing spontaneously arising tumors and whether immune therapy has the potential to become a meaningful treatment for human malignancies. Today, cytokines and antibodies are components of widely used cancer treatment regimens but also the cellular components of the immune system are being explored for their use in immunotherapy. Since T cells play a central role in cell-mediated immunity, therapy of human malignancies using T cells is being investigated in many disease settings. With recent progress in the understanding of the requirements for immune cell activation and their accumulation at tumor sites effective attacks against tumors engaging multiple components of the immune system are now possible.

Despite the efficiency of the immune system to recognize and eliminate transformed cells, cancers can develop due to mechanisms evolved by tumors to escape from surveillance of immune cells. Recent development in the understanding of the cellular and molecular mechanisms that regulate immune responses in the tumor microenvironment has opened new potential for cancer immunotherapy. However, for the translation of this knowledge into effective tumor immunotherapy the problem of tumors escape mechanisms has to be addressed.

Tumors escape mechanisms are multiple and several of them have been discovered over the last decades. Tumor antigens may not be specific enough for discrimination from normal host cells. Tolerance may be induced by insufficient costimulation of accessory molecules on lymphocytes. Tumors may downregulate molecules on antigen presenting cells to escape from T cell recognition or produce immune inhibitory molecules such as cytokines or messenger molecules. Furthermore, tumors can also employ a mechanism to escape from immune-mediated rejection which is based on modulation of the tumor vasculature leading to restriction of access of activated effector T cells to the tumor site.

Therefore, an effective cellular immunotherapy strategy should not only facilitate immune cell activation but also has to normalize tumor vasculature to allow for accumulation of immune effector cells at tumor sites.

1.1 Cellular Immunotherapy

T cells are able to recognize either unique tumor antigens, which can evolve by mutagenesis or viral oncogenesis, or self antigens which are overexpressed or are usually restricted to an exclusive developmental phase or tissue (Kawakami et al., 1994; Mandelboim et al., 1994). T cells recognize these antigens by using clonally distributed antigen receptors (T cell receptors, TCRs) that bind to the respective antigen in the context of major histocompatibility complex (MHC) proteins. For activation of the tumor directed effector functions, T cells have to be activated by bone marrow–derived antigen presenting cells (APCs) that present tumor-derived antigens and provide essential costimulatory signals (Huang et al., 1994). Sufficiently activated T cells recirculate through the blood stream, migrate through endothelium which expresses receptors and chemokines that match their homing pattern and gain access to the tumor microenvironment where they need to overcome inhibiting mechanisms elicited by the tumor. T cell derived cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) can arrest proliferation of tumor cells and inhibit neoangiogenesis which is needed for tumor growth. Finally, T cells are able to lyse tumor cells by inducing apoptosis via perforin/ granzyme B cytolysis and/or by engaging specialized death receptors (Suda et al., 1995; Trapani and Smyth, 2002). Consequently, for the use of T cells for immunotherapy, a tumor antigen has to be identified, the antigen has to be provided in an immunogenic format to induce strong tumor-specific responses and the frequencies of reactive T cells have to be enhanced. However, generating high frequencies of activated tumor reactive T cells alone either by stimulation of host T cells or by adoptive therapy has been shown to have limited success in immunotherapy, mainly because many tumors can escape from surveillance of immune cells. The following paragraphs describe recently developed vaccination and adoptive cell transfer approaches and improvements to existing protocols which have shown potential for the treatment of human tumors.

1.1.1 Stimulation of tumor-specific immune responses *in vivo*

Tumor antigens are usually poorly immunogenic because they are naturally presented in a repressive environment and they are mostly non-mutated proteins aberrantly expressed by the tumor which results in induction of tolerance (Sakaguchi et

al., 2001). For the generation of therapeutic vaccines activated APCs can be introduced into the patient to compensate for repressive factors from the tumor environment. This has been achieved by injection of cells such as by ex vivo antigen pulsed patient derived APCs or by transplantation of dead tumor cells which are modified to secrete factors that elicit local accumulation of APCs (Dranoff et al., 1993). Additionally, injecting activators of APC such as toll-like receptor (TLR) ligands or monoclonal antibodies specific to the costimulatory molecule CD40 on APCs with the antigen, or by injecting vectors that lead to expression of the antigen and a stimulus to the innate immune system have shown some success (Pardoll, 2002).

Rejection of established tumor masses has been demonstrated in murine studies with above mentioned approaches and clinical trials show tumor regressions for some patients (Karanikas et al., 2003; Nestle et al., 1998; Rosenberg et al., 1998). But in most vaccinated patients only weak or undetectable T cell responses to the tumor antigen were found and no clinical benefit could be demonstrated. However, during encounter with a replicating foreign pathogen, APC activation and antigen presentation have been shown to prolong much longer (reviewed in Baxter and Hodgkin, 2002). Therefore, maintaining APC activation and sustaining antigen presentation *in vivo* will likely be required for vaccines to counteract tumor escape mechanisms and to become more efficient.

Attenuation of negative checkpoint signals that limit T cell responses is another way to improve vaccines. Cytotoxic T lymphocyte antigen–4 (CTLA-4) is a cell surface molecule and binding to its ligand transmits inhibitory signals to T cells. Blocking of this negative regulator of T cell activation by administration of antibodies to CTLA-4 has shown marked effects in murine models (Tivol et al., 1995). In clinical trials, lymphocytic infiltration into tumors and significant tumor-specific immune responses were demonstrated, resulting in complete regressions of advanced disease in some patients (Phan et al., 2003; van Elsas et al., 2001). This example of effectively disrupting inhibitory signals demonstrates that further breaking tumor immune resistance may yield substantial therapeutic benefit.

Inhibitory signals to tumor-specific immune responses can be transmitted specifically by a specialized subset of T cells. T regulatory (Treg) cells are a subset of T cells capable to suppress T cell responses and were detected in settings of persistent anti-

gen stimulation in the absence of inflammatory signals, particularly in the presence of transforming growth factor beta (TGF-ß). Since these cells have been found to accumulate in some cancer patients (Woo et al., 2002), depletion of these cells *in vivo* can support the potential of T cell responses. Murine studies show potential for tumor therapy (Sutmuller et al., 2001), but as with abrogation of CTLA-4 signaling, systemic ablation of Treg cells increases autoimmunity. Therefore, for enhancing existing tumor immunotherapy protocols not only counteracting tumor escape mechanisms have to be attributed, but also local enhancement of immune effector cell accumulation and function is critical.

1.1.2 Adoptive transfer of T cells for tumor immunotherapy

Infusions of lymphocytes from a matched donor have been used with success for the treatment of leukemia. But the use of these allogeneic cell infusions for tumor immunotherapy is often associated with severe and life-threatening graft-versus-host disease (GVHD) syndromes (Childs et al., 2000). A better alternative has been to reinfuse *in vitro* expanded autologous tumor-reactive T cells back into tumor patients. Tumor-infiltrating lymphocytes can be used as an enriched source of tumor-reactive cells, but since these cells recirculate trough the body they can also be obtained from peripheral blood. Using this approach, tumor-reactive T cells have been expanded to large numbers *in vitro* and could be reinfused to get augmented *in vivo* frequencies (Dudley and Rosenberg, 2003). These therapies mediated regression and occasionally complete elimination of large disseminated tumor masses without occurrence of life-threatening toxicities, but only a fraction of patients responded. Again, this observation points to the requirement that the transferred cells not only have to be activated but also must persist to counteract tumor escape mechanisms and mediate an effective response.

It has been shown that lympho-depletion of patients before adoptive transfer of T cells can promote proliferation of transferred T cells, allowing to create an *in vivo* repertoire dominated by the effector population (Dudley et al., 2002). This treatment is probably taking advantage of endogenous homeostatic mechanisms that restore lymphocyte numbers after lymphopenia and eliminate host Treg cells. This exempli-

fies that tumor induced tolerance can be reverted and thereby large quantities of tumor reactive immune effector cells generated.

To improve adoptive therapy, T cell clones instead of polyclonal T cell lines can be used, because the specificity, avidity, and effector functions of infused cells can be improved. The transfer of antigen-specific CD8 positive T cell clones has been shown to be effective for prevention of viral infections and treatment of malignant disease (Walter et al., 1995; Yee et al., 2002). Infusion of genetically modified T cells with enhanced tumor recognition was shown to be feasible using integrating vectors. High-affinity chimeric transmembrane receptors with the external recognition structure of an antibody that allows MHC-independent antigen binding but uses a signaling domain of a T cell receptor have been used to improve T cell recognition of tumors in a murine study (Brentjens et al., 2003). However, recognition of tumor antigens by cytotoxic T cells is essential but not sufficient for effective cancer immunotherapy. So far, it has yet to be shown that T cell clones retain a functional phenotype, including *in vivo* cytolytic activity and the ability to travel to tumor sites without prematurely succumbing to apoptosis in the treatment of established human tumors which evolved a plethora of immune escape mechanisms.

The possibility to design T cells capable of circumventing many of the obstacles established by tumors could result in sufficiently activated T cells that can gain access to the tumor site. However, most studies failed at addressing the complexity of the tumor microenvironment which holds stroma cells and the tumor endothelium which represents a crucial impediment for T cell extravasation. The following section outlines the role of tumor vasculature in controlling lymphocyte influx to the tumor site as a central mechanism in tumor immune resistance.

1.2 Tumors escape from immunological effector mechanisms

1.2.1 Tumor resistance mediated by the tumor vasculature

The efficacy of tumor-specific cellular strategies decreases with tumor size, demonstrating that tumors possess mechanisms to escape immune destruction. A loss of antigen expression by the tumor and abnormal expression of growth factors were shown to down-regulate immune effector function (Pardoll, 2003). Furthermore, infil-

trating leukocytes are found in the tumor stroma and can contribute to tumor immune escape. The tumor stroma is a network of extracellular matrix that harbors inflammatory cells, such as macrophages, granulocytes and dendritic cells (DCs) (Dvorak, 1986) which are known to produce factors that promote tumorigenesis and may also contribute to immune evasion, for instance, by preventing DC maturation (Mantovani et al., 2002).

Adoptive transfer of ex-vivo-expanded, tumor-specific T cells was thought to be a solution to overcome poor activation of intrinsic effector cells, failure of vaccination or tumor resistance mechanisms (Dudley and Rosenberg, 2003), since tumor-reactive T cells are capable of migrating to and destroying the tumor tissue. But it has been shown that even fully activated tumor-specific T cells can fail to reject established, immunogenic tumors (Ganss and Hanahan, 1998; Ganss et al., 2002; Garbi et al., 2004) These studies demonstrated that the tumor endothelium as part of the microenvironment represents an impediment for T cell extravasation. Moreover, endothelial cells in the tumor microenvironment can support carcinogenesis by forming new blood vessels in the process of angiogenesis (Hanahan and Folkman, 1996).

Blood vessels are not only essential for nutrient delivery to the tumor but tightly control leukocyte extravasation and therefore require orchestration of multiple receptor-ligand interactions as well a favorable cytokine/chemokine micromilieu (Butcher, 1991). Moreover, ongoing angiogenesis induces profound morphological and molecular changes in tumor blood vessels (Ryschich et al., 2002; St Croix et al., 2000) and thus contributes to the tumor's intrinsic resistance to infiltration. Therefore, effective tumor immune strategies require both highly activated effector cells and need to modulate the tumor environment to be more permissive for infiltration by immune effector cells. In particular, normalization of the tortuous tumor blood vessel network is essential to enhance immune cell accumulation at the tumor site.

1.2.2 Abnormal Blood Vessel Architecture and Function in Tumors

Along with the development of the tumor microenvironment a tumor blood vessel network is formed characterized by an impaired angiogenesis regulation and continuous formation of neovessels.

Blood vessels in tumors have been shown to be dilated, twisted and heterogeneous in spatial distribution (Jain, 1988). They originate from normal microvessels which consist of arterioles, capillaries and venules, and form a well-organized, regulated and functional architecture (Jain, 2003). The structural hallmark of normal vasculature is an organized dichotomous branching, whereas in tumor vasculature unorganized multiple branches with uneven diameters are found (Chang et al., 2000; di Tomaso et al., 2005).

To allow for rapid exchange of molecules between tumor blood vessels and surrounding tissue large inter-endothelial junctions, increased numbers of sinusoid stretches, vesico-vacuolar channels, and a lack of normal basement membrane are often found (Winkler et al., 2004). In tumors perivascular cells such as pericytes and smooth muscle cells also show an abnormal morphology and have heterogeneous associations with tumor vessels.

The underlying mechanisms behind the formation of tumor blood vessels are not well understood, but the imbalance of pro- and anti-angiogenic factors which are released by both the tumor cells and the tumor infiltrate are considered to be a key contributor (Jain, 2005). The fluid pressure which is produced by proliferating tumor cells generates mechanical stress on the vessels in tumors, which is also thought to be one contributing factor in the conversion of normal vessels to tumor vessels (Padera et al., 2004).

Furthermore, tumor blood flow velocity is not equally distributed among different tumor vessels and can even reverse its direction. Therefore, tumor areas which are poorly or not at all perfused are frequently seen. The heterogeneity of tumor blood flow is considered to be the main barrier which hinders the delivery and efficacy of therapeutic agents to tumors.

Impaired angiogenesis regulation of tumor vessels is also responsible for reduced expression of leukocyte adhesion receptors and unresponsiveness to inflammatory stimuli. Consequently, this results in impaired lymphocyte infiltration of tumors.

1.2.3 Angiogenesis controls lymphocyte infiltration of tumors

Angiogenesis is required for the outgrowth and metastasis formation of tumors (reviewed in Carmeliet, 2005) and is regulated by stimulators and inhibitors. In normal resting vasculature angiogenesis is turned off, but tumors can stimulate angiogenesis through production of cytokines such as vascular endothelial cell growth factors (VEGFs) and fibroblast growth factors (FGFs) by the tumor cells. It has long been shown that tumor blood vessels have a limited capacity to express adhesion molecules and that tumor cells are involved in the modulation of the expression levels of adhesion receptors, through the production of angiogenic growth factors (Gamble and Vadas, 1988; Wu et al., 1992). Reduced expression of leukocyte adhesion receptors and unresponsiveness to most inflammatory stimuli has been termed tumor endothelial cell anergy (Griffioen et al., 1996). This anergy has been demonstrated to add to the tumors potential to abrogate immune surveillance mechanisms and escape from immunity, allowing tumor outgrowth and metastasis formation.

It was found that endothelial cells isolated from human tumors expressed significantly lower levels of adhesion molecules such as intercellular adhesion molecule-1 (ICAM1), which are involved in interactions between lymphocytes and the vessel wall and mediate lymphocyte transmigration (Griffioen et al., 1996; Melder et al., 1996). Studies of *in vitro* adhesion assays revealed that endothelial cells exposed to tumor derived growth factors were shown to be unresponsive to inflammatory stimuli caused by cytokines such as IL-1 (interleukin-1), IFN-γ, and TNF-α. These cytokines are known to induce an adhesive phenotype in mature endothelium by upregulation of adhesion molecules. However, endothelial cells driven into angiogenesis by tumor derived growth factors were refractory to these signals (Griffioen et al., 1996).

Multiple studies indicate that the *in vitro* exposure of endothelial cells to angiogenic growth factors reduces the adherence of leukocytes under static conditions (Nooijen et al., 1998) and both rolling and firm adhesion under dynamic conditions (Griffioen et al., 1998). *In vivo* studies using a FGF or VEGF delivery system showed that the exposure to these factors reduced adhesive properties of endothelial cells even after stimulation with TNF-α (Tromp et al., 2000). Moreover, in several *in vivo* models of highly angiogenic tumors it was shown that leukocyte rolling as well as firm adhesion to the vessel wall in the tumor vasculature was markedly reduced (Dirkx et al., 2003).

The concept of endothelial cell anergy in tumors clearly reveals the need to include treatment options into immunotherapy approaches that specifically normalize tumor blood vessels to allow enhanced lymphocyte interaction with the endothelium and tumor infiltration.

1.2.4 Breaking tumor-intrinsic resistance mechanisms

In the RIP1-Tag5 (RT5) mouse model of spontaneous tumors, the capability of tumor antigen reactive T cells to infiltrate malignant tissue is lost during tumor progression, resulting in tumor outgrowth. Moreover, the model is reflecting in many ways therapeutic failures in cancer patients because conventional therapeutic approaches known to enhance costimulation and antigen presentation *in vivo* or to increase the frequency of tumor antigen reactive T cells were proved to be unsuccessful (Ganss and Hanahan, 1998; Ganss et al., 2002; Garbi et al., 2004).

Angiogenesis in RT5 mice is the first step to multistage tumorigenesis where the quiescent tumor vasculature in early neoplastic lesions undergoes an angiogenic switch and gradually converts into a chaotic network of tumor vessels (Hanahan and Folkman, 1996). Remarkably, interactions between leukocytes and endothelium which are crucial for transmigration and extravasation are lost concomitant with the earliest stage of neovascularization, implying that angiogenesis creates a barrier for effector cell extravasation (Ryschich et al., 2002).

It was shown that irradiation as a proinflammatory stimulus in a non-lymphopenic environment rendered RT5 tumors accessible for massive infiltration by adoptively transferred, tumor antigen-specific effector T cells and resulted in complete rejection of well-established tumors over a period of weeks (Ganss et al., 2002). During the phase of regression the aberrant tumor vasculature was transformed into capillaries of normal appearance and correlated with strong induction of IFN-γ and secretion of angiostatic molecules by infiltrating macrophages. These findings demonstrate that complex interactions of immune and stromal cells can inhibit angiogenesis not by induction of endothelial cell death, but by reversing vessel abnormalities resulting in improved immunotherapy.

The present study employs the RT5 tumor model to demonstrate the use of a combination therapy which is able to enhance immunotherapy and which can be translated into the clinic for the treatment of human tumors. The following section outlines how tumor immune resistance is mediated by endothelial anergy in this model and the consequences for extravasation of lymphocytes to the tumor site.

1.3 RIP1-Tag5 as a model for autochthonous tumor growth

1.3.1 Spontaneous tumors arise from multistage carcinogenesis events

In the RT5 model as well as many human tumors the angiogenesis regulation is impaired and neovessels are formed continuously (reviewed in Bergers and Benjamin, 2003). The tumor blood vessels in this model show abnormal vessel architecture and function. Therefore, hemorrhages are frequently observed as the high vessel leakiness allows erythrocytes to pass unhindered into the tumor parenchyma; furthermore, normal blood flow in the tumors is impaired (Jain, 2003; Ryschich et al., 2002)

In RT5 mice the onco-gene SV40-Tag (Simian Virus 40 large T antigen) is expressed under the control of the rat insulin promoter (RIP). By binding of the onco-protein to the tumor suppressors transformation related protein 53 (trp53) and retinoblastoma 1 (rb1) these are inactivated causing transformation of SV40 expressing cells (Blouw et al., 2003). Expression of SV40-Tag is induced specifically in the beta-cells in the Islets of Langerhans and begins in adult animals at the age of 10 weeks (Hanahan, 1985). Over a time course of 20 weeks 2 percent of the roughly 400 islets develop to highly vascularized, solid tumors (Hanahan and Folkman, 1996). At the age of about 31 weeks animals die of hypoglycemia, due to high insulin production in the beta cell tumors (Ganss and Hanahan, 1998). The tumor development in these animals proceeds through defined stages: In normal islets the SV40-Tag expressing beta-cells show no enhanced proliferation. In the first stage of tumor progression the onco-gene expression causes a focal hyperproliferation of the beta-cells in about 50% of all islets, which itself is not sufficient for the development of solid tumors. The next stage is defined by growth of new blood vessels (neovascularization) in some of the hyperplatic islets leading to angiogenic islets at an age of 16 weeks. Finally, encapsulated

tumors arise from angiogenic islets, which invade into the exocrine pancreas at a low frequency (Ganss and Hanahan, 1998; Ryschich et al., 2002).

1.3.2 Failing immune destruction of non-tolerogenic tumors

In RT5 mice the SV40-Tag transgene is not expressed during embryonic development, it is not recognized as a self-antigen and the animals do not develop tolerance. Therefore, pre-neoplastic lesions are infiltrated by SV40-Tag specific lymphocytes (Ganss and Hanahan, 1998). Despite the presence of self-reactive T cells, insulitis does not lead to the immunological destruction of beta cells. Solid tumors are free of infiltrating lymphocytes and escape from tumor-specific immune responses in this model. Neither a higher frequency of tumor-specific T cells nor a more efficient T cell priming by co-expression of the costimulatory molecule B7.1 and SV40-Tag (Ganss and Hanahan, 1998), or adoptively transferred ex vivo activated tumor-specific CD4 or CD8 positive T cells (Garbi et al., 2004) lead to tumor eradication.

In RT5 mice functional cytotoxic lymphocytes can be generated throughout the tumor development, as it has been shown by immunization studies using SV40 Tag peptides, but a tumor controlling effect of this vaccination was only seen in early stages of tumor progression. Therefore, the lack of tumor elimination does not rely on a systemic induction of tolerance (Garbi et al., 2004). A defect in priming of tumor-specific lymphocytes as a result of low antigen amounts which can be presented in lymph nodes is unlikely, because adoptively transferred naive tumor-specific CD4 or CD8 positive T cells are shown to be successfully activated in the host (Ganss et al., 2002; Garbi et al., 2004)

Extravasation of lymphocytes into the tumor site is hindered already at the beginning of neovascularization in the hyperplastic beta-cell islets (Ryschich et al., 2002), demonstrating the effect of the angiogenic switch on endothelial properties that leads to expression of proteins which are typical for angiogenetic vasculature (Griffioen et al., 1996; St Croix et al., 2000). It is conceivable that angiogenesis is important for the tumor progression in RT5 mice taken the evidence that blocking of the angiogenic switch in premalignant lesions inhibits the formation of solid tumors (Bergers et al., 1999; Bergers et al., 2003).

During the tumor development a characteristic miromillieu is formed in the RT5 tumor. This is reflected by the morphological anomalies of the blood vessels, impaired leukocyte attachment to the endothelium and the appearance of hemorrhages. As a consequence lymphocytes are excluded from the tumors. It has been shown that lymphocytes can be recruited to the tumor and that the tumor blood vessels can be normalized by interference with the micromilieu. Irradiaiton (Ganss et al., 2002) or systemic administration of cytosine-phosphorothioate-guanine-containing oligodeoxynucleotides (CpG-ODN) (Garbi et al., 2004) induced a proinflammatory micromilieu which allowed infiltration of adoptively transferred T cell receptor transgenic (TCRtg) effector T cells into the tumor. These were able to eliminate solid tumors after massive infiltration (Ganss et al., 2002; Garbi et al., 2004). This combined radiation-/immunotherapy triggered a remodeling of the tumor vasculature which was reminiscent of that from normal tissue. This process was referred to as vascular normalization (Ganss et al., 2002). Treated tumors did not show lacunae or hemorrhages and showed normal vessel diameters. This normalization was ascribed to the induction of proinflammatory and angiogenesis-inhibiting factors from innate immune cells (Ganss et al., 2002).

Tumor vessel normalization by induction of a proinflammatory environment using irradiation is employed also in the present study to enhance immunotherapy in RT5 mice. The following section describes how irradiation especially with regard to application of low doses affects both the vasculature and the immune system.

1.4 Radiation therapy of tumors

1.4.1 Radiosensitivity of vascular tissue

Treatment of cancer patients with therapeutic ionizing radiation doses which are usually higher than 10 Gy and are typically used for treatment of most tumor entities at a dose of 50 Gy (Okunieff et al., 1995) is significantly compromised by the development of damage to normal tissue, which occurs within days after exposure (Bentzen, 1997; Kiltie et al., 1999).

The radiation response consists of a first wave of acute effects which occur within 24 hours and are dominated by apoptotic cell death of endothelial cells (Pena et al.,

1997). The second wave of late vascular effects occurs within months after irradiation. Frequently observed effects consist of capillary collapse, thickening of the basement membrane and capillary dilation (Pena et al., 2000).

Since capillaries are the most radiosensitive part of the vasculature, injury of capillaries by radiation is the main cause for tissue radiosensitivity (reviewed in Fajardo et al., 2001). One of the key characteristics of endothelial radiation response is leukocyte attachment, endothelial cell swelling, and increased capillary permeability (Jaenke et al., 1993). As a consequence of irradiation, detachment of endothelial cells from the basal lamina, cell death and therefore loss of entire capillary segments are often observed, resulting in tissue ischemia or regrowth of lost vessels in some organs (reviewed in Fajardo, 1989). Capillaries also show more morphological changes than larger vessels after irradiation making a shift in size distribution to larger diameters (Dimitrievich et al., 1984).

On a molecular level, within the first wave of irradiation induced changes in endothelial cells, expression of structural proteins can be detected in these cells, which affect their physiological appearance (O'Connor and Mayberg, 2000). The hallmarks of this process are alterations in the distribution of filamentous actin, cell retraction and a dose-dependent increase in trans-endothelial flux of low–molecular-weight solutes (Friedman et al., 1986; Waters et al., 1996). Furthermore, irradiation induces alterations in the synthesis and secretion of a variety of growth factors and chemoattractants and an upregulation of injury markers is frequently detected (Nicolson et al., 1991). Finally, a fraction of the surviving endothelial cells after irradiation exposure form giant cells which are arrested in cell cycle and are permanently unable to proliferate (Rubin et al., 1989).

Although, tumor treatment using high doses of irradiation is associated with the risk of induction of toxicities especially for gastro-intestinal tumors, it is a “gold standard” treatment for many malignancies. The success of this treatment does at least in part rely on its immunostimulatory effects.

1.4.2 Immunostimulatory effects of ionizing radiation

Current success of radiotherapy relies on the well-established ability to kill cancer cells and other cells within the tumor stroma, including endothelial cells and intratumoral lymphocytes (Watters, 1999). One explanation is based on a model that predicts increased tumor antigen release upon irradiation which induces tumor cell killing and tumor antigen uptake by DC and presentation to T cells (Larsson et al., 2001). The role of radiotherapy for promotion of tumor antigen presentation by DC has been explored in preclinical studies. These studies revealed that tumor immunity can be elicited *in vivo* when tumor irradiation is combined with a DC transfer or an administration of DC growth factors to increase DC numbers in tumor-bearing mice (Chakravarty et al., 1999; Demaria et al., 2004). It has yet to be shown that radiotherapy on its own is able to enhance tumor immunity.

An optimal activation of T cells by DC presenting tumor-derived antigens can only be achieved in the presence of inflammatory or “danger” signals. Danger signals which are generated upon radiation exposure are thought to act by stimulating DC to mature so that they can efficiently present antigens, but their nature remains largely undefined (reviewed in Bianchi, 2007; McBride et al., 2004). The cytokines IL-1 and TNFα and other inducers of DC maturation can be induced by radiation both *in vitro* and *in vivo* (Hong et al., 1999; Rieser et al., 1997; Steinauer et al., 2000). Therefore, radiotherapy might provide at least some of the necessary maturation signals which improve T cell–mediated tumor immunity.

Another effect of irradiation which may improve tumor immunity might influence the effector phase of tumor cell killing. Radiation therapy has been shown to upregulate expression of death receptors, such as TNF receptor superfamily member 6 (Fas), but also MHC I and costimulatory molecules on tumor cells. This might enhance their tendency to either die or be recognized by immune effector cells (Chakraborty et al., 2003; Garnett et al., 2004; Sheard, 2001). It has been shown that after irradiation mouse colon adenocarcinoma tumor cells are sensitized to killing by adoptively transferred T cells trough irradiation induced upregulation of Fas on the tumor cells which can then be cross-linked by Fas-Ligand on T cells (Chakraborty et al., 2004).

Additionally, it was reported that both APCs and effector T cells show improved homing to the tumor after irradiation (Ganss et al., 2002; Nikitina and Gabrilovich, 2001).

This effect may be elicited by radiation induced inflammatory signals and by changes in extracellular matrix proteins and in the expression of adhesion molecules by endothelial cells (Hallahan et al., 2003; Hallahan and Virudachalam, 1999; Ryschich et al., 2003). One report highlights the distribution of cell adhesion molecules to the vascular lumen after irradiation of tumor vessels but not normal vessels, an effect which might increase the entry of effector T cells into tumors (Tanigawa et al., 2001).

While the potential for radiotherapy to elicit tumor immunity has been described in numerous reports, clinical success has yet to be demonstrated. However, strategies tested in preclinical studies have shown promise in enhancing tumor immunity by combining radiation with recently developed approaches in the immunotherapy field (reviewed in Antonia et al., 2004). Low-dosed (LD) radiotherapy could be an alternative to enhance immunotherapy for human cancer when treatment with high doses is not applicable due to high associated risk of organ toxicity.

1.4.3 Dichotomy of the irradiation dose response

The molecular responses following ionizing irradiation and the subsequent radiation-related events have started to be explored in detail in the past years (Dent et al., 2003; Hallahan et al., 1995). The upregulation of genes implicated in recognition and repair of damaged DNA and in regulation of cell death have been recognized as early molecular events. Later events include the induction of inflammation, which is under the control of transcription factors such as NF-κB (Hong et al., 1999; Weichselbaum et al., 1994). These factors are required for the expression of immune effector molecules such as cytokines, adhesion molecules and enzymes such as inducible nitric oxide synthase (iNOS) as well as molecules involved in the induction of cell death or DNA repair (Kracht and Saklatvala, 2002; Pahl, 1999). Although the induction of transcription factors and cytokines has been described after high doses of irradiation (e.g. 7–50Gy), there are only few reports about their activation by low-dose (LD) irradiation, which is usually applied in fractions of up to 2 Gy in a total dose of up to 12 Gy.

The relationship between the radiation dose and inflammatory response is however dichotomous. Radiation doses as low as 1 Gy are sufficient to exert pro-inflammatory effects (Hong et al., 1999), whereas lower doses reveal anti-inflammatory activity im-

plicating that complex mechanisms seem to differentially operate at different doses and times (Joiner et al., 2001).

1.4.4 Effects of LD irradiation on endothelial cells

Since the adhesion of leukocytes to endothelial cells represents an initial step in the transmigration process, several authors analyzed the effect of irradiation on the leukocyte adhesion using dynamic and static experimental conditions ((Hildebrandt et al., 2002; Kern et al., 2000; Roedel et al., 2002). In a static adhesion assay, where integrin mediated adhesion dominates, radiation doses of 0.3 - 0.6 Gy reduced the adhesion of leukocytes to endothelial cells but it was increased with radiation doses of 1 - 3 Gy. Higher doses than 3 Gy again reduce the adhesion of leukocytes to endothelial cells. The same was proven in a dynamic adhesion assay, where selectin mediated adhesion was enhanced with radiation doses of 1 - 3 Gy compared to lower doses (Hildebrandt et al., 2002; Rodel et al., 2004). An attenuated expression of the cell adhesion molecule E-selectin was documented with irradiation doses less than 1 Gy but not with more than 1 Gy (Hildebrandt et al., 2002; Roedel et al., 2002). Additionally an induction of ICAM1 by irradiation with doses higher than 4 Gy has been reported.

Endothelial cells play a pivotal role in the transmigration process of leukocytes because they are both able to recruit leukocytes and have the capacity to express cytokines and growth factors. The cytokine profile of stimulated endothelial cells following LD irradiation showed an elevated expression of TGF-ß at an irradiation dose of less than 1 Gy but was normalized using 2-3 Gy (Roedel et al., 2002). Several lines of evidence suggest that TGF-ß directly or indirectly mediates the decreased leukocyte adhesion following LD irradiation. For example, treatment using anti-TGF-ß antibody restored the adhesion of leukocytes to irradiated endothelial cells.

There is also considerable evidence for the contribution of the transcription factor NF-κB in the cellular responses to irradiation as well as in inflammatory processes. In activated human endothelial cells, LD irradiation resulted in the induction of NF-κB DNA binding and transcriptional activity at doses from 0.25 to 2 Gy (Prasad et al., 1994). By specific blocking of the transcriptional activity of NF-κB (Miagkov et al.,

1998), the induction of inflammatory cytokines such as IL-1 and TNFα was abrogated. These findings demonstrate that NF-κB governs inflammatory cytokine responses in endothelial cells which can be enhanced using LD irradiation.

Whereas irradiation with high doses is established to exert pro-inflammatory effects, LD radiotherapy fractions below 1 Gy are clinically well known to exert anti-inflammatory and analgesic effects on several inflammatory diseases and painful degenerative disorders. Above mentioned studies show, that irradiation doses of 2 Gy or higher have the potential to enhance lymphocyte binding to endothelium and induce proinflammatory responses. This radiation regimen should therefore be most effective to enhance cancer immunotherapy in a setting where high dose radiotherapy can not be used.

2 Objectives

Several studies which employed vaccination or adoptive cell therapy to treat tumors have shown that the generation of high frequencies of activated tumor reactive T cells alone has limited success in immunotherapy. It has been shown that this mainly depended on the ability of tumors to escape from immune destruction engaging several mechanisms. Tumors can control of extravasation of immune effector cells at the level of tumor endothelium which forms the microvasculature in the tumor microenvironment. Therefore, tumor microvessels show reduced expression of leukocyte adhesion receptors and unresponsiveness to conventional inflammatory stimuli. Consequently, this results in impaired lymphocyte infiltration of tumors. Modulation of the tumor environment has been shown to render it more permissive for infiltration by immune effector cells.

To create a preclinical system that allows evaluation of the combination of immunotherapy with irradiation the following objectives were formulated:

i. Establishment of a protocol for isolation and *in vitro* cultivation of human pancreatic tumor derived microvascular endothelial cells.
ii. Titration of the minimum effective dose of gamma irradiation needed to induce an activated phenotype of endothelial cells *in vitro*.
iii. Establishment of a immunotherapeutic treatment in a mouse model of spontaneous autochthonous tumor growth using adoptive transfers of CD4 and CD8 positive TCRtg tumor-specific T cells
iv. Application of local low dose irradiation of tumors *in vivo* and characterization of the microvasculature and infiltrating T cell subsets with and without adoptive transfer of T cells
v. Defining pathways that can regulate tumor infiltration and respond to irradiation treatment and testing their importance *in vivo* by abrogation of cell populations and / or blockage of effector mechanisms
vi. Employment of studies of tumor growth intervention and survival
vii. Comparison of adoptive transfers with alternative strategies to elicit tumor infiltration

Materials and Methods

2.1 Materials

2.1.1 Chemicals and enzymes

Product	Source of Supply
β-mercaptoethanol	Sigma, Taufkirchen
Aceton	Sigma, Taufkirchen
Agarose	GibcoBRL, Karlsruhe
Biocoll	Biochrom, Berlin
Bovine serum albumin (BSA)	Roche Diagnostics GmbH, Mannheim
Collagenase Typ1 CLS1	Cell Systems, Heidelberg; D
Deoxynucleotides (dNTPs)	Bioline, Luckenwalde
Dispase I	Roche, Darmstadt; D
EDTA (versen)	Biochrom, Berlin
Ethanol	VWR, Darmstadt
Ethidiumbromid	Roth, Karlsruhe
Fetal calf serum (FCS)	PAN Biotech, Aidenbach
Freund's Adjuvant, Incomplete	Sigma, Taufkirchen
Glycerol gelatine, Kaiser's	Merck KGaA, Darmstadt
KETANEST S 25 mg/ml	Pfizer Pharma GmbH, Berlin
Marker 1kb DNA-ladder	GibcoBRL, Karlsruhe
Phosphate buffered salt solution (DPBS), Dulbecco's	Sigma, Taufkirchen
Proteinase K	Roche Diagnostics GmbH, Mannheim
RPMI-1640	Sigma, Taufkirchen
Rompun 2%	Bayer HealthCare, Leverkusen
Taq-DNA-Polymerase	Bioline, Luckenwalde
Tris-Acetat-EDTA (TAE)	GibcoBRL, Karlsruhe
Trizol	Invitrogen, Darmstadt
Tween20	Sigma, Taufkirchen

2.1.2 Laboratory supplies

Product	Source of supply
Cell strainer	NUNC, Wiesbaden
Cryomold tissue molds	Sakura Finetek Germany GmbH, Staufen
Cryotubes (1.8 ml)	Corning B.V., Schipol-Rijk, Netherlands
Cover Slips (24 x 50 mm)	R. Langenbrinck, Teningen, Germany
Combitips	Eppendorf, Hamburg, Germany
Cell Culture Test Plates	TPP, Trasadingen, Switzerland
DAKO Pen	DAKO Diagnostika, Hamburg
Disposable syringes (1-50 ml)	BD Pharmingen, Heidelberg, Germany
Disposable scalpels	PfM AG, Köln, Germany
Disposable pipettes	Renner, Darmstadt, Germany
FACS-tubes	Greiner, Frickenhausen, Germany
MACS columns	Miltenyi, Bergisch-Gladbach
Parafilm	American National Can Company, USA
Petri Dishes (tissue culture-treated)	Biochrom, Berlin, Germany
Pipette tips (2, 20, 100, 200, 1000 µl)	Gilson, Bad Camberg, Germany
Plastics	Corning B.V. Life Sciences, Amsterdam, The Netherlands
Safe-Lock Reaction tubes (0.5 ml, 1 ml)	Eppendorf, Hamburg, Germany
Sterile-filter (0.22 µm)	Millipore, Molskeim, France
Tissue-Tek® O.C.T™ Compound	Sakura Finetek Germany GmbH, Staufen
TPP tissue culture flasks (T75)	Sigma Aldrich, Deisenhofen, Germany
Tubes (15 ml, 50 ml)	Biochrom, Berlin, Germany

2.1.3 Media and buffers

Product	Composition	
FACS staining buffer	1x	PBS
	0.5% (w/v)	BSA
	0.01% (w/v)	NaN3

Product	Composition	
ACK lysis buffer	0.15 mM	NH4Cl
	10 mM	KHCO3
	0.1 mM	Na2EDTA
		pH 7.3
Tail buffer	100 mM	Tris-HCl, pH 8.5
	5 mM	EDTA
	0.2% (w/w)	SDS
	200 mM	NaCl
TE buffer	10 mM	Tris
	1 mM	EDTA
		pH 8.0
ECBM	ECBM MV	10 ml
	HEPES	10 mM
	Penicillin	50 µg/ml
	Gentamycin	50 µg/ml
	bFGF	560 U/ml
	Hydrocortisone	100 U/ml
		pH 7.4

2.1.4 Peptides and primers

All listed peptides were produces at the Genomics & Proteomics Core Facility of the German Research Center (Heidelberg)

Protein	Peptide	Sequence
SV40 tag	362–384	TNRFNDLLDRMDIMFGSTGSADI
	560–568	SEFLLEKRI

All listed primers were purchased from Thermo Fisher Scientific GmbH (Dreieich).

Primer name	**Sequence (5' → 3')**
Tag1	GGA CAA ACC ACA ACT AGA ATG CAG
Tag2	CAG AGC AGA ATT GTG GAG TGG
ACH-1	CCA GAT TGC AGT TAT GAG GAC AGC
ACH-2	CTT GAC TAG TAT TAG CTT GGT CCC AGA GC
TGB-1	CCC TCA TTG TCC CAG AGG GAG CCA TGA C
TGB-2	CCC CCT CCG AAT GTG AGC TTG GCA CCT GC

2.1.5 Primary antibodies

2.1.5.1 Antibody reactivity: human

Name	**Host**	**Clonality**	**Supplier, cat#**
ADAM15	goat	pab	R&D Systems, AF935
MAdCAM1	goat	pab	Santa Cruz Biotech, sc-16004
Integrin beta1	mouse	mab	Santa Cruz Biotech, sc-9970
Integrin beta7	rabbit	pab	Santa Cruz Biotech, sc-15330
Tissue Factor	mouse	mab	Calbiochem, 612161
CD6	mouse	mab	Santa Cruz Biotech, sc-7320
TECK	goat	pab	R&D Systems, AF334
Flt1	rabbit	pab	Neomarkers, RB-1527-P0
E-Selectin	goat	pab	Santa Cruz Biotech, sc-6937
HCAM	rabbit	pab	Santa Cruz Biotech, sc-7051-R
ICAM1	rabbit	pab	Santa Cruz Biotech, sc-7891
ICAM2	goat	pab	Santa Cruz Biotech, sc-1512
CD107a	mouse	mab	BD Biosciences, 555798
VCAM1	mouse	mab	Santa Cruz Biotech, sc-52620
P-Selectin	rabbit	pab	USBiological, C2415-04A
ALCAM	goat	pab	Santa Cruz Biotech, sc-8548

2.1.5.2 Antibody reactivity: Simian Virus 40

Name	Host	Clonality	Supplier, cat#
SV40 Tag	rabbit	pab	Santa Cruz Biotech, sc-20800

2.1.5.3 Antibody reactivity: Murine

Name	Host	Clonality	Supplier, cat#
MAdCAM1	rat	mab	Santa Cruz Biotech, sc-19604
E-Selectin	rabbit	pab	Santa Cruz Biotech, sc-14011
ICAM1	rat	mab	Santa Cruz Biotech, sc-52553
VCAM1	rat	mab	AbD Serotec, MCA2297
P-Selectin	goat	pab	Santa Cruz Biotech, sc-6941
CD31	Rat	IgG_{2a}	BD Biosciences, 550274
FoxP3	rat	IgG_{2a}	eBioscience, 14-5773
CD4	rat	IgG_{2a}	BD Biosciences, 553647
CD8 Alpha	rat	mab	AbD Serotec, MCA1108XZ
CD3-e	goat	pab	Santa Cruz Biotech, sc-1127

2.1.6 Secondary antibodies

Reactivity	Conjugate	Host	Format	Supplier, cat#
hamster IgG	DyLight®549	goat	F(ab')2	AbD Serotec, STAR104D549
rabbit IgG	Alexa Fluor® 488	chicken	Whole ab	Invitrogen, A-21441
rabbit IgG	Alexa Fluor® 594	chicken	Whole ab	Invitrogen, A-21442
goat IgG	Alexa Fluor® 488	chicken	Whole ab	Invitrogen, A-21467
goat IgG	Alexa Fluor® 594	chicken	Whole ab	Invitrogen, A-21468
rat IgG	Alexa Fluor® 594	chicken	Whole ab	Invitrogen, A-21471

2.1.7 Mice

RT5 mice (kindly provided by N. Garbi, German Cancer Research Center, Heidelberg, Germany) express the SV40 T Antigen (Tag) under control of the rat insulin promoter (RIP) (Hanahan, 1985) and have been generated in the C3HeB/Fe back-

ground. Tag expression in RT5 mice starts at 10 weeks of age and leads to the formation of insulinomas and premature death at ~30 weeks. In indicated experiments, the F1 generation of RT5/C3H mice was used.

Mice transgenic for a TCR that recognizes Tag presented by the MHC class I molecule H2-Kk (kindly provided by N. Garbi, German Cancer Research Center, Heidelberg, Germany) are referred to as TCRCD8. TCRCD8 mice have been backcrossed on the C3HeB/Fe background and are maintained as a homozygous colony.

TagTCR1 mice bear a transgenic TCR specific for the SV40 T Ag (Forster and Lieberam, 1996). TagTCR1 mice express the H2-ABk-restricted TCR for Tag (kindly provided by N. Garbi, German Cancer Research Center, Heidelberg, Germany) and have been backcrossed on the C3HeB/Fe strain for ~30 generations. Peripheral lymphocytes represent 10% transgenic CD4 positive T cells with a normal CD4:CD8 T-cell ratio.

NOD/Scid mice were purchased from Charles River WIGA (Sulzfeld, Germany).

All mice were kept under specific pathogen-free conditions at the German Cancer Research Center. Animal experiments were approved and authorized by local government. Experiments were performed with 6- to 12-week-old mice, unless otherwise stated.

2.1.8 Equipments

Product	**Source of supply**
Biological Safety Cabinet	SterilGARD Hood Baker, Stanford, USA
Centrifuge (Minifuge T)	Heraeus, Hanau, Germany
FACS Calibur	Becton Dickinson, Heidelberg, Germany
FACS Canto II	Becton Dickinson, Heidelberg, Germany
Freezer (-20°C)	Liebherr, Ochsenhausen, Germany
Freezer (-80°C)	Forma Scientific, USA
Glass Pipettes	Hirschmann, Eberstadt, Germany
Glassware	Schott, Mainz, Germany
Incubator	Nuaire, Plymouth, USA

Product	Source of supply
Magnetic Particle Concentrator	Dynal, Hamburg, Germany
Micropipettes (2-1000 µl)	Gilson, Bad Camberg, Germany
Milli-Q Water Purification Device	Millipore, Eschborn, Germany
Multichannel Pipettes (8, 12 channels)	Rainin, Leiden, Netherlands
Neubauer counting chamber (0.1 mm)	Brand, Wertheim, Germany
OneTouch Ultra 2	LifeScan, Neckargemünd, Germany
TM separator	Miltenyi Biotech, Bergisch Gladbach, Germany
Pipetboy	Brand, Wertheim, Germany
Axioplan 2 Microscope	Carl Zeiss, Jena, Germany
Leica CM3050s cryo-microtome	Leica Microsystems, Wetzlar, Germany
Refrigerator	Liebherr, Ochsenhausen, Germany
Roller-mixer RM5	Karl Hecht GmbH, Sondheim, Germany
Spectrophotometer	Titertek Labsystems, Helsinki, Finland
Tabletop centrifuge	Heraeus, Hanau, Germany
Vortexer (Reax 2000)	Heidolph, Schwabach, Germany
Water bath (SW21)	Julabo, Seelbach, Germany

2.1.9 Software

Product	Source of supply
CELLQuest Pro (4.02)	Becton Dickinson, Heidelberg, Germany
FACS Diva	Becton Dickinson, Heidelberg, Germany
FlowJo (6.2)	Tree Star, San Carlo, USA
Graphpad Prism 5	GraphPad Software Inc., San Diego, USA
Windows XP	Microsoft, Redmond, USA
Office 2003	Microsoft, Redmond, USA

2.2 Methods

2.2.1 Murine studies

2.2.1.1 Adoptive Transfer

Donor T cells for adoptive transfers were derived from Tag-TCR1 and TCRCD8 mice. To isolate CD4 positive TCRtg T cells lymph nodes of Tag-TCR1 mice at the age of 6-8 weeks were used. The animals were sacrificed by cervical dislocation and inguinal, mesenteric, brachial, axillary and superficial cervical lymph nodes were excised in a sterile environment and kept in RPMI-1640 on ice until further use. To isolate CD8 positive TCRtg T cells the spleen of TCRCD8 mice at the age of 6-8 weeks was used. The animals were sacrificed by cervical dislocation and the spleen was excised in a sterile environment and kept in RPMI-1640 on ice until further use.

Lymph nodes and spleen were minced using a scalpel and the tissue fragments were placed into the cell strainer. Using the plunger end of the syringe, the respective tissue fragments were pressed through the cell strainer into a petri dish. The cell strainer was rinsed with 5mL RPMI-1640 and discarded. The suspended cells were transferred to a 15mL conical centrifugation tube and spun at 800xg for 3 minutes. The supernatant was discarded and the cell pellet resuspended in 1mL ACK lysis buffer. After incubation at RT for 5-10 minutes 9mL RPMI-1640 was added and the tube was spun as before. The supernatant was again discarded and the pellet resuspended in 3mL RPMI-1640. Finally the cells were counted using a hemocytometer.

Of the respective cell suspension 1.5 x 10^7 cells per six-well plate were cultured in RPMI-1640 medium supplemented with 10% FCS, 2 nM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.05 mM 2-ME, and 10 U/ml of rIL-2. TagTCR1 lymph node cell cultures received 25 nM Tag peptide 362–384 and TCRCD8 splenocytes received 25 nM Tag peptide 560–568. Cultures were incubated for 72h.

Ten days after irradiation, 5.0 x 10^6 *in vitro* activated TagTCR1 cells or TCRCD8 cells were injected intravenously or intraperitoneally in RT5 mice or NOD/Scid mice.

2.2.1.2 Organ removal

Seven days after adoptive transfer of T cells animals were sacrificed by cervical dislocation. The abdominal region was disinfected with 70% ethanol and the organs were removed under aseptic conditions. For histology all organs were frozen in Tissue-Tek O.C.T. embedding medium and stored at -80°C.

2.2.1.3 Anesthesia of mice

Anesthesia was used to produce a surgical level of anesthesia lasting 15-30 minutes and sedation of 1-2 hours. To produce 10ml anesthetic mixture consisting of 100mg ketamin and 10mg xylazine, 4 ml KETANEST S (concentration 25 mg/ml, Pfizer Pharma GmbH, Berlin) and 0.5 ml Rompun 2% (concentration 20mg/ml, Bayer HealthCare, Leverkusen) were mixed with 5.5 ml PBS. Using a syringe (1 ml) and a needle (23-25 gauge 5/8 inch) 0.1ml per 10 gm of body weight were injected intraperitoneally.

2.2.1.4 Animal irradiation

At an age of 24 weeks RT5 mice were anesthetized and irradiated with 0.5, 1, 2, or 6 Gy from a Gammatron Cobalt 60 therapy unit (Siemens, München, Germany) at a dose rate of 0.4 Gy/min. Irradiation was directed against the visceral region by shielding caudal and cranial areas of the animal using a lead apron with 3mm thickness. Subcutaneous tumors in NOD/Scid mice were irradiated with a dose of 2 Gy using the same therapy unit.

2.2.1.5 Surgical procedures

Mice were anesthetized to attain a surgical level of anesthesia. The anesthetic effect was verified by checking the plantar reflexes. The operation area was prepared by washing the surgical and adjacent area with 70% ethanol. The fur of mice was removed using a razor and the surgical area was carefully cleaned using 70% ethanol. All surgical procedures were performed in a sterile environment.

Experimental splenectomy in the mouse: At the left hypochondrium a 1.5 cm long incision was made using surgical scissors through skin and peritoneum. The spleen was lifted gently using blunt-ended forceps until the hilum was visible. A single knot was used to tie off the artery with absorbable monofilament suture (MonoPlus, B.Braun, Germany) by looping the suture through the mesentery, thereby ligating the artery that enters the spleen at the superior polus. Another single knot was made to tie off the efferent venule, which exits the spleen at the inferior polus. Connective tissue was cut away and the spleen was removed. The peritoneum was closed using absorbable monofilament suture (MonoPlus, B.Braun, Germany). The skin was closed using Ethicon Mersilene 3-0 suture (Johnson & Johnson, Neuss, Germany).

After the operation the animals were left to rest for 7 days.

Tumor allografts: Multiple tumors from RT5 mice were excised in a sterile environment and separated from non-malignant tissue in RPMI-1640 on ice using a scalpel. NOD/Scid mice were anesthetized, a dorsal skinfold was formed with blunt-ended forceps and a 1.5 cm incision was made using surgical scissors through the skin. Forceps were used to form a pocket under the skin and a single tumor was inserted. The skin was closed using Ethicon Mersilene 3-0 suture (Johnson & Johnson, Neuss, Germany).

After the operation the animals were monitored regularly for tumor growth. Mice bearing tumors which outgrew to a volume bigger than 64 mm^3 were selected for the irradiation protocol with a dose of 2 Gy and infusion of TCRtg CD8 positive T cells.

1400W slow release mini-osmotic pumps: The iNOS inhibitor 1400W (N-[[3-(Aminomethyl)phenyl]methyl]-ethanimidamide dihydrochloride) (Tocris Bioscience, United Kongdom) was used at a concentration of 240 µg/µl in PBS, 200µl of the solution were filled into a ALZET mini-osmotic pump model 2002 (ALZET Osmotic Pumps, Cupertino, USA) which provided a flow rate of 0.5 µl/h. This resulted in a dose of 144 mg/kg/d (= 6mg/kg/h) over the duration of 2 weeks.

Filling the pump was accomplished with a small syringe (1.0 ml) and the provided blunt-tipped, 27 gauge filling tube. First, the solution was drawn into the syringe and the filling tube was attached. Then, the filling tube was inserted through the opening

at the top of the pump and the pump was filled. Excess solution was wiped off and the flow moderator inserted until the white flange was flush with the top of the pump.

Once the animals were anesthetized, and prepared for surgery, a 1.5 cm wide mid-scapular incision was made using a scalpel, which allowed placing the pump on the dorsal side of the animal. A hemostat was inserted into the incision and the subcutaneous tissue was spread to create a 2.5 cm long pocket for the pump. A filled pump was inserted into the pocket, with the delivery portal first to minimize interaction between the compound and the healing of the incision. The skin was closed using Ethicon Mersilene 3-0 suture (Johnson & Johnson, Neuss, Germany).

2.2.1.6 Administration of CLIP

Clodronate was a gift from Roche (Roche Diagnostics GmbH, Mannheim, Germany) and was encapsulated in liposomes (kindly conducted by N. van Rooijen, VUMC, Amsterdam, The Netherlands) to create CLIP. For the construction of control liposomes, PBS was encapsulated. Other reagents for preparation of liposomes were phosphatidylcholine (Lipoid GmbH, Ludwigshafen, Germany) and cholesterol (SIGMA Chem.Co. USA). For long term administration of CLIP, intra-peritoneal injections were given every 5 days. For the first injection 200µl were applied, the following injections used 100µl each.

2.2.2 Methods of molecular biology

2.2.2.1 Isolation of chromosomal DNA

Mouse tail biopsies from 3 week old animals were digested for 2 hours with 0.2 mg/ml Proteinase K in tail buffer at 55°C. The suspension was diluted with 300µl ddH_2O and the samples were boiled at 95 °C for 5 minutes. The samples were cooled down to room temperature and cellular debris was removed by centrifugation (13.000 rpm/10 min). From the supernatant 3 µl were used as template for the PCR amplification.

2.2.2.2 PCR amplification of genomic DNA

The polymerase chain reaction (PCR) was used to amplify a DNA sequence from the transgene of interest, which is specific for every mouse strain used. Repeated cycles of melting, annealing and synthesis lead to exponential amplification respective sequence.

The reaction mix was set up as follows:

Reagent	**Volume**	**Final concentration**
dNTP-Mix (2.5 mM each)	4.0 µl	0.2 mM
Primer βm1 (100 pmol/µl)	0.2 µl	0.4 pmol/µl
Primer βm2 (100 pmol/µl)	0.2 µl	0.4 pmol/µl
Taq Polymerase (5 units/µl)	0.5 µl	0.05 units/µl
5x reaction buffer	10.0 µl	1x
MgCl2 (50 mM)	2.5 µl	2.5 mM
DNA Template	3.0 µl	
ddH_20	ad 50 µl	

The PCR program mix was set up as follows:

Step	**Time**	**Temperature**
I) Denaturation	60 sec	94°C
II) Annealing	30 sec	60°C
III) Elongation	120 sec	72°C
Go to step I; 35 cycles		
IV) Finalizing	10 min	72°C

2.2.2.3 Analytical agarose gel electrophoresis

Separation of the DNA fragments according to their size was carried out in a 1.5 % Agarose gel (0.2 µg/ml ethidiumbromid) in TAE buffer using an electric field. 10 µl of the PCR product were used, a standard 100 bp DNA ladder allowed evaluation of DNA fragment size. UV-irradiation at 280 nm of the gels was used to detect fluorescent DNA bands.

2.2.3 Cell culture methods

All types of cells were cultivated in sterile incubators at 37°C with a CO2-concentration of 5% (v/v).

2.2.3.1 Isolation and culture of human microvascular endothelial cells

Microvascular endothelial cells were isolated from samples of pancreatic tumors. Tissue samples were washed in phosphate-buffered saline (PBS; Invitrogen, Karlsruhe, Germany), minced mechanically into small pieces (~3 mm^2), and resuspended with endothelial cell basal medium (ECBM) + supplement. The suspension was filtered using 40-µm cell strainers (Falcon BD, Heidelberg, Germany), and the resulting cells and cell aggregates were washed with PBS. Endothelial cells were magnetically isolated from this cell population using anti–CD31-Dynabeads (Dynal). Isolated endothelial cells were used immediately or transferred to gelatin-coated (2%) cell culture flasks (Biochrom) and cultured in supplemented ECBM until passage three. During endothelial cell culture, endothelial cells derived from primary pancreatic carcinomas were supplemented with 50 µg/mL of autologous tumor cell lysate to maintain the tumor endothelial phenotype during culture. Tumor tissue lysates were prepared by mechanical homogenization using an Ultra-turrax T8 disperser (IKA, Werke Staufen, Germany). Tumor lysates were centrifuged (20 minutes at 30000g) to remove cell debris and organelles. Protein concentrations of the lysate supernatants were determined by Bradford assay (BioRad, München, Germany). Endothelial cells were always washed carefully with PBS to remove traces of cell lysates before use in subsequent experiments.

2.2.3.2 Quantification of viable cells

A Neubauer hemocytometer was used for the quantification of viable cells. The respective cell suspension was mixed with an equal volume of trypan blue [0.4% (w/v) tyrypan blue in PBS] and filled into the chamber. The counting chamber was then placed on the microscope stage and the counting grid was brought into focus at low power. Viable cells remained opaque and were counted in the 1mm center square and the four corner squares.

Each square of the hemocytometer represents a total volume of 10^{-4} cm^3. Since 1 cm^3 is equivalent to 1 ml, the subsequent cell concentration per ml was determined using the following calculation:

Cells / ml = count per square x the dilution factor x 10^4

2.2.3.3 Passage of adherent cells

When confluence was reached, adherent cells were passaged under sterile conditions. After the removal of cell culture medium, cells were washed with Dulbecco´s PBS and detached from the flask by adding EDTA/trypsin. The reaction was quenched with RPMI-1640 + 10% (v/v) FCS after 5 minutes of incubation at 37°C. The detached cells were spun down at 1400 rpm for 10 min, the pellet was resuspended in endothelial cell basal medium + supplement and finally distributed to 3 tissue culture flasks.

2.2.4 Immunological methods

2.2.4.1 Flow cytometry

Single-cell suspensions of tumor tissue (10^7 cells per well) were blocked with polyclonal human immunoglobulins (Endobulin, 2.5 mg/mL; Baxter Oncology, Frankfurt, Germany) and incubated with one of the following anti-human antibodies: beta1 integrin (1:100); or beta7 integrin (1:100), E-selectin (1:100), ICAM1 (1:100), ICAM2, MAdCAM1 (both 1:100), CD166 (1:100), CD6 (1:100); P-selectin (1:200); or VCAM1 (1:100) for 30 minutes on ice. Antibodies were detected by respective chicken anti-mouse, chicken anti-goat, or chicken anti-rabbit secondary antibodies (1:400). Dead cells, which were labeled with 1 µg/mL of propidium iodide immediately before flow cytometry, were excluded from analysis. Recordings were made from at least 1.0 x 10^5 cells on a FACS-Calibur and FACS-Canto II flow cytometer using FlowJo 6.2 software.

2.2.4.2 Immunohistochemistry

For histological analysis organs were snap-frozen in liquid nitrogen and cryosections of 5 µm thickness were produced using a cryotome. Sections were transferred to glass slides, air dried and fixed in ice-cold aceton for 10 min. Sections were used immediately after fixation for immunostaining.

A water repellant wax pen (DAKO pen) was used to create a boundary around the tissue sections on the glass slide. The immunostaining procedure was performed in a dark, humid staining chamber. Sections were rehydrated in PBS for 10 min and Fc receptors were saturated by blocking for 20 min with 1% normal chicken serum solution. Sections were incubated for 45 minutes with 100 µl of one of the following diluted detection antibodies each: CD31, FoxP3, CD4, CD8, CD3 or SV40 Tag (all 1:50). Subsequently the tissue sections were washed 3 times for 3 min in PBS. Fluorophore-conjugated secondary antibodies were diluted 1:200 in PBS and incubated for 45 minutes on the tissue sections with 100 µl each. Thereafter the sections were washed 3 times for 3 min in PBS. Finally the sections were mounted in glycerol gelatin and cover slips were mounted on the slides.

2.2.5 Statistical Analyses

P values were calculated by using two-sided Student's t test; for survival calculations Log-Rank tests were used. P less than .05 was considered to be statistically significant. Tumor growth experiments were tested using Spearman's rank correlation. Given the monotonic relation of age and blood glucose in this mouse model and a total of 16 XY pairs, a threshold value of r was set at -0.5 to indicate a significant difference in the course of blood glucose decrease.

3 Results

3.1 *In vitro* response to LD radiation

3.1.1 LD irradiation response of human tumor derived HPMEC

Endothelial cells are the first barrier of activated tumor-specific T cells that home to tumors (Buckanovich et al., 2008). In many tumors the malignant cells drive the endothelium into constant angiogenesis by secretion of angiogenic factors which finally induce endothelial cell anergy (Fukumura et al., 1998; Griffioen et al., 1999). Thus, T cells are unable to transmigrate into the tumor parenchyma to perform their effector functions such as release of anti-angiogenic cytokines or lysis of tumor cells. Since it was reported that irradiation can induce an inflammatory tumor environment allowing adoptively transferred T cells to transmigrate into the tumor (Ganss et al., 2002), direct irradiation of pancreatic tumor derived HPMEC (human primary microvascular endothelial cells) was performed to elucidate the role of radiation induced changes in expression patterns of endothelial factors associated with leukocyte transmigration.

Pancreatic tissue from patients with histologically confirmed primary pancreatic carcinoma was collected during pancreatectomy. HPMEC were isolated from resected tumor tissue as described (Nummer et al., 2007) and expanded for 4 passages. Additionally, primary endothelial cells from human umbilical vein (HUVEC) were expanded for 4 passages and incubated with tumor lysate from pancreatic carcinoma patients. Both cell types were allowed to divide until 90% confluency was reached. Subsequently, cells were subjected to irradiation doses from a linear accelerator from 0.5 up to 6 Gy in a sterile container and analyzed by flow cytometry after 4 hours, 18 hours, 1 day or 2 days, respectively. Cell Culture medium was not changed to leave growth factor levels undisturbed. The frequencies of cells double-positive for the endothelial cell marker CD31 and ADAM15, MAdCAM1, Integrin β1, Integrin β7, Tissue Factor, CD6, CCL25, Flt-1, CD62E, HCAM, ICAM1, ICAM2, CD107a, VCAM1, CD62P or ALCAM were measured.

Radiation induced cell apoptosis as detected by flow cytometric measurement of cell shape and granularity was not significant for irradiation doses less than 3 Gy, but detectable after irradiation using a dose of 3 Gy (36% of all cells) or 6 Gy (61% of all cells) (not shown). **Figure 1** depicts a representative LD irradiation response of the

expression profile of ICAM1 in HPMEC from one pancreatic carcinoma patient. The frequency of double positive endothelial cells depends on both the irradiation dose and the time point of analysis. The numbers of ICAM1 expressing cells increased in the unirradiated fraction until one day after treatment and then stabilized at that level, an artificial phenomenon that relies on deprivation of medium growth factors and overgrowth of the plastic surface. Therefore, for all following experiments the time point of analysis at 2 days after irradiation was chosen. With regard to irradiation dependency, ICAM1 expression increased (21% or 18%, respectively) at day 2 when irradiated with 3 or 6 Gray compared to the unirradiated fraction demonstrating the activating effect of LD irradiation on human tumor derived microvascular endothelial cells.

3.1.2 LD irradiation induces human tumor derived HPMEC to specifically upregulate lymphocyte transmigration associated molecules

To study the influence of irradiation on tumor vasculature, primary endothelial cells were cultured from pancreatic cancer patient derived tumors. Since it is well-known that even genetically normal primary cells placed in cell culture quickly lose their differentiated gene expression pattern and phenotype (Mooney et al., 1992), tumor microenvironment was mimicked by adding tumor lysate from each tumor to the respective cultures. **Figure 2** summarizes the irradiation induced effects in HPMEC. Significant differences could be detected in the expression patterns of P-selectin and the Ig-superfamily cell adhesion molecules ICAM1, ICAM2, MAdCAM1, VCAM1 and ALCAM. Interestingly, significantly higher frequencies of both ICAM1 and ICAM2 expressing cells were observed when treated with the highest irradiation dose (6Gy) and when treated with an intermediate dose of 2Gy. Significantly higher frequencies of VCAM1 and ALCAM expressing cells were observed when cells were treated with the highest irradiation dose (6Gy) and when treated with an intermediate irradiation dose of 1Gy. Additionally, numbers of ALCAM expressing cells were increased after 3Gy irradiation. The frequencies of both MAdCAM1 and P-selectin expressing cells were increased only when cells were treated with the highest irradiation dose (6Gy). For all increased expression patterns that were observed, treatment with the highest dose of 6Gy showed the strongest effect, the effect of the intermediate dose was

weaker. In summary, in human tumor derived HPMEC many molecules associated with lymphocyte transmigration displayed a differential activation pattern after LD irradiation.

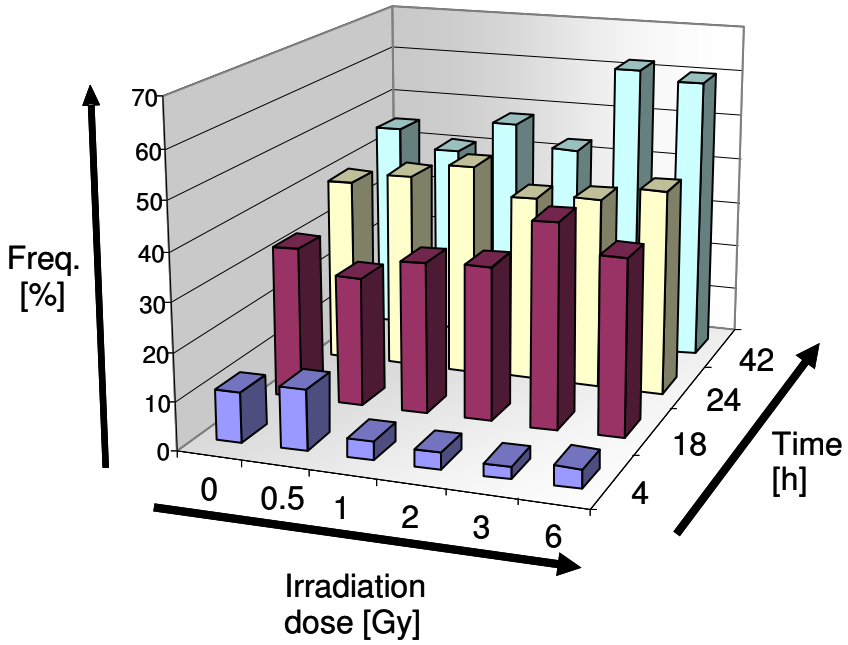

Figure 1. Representative LD irradiation response of ICAM1 in HPMEC. HPMEC were isolated from one resected pancreatic carcinoma tissue as described. After cultivation to subconfluent cell layers, HPMEC were subjected to irradiation doses of 0.5, 1, 2, 3 or 6 Gy and analyzed by flow cytometry after 4 [blue], 18 [magenta], 24 [yellow] or 42 [cyan] hours for the expression of CD31 and ICAM1. The frequency of double-positive cells is shown.

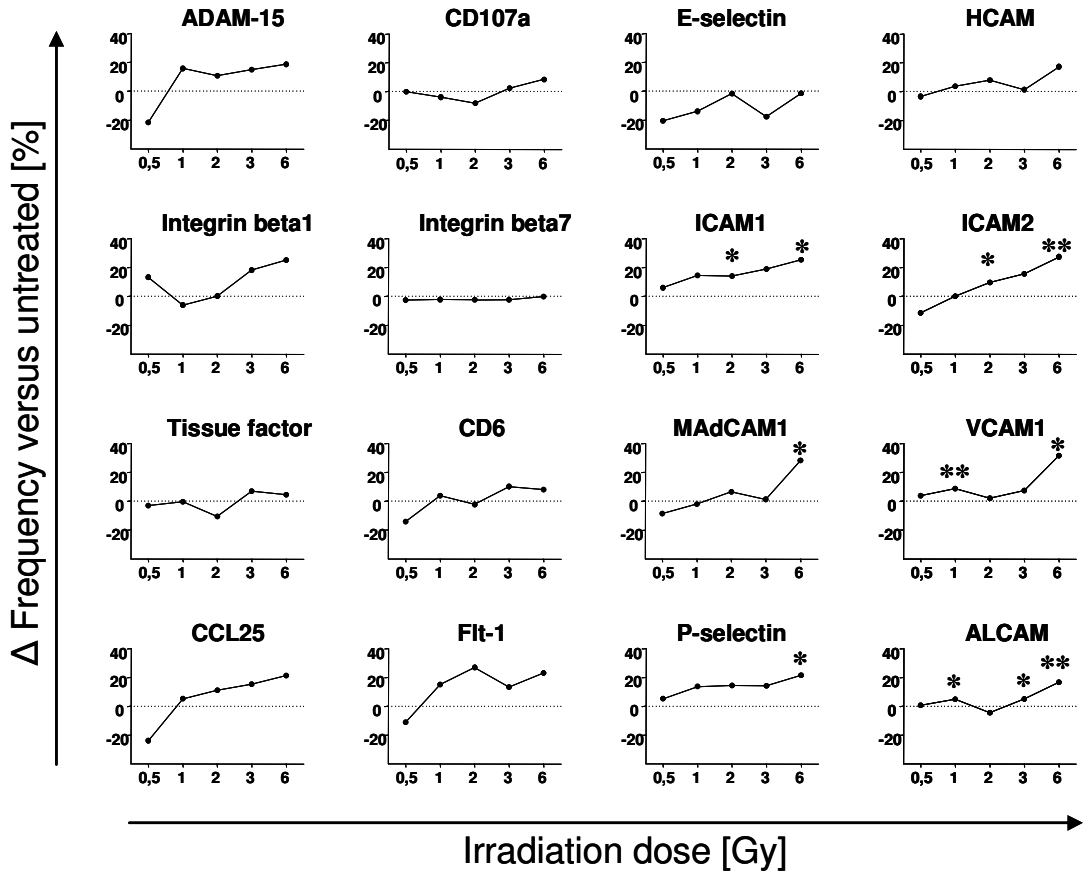

Figure 2. LD irradiation response of endothelial cell molecules in HPMEC. After cultivation to sub-confluent cell layers, HPMEC (n=4) were analyzed untreated or treated with irradiation doses of 0.5, 1, 2, 3 or 6 Gy and analyzed by flow cytometry after 2 days for the expression of CD31 and one of the indicated molecules. The difference in frequency of double-positive cells between untreated and irradiated cells is shown.

** $P < 0.05$; * $P < 0.1$ (two-tailed Student's t-test)

3.1.3 HUVEC display minimal responsiveness to LD irradiation treatment

Additionally to human tumor derived HPMEC, a different source of endothelial cells was used in order to analyze the impact of LD irradiation on a non-tumorous endothelial cell subset. HUVEC are cultured from cord of newborns and are widely used as a model for endothelial cell functions. However, the properties of untreated HUVEC do not represent the responses in physiopathology and toxicity related to the different types of endothelial cells found in an organism, especially tumor derived mi-

crovascular cells. Therefore, HUVEC were pulsed with tumor lysate since it was demonstrated that this treatment induces an adhesion molecule expression pattern in part resembling that of tumor derived microvascular cells (Nummer et al., 2007), making them better suited for studies of irradiation dependant adhesion molecule expression studies.

There was no detectable ICAM1 expression in HUVEC whether treated with irradiation or not (**figure 3**). Expression on more than 10% of all cells was limited to VCAM1 and Integrin beta 1. VCAM1 was upregulated in HUVEC pulsed with tumor lysate in 39% of the cells but not in unpulsed cells (not shown). The frequency of VCAM1 expressing cells decreased with an irradiation dose of 1Gy to 14% of the cells but not with any other dose. Integrin beta 1 was upregulated in HUVEC pulsed with tumor lysate in 51% of the cells. Irradiation induced expression of Integrin beta 1 on more than 79% of all cells, regardless of the irradiation dose. All other molecules analyzed showed expression on less than 10% of all cells and were expressed independently of irradiation treatment without statistically relevant variations. Taken together, HUVEC showed only minimal responsiveness to irradiation treatment.

3.2 Combination treatment using LD radiation and Immunotherapy

3.2.1 Local LD irradiation affects tumor microvessel morphology and cell adhesion molecule expression

Due to the change in adhesion molecule expression on human tumor derived HPMEC upon low dose irradiation (**figure 2**), it was hypothesized that locally applied low dose irradiation was sufficient to create a niche favoring immune effector cell entry to a tumor without inducing toxic side effects. To test this hypothesis *in vivo*, the RIP1-tag5 (RT5) mouse model was chosen for its ability to spontaneously develop non-tolerogenic tumors. Several irradiation doses were tested for their effectiveness to activate tumor endothelium *in vivo*. This was accomplished by LD irradiation of whole tumors by abdominal radiation treatment of RT5 mice. Tumors were excised after 1 week and analyzed by immunohistochemistry for the expression of the endothelial marker CD31 and P-selectin, E-selectin, ALCAM, VCAM1, ICAM1 or

MAdCAM1. The expression levels of E-/P-selectin and all the Ig-superfamily cell adhesion molecules but CD31 were lower than the detection limit of fluorescence microscopy (not shown). CD31 positive endothelial cells were analyzed by computer assisted measurement in the tumor and in normal surrounding pancreatic tissue for the total area, the mean diameter and the mean expression level of CD31 (**figure 4**). Tumor microvessel morphology and expression of the cell adhesion molecule CD31 was affected compared to irradiated normal tissue when tumors were irradiated with a dose of 6 Gy but not with 2 Gy. Irradiation rendered endothelial cells in tumors longer, the total area of the tissue covered by endothelial cells in tumors increased and the expression level of CD31 of tumor endothelial cells increased compared to endothelial cells from irradiated normal tissue.

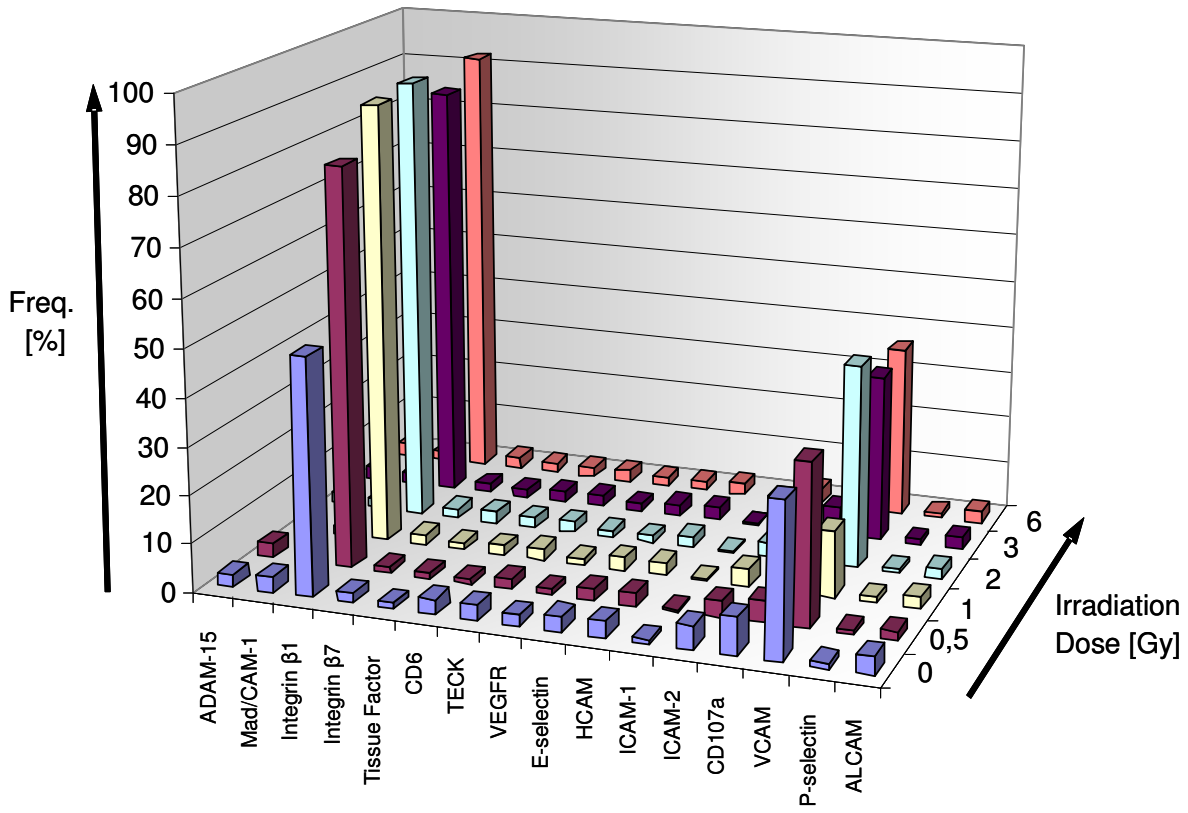

Figure 3. LD irradiation response of endothelial cell molecules in tumor lysate treated HUVEC. After cultivation to sub-confluent cell layers, HUVEC (n=4) were analyzed untreated [0, blue] or treated with irradiation doses of 0.5 [magenta], 1 [yellow], 2 [cyan], 3 [lilac] or 6 Gy [orange] and analyzed by flow cytometry after 2 days for the co-expression of CD31 and one of the indicated molecules. The frequency of double-positive cells is shown.

* $P < 0.05$ (two-tailed Student's t-test)

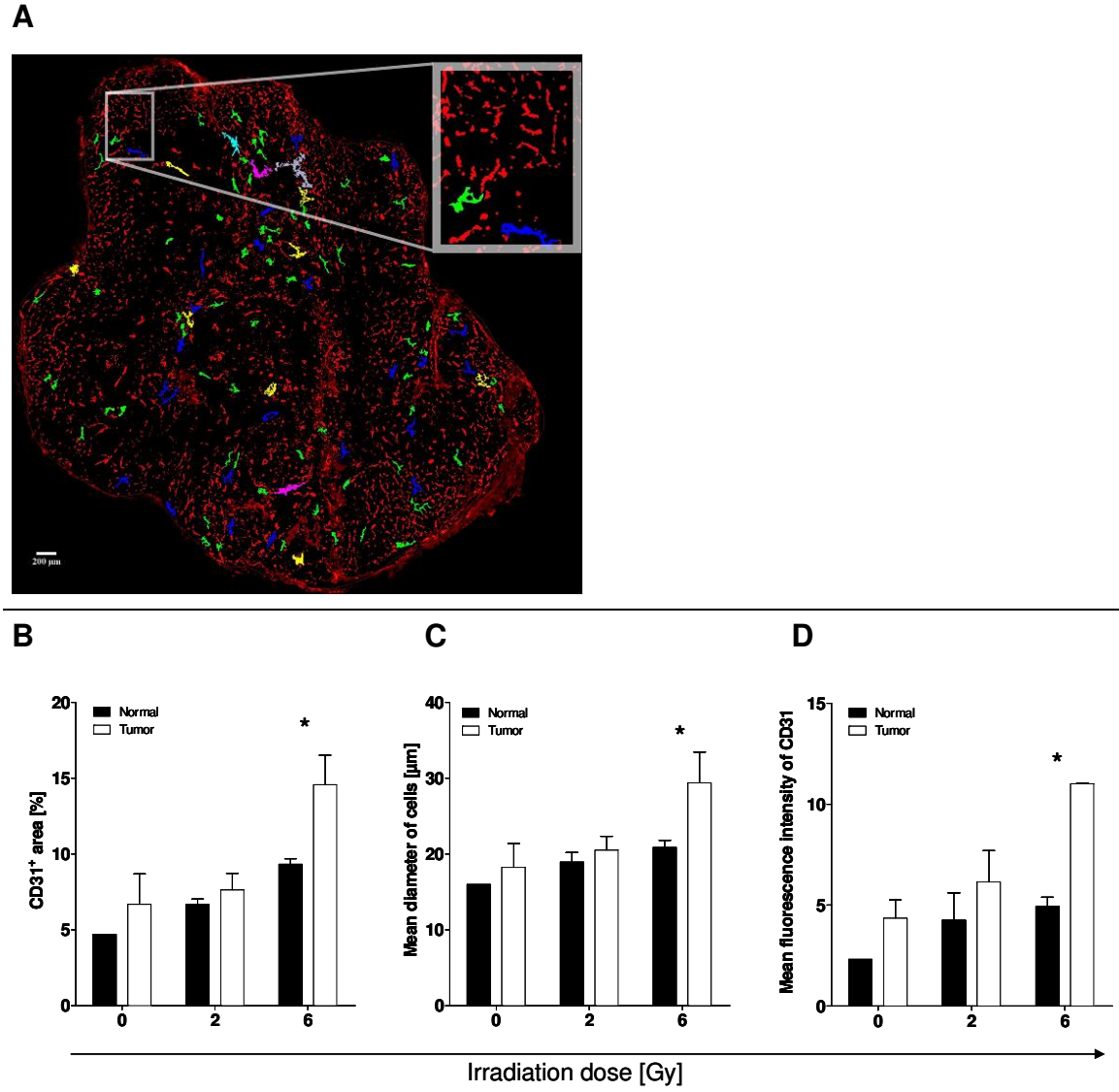

Figure 4. LD irradiation response of endothelial cells in insulinomas. Tumors and normal pancreatic tissue of RT5 mice (n=8) were left untreated or were treated with irradiation doses of 2 or 6 Gy, excised after one week and analyzed by immunohistochemistry for the expression of CD31. (**A**) Single immunofluorescence pictures were assembled into one image per tumor. CD31 positive endothelial cells were analyzed for (**B**) the fraction of total tissue area, (**C**) their mean diameter, and (**D**) the mean fluorescence intensity (MFI) of the molecule CD31 by computer assisted measurement.

* $P < 0.05$; (two-tailed Student's t-test). Color coding for mean diameter in (**A**): red <80 µm, green 80-120 µm, yellow 120-160 µm, blue 160-200 µm, turquoise 200-240 µm, lilac 240-280 µm, white >280 µm [cut-off]

3.2.2 Local LD irradiation renders solid RT5 tumors accessible for host T cell infiltration

Tumor-specific host T cells can be found in RT5 mice, since expression of the oncogene begins in adult life and leads to autoimmunity and lymphocytic infiltration of premalignant lesions (Ganss and Hanahan, 1998). To investigate whether LD irradiation alone does affect recruitment of host T cells to the tumor although no direct evidence was obtained about an increased activation of tumor microvessels after LD irradiation, the same tumors were analyzed for infiltrating T cell subsets (**figure 5**). Unirradiatetd tumors showed only base line levels of T cell infiltration. Significantly higher numbers of both CD3 positive T cells and FoxP3 positive T regulatory cells were found in irradiated tumors. However, this was observed only after irradiation with 0.5 Gy, whereas all other doses did not show this effect. Although local LD irradiation made solid RT5 tumors accessible to tumor-specific host T cell infiltration, the frequencies of infiltrated T cells were low, probably because activation of tumor endothelium could not be induced as indicated before by the lack of upregulation of transmigration relevant molecules.

It has been demonstrated that immunotherapeutic treatment of tumors in a murine tumor model system could only induce significant expression of ICAM and VCAM on tumor endothelium when radiation therapy and T cell transfer were combined, but not with either regimen alone (Quezada et al., 2008), suggesting that infiltrating T cells could be an important factor in further increasing tumor vessel activation. Therefore, a therapeutic approach using a combination of local LD irradiation and adoptive transfers of tumor-specific T cells was employed to induce T cell infiltration and destruction of RT5 tumors.

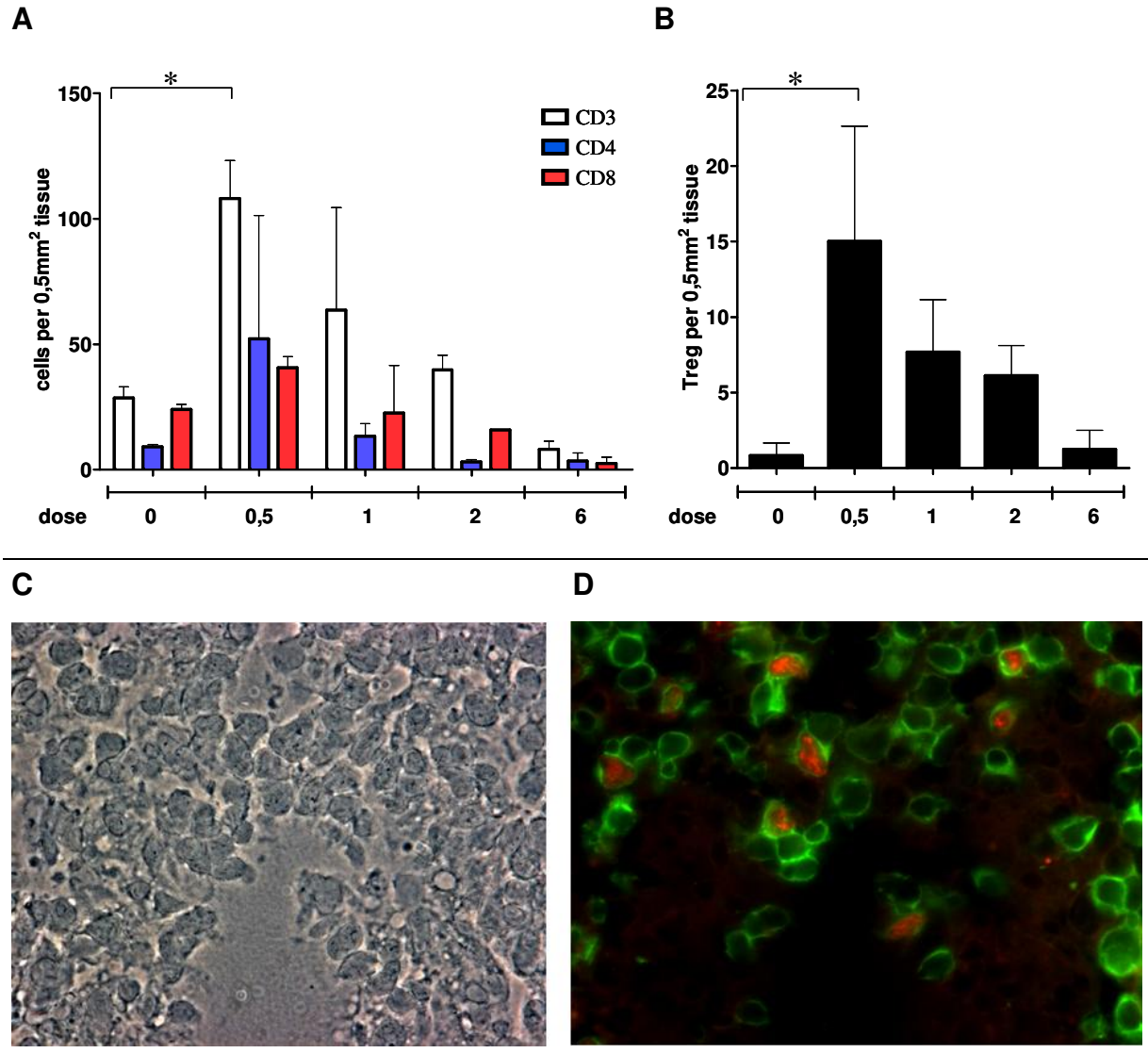

Figure 5. Tumor infiltration by host T cells after LD irradiation. Tumors of RT5 mice (n=8) were left untreated or were treated with irradiation doses of 0.5, 1, 2 or 6 Gy, excised after one week and analyzed by immunohistochemistry for CD3 (white bar), CD4 (blue bar) and CD8 positive (red bar) T cells (**A**) and for CD3 FoxP3 double positive Treg (**B**). A representative immunohistochemical staining of Treg is depicted in panel **C** and **D**, the former showing a phase contrast and the latter showing a fluorescence image (red: FoxP3, green: CD3).

* $P < 0.05$; (two-tailed Student's t-test).

3.2.3 Combination of local LD irradiation and transfer of tumor-specific T cells into tumor-bearing hosts induces massive T cell infiltration of RT5 tumors

T cell receptor transgenic (TCRtg) CD4 and CD8 positive T cells specific for the surrogate tumor antigen SV40 Tag were recovered from spleen and lymph nodes of donor mice and activated *in vitro* using IL-2 and feeder cells pulsed with MHC class I and class II restricted peptides (**figure 6**). Pre-activated T cells were adoptively transferred 10 days after LD irradiation treatment of RT5 animals. Tumors were excised 1 week after T cell transfer and analyzed by immunohistochemistry for the infiltration of CD3 positive T cells, CD4 positive T cells, CD8 positive T cells and CD3 FoxP3 double-positive T regulatory cells (Treg). Transfer of either CD4 or CD8 positive T cells without irradiation treatment resulted in higher numbers of T cells in the tumors than in untreated mice (**figure 7 A, B**); the frequency of CD4 positive T cells increased to 3 times the base line infiltration (32 vs. 13 cells per area), the frequency of CD8 positive cells increased to 9 times the base line infiltration (219 vs. 24 cells per area). When mice were subjected to irradiation treatment in combination with T cell transfer an additional boost of T cell infiltration for both transfer groups was detected; the frequency of CD4 positive cells increased up to 3 times the frequency in the unirradiated group (101 vs. 32 cells per area), the frequency of CD8 positive cells increased up to 3 times the frequency in the unirradiated group (701 vs. 219 cells per area). Additionally to the increased infiltration of the transferred T cell fraction, concomitant infiltration was observed, leading to high numbers of total CD3 positive T cells. The strongest total T cell infiltration was observed when CD4 or CD8 positive T cells were transferred into mice locally irradiated with a dose of 1 Gy, respectively (920 or 200 cells per area).

Concomitant tumor infiltration of CD4 positive host T cells after transfer of CD8 positive TCRtg T cells was most pronounced in the case of radiation treatment of RT5 mice with 0.5, 1 or 2 Gy (**figure 7 D**). Numbers of Foxp3 positive T regulatory cells were enhanced only when the lowest dose of 0.5 Gy was applied. Concomitant tumor infiltration of CD8 positive host T cells after transfer of CD4 positive TCRtg T cells was most pronounced when mice received radiation treatment of 1 or 2 Gy (**figure 7**

C). There was no enhanced infiltration of Foxp3 positive T regulatory cells in any of the settings after transfer of CD4 positive TCRtg T cells.

The results of this study proved that the transfer of tumor-specific T cells into previously low-dose irradiated tumor-bearing hosts, which probably increased tumor vessel activation as demonstrated in other studies (Ganss et al., 2002; Quezada et al., 2008), is able to induce massive T cell infiltration of RT5 tumors. To assess the capacity of T cells to interfere with tumor growth, treatment response and survival were analyzed.

3.2.4 Treatment response after massive T cell infiltration of RT5 tumors is T cell subset dependent

It was reported that after repeated adoptive transfers of TCRtg T cells into irradiated host RT5 mice reduced hemorrhages in the tumor mass can be observed and can be used as a measure of the treatment response (Ganss et al., 2002; Hamzah et al., 2008). To investigate the treatment response in RT5 mice after LD irradiation and adoptive transfer of TCRtg T cells, tumors were excised, photographed and then preserved for immunohistological use. Representative photographs are depicted in **figure 8B,** showing a whole pancreas with two highly hemorrhagic tumors (blue arrows) from the untreated control group in the upper panel and another pancreas with two non-hemorrhagic tumors (blue arrows) from the 2 Gy irradiated CD8 positive T cell transfer group in the lower panel. Transfer of TCRtg CD4 positive T cells into low-dose irradiated RT5 mice resulted in a treatment response which was 10% for the animals that were irradiated with an intermediate dose (1 or 2 Gy, respectively), the other irradiation doses showed no difference compared to control. Transfer of TCRtg CD8 positive T cells into unirradiated RT5 mice alone resulted in 23% of all tumors becoming non-hemorrhagic, significantly higher values were obtained with irradiation doses of 1, 2 or 6 Gy (75%, 50% or 63%). Consequently, a single injection of TCRtg CD4 positive T cells can induce a mild treatment response and is not likely to interfere with tumor growth, whereas a single injection of TCRtg CD8 positive T cells can induce a strong treatment response.

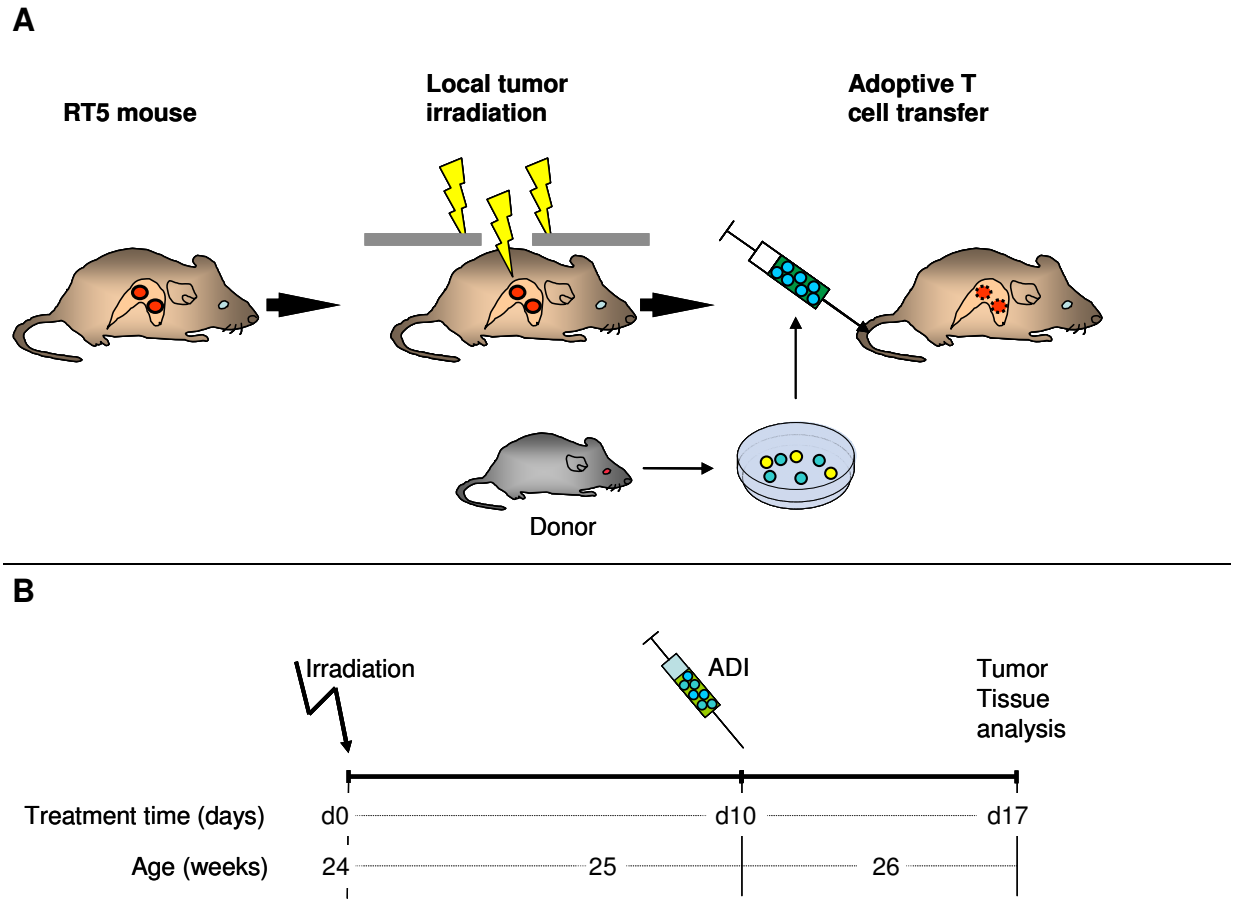

Figure 6. Local low dose irradiation protocol. **(A)** 24 weeks old RT5 mice were irradiated with 0.5, 1, 2, or 6 Gy from a Gammatron therapy unit. Irradiation was directed against the visceral region by shielding caudal and cranial areas of the animal using a lead apron. Lymphocytes were isolated from lymph nodes or spleen of donor mice (TagTCR1, TCRCD8) and were cultured in medium supplemented with 10 U/ml of rIL-2. TagTCR1 lymph node cell cultures received 25 nM Tag peptide 362–384, TCRCD8 splenocytes received 25 nM Tag peptide 560–568. Cultures were incubated for 72h. (**B**) Ten days after irradiation, 5.0 x 10^6 *in vitro* activated TagTCR1 cells or TCRCD8 cells were injected i.v. or i.p (ADI).

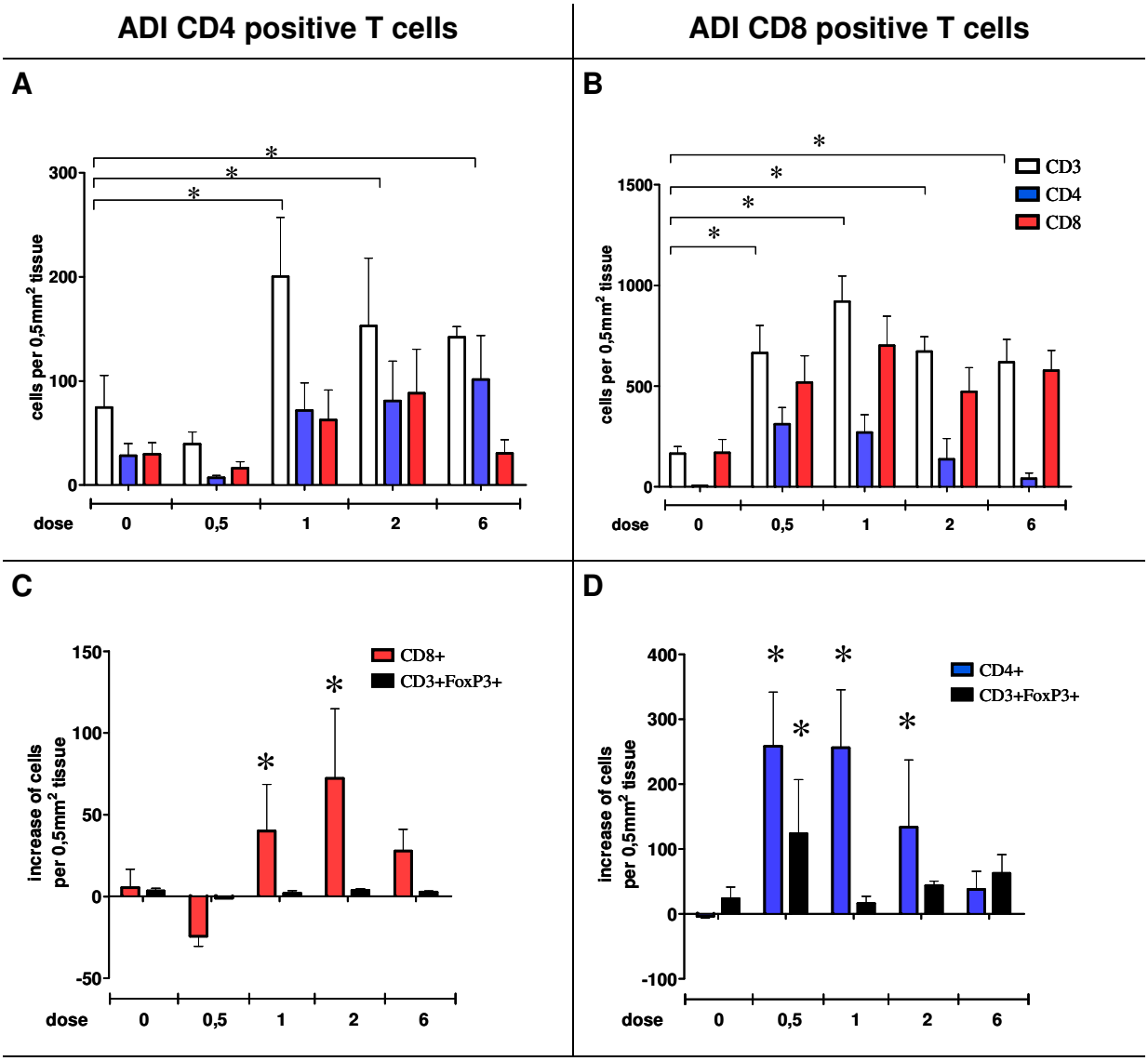

Figure 7. Tumor infiltration by transferred TCRtg T cells **(A, B**) and concomitant infiltration by host T cells (**C ,D**). RT5 mice (A:n=13, B:n=22) were left untreated or were treated with local tumor irradiation doses of 0.5, 1, 2 or 6 Gy and received after 10 days i.p. injections of TCRtg CD4 (**A**) and CD8 positive (**B**) T cells. Tumors were excised after one week and analyzed by immunohistochemistry for CD3 (white bar), CD4 (blue bar) and CD8 positive (red bar) T cells and for CD3 FoxP3 double positive Treg (black bar).

* $P < 0.05$; (two-tailed Student's t-test).

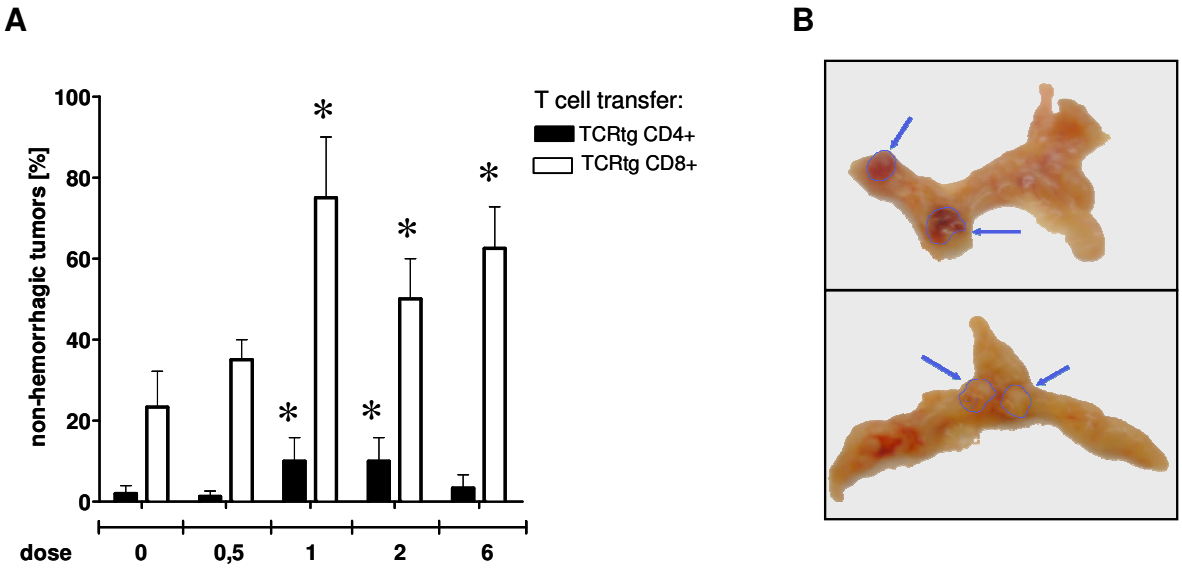

Figure 8. Treatment response after local LD irradiation. RT5 mice (n=35) were left untreated or were treated with local tumor irradiation doses of 0.5, 1, 2 or 6 Gy and received after 10 days i.p. injections of TCRtg CD4 and CD8 positive T cells, respectively. Tumors were excised after one week and analyzed for hemorrhages. The percentage of all non-hemorrhagic tumors is shown in **A**, panel **B** depicts two representative whole pancreata with hemorrhagic (top, arrows) and non-hemorrhagic tumors (bottom, arrows).

* $P < 0.05$; (two-tailed Student's t-test).

3.2.5 T cell infiltration of RT5 tumors does not depend on stimulation of lymphatic organs by irradiation

Using the irradiation protocol established in this study in combination with T cell based immunotherapy of spontaneous tumors, highly infiltrated tumors were generated showing a strong treatment response already after administration of a single injection of TCRtg CD8 positive T cells. Irradiation of the whole pancreas with adjacent organs could however induce artifacts. Since the irradiated tissue area does contain non-malignant tissue and especially lymphatic tissue such as the spleen and lymph nodes control experiments have been conducted to exclude activation of lymphocytes in these organs. Local LD irradiation with a dose of 2 Gy was used in combination with adoptive T cell transfer of TCRtg CD8 positive T cells to treat RT5 mice which have been splenectomized one week before treatment. Tumor infiltration by T

cells as read-out system was assessed using immunohistochemistry (**figure 9**). No significant differences were detected between the standard treatment of LD irradiation with a dose of 2 Gy and adoptive T cell transfer of TCRtg CD8 positive T cells and the splenectomy group in terms of infiltration by CD3 positive T cells. To confirm that irradiation has to be focused only at the tumor and effects to other lymphatic tissues such as lymph nodes are inconsequential, a subcutaneous allotransplant system was established that allowed irradiation of the tumor without irradiating adjacent organs. In this system tumor fragments from RT5 mice were transplanted subcutaneously in NOD-Scid mice which were used because of their impaired T, NK and B cell function and because they fail to stimulate complement activity making them unable to reject an allotransplant. Multiple tumors from RT5 mice were excised and transplanted in NOD-Scid mice, from these 5 tumors continued to outgrow to a volume bigger than 64 mm^3 and were selected for the irradiation protocol with a dose of 2 Gy and infusion of TCRtg CD8 positive T cells. The control group which was not irradiated but did receive T cells had completely uninfiltrated tumors, whereas the group that additionally received irradiation had infiltrated tumors although the amount of T cells (22 cells per area) was considerably lower than the baseline infiltration of transferred TCRtg CD8 positive cells in the RT5 model (165 cells per area, **figure 7**).

The independency of stimulation of lymphatic organs by irradiation makes it possible to further analyze the mechanism behind the direct activation activation of the tumor stroma by irradiation and to characterize the involved cell types.

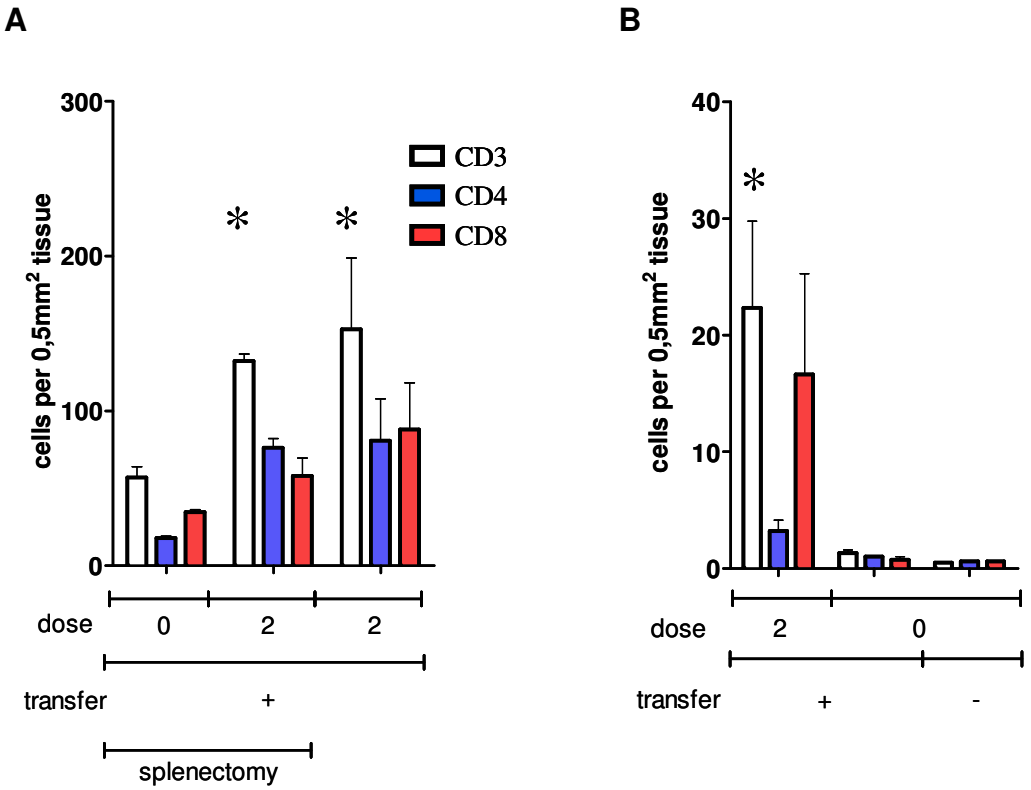

Figure 9. Tumor infiltration by transferred TCRtg T cells in splenectomized RT5 mice (**A**) and allo-transplanted NOD-Scid mice (**B**). **A**) RT5 mice (n=7) were splenectomized one week before treatment, left untreated or treated with a local tumor irradiation dose of 2 Gy and received after 10 days i.p. injections of TCRtg CD4 positive T cells. **B**) NOD-Scid mice (n=5) received tumor fragments from RT5 mice by subcutaneous transplantation, were left untreated or were treated with a local tumor irradiation dose of 2 Gy and received after 10 days i.p. injections of TCRtg CD8 positive T cells. **A, B**) Tumors were excised after one week and analyzed by immunohistochemistry for CD3 (white bar), CD4 (blue bar) and CD8 positive (red bar) T cells.

* $P < 0.05$; (two-tailed Student's t-test).

3.3 Innate immune cells mediate therapy outcome

3.3.1 Macrophage ablation prevents tumor infiltration by adoptively transferred T cells

The above mentioned results strongly indicate the requirement of low dose irradiation of the tumor to allow for the effective infiltration of transferred tumor reactive T cells. However, the target population of tumor residing cells that is capable to break the tumors resistance to infiltration is not identified. Several studies have shown that low dose irradiation can stimulate macrophages (Ibuki and Goto, 1997; Knoops et al., 2007) and that macrophages have been identified as regulators in the context of lymphocytic tumor infiltration (reviewed in Allavena et al., 2008). Therefore, investigations were undertaken to block macrophage cells or cell functions to interfere with the treatment protocol.

Clodronate loaded liposomes (CLIP) were used to deplete macrophages *in vivo*. RT5 mice were treated using LD irradiation with 2 Gy and adoptive T cell transfer of TCRtg CD4 or CD8 positive T cells and received CLIP or control liposome injections over the time span of the experimental procedure. Liposomes were administered intra-peritoneally and tumor infiltration by T cells as was assessed using immunohistochemistry (**figure 10**). Tumors from irradiated mice treated with control liposomes and TCRtg CD4 positive T cells had strongly infiltrated tumors with a total T cell count of 108 CD3 positive T cells 68 CD4 positive T cells and 48 CD8 positive T cells per tissue area. Tumors from irradiated mice treated with control liposomes and TCRtg CD8 positive T cells had strongly infiltrated tumors with a total T cell count of 480 CD3 positive T cells and 471 CD8 positive T cells. Administration of clodronate loaded liposomes instead of control liposomes together with TCRtg CD4 or CD8 positive T cells in irradiated RT5 mice reduced the numbers of total infiltrating T cells to numbers as low as 15 CD3 positive T cells or 136 CD3 positive T cells per area, respectively. There was no significant difference in respect to T cell tumor infiltration in animals that received CLIP and TCRtg CD4 or CD8 positive T cells whether animals were irradiated or not.

The depletion experiments demonstrated that tumor residing macrophages may be directly affected by local LD irradiation *in vivo*, because after macrophage ablation massive tumor infiltration by T cells was no longer possible.

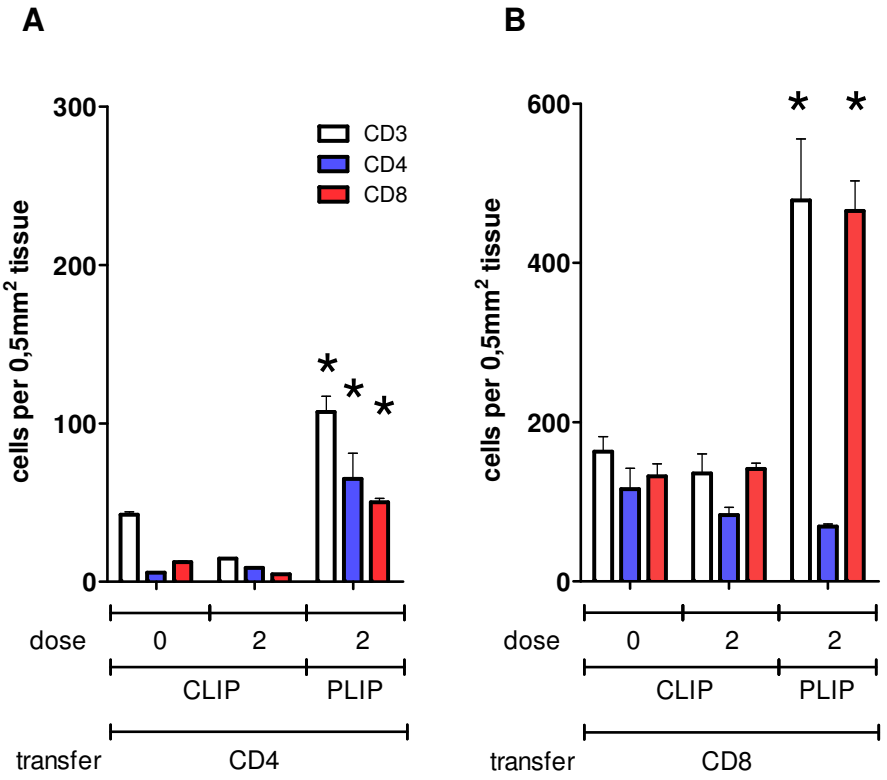

Figure 10. LD irradiation triggered tumor infiltration by transferred TCRtg T cells is abrogated by depletion of macrophages. RT5 mice (n=15) were given repeated injections of clodronate (CLIP) or saline (PLIP) loaded liposomes i.p. starting one week before treatment, were left untreated or treated with a local tumor irradiation dose of 2 Gy and received after 10 days i.p. injections of TCRtg CD4 (**A**) or CD8 (**B**) positive T cells, respectively. Tumors were excised after one week and analyzed by immunohistochemistry for CD3 (white bar), CD4 (blue bar) and CD8 positive (red bar) T cells.

* $P < 0.05$; (two-tailed Student's t-test).

3.3.2 iNOS regulates tumor infiltration by adoptively transferred CD8 positive T cells

This study demonstrated, that low dose irradiation can induce tumor derived endothelial cells to upregulate lymphocyte transmigration associated factors *in vitro* and that macrophage depletion abrogates massive T cell tumor infiltration induced by local LD irradiation and adoptive T cell transfer *in vivo*. Yet, there is no evidence for a regulation of endothelial cell function by macrophages. One major regulator of the endothelial cell layer permeability is macrophage derived nitric oxide (NO) (Fukumura

et al., 1997). Blocking of NO synthase therefore may interfere with the macrophages ability to control endothelial cell function. **Figure 11** shows the impact of the inducible nitric oxide synthase (iNOS) inhibiting drug 1400W administered trough slow-release micro-osmotic pumps implanted in RT5 mice on T cell tumor infiltration. The drug 1400W is active *in vivo* and was released trough the implants over a time span of 2 weeks. The treatment protocol of LD irradiation with a dose of 2 Gy and adoptive T cell transfer of TCRtg CD4 or CD8 positive T cells was used for RT5 mice which received 1400W or saline loaded pumps. T cell tumor infiltration by CD3, CD4 or CD8 positive T cells was not impaired in irradiated mice given CD4 positive T cells and 1400W according to statistical testing, although absolute numbers were lower than in the control group which received saline loaded pumps. However, a clear impairment was detected in the CD8 positive T cell transfer system, when 1400W loaded pumps were applied before treatment. In this group the infiltration of total CD3 and CD8 positive T cells was 4 times lower than in the control group which received saline loaded pumps (160 vs. 665 CD3 positive T cells per area, 114 vs. 460 CD8 positive T cells per area). Implantation of saline loaded pumps did not have a statistically relevant effect on T cell tumor infiltration of any subset compared to T cell tumor infiltration counts in the control group which did not receive an implant.

These results show the relevance of NO – which is probably derived from tumor residing macrophages – in regulating massive tumor infiltration by adoptively transferred CD8 positive T cells into low-dose irradiated RT5 mice.

3.3.3 Macrophage depletion derogates the ability of adoptively transferred CD4 positive T cells to induce reduction of the tumor mass and affects survival

Tumor reactive T cells that can circulate to the tumor have the ability to induce reduction of the tumor mass by direct lysis of tumor cells or cytokine mediated effects (Shankaran et al., 2001; Street et al., 2001; van den Broek et al., 1996). In the present study it was tested whether the use of LD irradiation with 2 Gy and adoptive T cell transfer of TCRtg CD4 or CD8 positive T cells in RT5 mice can interfere with tumor growth and how ablation of macrophages using clodronate loaded liposomes (CLIP) influences T cell effector functions (**figure 12** and **figure 14**). Tumor growth

was measured indirectly using amperometric capillary blood glucose measurement. Reduction of insulin producing tumor cells was indicated by higher than normal levels of blood glucose (>80mg/dL) after irradiated animals received the first infusion of TCRtg CD4 positive T cells but no clodronate loaded liposomes (38 mg/dL over normal blood glucose, week 25) or the same treatment and additional injections of saline loaded liposomes (31 mg/dL over normal blood glucose, week 26). In all other treatment groups no reduction of tumor cells after the first infusion of T cells were detected as their blood glucose levels were equal to or lower than normal. After the second infusion of T cells blood glucose levels failed to be normalized in all groups observed. Instead, the same two treatment groups that showed reduction of insulin producing tumor cells after the first infusion of TCRtg CD4 positive T cells showed stabilized blood glucose levels after the second infusion for up to 2 weeks. The above mentioned results were tested using Spearman's rank correlation. Given the monotonic relation of age and blood glucose in this mouse model – since blood glucose decreases with age – and a total of 16 XY pairs, a threshold value of r was set at -0.5 to indicate a significant difference in the course of blood glucose decrease. According to this test, only the group that received infusions of TCRtg CD4 positive T cells but no clodronate loaded liposomes after irradiation responded to the treatment ($r = -0{,}359$).

In summary, the reduction in insulin producing tumor cells could be detected only in the groups treated with the combination of LD irradiation and T cell transfer without injections of CLIP. No such effect was detected when CLIP were added to the combination treatment, when CLIP and T cell transfers were applied or in the mono-therapy conditions. Therefore, the macrophage depletion which abrogated the massive T cell infiltration into tumors that received combination treatment of LD irradiation and T cell transfer also derogated the ability of T cells to induce reduction of the tumor mass.

It was investigated, whether the observed transient normalization of blood glucose levels and the delayed decrease after the T cell infusions would lead to benefit in survival of RT5 mice (**figure 13**). Statistically significant benefit was assessed by Log-rank tests indicating improved survival for the group of animals that received LD irradiation with a dose of 2 Gy and adoptive T cell transfer of TCRtg CD4 positive T cells but no liposome injections ($p=0.0039$ vs. untreated group) and for the group that received the same treatment and saline loaded liposomes ($p=0.0296$ vs. untreated

group). The median survival of these groups was 6 or 4 weeks longer than that of the untreated group, respectively.

Improved survival could be observed only in the groups treated with the combination of LD irradiation and T cell transfer without injections of CLIP. When CLIP were added to the combination treatment or when CLIP and T cell transfers were applied or in the mono-therapy conditions survival was not improved. These survival experiments reflect the results gained from the monitoring of tumor cell death via measurements of blood glucose, in which only the combination of LD irradiation and T cell transfer without injections of CLIP was able to induce reduction in insulin producing tumor cells (**figure 12**), demonstrating the role of tumor infiltrating T cells in tumor regression.

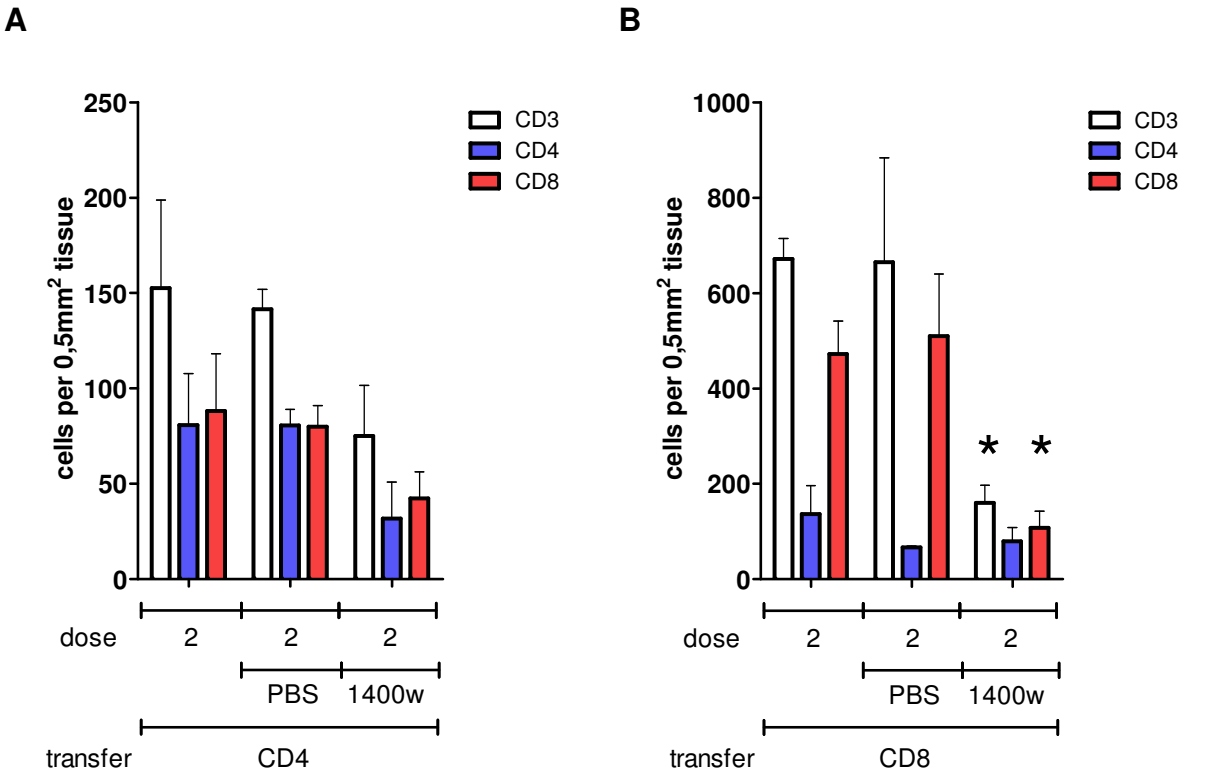

Figure 11. LD irradiation triggered tumor infiltration by transferred TCRtg T cells is abrogated by iNOS blocking. RT5 mice (n=21) received 1400W or saline loaded as a subcutaneous implant one day before treatment. Mice were left untreated or treated with a local tumor irradiation dose of 2 Gy and received after 10 days i.p. injections of TCRtg CD4 (**A**) or CD8 (**B**) positive T cells, respectively. Tumors were excised after one week and analyzed by immunohistochemistry for CD3 (white bar), CD4 (blue bar) and CD8 positive (red bar) t cells.

* $P < 0.05$; (two-tailed Student's t-test).

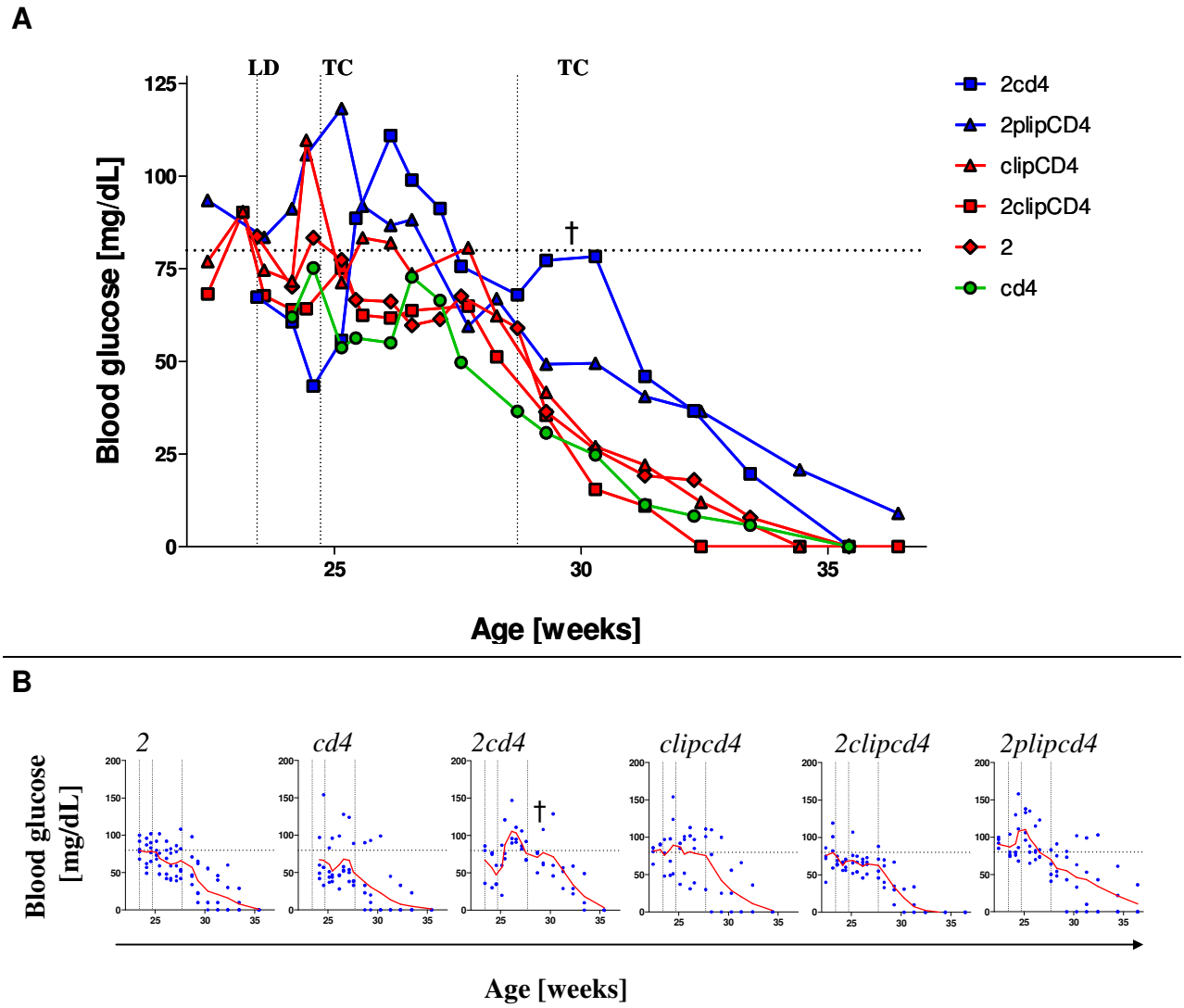

Figure 12. Tumor growth after LD irradiation and adoptive transfer of TCRtg CD4 positive T cells with and without macrophage ablation. Tumor growth was measured indirectly using an amperometric capillary blood glucose reader once weekly in RT5 mice treated with a local tumor irradiation dose of 2 Gy and received i.p. injections of TCRtg CD4 10 days later (2cd4), in mice with the same treatment plus clodronate (2clipcd4) or saline loaded liposomes (2plipcd4), and in mice that received only the irradiation (2), only the T cell infusion (cd4), or clodronate (2clipcd4) loaded liposomes and T cells (clipdcd4). **A** depicts the time course of blood glucose of all treatment groups as means, **B** shows each replicate from each group. (**A, B**) Horizontal dotted line: Normal blood glucose level at 80 mg/dL; 1st vertical dotted line: 2Gy irradiation (LD); 2nd and 3rd vertical dotted line: intravenous T cell infusions (TC).

† $r < -0.5$; (Spearman's rank correlation test).

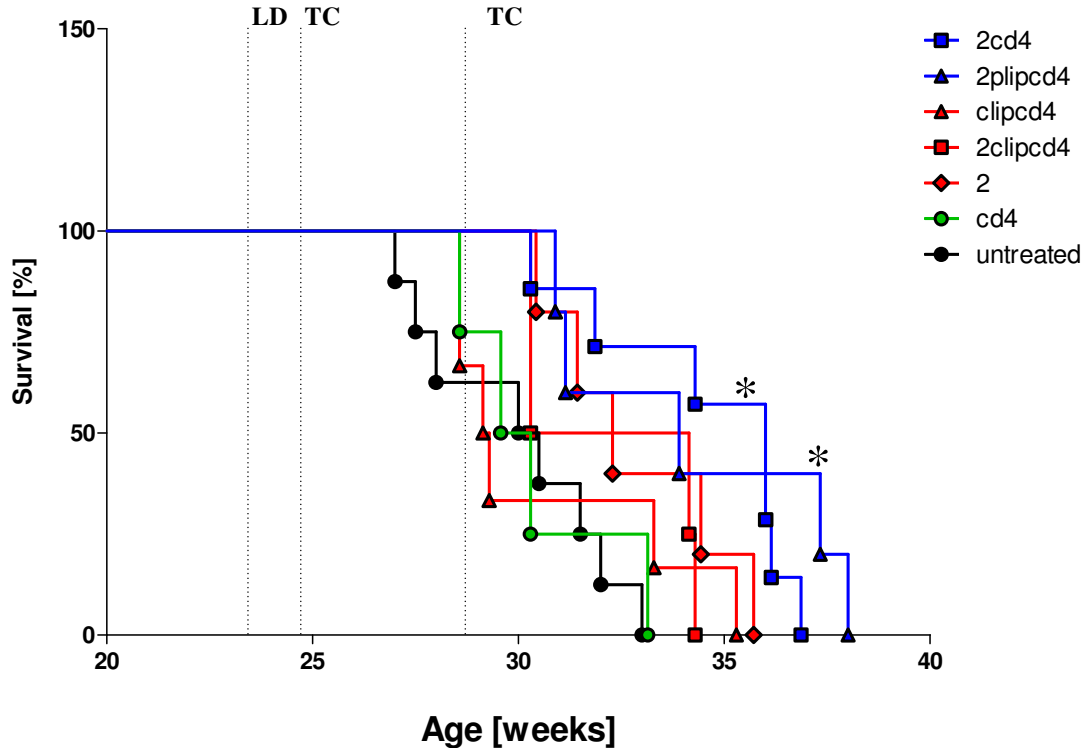

Figure 13. Survival of RT5 mice after LD irradiation and adoptive transfer of TCRtg CD4 positive T cells with and without macrophage ablation. RT5 mouse survival is shown for animals treated with a local tumor irradiation dose of 2 Gy and received i.p. injections of TCRtg CD4 10 days later (2cd4), in mice with the same treatment plus clodronate (2clipcd4) or saline loaded liposomes (2plipcd4), and in mice that received only 2 Gy irradiation (2), only a CD4 positive T cell infusion (cd4), or clodronate (2clipcd4) loaded liposomes and T cells (clipdcd4). 1st vertical dotted line: 2Gy irradiation (LD); 2nd and 3rd vertical dotted line: intravenous T cell infusions (TC).

* $P < 0.05$; (Mantel-Cox test).

3.3.4 Macrophage depletion can interfere with or enhance treatment of RT5 mice using adoptive transfer of CD8 positive T cells

Since infusions of TCRtg CD8 positive T cells displayed a stronger infiltration of pancreatic tumors and a more pronounced treatment response (**figure 7**, **figure 8**), their effect on tumor growth and survival was investigated. Upon administration of LD irradiation and infusion of TCRtg CD8 positive T cells but no liposomes, blood glucose levels were higher than normal (40 mg/dL over normal blood glucose, week 27) indicating a reduction in insulin producing cells (**figure 14**). Addition of saline loaded li-

posomes to this regimen also resulted in higher than normal (41 mg/dL over normal blood glucose, week 25) blood glucose levels as did injections of clodronate loaded liposomes in unirradiated animals that received infusion of TCRtg CD8 positive T cells (47 mg/dL over normal blood glucose, week 27). All other groups did not show signs of reduced numbers of tumor cells after T cell infusion as their blood glucose levels were lower than normal. A second infusion of T cells was not applied. The three groups that showed reduction of insulin producing tumor cells after the T cell infusion showed retardation in glucose decline for up to 2 weeks compared to control. The above mentioned results were tested using Spearman's rank correlation. According to this test, the three above mentioned groups responded to the treatment ($r < -0.5$).

Reduction in insulin producing tumor cells was detected not only in the groups treated with the combination of LD irradiation and T cell transfer without injections of CLIP but also in the group of unirradiated animals treated with injections of clodronate loaded liposomes and TCRtg CD8 positive T cells. No such effect was detected when CLIP were added to the combination treatment or in the mono-therapy conditions. Therefore, the outcome of macrophage depletion which abrogated the massive T cell infiltration into tumors that received combination treatment of LD irradiation and T cell transfer can interfere with treatment or serve by itself as a stimulatory condition that leads to tumor cell death despite of inducing a weak T cell infiltration.

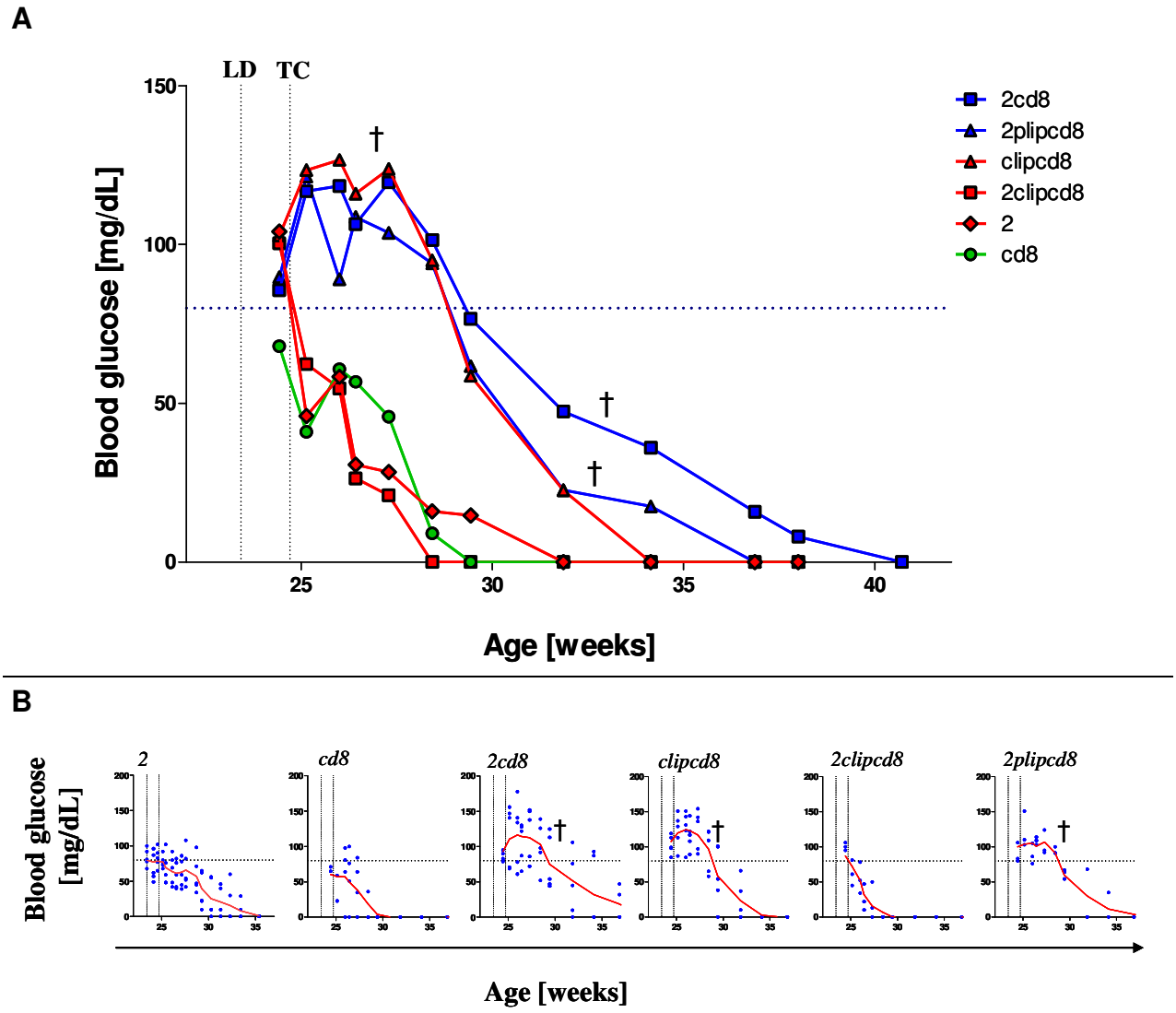

Figure 14. Tumor growth after LD irradiation and adoptive transfer of TCRtg CD8 positive T cells with and without macrophage ablation. Tumor growth was measured indirectly using an amperometric capillary blood glucose reader once weekly in RT5 mice treated with a local tumor irradiation dose of 2 Gy and received i.p. injections of TCRtg CD8 10 days later (2cd8), in mice with the same treatment plus clodronate (2clipcd8) or saline loaded liposomes (2plipcd8), and in mice that received only the irradiation (2), only the T cell infusion (cd8), or clodronate (2clipcd8) loaded liposomes and T cells (clipcd8). **A** depicts the time course of blood glucose of all treatment groups as means, **B** shows each replicate from each group. (**A, B**) Horizontal dotted line: Normal blood glucose level at 80 mg/dL; 1st vertical dotted line: 2Gy irradiation (LD); 2nd vertical dotted line: intravenous T cell infusion (TC).

† $r < -0.5$; (Spearman's rank correlation test).

The effect of transient normalization of blood glucose levels and delayed decrease after infusions of TCRtg CD8 positive T cells was investigated in terms of benefit in survival of RT5 mice (**figure 15**). Although blood glucose levels measurements indicated a treatment response for the group of animals that received LD irradiation with a dose of 2 Gy and adoptive T cell transfer of TCRtg CD8 positive T cells but no liposome injections, Logrank tests indicated no benefit in survival. Likewise, there was no survival advantage for the group that received the same treatment and saline loaded liposomes, but mice treated with clodronate loaded liposomes and T cells lived significantly longer compared to the untreated group ($p=0.0212$). All other groups showed no advantage in survival compared to the untreated group. The median survival of the group treated with clodronate loaded liposomes and T cells was 3 weeks longer than that of the untreated group and only one week for the group of animals that received LD irradiation with 2 Gy and adoptive T cell transfer.

Improved survival was only observed upon treatment with clodronate loaded liposomes and T cells. These results do not reflect the results gained from the monitoring of tumor cell death via measurements of blood glucose, in which the combination of LD irradiation and T cell transfer without injections of CLIP was able to induce reduction in insulin producing tumor cells (**Figure 14**).

3.4 Vaccination with SV40 Tag peptides induces strong T cell tumor infiltrates

Finally, a peptide based homologous prime-boost vaccination protocol was established to substitute for the infusion of T cells from congenic donors (**figure 16**). In short, MHC class I and class II restricted peptides from the SV40 Tag protein were emulsified and injected under the neck skin of RT5 mice once before and once after treatment with 2 Gy irradiation. A significant increase in total CD3 positive tumor infiltrating T cells was detected upon administration of a single peptide priming injection of MHC class II restricted peptides in animals irradiated with a dose of 6 Gy (8 vs. 50 cells per area; $p=0.05$) or both priming and boost injections (8 vs. 62 cells per area; $p=0.004$). Irradiation using a dose of 2 Gy was not sufficient to allow for infiltration of increased numbers of CD3 positive T cells, both in the prime and in the prime-boost setting using MHC class II restricted peptides. In all tumors analyzed, the numbers of

infiltrated CD4 positive T cells was low, compared to the effect of adoptively transferred activated CD4 positive T cells (**figure 7A)**. However, a single vaccination of RT5 mice using MHC class I restricted peptides in combination with local tumor irradiation with a dose of 6 Gy resulted in significantly higher numbers of CD3 (100 vs. 8 cells per area, p=0.016), CD4 (20 vs. 3 cells per area, p=0.008), and CD8 positive T cells (98 vs. 3 cells per area, p=0.018) compared to control. A second boosting vaccination also resulted in higher numbers of CD3 (195 vs. 8 cells per area, p=0.023), CD4 (31 vs. 3 cells per area, p=0.017), and CD8 positive T cells (191 vs. 3 cells per area, p=0.021) compared to control. However, combination of prime-boost vaccination and local tumor irradiation with 2 Gy resulted in significantly higher numbers of infiltrated CD3 positive T cells (161 vs. 40 cells per area, p=0.043). Priming vaccination alone did not alter the levels of infiltrating T cells.

Tumors with strong T cell infiltrates could be recovered from RT5 mice using this homologous prime-boost vaccination protocol, however, the use of adoptively transferred T cells resulted in tumors which were infiltrated 3 to 5 times more with T cells (**figure 7**). Moreover, the use of MHC class II restricted peptides failed to induce tumor infiltrating CD4 positive T cells.

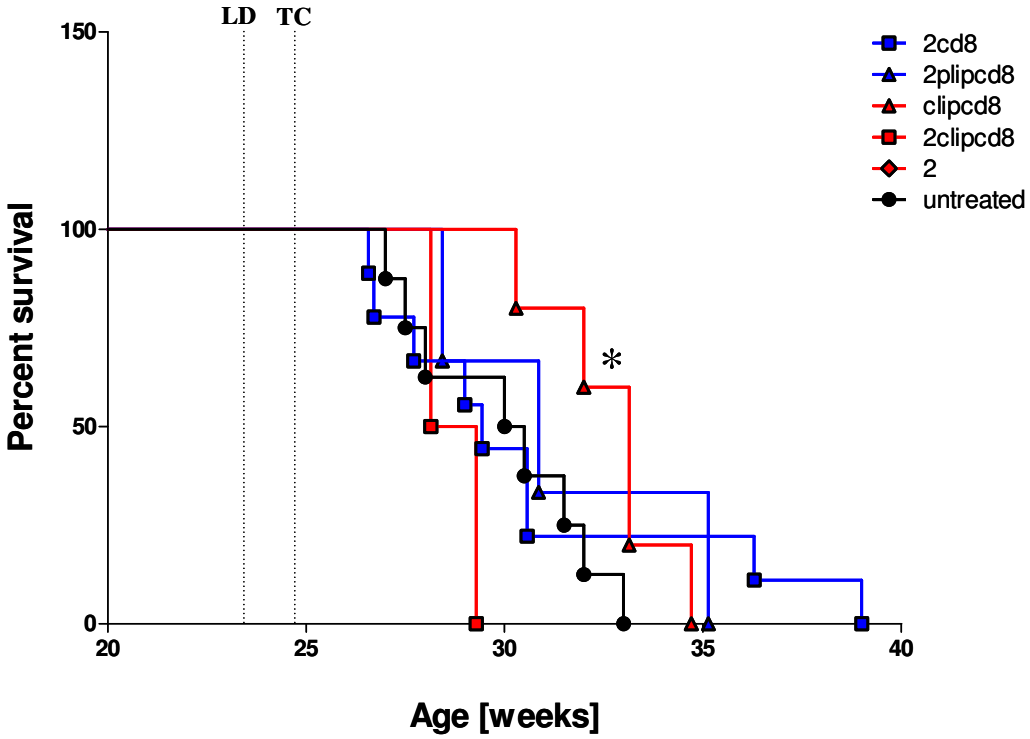

Figure 15. Survival of RT5 mice after LD irradiation and adoptive transfer of TCRtg CD8 positive T cells with and without macrophage ablation. RT5 mouse survival is shown for animals treated with a local tumor irradiation dose of 2 Gy and received i.p. injections of TCRtg CD8 10 days later (2cd8), in mice with the same treatment plus clodronate (2clipcd8) or saline loaded liposomes (2plipcd8), and in mice that received only 2 Gy irradiation (2), only a CD8 positive T cell infusion (cd8), or clodronate (2clipcd8) loaded liposomes and T cells (clipdcd8). 1st vertical dotted line: 2Gy irradiation (LD); 2nd vertical dotted line: intravenous T cell infusion (TC).

* $P < 0.05$; (Mantel-Cox test).

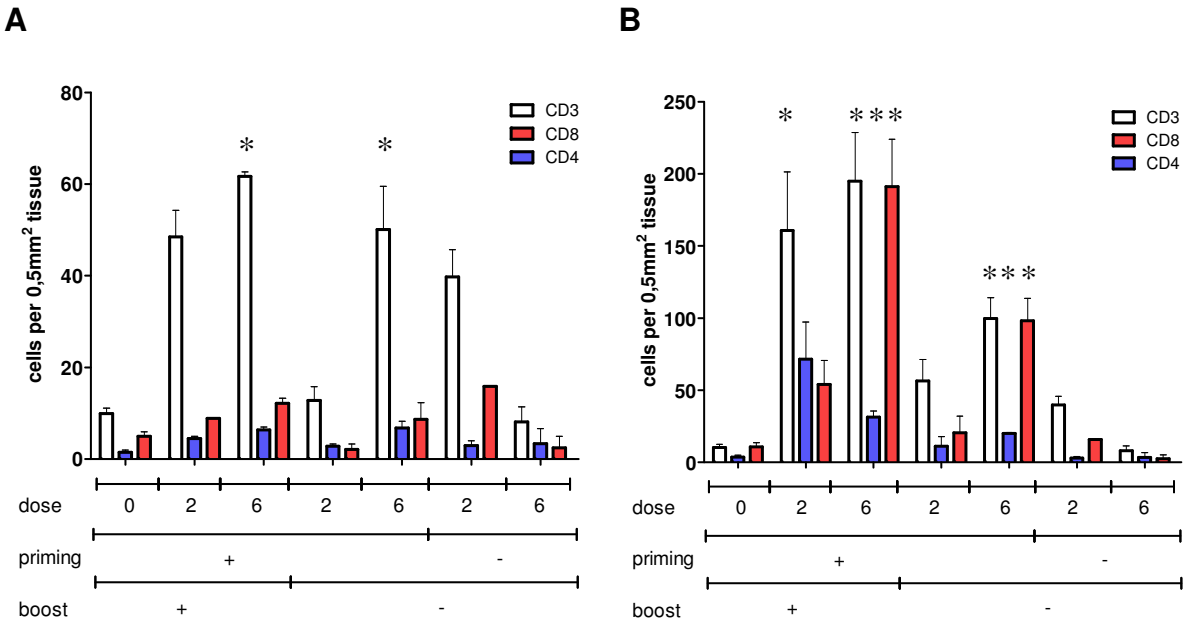

Figure 16. LD irradiation triggered tumor infiltration by peptide based homologous prime-boost vaccination. MHC class I (**B**) and class II (**A**) restricted peptides from the SV40 Tag protein were emulsified and injected under the neck skin of RT5 mice (**A**, n=13; **B**, n=17) in some groups. Mice were treated with a local tumor irradiation dose of 2 Gy or 6 Gy and received either no vaccination, vaccination one week before irradiation or both one week before and one week after irradiation. Tumors were excised after one week and analyzed by immunohistochemistry for CD3 (white bar), CD4 (blue bar) and CD8 positive (red bar) t cells.

* $P < 0.05$; (two-tailed Student's t-test).

4 Discussion

An effective T cell response against tumors requires an activated tumor stroma (Ganss et al., 2002; Garbi et al., 2004). As various studies have shown, the tumor stroma is crucially important in the setting of immunotherapy, as it influences the local priming of T cells (Spiotto et al., 2002), access of lymphocytes into tumor tissue (Ganss et al., 2002; Garbi et al., 2004) and acts as direct target for immune destruction (Qin and Blankenstein, 2000; Qin et al., 2003). Blood vessels, as a major component of the tumor stroma, have long been recognized as targets for cancer therapies (Folkman, 1972). Although endothelial cells regulate the influx of lymphoid cells into tissues, it is unclear which factors dictate the state of both activation and anergy of endothelial cells in tumors. This work demonstrates for the first time a correlation between local LD irradiation induced NO release from activated tumor stroma cells, most likely macrophages, and infiltration of tumor-specific effector T cells which was probably governed by functional changes in the tumor endothelium.

4.1 *In vitro* response to LD radiation

4.1.1 Intermediate irradiation doses of 1 to 2 Gy induce activation of tumor endothelium *in vitro*

It was reported that irradiation induced tumor infiltration relies on the generation of an inflammatory tumor environment allowing adoptively transferred T cells to transmigrate into the tumor (Ganss et al., 2002). This inflammatory tumor environment is characterized by endothelial cells, which have been activated by inflammatory cytokines to express adhesion molecules and synthesize chemokines and lipid chemoattractants that are presented their luminal surface (reviewed in Ley et al., 2007). To elucidate the role of radiation induced endothelial activation manifested in enhanced expression of endothelial adhesion molecules, direct irradiation of pancreatic tumor derived HPMEC was performed. Treatment of *in vitro* cultured HPMEC with LD irradiation induced an activated phenotype in HPMEC as confirmed by flow cytometric measurement of the expression of extra-cellular endothelial molecules (**figure 2**). This activation was manifested in terms of enhanced expression of the cell surface molecules P-selectin, ICAM1, ICAM2, MAdCAM1, VCAM1 and ALCAM. Interestingly,

irradiation using the highest dose (6Gy) induced the strongest upregulation in all of the tested molecules, whereas the intermediate doses (1 or 2 Gy, respectively) resulted in an intermediate activation in most of those factors.

The above mentioned results are in accord with findings in the field of treatment of chronic inflammatory malignancies using low dose irradiation, which demonstrate that irradiation doses of 1 Gy or lower have immunosuppressive properties and induce endothelial anergy, characterized by low expression of endothelial cell adhesion molecules and low lymphocytic binding strength (Hildebrandt et al., 2002; Kern et al., 2000; Roedel et al., 2002). Those studies also highlight that irradiation doses of 1 to 2 Gy can activate endothelial cells which in turn is characterized by upregulation of endothelial cell adhesion molecules. However, tumor derived endothelial cells are already in a state of reduced expression of leukocyte adhesion receptors and unresponsiveness to inflammatory stimuli (Griffioen et al., 1996; Nooijen et al., 1998; Wu et al., 1992). In the present study, the intermediate irradiation doses of 1 to 2 Gy were able to convert tumor endothelial cells back to a normal activation state and higher doses further activated it. This indicated that intermediate irradiation doses are already able to restore transmigration relevant functions in tumor endothelial cells, but higher irradiation doses may induce a highly activated endothelium reminiscent of that in inflammatory conditions. A highly activated endothelium may be able to greatly enhance influx of immune effector cells into tumor tissue (reviewed in Danese et al., 2007). However, *in vivo* application of irradiation doses of 6 Gy or higher usually result in severely reduced numbers of lymphocytes because of radiation toxicity (Ozsahin et al., 2005; Trowell, 1952) and thus may deplete tumor-specific T cells. Therefore, activation of tumor endothelium using intermediate irradiation doses of 1 to 2 Gy would be more suited in a setting of immunotherapy using T cells, since it was shown to convert tumor endothelial cells from anergy back to a normal activation state without causing substantial toxic effects.

Although, the results from this *in vitro* model imply that LD irradiation could be used to enhance infiltration of immune cells into tumors, the relevance of endothelial cell anergy has so far not been explored in detail for human tumors. In a study of breast cancer in patients with ductal carcinoma and in patients with medullary carcinoma, the latter group was characterized by tumors with increased amounts of infiltrated leukocytes in the tumor, when compared to tumors of patients with ductal carcinomas

(Bouma-ter Steege et al., 2004). Intriguingly, higher amounts of angiogenic factors were detected in ductal carcinomas, most evident in the expression levels of VEGF, which has been shown to synergize with other angiogenic growth factors such as FGF, to downregulate ICAM1 thereby inducing endothelial anergy. In another study, analysis of tumor tissues of patients with colorectal carcinoma showed a negative correlation between expression of VEGF or the number of proliferating endothelial cells and leukocyte infiltration (Baeten et al., 2006). Therefore, LD irradiation which has been shown to activate endothelium in the present study may hold potential to normalize tumor vasculature and thereby induce leukocyte infiltration for the treatment of human tumors.

4.2 Combination treatment using LD radiation and immunotherapy

The use of combined treatment regimens has been used to enhance cancer therapy in different experimental setups. In one study, the reagent group cytosine-phosphorothioate-guanine-containing oligodeoxynucleotides (CpG-ODN) was used. CpG-ODN is primarily known to enhance vaccination strategies by promoting DC maturation (Brunner et al., 2000) or by blocking regulatory T cells (Yang et al., 2004), but it has also been shown to render RT5 tumors permissive for infiltration by pre-activated effector T cells (Garbi et al., 2004). It was demonstrated that CpG-ODN supported the induction phase of an immune response (Kawarada et al., 2001), and was taken up by tumor-resident macrophages which in turn triggered up-regulation of adhesion molecules on endothelial cells. Similarly, there is evidence that other efficient therapeutic agents such as cyclophosphamide not only enhance the efficacy of adoptive transfers (Dudley et al., 2002) but also act on tumor-resident cells (Hanahan et al., 2000; Ibe et al., 2001). Therefore, the combination of treatments which target the tumor as well as the tumor microenvironment holds potential for future immunotherapy protocols.

4.2.1 Infiltrating T cells are required for sufficient activation of tumor vasculature *in vivo*

In the present study, LD irradiation was used to enhance immunotherapy. Irradiation was chosen to improve an established adoptive transfer protocol, since it has been demonstrated to promote tumor immunity by enabling transferred effector cells to proliferate and to persist *in vivo* (Dummer et al., 2002; North, 1986). Moreover, irradiation has been shown to have profound effects on the anergy of tumor endothelium and, hence, effector cell extravasation (Ganss et al., 2002).

In order to test several irradiation doses for their effectiveness in an immunotherapeutic treatment situation *in vivo*, LD irradiation of whole tumors was carried out by abdominal radiation treatment of RT5 mice. Afterwards, tumors were excised and analyzed by immunohistochemistry for the expression of the endothelial marker CD31 and the transmigration associated factors P-selectin, E-selectin, ALCAM, VCAM1, ICAM1 and MAdCAM1.

Local LD irradiation did not cause irreversible damage to microvascular cells as supported by the observed full revascularization after therapy. Moreover, local LD irradiation influenced the morphology of endothelial cells in terms of elongation and induced enhanced intercellular binding strength as indicated by an increased expression of the molecule CD31 when the highest dose (6 Gy) was applied (**figure 4**). These observations indicated that endothelial cells reverted from an angiogenic to a resting phase, a process which is usually accompanied by dilatation of surviving vessels (Dimitrievich et al., 1984). Interestingly, no expression of transmigration associated factors was found on endothelial cells after radiation therapy. In a report about tumor treatment using radiation therapy and T cell transfer, significant expression of ICAM and VCAM was observed only when both regimens were combined, but not when radiation alone was used (Quezada et al., 2008). This suggests that infiltrating T cells are, at least in part, an important factor in further increasing vasculature activation. Moreover, corroborating evidence for this hypothesis can be gained from a study of tumor-specific T cells in a murine tumor model showing that combined TNF-α and IFN-γ signalling induced secretion of antiangiogenic chemokines and prevented tumor angiogenesis (Müller-Hermelink et al., 2008). Therefore, in the present study a

therapeutic approach using a combination of local LD irradiation and adoptive transfers of tumor-specific T cells for the treatment of tumor-bearing mice was employed.

4.2.2 Local LD irradiation and adoptive transfer of activated tumor-specific CD4 or CD8 positive T cells induces massive tumor infiltration of both T cell subsets

Immunotherapeutic treatment of cancer is frequently hindered by insufficient infiltration as a result of the tumors intrinsic resistance to t cell infiltration and effector function. However, this barrier can be overcome by inducing an activated tumor microenvironment utilizing whole body irradiation in mice. In a report from Ganss and colleagues a combination of whole body irradiation and adoptive transfers of tumor-specific T cells triggered an inflammatory response at the tumor site (Ganss et al., 2002). This treatment regimen resulted in complete regression of established, highly vascularized tumors over a period of weeks.

In the present study, the same transgenic mouse model was employed. Following *in vitro* activation, tumor-specific TCRtg T cells derived from donor mice were injected into RT5 mice previously irradiated with doses ranging from 0.5Gy to 6Gy. Immunohistochemical assessment revealed that tumors from mice treated with local LD irradiation and adoptive transfer of activated tumor-specific CD4 or CD8 positive T cells induced massive tumor infiltration of both T cell subsets (**figure 7**). T cells transferred into animals without previous local LD irradiation caused only mild tumor T cell infiltration, comparable to the extent of spontaneous T cell infiltration observed when tumors were treated using irradiation only (**figure 5**).

In the CD4 positive T cell transfer setup, all irradiation doses except the dose of 0.5 Gy resulted in massive tumor infiltration of both CD3 and CD4 positive T cells. As seen in the vitro experiments of this study, LD irradiation of 0.5 Gy was also not sufficient to activate HPMEC. The significance of this finding is bolstered by observations in the field of treatment of chronic inflammatory malignancies using low dose irradiation, which demonstrated that LD irradiation of 0.5 Gy induced endothelial anergy also in their models (Hildebrandt et al., 2002; Kern et al., 2000; Roedel et al., 2002). The dependency of T cell tumor infiltration on the activation of the endothelium be-

comes more evident, when the increase of CD4 positive T cell infiltration along with higher irradiation doses and the observation of the highest CD4 positive T cell infiltration with the highest irradiation dose (6 Gy) are taken into consideration. This tendency was also detectable in the vitro experiments of this study, in which 6 Gy was the most potent activator of HPMEC. Therefore, it can be assumed that tumor infiltration of CD4 positive T cells is dependent on the activation status of the endothelium. However, when CD8 positive T cells were transferred into irradiated hosts, an irradiation dose of 0.5 Gy was sufficient to induce massive T cell infiltration, indicating a difference in the homing capacities of T cell subsets. There is only sparse information available about differential homing of CD4 positive and CD8 positive T cells to tissues. One study highlights that a substantial proportion of CD4 positive effector T cells migrates to the small intestine in a CCR9-independent fashion, whereas CD8 positive effector T cells do not (Stenstad et al., 2006). Likewise, it would be possible that tumor infiltration of CD4 positive T cells is dependent on the activation status of the endothelium, because expression patterns of homing receptors on CD4 positive T cells may be differentially expressed.

Along with CD4 positive T cell infiltration, concomitant infiltration of host CD8 positive T cells was observed and resulted in absolute numbers of tumor infiltrating T cells equal to those of the CD4 positive T cell subset. Concomitant CD8 positive T cell infiltration was impaired when the dose of 0.5 Gy was applied, pointing at the role of CD4 help which was reduced since levels of CD8 positive T cells were also reduced at this dose. Application of an irradiation dose of 6 Gy also resulted in lower frequencies of concomitantly tumor infiltrating CD8 positive T cells, probably a result irradiation toxicity on the host subset.

Treatment of RT5 animals with local LD irradiation and transfer of CD8 positive T cells resulted in a massive tumor infiltration of both CD3 and CD8 positive T cells regardless of the irradiation dose applied. Irradiation with 0.5 Gy induced a marked recruitment of host Treg to the tumor. The same effect of Treg recruitment after irradiation with a dose of 0.5 Gy has been described in the context of treatment of autoimmune malignancies such as rheumatoid arthritis and encephalomyelitis. In these studies, irradiation induced low-intensity myeloablation mediated Treg activation and increment in the proportion of Tregs despite the overall reduction in lymphocyte counts (Tsukimoto et al., 2008; Weng et al., 2010). Additionally, it was shown

that Treg can dampen tumor-specific immune responses (Ghiringhelli et al., 2004; Steitz et al., 2001) and accumulate in tumor beds and tumor draining lymph nodes, leading to tumor progression (Ghiringhelli et al., 2005). Therefore, although effectively recruiting CD3 and CD8 positive T cells to the tumor, the treatment regimen using 0.5 Gy would not be an adequate solution for the therapy of cancer where a sustained T cell effector response would be beneficial.

Surprisingly, in the present study the irradiation dose of 0.5 Gy was not only sufficient to induce infiltration of both CD3 and CD8 positive T cells, but induced the highest concomitant infiltration of CD4 positive T cells. Higher doses of irradiation impaired the effectiveness of recruitment of host CD4 positive T cells to the tumor.

4.2.3 Combination treatment efficacy relies on CD8 positive T cells for vascular normalization of the tumor endothelium

As mentioned above, the report from Ganss and colleagues described a combination of whole body irradiation and repetitive adoptive transfers of tumor-specific T cells as responsible for the induction of an inflammatory response at the tumor site (Ganss et al., 2002). In their study, irradiation was used to render solid tumors accessible for effector cell infiltration, which coincided with conversion of the heterogeneous, tortuous tumor vasculature into a homogeneous network of capillaries as found in normal tissue, a process which was called vascular normalization. As a result, complete regression of established, highly vascularized tumors over a period of weeks was achieved. Since normalization of the vasculature was found to be a prerequisite to break tumor growth promotion and to optimize access of anticancer immune cells, normalization of the tumor vasculature was used as a measure of treatment response in the present study

The treatment response was quantified by means of vascular normalization events in all tumors (**figure 8**). Normalization was induced most effectively by transferred CD8 positive T cells when the irradiation dose was equal to or higher than 1 Gy. Since CD8 positive T cells are generally required for vascular normalization (Hamzah et al., 2008) it is not surprising that CD4 positive T cells were not as potent at inducing normalization in the tumors, because of the relatively low levels of CD8 positive T

cells which were recruited to the tumor. As vascular normalization is a prerequisite for immune destruction (Hamzah et al., 2008), the weak treatment response using CD4 positive T cells indicates that repetitive administrations of CD4 positive T cells would be required to interfere with tumor growth.

The idea of vascular normalization of the tumor endothelium as a prerequisite for tumor destruction has received much attention in the past years. Several studies are indicating that immunotherapy does at least not fully rely on the direct killing of tumor cells. In an mouse model using IFN-γ- and IFN-γ receptor-knockout variants, rejection of transplanted tumors by CD4 positive or CD8 positive T cells was not mediated by direct tumor cell killing, but by secretion of IFN-γ which in turn inhibited angiogenesis (Qin and Blankenstein, 2000; Qin et al., 2003). Other studies have shown that cytokine mediated tumor rejection targeted tumor-resident fibroblasts that indirectly interfered with angiogenesis (Schuler et al., 1999) and that cytokines were critical in the prevention of spontaneous carcinogenesis by reducing angiogenic activities (Nanni et al., 2001). Finally, the concept of vascular normalization using immunotherapy was demonstrated in RT5 mice where inflammation in the right context led to vessel normalization (Ganss et al., 2002), demonstrating that angiogenesis is reversible and that vascular cells can change the dynamics from a tumor-favoring to a tumor-antagonistic environment during immunotherapy.

However, the complex cascade of events that leads to reduced angiogenesis and permits effector T cell entry into tumors is still not understood. Elucidating the mechanisms will allow a specific targeting of the tumor environment and further improve therapeutic efficacies by providing proinflammatory factors locally rather than systemically.

4.2.4 Massive T cell tumor infiltration can be achieved by a focused irradiation of the tumor mass

Systemic immunomodulatory effects can be induced by local irradiation of lymphoid tissues which is manifested mainly in terms of production of pro-inflammatory cytokines and other inducers of DC maturation (Hallahan et al., 1989; Ishihara et al.,

1993). To exclude enhanced T cell activation induced in irradiated spleen or lymph nodes, two different experimental approaches were used (**figure 9**).

In the first approach, TCRtg T cells were transferred into locally irradiated splenectomized RT5 mice to prevent that the transferred T cells get further activated and that host T cells get activated by irradiation induced mature splenic dendritic cells. Highly infiltrated tumors were recovered from these mice indicating that irradiation of the spleen was not required.

In the second approach, TCRtg T cells were transferred into NOD/Scid mice that have received an allo-transplanted RT5 tumor sub-cutaneously. Irradiation of solely the tumor which was located beneath the skin, but not of abdominal organs or lymph nodes was achieved in this setup. Although the amount of recovered T cells from the subcutaneous tumors were much lower, there was a striking difference between the irradiated and unirradiated groups that were treated with adoptive T cell transfers, suggesting the crucial role of irradiation of the tumor but not other tissues for T cell tumor infiltration. Considering that massive tumor T cell infiltration can be achieved by a focused irradiation of the tumor mass, the local LD irradiation regimen in combination with transfer of TCRtg T cells should be widely applicable and complications involving damage of organs could be minimized.

4.3 Innate immune cells mediate therapy outcome

4.3.1 Depletion of macrophages abrogates LD irradiation triggered tumor infiltration by transferred TCRtg T cells

Two reports that used the RT5 mouse model demonstrated that inflammatory stimuli like whole body irradiation (Ganss et al., 2002) or systemic application of adjuvants (Garbi et al., 2004) were sufficient to allow dramatic infiltration of tumor-specific T cells into pancreatic tumors and were linked to activation of tissue-resident macrophages which probably induced a strong up-regulation of adhesion molecules on blood vessel endothelia. Moreover, several studies have shown that LD irradiation is able to stimulate macrophages (Ibuki and Goto, 1997; Knoops et al., 2007). Given the pivotal role of macrophages in the context of lymphocytic tumor infiltration (reviewed in Allavena et al., 2008) and their proneness to be activated by irradiation,

depletion experiments were carried out using the bisphosphonate clodronate. Bisphosphonates are compounds used in the clinic to prevent or inhibit development of bone metastases and for the therapy of inflammatory diseases such as rheumatoid arthritis and osteoarthritis (Rogers et al., 2000; Ross et al., 2004). The use of bisphosphonates as antiangiogenic agents has been found to suppress solid tumour growth and metastases (Giraudo et al., 2004). With the encapsulation of clodronate into liposomes, an efficient reagent for the selective depletion of macrophages has been developed and successfully applied in several immunological studies (Seiler et al., 1997; Tyner et al., 2005). In the present study, clodronate loaded liposomes (CLIP) were used to deplete macrophages *in vivo*. Liposomes are multi-lamellar nano-particles taken up by phagocytic cells of the reticuloendothelial system. Following intra-peritoneal injection of CLIP, apoptosis is induced in phagocytic cells in the peritoneum resulting in depletion of these cells. Liposomes can not pass trough membranes but reach the blood stream from the peritoneum via the parathymic lymph node. CLIP were shown to efficiently deplete macrophages but to some extend also monocytes in the blood stream and myeloid DC in the spleen (Gregoriadis, 2006).

Using this approach, LD irradiation triggered tumor infiltration by transferred TCRtg T cells was abrogated by depletion of macrophages (**figure 10**). Using immunohistochemical methods, it was assessed whether tumor residing macrophages were depleted (not shown). Although, CLIP were used to deplete macrophages systemically no difference in macrophage frequencies could be detected, raising the question, whether the tumor-resident macrophage population was depleted. CLIP have been shown to reach tumor vessels (Zeisberger et al., 2006), but usually cannot pass through endothelial cells. Since CLIP were injected into the peritoneum in this study, they must have depleted peritoneal macrophages first. CLIP can then leave the peritoneal cavity via lymph nodes and reach the blood stream where they deplete monocytes (Gregoriadis, 2006). Circulating to various organs, CLIP can deplete other subsets of the reticuloendothelial system such as macrophages in the liver, spleen and bone marrow (Gregoriadis, 2006). Given that CLIP therapy will thereby deplete most subsets of the reticuloendothelial system and phagocytic cells in the peritoneum and blood stream, it is likely that the effect of macrophage depletion on LD irradiation trig-

gered tumor infiltration can be attributed to systemic effects of macrophage depletion rather than local effects of macrophage depletion in the tumor.

Regardless of whether systemic or local effects may have interfered with the therapy outcome, this study clearly highlights the role of macrophages in mediating tumor permissiveness to T cell infiltration. It may be assumed that LD irradiation activated macrophages which probably induced an up-regulation of adhesion molecules on tumor microvessels and that macrophage depletion interferes with this process.

4.3.2 Tumor infiltration by CD8 positive T cells highly depends on iNOS activity

It was reported that vascular normalization coincides with massive lymphocytic tumor infiltration after irradiation treatment (Ganss et al., 2002) and that gamma irradiation can induce enhanced NO generation from macrophages (Ibuki and Goto, 1997) which is known to be one major regulator of the endothelial cell layer permeability (Fukumura et al., 1997). Therefore, it was tested whether blocking of inducible nitric oxide synthase (iNOS) may interfere with the macrophages ability to control endothelial cell function. The iNOS inhibiting drug 1400W (N- [[3- (Aminomethyl) phenyl] methyl] -ethanimidamide) was administered continuously by sub-cutaneously implanted slow-release mini-osmotic pumps. The drug 1400W is a slow, tight binding, potent and highly selective inhibitor of iNOS, which is cell-permeable, active *in vivo* and able to reduce NO levels systemically (Garvey et al., 1997). Release of the drug from the pumps was ensured for the whole time of the experimental procedures.

Levels of CD3 and CD8 positive T cells in the tumors were significantly reduced in those animals that received TCRtg CD8 positive T cells and LD irradiation, a weaker effect was detected in animals that received TCRtg CD4 positive T cells and LD irradiation (**figure 11**). As reported by others (Forster and Lieberam, 1996; Garbi et al., 2004), TCRtg tumor-specific CD4 and CD8 positive T cells take different routes when infiltrating insulinomas. This finding together with the observed differential dependence on iNOS activity in the present study points to the existence of two different mechanisms which are responsible for the infiltration of CD4 and CD8 positive T cells into the tumor. The mechanism that controls infiltration of CD4 positive T cells seems

to be at least partly independent of NO induced endothelial cell activation of tumor vessels, whereas infiltration of CD8 positive T cells depends highly on iNOS activity, as highlighted by the iNOS inhibition experiments of the present study.

The results presented here show for the first time the crucial role of macrophage derived NO for the recruitment of adoptively transferred T cells to the tumor. However, depletion of macrophages had a more pronounced effect on tumor infiltration of both CD4 and CD8 positive T cells. Therefore, it can be assumed that macrophages control T cell infiltration not only by NO production.

4.3.3 Combination treatment can result in reduction of tumor cells and improved survival, depending on the transferred T cell subset

Since the present results show that macrophages are heavily involved in the recruitment of T cells to the tumor, experiments have been performed to monitor tumor growth as well as survival under the influence of macrophage depleting liposomes. When CD4 positive T cells were transferred, normalization of blood glucose levels and improved survival was achieved only using the combination of LD irradiation and T cell transfer with or without the additional treatment with control liposomes (**figure 12** and **figure 13**). This result was in line with the immunohistological observation of T cell infiltration, which was strongly pronounced only when irradiation and T cell transfer were combined and declined when clodronate liposomes where administered. Nevertheless, since only two infusions of T cells were given, tumor growth was only transiently delayed. Ganss and colleagues reported tumor free mice when 7 infusions were applied (Ganss et al., 2002) and showed that after irradiation and adoptive transfer, highly activated tumor-specific lymphocytes were initially found at tumor and tumor-draining sites but then disappeared because of activation-induced cell death (Webb et al., 1990; Zhang, 1996). Therefore, in the present study the small remaining T cell population that infiltrated the tumor after the last T cell infusion was given was probably unable to prevent progressive tumor growth and host T cells failed to uphold tumor immunity.

When CD8 positive T cells were transferred, normalization of blood glucose levels was achieved not only using the combination of LD irradiation and T cell transfer with

or without the additional treatment with control liposomes, but also in the group with unirradiated animals that received clodronate liposomes and T cell transfer (**figure 14**). Remarkably, the latter group was the only one to show improved survival (**figure 15**). This result is not in line with previous observations since the time span of normalized blood glucose levels was 5 weeks which is longer than what was observed in the CD4 positive T cell transfer experiments and immunohistological observations of T cell infiltration showed much higher numbers of tumor infiltrating T cells than in the CD4 positive T cell transfer experiments. Additionally, until week 30 which is the end point of normalization of blood glucose levels, about half of the animals had died in the treatment groups which had received the combination of LD irradiation and T cell transfer with or without the additional treatment with control liposomes. Taken together, it seems likely that the animals did not die of tumor-induced hypoglycemia but of side effects of the therapy. Complications such as sepsis as a result of the insulitis, leading to the overproduction of proinflammatory cytokines and causing systemic inflammation may have led to animal death. Furthermore, massive lymphoproliferation caused by the transferred T cells could lead to fatal multiorgan tissue destruction as described in a study that used blocking of the negative T cell regulator CTLA-4 (Tivol et al., 1995).

Normalized blood glucose levels and improved survival were detected in the group with unirradiated animals that received clodronate liposomes and T cell transfer. Notably, immunohistological examinations proved that treatment with this regimen did not result in massive T cell tumor infiltration but in numbers of infiltrating T cells comparable to those seen in unirradiated animals that received just the T cell transfer. This indicates that massive infiltration of T cells is not a prerequisite for tumor cell death and that use of clodronate liposomes may have multiple effects which may increase activation of T cells or may work by a T cell independent effect. Zeisberger and colleagues showed that tumor treatment with clodronate encapsulated in liposomes alone resulted in significant inhibition of tumor growth accompanied by a drastic reduction in blood vessel density in the tumor tissue (Zeisberger et al., 2006). This effect in combination with the infusion of pre-activated T cells may have favored tumor cell death without massive infiltration of the tumor by T cells which was detected in this study.

Furthermore, it has been reported that treatment with clodronate encapsulated in liposomes not only depletes macrophages in mouse tumor models efficiently (Zeisberger et al., 2006), but also results in a significant reduction in peripheral blood total MDSCs as well as tumor-associated MDSCs and Gr-1$^+$ MDSCs (Priceman et al., 2010). Thus, aforementioned increased activation of T cells which was elicited by the use of clodronate liposomes may be triggered by the loss of inhibiting effects from MDSC or MDSC-like macrophages in the tumor. Taken together, the treatment of tumors using the combination of clodronate loaded liposomes and T cell transfer may be a promising alternative treatment option but still needs more investigation.

4.3.4 Local LD irradiation renders pancreatic tumors permissive to infiltration by activated host T cells after peptide vaccination

The RT5 mice do not express Tag before 6 weeks of age and have preserved the ability to efficiently generate effector T cells, if vaccinated before tumor development (Garbi et al., 2004; Otahal et al., 2006; Ye et al., 1994). Ganss and colleagues showed that these mice could be vaccinated using Tag protein plus CpG oligonucleotides as adjuvant before Tag was expressed in the pancreas resulting in highly infiltrated tumors (Garbi et al., 2004). At the time point when neoplastic transformation of beta-cells had occurred and tumor-induced neo-angiogenesis had started, vaccination failed in most of the mice. At late stage when solid tumors had formed, vaccination completely failed. However, throughout the whole time Tag-specific effector T cells were induced by vaccination as demonstrated by *in vivo* kill assays. The combination of CpG oligonucleotides and infusion of activated Tag-specific lymphocytes resulted in progressive insulitis. Thus, it seems that pancreatic tumors are resistant to activated host T cells even when a potent proinflammatory stimulus able to support an ongoing adoptive immune response is given systemically.

To assess the capacity of host T cells to infiltrate the tumor tissue after LD irradiation treatment, a peptide based homologous prime-boost vaccination protocol was used (**figure 16**). Both vaccination approaches using MHC class I and MHC class II restricted peptides resulted in enhanced numbers of CD3 positive T cells preferentially for the 6 Gy irradiation setting, probably as a consequence of higher activation of endothelial cells, as described for *in vitro* irradiated HPMEC in this study. However, the

absolute numbers where lower than in the corresponding group of the adoptive T cell therapy groups reflecting the considerable lower numbers of specific T cells in the non-transgenic host compartment.

Surprisingly, vaccination with MHC class II restricted peptides had no impact on the numbers of CD4 positive T cells, raising the question about the subset of T cells which infiltrated the tumor. It was reported that TCRα/β^{+} CD4/CD8 double-negative T cells have the ability to specifically down-regulate immune responses towards allo-antigens both in humans and mice (Londei et al., 1989; Zhang et al., 2000). Double-negative T cells were shown to be found in lymphoid as well as in non-lymphoid tissues and have been shown to take up allo-MHC peptide complexes from antigen-presenting cells. Since the majority of the tumor infiltrating T cells in the RT5 tumors have been identified as CD4/CD8 double-negative, this result points at a putative mechanism that leads to the recruitment of this suppressive T cell subset to the tumor, when high tumor antigen amounts are available for MHC class II molecules on antigen presenting cells.

Vaccination of 6 Gy irradiated animals with MHC class I restricted peptides resulted in highly infiltrated tumors of both CD4 and CD8 positive T cells and the second boosting vaccination showed a clear enhancement in T cell numbers. This demonstrates the predominant capacity of activated CD8 positive T cells to infiltrate the tumor as it was demonstrated in the context of an adoptive T cell transfer in this study and as it has been demonstrated as a spontaneous event (Sato et al., 2005) and after therapy of human tumors (Yee et al., 2002). Thus, it can be concluded that pancreatic tumors are permissive to infiltration by activated host T cells when the tumors are treated with local LD irradiation.

4.4 Conclusion

The present study illustrates for the first time the unexpected role of macrophages and macrophage derived iNOS activity for the recruitment of T cells to the tumor site. Combination treatment of tumor-bearing mice using local low dose irradiation and adoptive T cell transfer induced a massive influx of T cells into the tumor. This effect correlated with the presence of macrophages in the tumor micromilieu which mediated tumor infiltration of T cells by production of NO. Depletion of this cell population or suppression of iNOS prevented the treatment effect.

The author believes that local LD irradiation treatment holds potential for the development of novel anticancer combination therapies, because of its ability to create an activated tumor microenvironment to selectively enrich immune effector cells facilitating immune-mediated tumor destruction without elevated risk of causing adverse effects. While the enhancing effect of irradiation on adoptive T cell therapy has been reported (Ganss et al., 2002), lethal whole body irradiation was required in that study and involved bone marrow transplantation, making it excessively elaborate and unsafe for the use in the clinic. Moreover, this study has proven that the combination of local LD irradiation and cellular therapy as means of enhancing both the activity of innate immune cells and the frequency of tumor-specific T cells can induce tumor immune destruction. While the former was required to make tumors permissive for T cell infiltration, the latter ensured regression of tumors. Since there was no evidence for complete regression of the malignancy, the requirement of enhanced persistence of effector cells has to be addressed in future trials. Furthermore, the study shows that activated host T cells are able to infiltrate tumors after peptide vaccination and local LD irradiation. Therefore, the use of adjuvants to enhance vaccination efficacy in combination with local LD irradiation may further improve current therapy protocols.

These findings could be translated into an immunotherapeutic treatment of advanced carcinomas especially of the gastro-intestinal (GI) tract. While high-dose radiotherapy is a powerful anticancer treatment approach, it is associated with severe side effects that drastically limit its therapeutic capacity for cancer treatment in the GI tract. Using LD irradiation, the outcome of radiotherapy could be substantially improved due to a selective reduction of damage to the gastro-intestinal organs.

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

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Tobias Seibel