# Prediction, Diagnosis and Treatment of Experimental Graft-versus-Host Disease

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An investigation of genomic, molecular and cellular factors in graft-*versus*-host disease and mesenchymal stromal cell therapy in a rat model of allogeneic stem cell transplantation

by

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*Journal of Leukocyte Biology* 90(1): 177-187

II. Novota P, Sviland L, Zinöcker S, Stocki P, Balavarca Y, Bickeböller H, Rolstad B, Wang XN, Dickinson AM, Dressel R (2008)
**Correlation of Hsp70-1 and Hsp70-2 gene expression with the degree of graft-versus-host reaction in a rat skin explant model**.
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# Abbreviations

| | |
|---|---|
| **APC** | antigen-presenting cell |
| **BCR** | B cell receptor |
| **BM** | bone marrow |
| **BMT** | bone marrow transplantation |
| **CD** | cluster of differentiation |
| **CFU** | colony-forming unit |
| **DLI** | donor lymphocyte infusion |
| **ECP** | extracorporeal photopheresis |
| **G-CSF** | granulocyte colony-stimulating factor |
| **GvH** | graft-*versus*-host |
| **GvHD** | graft-*versus*-host disease |
| **GvHR** | graft-*versus*-host reaction |
| **GvL** | graft-*versus*-leukemia |
| **GvT** | graft-*versus*-tumor |
| **H** | histocompatibility |
| **HCT** | hematopoietic cell transplantation |
| **HLA** | human leukocyte antigen |
| **HSP** | heat-shock protein |
| **HSC** | hematopoietic stem cell |
| **Ig** | immunoglobulin |
| **IL** | interleukin |
| **iNOS** | inducible nitric oxide synthase |
| **KIR** | killer cell immunoglobulin-like receptor |
| **LRC** | leukocyte receptor complex |
| **LPS** | lipopolysaccharide |
| **MAPC** | multipotent adult progenitor cell |
| **MHC** | major histocompatibility complex |
| **MLR** | mixed lymphocyte reaction |
| **MSC** | mesenchymal stromal cell |
| **MUD** | matched unrelated donor |
| **NKC** | natural killer gene complex |
| **NKR** | natural killer cell receptor |
| **PB** | peripheral blood |
| **RIC** | reduced-intensity conditioning |
| **SLT** | secondary lymphoid tissue |
| **$T_C$** cell | cytotoxic T cell |
| **$T_E$** cell | effector T cell |
| **$T_H$** cell | helper T cell |
| **$T_R$** cell | regulatory T cell |
| **TCD** | T-cell depletion |
| **TCR** | T cell receptor |
| **TGF** | transforming growth factor |
| **TLR** | Toll-like receptor |
| **TNF** | tumor necrosis factor |
| **TRM** | transplant-related mortality |
| **UCB** | umbilical cord blood |

# Foreword

In the constant struggle for natural resources, all life has to defend itself against other life that threatens to encroach on its domain – or perish. Microbial parasites (viruses, bacteria, fungi, protozoa, worms) were among the earliest life forms on our planet and will continue to endanger the health and survival of their involuntary hosts. One such type of parasite, *Mycoplasma*, a class of bacteria that are able to enter and survive in association with eukaryotic cells, has literally infiltrated this work and became – true to their nature – an unintended topic hosted in this thesis.

All living organisms, by virtue of the successful survival of their ancestors, have in some way adapted to the evolutionary pressures of disease-causing agents. Every kind of multicellular organism, in which different cell types cooperate to fulfill specialized functions, has some type of immunity, *i.e.* the capacity to stay free from biological invasion and infection. Vertebrates, including the humankind, evolved to develop sophisticated defense systems composed of distinct molecules and highly specialized cell types which together recognize dangerous biological structures and initiate counter-measures to rid their bodies of such undesired presence.

In order to effectively fight off disease-causing microorganisms ('pathogens'), the cells of the immune system (for short, 'immune cells') must be able to distinguish, in anthropologic terms, 'harmful' from 'harmless' so as not to attack the body's own parts. In other words, immune cells tolerate 'self' while retaining the ability to detect and destroy 'foreign' or 'non-self'. It is therefore practical to begin with a description of the defense system that makes the important distinction between 'self' and 'non-self' in *Homo sapiens*, which may be viewed as exemplary for other mammals. The human immune system will be explained in more detail in the introduction of this thesis.

When the immune system fails to make these essential distinctions, diseases occur. 'Non-foreign' but dangerous cells are not controlled and as a result cancer will develop. Normal 'self' cells are mistakenly attacked and autoimmune disease may manifest. Both conditions can be life-threatening and both can, in many cases, be treated by replacing the defective cells with those from a healthy person and establishing a new, functional immune system. The procedure by which this is made possible, allogeneic stem cell transplantation, is the superordinate topic of this thesis.

The issue of compatibility between 'self' and 'non-self' is of importance in the transplantation of cells, tissues or whole organs from a donor to a sick person; the patient's immune cells will recognize incompatible cells as foreign and reject them. Conversely, when immune cells enter a defense-less body (due to disease or artificially rendered 'immune compromised') *via* a transplanted graft, their natural function in the foreign environment of the host organism is to inactivate and destroy unknown 'non-self' cells. In this way, transferred donor immune cells which attack the recipient of the graft give rise to the so-called graft-*versus*-host disease, a man-made immune disorder necessitated by stem cell transplantation as the only available treatment option for many patients suffering from severe malignancies, but frequently fatal in itself.

My intention with the writing of this thesis was, apart from the obvious objective to satisfy the demands for the doctoral dissertation set by the faculty, to give a summary of the basic underlying principles of bone marrow transplantation, graft-*versus*-host disease and mesenchymal stromal cell therapy as the main subjects of my doctoral research, and to provide a comprehensive starting point for new PhD candidates who are about to embark on this exciting field of science.

# Introduction

## *Chapter I. Generation of blood and immune cells in the human body*

Before laying out the main principles of immunity and going into the basic mechanisms of immune recognition in health and disease, it is instructive to illustrate how immune cells are generated in the human body and to describe briefly what their principal functions are.

The immune system is an integral component of the hematopoietic system. Hematopoiesis, the generation of red blood cells, platelets and leukocytes, is initiated in prenatal life in blood islands of the yolk sack, and later in the fetal anlage of the liver, spleen and lymph nodes. After birth, hematopoiesis occurs mainly in the bone marrow (BM). It has been known since the 1950s that mammalian blood cells derive from the spleen and marrow, but it was a major scientific breakthrough when Till, McCulloch and colleagues were able to demonstrate some 50 years ago that a single clonal BM cell (then termed the 'colony-forming unit spleen', CFU-S) could give rise to all cells of the murine blood system [reviewed by Weissman and Shizuru 2008]. Abramson *et al.* later formally confirmed the proposed existence of the so-called 'hemacytoblast' [Abramson 1977], which was renamed to 'hematopoietic stem cell' (HSC), as the founder cell of all blood cells (Figure 1). The biological process by which daughter cells with different properties are produced through self-replication of stem cells has been termed 'asymmetric cell division'. It is believed that one daughter cell becomes an identical copy of the original cell during cell division and thereby, serves to replenish the cell pool ('self-renewal'). Conversely, the other daughter cell will undergo biological changes during further cell divisions in a process termed 'differentiation' and become the progenitor for a family of cell offspring, eventually giving rise to all mature (terminally differentiated) cells of the blood system.

Stroma consists of various cell types, including vascular endothelial cells, reticular cells and mesenchymal stromal cells (MSC), which create the physical niches for stem and

progenitor cells. HSC quiescence, self-renewal and differentiation is controlled and maintained through the expression of intercellular messenger molecules (cytokines), cell-adhesion molecules and hematopoietic growth factors by stromal resident cells [Ehninger and Trumpp 2011]. In response to such signals, HSC reside in, migrate from and return to their hematopoietic niches in the BM [Bhattacharya 2006] and are thus able to repopulate the blood system in the same organism or a different one ('functional reconstitution'). Migratory primitive blood progenitor cells are normally found at very low numbers in the circulation.

## I.1 Mature blood cells derive from a common progenitor in the bone marrow

The multipotent HSC gives rise to early hematopoietic progenitor cells. Hematopoiesis is separated into two main lineages of blood cells, *i.e.* the lymphoid and the myeloid lineage. Accordingly, two progenitor cell types represent the origins of these branches, the common lymphoid and the common myeloid progenitor cell, respectively (*cf.* Figure 1).

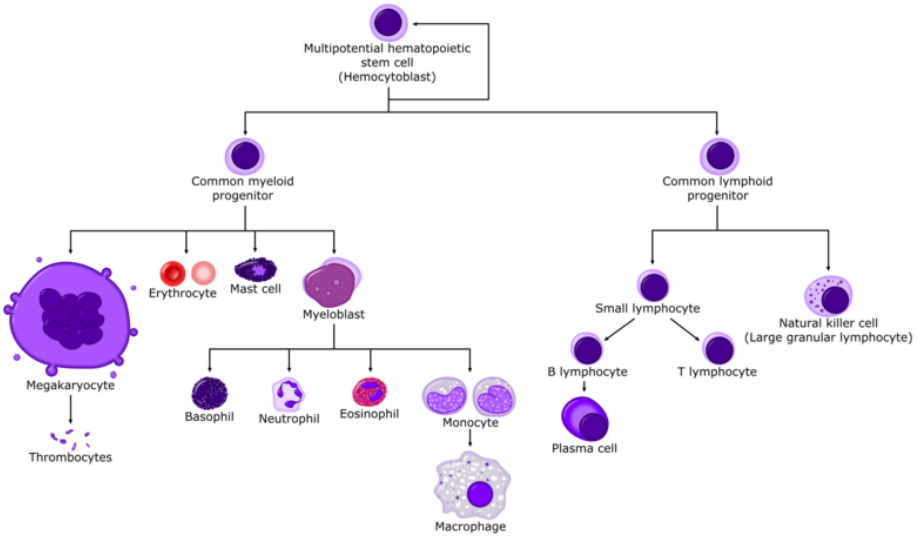



Figure 1 | **Hematopoiesis**
A schematic drawing of the classical model of hematopoiesis, the generation of the blood system, in the human body. All mature blood cells derive from a multipotent progenitor cell, the HSC.

Myeloid cells and their progeny (erythrocytes, granulocytes, megakaryocytes, monocytes, and macrophages) are produced in the BM and in the peripheral blood (PB) [Janeway 2005]. The myeloid stem branches off into the granulocyte and monocyte lineages. Granulocytes are polymorphonuclear cells commonly divided into basophils, eosinophils, and neutrophils. These are known to kill bacteria and worm parasites either by direct cell lysis through antimicrobial compounds called granules, which are stored in subcellular compartments and can be secreted upon activation, or by engulfing and ingesting the target cells altogether (phagocytosis).

Macrophages and myeloid dendritic cells are the descendants of blood-borne, circulating monocytes. Macrophages are present in peripheral tissues where they take up invading microorganisms and small particles in vesicles (endocytosis) and alert other parts of the immune system, leading to an influx of neutrophils and other effector cells. Tissue-resident dendritic cells have the ability to leave the site of infection and transport the internalized pathogen to nearby lymphoid tissues where they activate other immune cells to become involved in the ongoing immune response. Dendritic cells have a marked morphology which is believed to maximize the cell surface area where antigens are presented to other immune cells (*cf.* Chapter II) [Janeway 2005].

Together, these components have critical roles in direct defense mechanisms of the immune system. In addition to their functions in generating and supporting inflammatory processes, they have the ability to initiate 'adaptive' immune responses (*cf.* Chapter II) against persistent infections [Parham 2005].

Lymphocytes are the cells of the lymphoid lineage of white blood cells. Lymphocytes comprise T cells, B cells and natural killer (NK) cells, which have critical functions in the body's recognition of and defense against infections and disease.

NK cells play a central role in the primary immune surveillance against virus-infected and other aberrant or neoplastic (tumor) cells. Like other components of the 'innate' immune system (*cf.* Chapter II), NK cells broadly recognize typical, conserved structural components of pathogens and react immediately by contact-dependent lysis of the target cell (*e.g.* through degranulation, *i.e.* the secretion of cell wall-perforating proteins such as perforin). They are generally assumed to be non-specific and short-lived, but recently, characteristics of these cells have been discovered [Sun 2009; Paust 2010] that can be interpreted as the ability to adapt to previous encounters of the infection ('immunological memory'): This quality was hitherto considered as an exclusive feature of 'adaptive' immune cells (explained in more detail in Chapter II).

T and B cells of the lymphoid lineage form the main constituents of the so-called adaptive immune system. They can be long-lived and respond relatively slowly to primary infections, using highly specific immune recognition mechanisms. T cells have primarily cell-killing or cell-activating functions. B cells are lymphocytes which are specialized to produce, assisted by T cells, large amounts of soluble defense molecules (immunoglobulins, Ig) against infections. T cells and B cells arise and mature in the primary (central) lymphoid tissues, *i.e.* the thymus and the BM, respectively, hence their names. After completed maturation, these cells circulate in the bloodstream and the lymphatic system. They pass through and accumulate in high numbers in the secondary (peripheral) lymphoid tissues (SLT) where they may become activated by antigen-presenting cells (APC) (*cf.* Chapter II).

Dendritic cells have their origins both in the lymphoid and the myeloid lineage and are separated into different subtypes (*e.g.* plasmacytoid and myeloid dendritic cells) [Ziegler-Heitbrock 2010].

### I.2 The plasticity of blood cell differentiation and development makes a modification of the classical model of hematopoiesis necessary

The presented dichotomic scheme of hematopoiesis can assist our understanding of the process by illustrating sequential developmental events which lead to the formation of terminally differentiated, fully mature and functional blood cells. However, the model of myeloid *versus* lymphoid lineage commitment is overly simplistic as it suggests that blood cells develop in a strictly linear fashion. The model does not account for the diversity of seemingly 'committed' cell types to transgress into other distinct lineages under different physiological conditions [Buza-Vidas 2007; Doulatov 2010; Kawamoto 2010]. For example, T cell progenitors, although categorized as lymphoid cells, retain considerable myeloid potential [Wada 2008; Bell and Bhandoola 2008]; likewise, NK cells as well as macrophages and dendritic cells may derive either from the myeloid or the lymphoid branch [Doulatov 2010; Grzywacz 2011]. These studies demonstrated the capacity of 'trans-differentiation' at certain developmental stages and challenge the current concept of rigid unidirectionality in hematopoiesis. Thus, the complexity and plasticity of hematopoietic cell development is likely underappreciated for the time being.

## *Chapter II. The two arms of the immune system*

### II.1 Innate immune cells use germline encoded receptors for recognition of common pathogenic structures, adaptive immune cells express recombined receptors which are highly specific for their antigens

The term 'innate' summarizes the inherent nature of immune cell types and humoral components (*e.g.* complement molecules that tag and lyse pathogens by attachment to their plasma membrane in a process termed 'opsonization') of this category to detect and react to common pathogenic features promptly and broadly, and without further adaptation to the particular infection at hand. Their mode of action is unchanged in recurring immune responses. Innate immune cells are activated upon binding of exogenous biological structures

such as bacterial flagellin, lipopolysaccharide (LPS), DNA or RNA to germline encoded receptors termed Toll-like receptors (TLR) [Medzhitov 2001].

Adaptive immunity, on the other hand, has the capacity to form its immune response specifically to the respective pathogen, and gets help from the innate immune system to fight an infection. When becoming activated and engaged in an immune response, only a small number of T and B cells have the appropriate specificity to participate in fighting the particular infectious agent. These cells will expand and adapt their response in its course. The processes of activation, expansion and adaptation of the responsive T and B cell clones require longer time (several days) than the immediately ensuing innate immune responses (several minutes to hours) [Janeway 2005]. By adaptation to a specific infectious agent, adaptive immune cells are able to achieve long-lasting protection against renewed challenges of the same pathogen. This capacity to 'remember' is facilitated by a small pool of modified, long-lived cells which persist in the individual after an infection has been successfully eliminated and are capable of improved clearance of secondary infections, *i.e.* they exhibit immunological memory. Conversely, the adaptive immune system remains 'naïve' to pathogens it has never previously encountered.

## II.2 T cells and B cells rearrange genes to generate highly specific receptors for foreign antigen detection

Mature T and B cells express specialized receptors on their cell surface. B cells express a membrane-bound Ig molecule (also called the B cell receptor, BCR) consisting of two heavy and two light chains. T cells display the T cell receptor (TCR) comprised of one α and one β protein chain on their cell surface together with several signaling molecules in the TCR:CD3 complex. B cells, when activated, differentiate to become so-called plasma cells which secrete high copy numbers of their surface Ig as soluble molecules (antibodies). Individual T cells and B cells, respectively, express TCR and BCR of a certain type which have unique binding specificity for a particular biological structure (the antigen). The form of the receptor is

determined during T cell and B cell development in a random process of rearrangement of genes which make up the receptor subcomponents (‘somatic rearrangement’). Junctional diversity during rearrangement of gene segments and somatic hypermutation can further extend the diversity of the array of BCR specificities and enhance their fine-tuning to a particular antigen [Janeway 2005].

The binding site of one particular receptor type forms an inverted negative pattern which closely matches the shape of its cognate antigen and thereby, defines the receptor specificity. Together, the sum of structurally related but highly specific receptor molecules can bind to a virtually infinite range of possible, and potentially harmful, biological structures. This creates the diversity of cell clones with distinct ligand-binding affinities within the adaptive immune system.

Adaptive immunity is not restricted to pathogen defense, however, but can detect any foreign entity in the body, at least in theory. This concept is illustrated by allergies, manifestations of immunological hyperreactivity to substances that are hardly dangerous *per se*, but cause complications for the affected person [Janeway 2005].

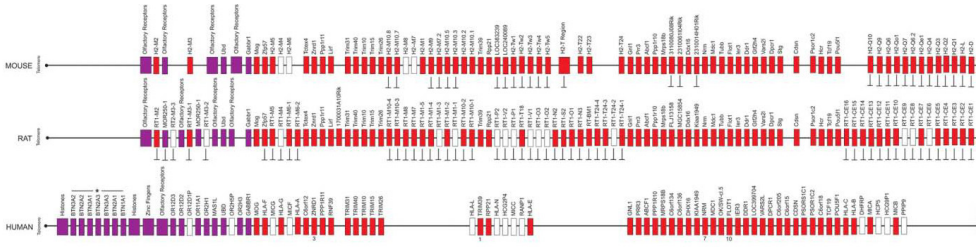
How does the adaptive immune system, which lacks innate receptors designed for the recognition of common pathogen-associated molecular patterns, make the distinction between harmless and potentially hazardous agents and decide whether they should be ignored or eliminated? How does the healthy body with its competent immune system avoid attacking its own cells? Why does it not tolerate other human cells or tissues as is often witnessed after the transplantation of organs? The presentation of a group of highly variable proteins which indicate the identity of a cell and convey the presence of infections to the immune defense system addresses these questions in the following chapter.

## II.3 The major histocompatibility complex determines the 'immunological identity' of an individual

Every cell of the human body (and likewise of any other vertebrate organism) carries a set of signature molecules that identifies it as normal, *i.e.* it is marked as 'self'. These cell surface proteins are encoded within a special gene region, the major histocompatibility complex (MHC). Tissues with a certain MHC expression pattern are not compatible (because they are 'non-self') with another individual of a different MHC makeup. Thus, the MHC determines the 'biological identity' of a given individual, which is of importance not least in clinical transplantation.

The MHC forms a large genomic segment of contiguous genes which are coinherited as haplotypes (genes located next to each other on the same chromosome). Some MHC genes have the largest number of alleles (*i.e.* different versions of a gene present in the genetic pool of the population) that are known [Kelley 2005b]. This high degree of genetic polymorphism (*cf.* subchapter III.6, p.26*ff.*) renders a set of MHC proteins expressed in a given individual highly distinct within the population. Exceptions to this are pairs of identical twins who share the same genes, including the MHC, between them, and syngeneic individuals of inbred

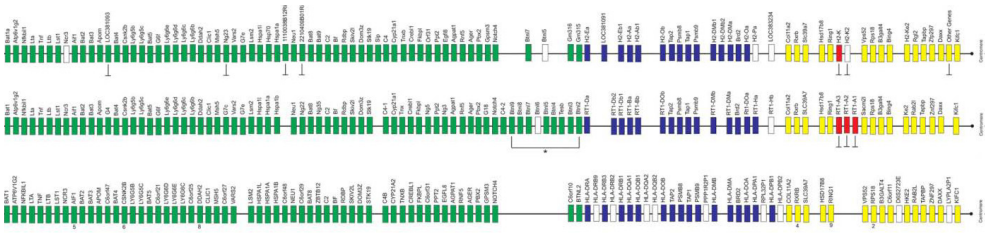
Figure 2 | **Comparison of the major histocompatibility complexes on human chromosome 6** (HLA, bottom)**, mouse chromosome 17** (H-2, top) **and rat chromosome 12** (RT1, center), *p.*8-9
The subregions of the MHC are decoded by color (**red**, class I; **velvet**, extended class I; **blue**, class II; **yellow**, extended class II; **green**, class III, contains *e.g.* complement, HSP70 and TNF genes). Individual genes are schematically shown as boxes in telomer-centromer orientation (from left to right, not drawn to scale). Rat and mouse carry the classical class I genes *RT1-A* and *H-2K*, respectively, at the centromeric end of their MHC, in contrast to the telomeric position of *HLA-A, -B* and *-C* in human.
Adapted with permission from Springer: Kelley, Walter & Trowsdale (2005), *Immunogenetics* 56



animal strains such as the laboratory rats used in experiments in this work. The human, rat (*Rattus norvegicus*) and mouse (*Mus musculus*) MHC gene regions are schematically depicted in Figure 2.

In humans, MHC genes have been termed human leukocyte antigens (HLA). The rat and mouse MHC regions are denoted RT1 and H-2, respectively [Kelley 2005b]. The HLA, RT1 and H-2 complexes share some overall similarities (cf. Figure 2). Three major gene clusters divide the MHC into class I (MHC-I), class II (MHC-II) and class III (MHC-III) regions, where the MHC-I and -II molecules are directly involved in antigen presentation. MHC-III genes often have functions related to the immune response. In humans, HLA-A, -B and -C are the MHC-I molecules and HLA-DR, -DQ, and -DP the MHC-II molecules which are constitutively expressed by APC. The rat has its MHC-I region physically divided into 'classical' (*RT1-A*, located at the centromere end of the complex) and 'non-classical' (*RT1-CE/M/N*, at the telomere end) genes. The class II genes are denoted *RT1-B/D* in the rat genome.

On the protein level, MHC molecules are plasma membrane-anchored glycoproteins that consist of two associated polypeptides. MHC-I molecules are made up of one large protein (the heavy chain) composed of three Ig-like domains which binds β-microglobulin (encoded by a single gene outside the MHC). MHC-II molecules are composed of two gene products, the α- and β-chain, that combine to form a heterodimeric protein on the cell membrane [Janeway 2005].



## II.4 MHC molecules signal infections to the immune system

The essential immunological function of HLA proteins is to bind short peptides and present them on the cell surface. Peptides can be the degradation products of endogenous cellular proteins or external pathogenic elements, *e.g.* from viral or bacterial infections. A cell can thereby display a representative sample of its internal content on a selection of MHC-I molecules distributed on its surface. MHC-II molecules are distinct in that they are loaded with and present peptides derived from the extracellular space and are expressed only on certain cell types (see below).

Viral and some bacterial infections have an intracellular life cycle and often target certain cell types. MHC-I loading with cytosolic peptides takes place in the endoplasmatic reticulum of the cell. After peptide loading, the MHC-I protein is transferred to the outer cell membrane where it can serve as a ligand for T cells expressing the corresponding TCR for the specific MHC-I:peptide complex. In this way, viral and bacterial peptides, just as other intracellular breakdown products, are exposed to detection by the immune system on the infected cell. Virtually all nucleated cells of the body, with the exception of red blood cells, express MHC-I molecules on their cell surface [Janeway 2005].

Elimination of exogenous bacteria and other parasites involves various immune defense mechanisms. The innate immune system has the ability to detect and neutralize pathogens in the extracellular space. Whole pathogens, their cell fragments or soluble products (*e.g.* bacterial toxins) are taken up by phagocytosis directly or, opsonized with complement or antibody, through specific surface receptors on phagocytes. The pathogens and their products are then internally degraded within the endosomal compartments of the phagocytic cell. Endosomes fuse with intracellular compartments where available membrane-bound MHC-II molecules can bind degraded pathogenic leftovers in the form of short peptides. The assembled MHC-II:peptide complex is transported to the cell surface where it may be

recognized by T cells. APC have the ability to take up, process and load antigens from the extracellular space onto MHC-I molecules by an unknown mechanism, thus making these peptides available also in the MHC-I presentation pathway [Janeway 2005]. This prosess is termed 'cross-presentation'.

B cells, dendritic cells, macrophages and microglia in the brain, which are all involved in immune responses against extracellular pathogens, constitutively express MHC-II on their cell surface. Thymic epithelial cells which are required for negative selection of autoreactive T cell clones may also express MHC-II [Janeway 2005].

Self-reactive T cells which can bind to peptides derived from normal endogenous protein (self-antigen) in the context of MHC molecules are normally not present in the peripheral T cell pool as these cells are removed early during T cell development in the thymus [Klein 2009]. Immature T cells which have a high binding affinity for self-antigen presented on thymic epithelial cells undergo controlled cell death by induced apoptosis. The rigorous negative selection of autoreactive T cell clones is a critical safeguard to ensure immunological self-tolerance, and failure to dispose of these cells during maturation can result in autoimmune disease [Anderson 2002].

T cells require a coreceptor for TCR binding of the MHC molecule. T cells which recognize foreign antigen presented in the context of MHC-I molecules are 'cytotoxic' T ($T_C$) cells; they express the CD8 coreceptor [Norment 1988]. T cells which engage the CD4 coreceptor for MHC-II:peptide complex binding [Doyle and Strominger 1987] are called 'helper' T ($T_H$) cells.

To activate a naïve T cell, an additional costimulatory signal is required and provided by a separate receptor-ligand interaction, *e.g.* the CD28 receptor on T cells and its ligands CD80 and CD86; or CD40 ligand and CD40 on APC. In the absence of a costimulatory signal, non-responsiveness (anergy) may be induced in the responding T cell.

Activation of CD4-expressing ($CD4^+$) T cells by APC mostly takes place in SLT, typically the draining lymph node nearest to the site of infection. $CD4^+$ $T_H$ cells respond to activation with proliferation, expansion and differentiation to effector T ($T_E$) cells, which typically secrete proinflammatory cytokines, such as interferon (IFN)γ and tumor necrosis factor (TNF)α, and chemotactic cytokines (chemokines) to attract other leukocytes. Activated helper cells may further mediate the recruitment of innate immune cells such as neutrophils and macrophages; the activation of APC effecting upregulation of MHC expression and thereby increased presentation of antigen; and the proliferation and differentiation of B cells into antibody-producing plasma cells. In this way, $T_H$ cells help counteract persisting infections which are not effectively cleared by the innate immune system.

$CD4^+$ T cells also play a role in the activation of $CD8^+$ T cells. As mentioned above, APC can present extracellular antigens in the context of MHC-I which are recognized by $T_C$ cells. A $CD4^+$ $T_H$ cell which contacts the same APC *via* TCR:MHC-II interaction can help to activate the $CD8^+$ $T_C$ cell. $T_C$ cells expressing the apposite TCR for the specific peptide antigen on display can thereby become involved in the ongoing immune response.

$T_C$ cells may become activated directly by MHC-I presentation of viral antigen on dendritic cells, leading to lysis of infected target cells. TCR and coreceptor engagement on the $T_C$ cell results in an intracellular signaling cascade that activates degranulation or alternative killing pathways (*e.g.* through CD95/CD95 ligand binding). A well-appreciated strategy used by intracellular pathogens to evade $T_C$ cell responses is to effect downregulation of MHC-I surface expression.

### II.5 Various subtypes of leukocytes are defined by their cell phenotype and 'innate' or 'adaptive' functionality

We have seen that T cells can be divided into functionally different subtypes by use of phenotypic markers (mostly surface proteins expressed on certain types of cells), *e.g.* mature T cells which express either CD4 or CD8 molecules together with the rearranged TCR on the

cell surface. These two T cell subsets are characterized by their typical patterns of cytokine production, cytotoxic activity and stimulation of other cells. Other cell types besides the already mentioned CD4 $T_H$ cells and CD8 $T_C$ cells cells have been defined according to their distinct features and phenotypes. Some of these cell types will become important in later chapters of the thesis and are therefore briefly introduced.

The expression of surface proteins on naïve CD4 and CD8 T cells is altered upon antigen binding; activated T cells upregulate CD44 and downregulate CD62L on the cell surface. A fraction of the cells which are selected and proliferate during an immune response do not undergo apoptosis after its completion and persist in the circulation as 'memory' T cells. T cells with antigen-recall properties are the 'central memory' T cells (typically expressing $CD44^+CCR7^+CD62^{hi}$) located in SLT and 'effector memory' T cells ($CD44^+$ $CCR7^-CD62^{lo}$) present in the periphery.

$T_H$ cells are traditionally divided into two main subtypes, $T_H1$ and $T_H2$ cells, which differ in their cytokine requirements and secretion patterns, as well as the type of adaptive immune response they promote [Krenger and Ferrara 1996]. More recently, a new subtype of $T_H$ cell (which can be either $CD4^+$ or $CD8^+$), referred to as the $T_H17$ cell, has been defined by its characteristic secretion of interleukin (IL)17, IL21 and IL22 when stimulated with transforming growth factor (TGF)β and IL6. This cell type is thought to have important roles in immune regulation and pathological conditions, including infectious and autoimmune diseases [Weaver 2007].

'Regulatory' T ($T_R$) cells have the capacity to suppress immune cell activation. Thymic-derived $T_R$ cells, which express CD4 and CD25 surface markers and the transcription factor FoxP3, are thought to have 'natural' suppressive capacity [Hori 2003] without the requirement for priming of this property. Notably, peripheral $CD4^+$ T cells can be induced to express FoxP3 by TGFβ and IL1 stimulation and assume regulatory functions ('inducible'

$T_R$ cells) [Chen 2003]. $T_R$ cells can inhibit $T_E$ cells and APC through cell contact or secretion of antiinflammatory cytokines, *e.g.* IL10. The lack of $T_R$ cells can cause autoimmune disease [Sakaguchi 1995; reviewed by Sakaguchi 2008; Buckner 2010]. Recent studies have shown that $T_R$ cells may differentiate into IL17-producing $CD4^+$ T cells *in vivo* [Yang 2008; Koenen 2008], shifting the balance further towards autoreactivity. Novel functional subtypes of regulatory cells within the T cell population have been described recently [Buckner 2010].

NKT cells display a T lymphocyte-like phenotype with surface expression of CD3 and TCR together with NK cell receptors. Some NKT cells display semi-invariant TCR molecules with a limited repertoire that binds the non-classical MHC-I ligand CD1 [Berzins 2011]. NKT cells are considered as innate immune cells which mediate crucial immunomodulatory properties in different disease settings [Berzins 2011].

NK cells have been associated with the innate immune defense (see the following subchapter). However, according to recent reports, NK cells display certain properties of plasticity and flexibiliy, such as education for self-tolerance, adaptation to the cell-molecular environment and immunological memory [Vivier 2011].

The rigid distinction between adaptive and innate immune responses is becoming more difficult to uphold as both arms of the immune system are found to be closely regulated and interdependent; phenotypically defined cell types may combine dichotomic functions that were historically strictly classified as either innate or adaptive characteristics.

### II.6 Natural killer cells are large granular lymphocytes that can reject infected, transformed and transplanted allogeneic hematopoietic cells

NK cells are large granular lymphocytes that make use of a range of receptor types to distinguish normal from aberrant cells. Unlike the TCR and BCR, NK cell receptors (NKR) do not undergo somatic rearrangement, but are encoded in the germline. The natural killer gene complex (NKC; Figure 3) and leukocyte receptor complex (LRC) gene regions encode several families of NKR. Most defined NKR have specificities for MHC class I molecules,

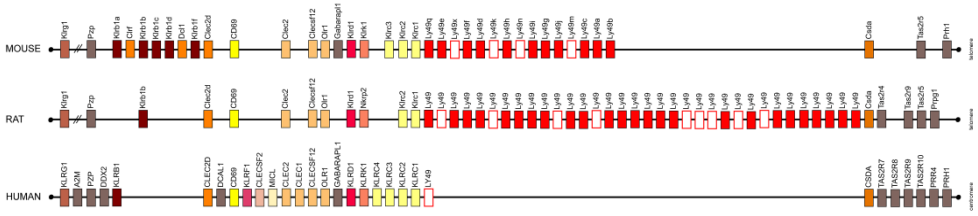
such as human classical HLA-A, -B, -C, and non-classical HLA-E, -G, or the non-classical class I molecules RT1-C/E in the rat. Some receptors can bind to other MHC-related ligands, such as MIC-A and -B. NK cells can thereby monitor target cells for the presence of surface MHC-I and stress-induced ligands.

Among the receptors utilized by human NK cells are the killer cell Ig-like receptors (KIR) located in the LRC [Yokoyama and Riley 2008]. Rodents have evolved a number of C-type lectin-like genes within the NKC, the Ly49 family, which fulfill similar functions [Yokoyama and Plougastel 2003]. Both gene families comprise receptors with inhibitory and activating functions that regulate NK activity.

Inhibitory receptors contact MHC-I alleles expressed on normal cells and thereby arrest NK cell effector functions (*i.e.* release of cytotoxic perforin/granzyme and cytokines). Target cells which do not express MHC-I ligands have a 'missing self' phenotype and are therefore susceptible to NK cell-mediated lysis due to the failure to inhibit NK cell activation [Kärre 1986]. In this way, downregulation of MHC-I expression as an immune evasion strategy against recognition by $T_C$ cells is counteracted by NK cell surveillance.

Activating NKR may recognize stress-induced ligands such as MIC-A and MIC-B through the NKG2D receptor [Bauer 1999], or MHC-like viral decoy molecules such as m157

Figure 3 | **Comparison of the natural killer complexes on human chromosome 12** (bottom), **mouse chromosome 6** (top) **and rat chromosome 4** (center)
Colors decode the different lectin gene families on the NKC (**gray** indicates genes that are not considered NK receptors, **white** are pseudogenes). Individual genes are schematically shown as boxes (circles indicate the centromere/ telomere ends, left to right, not drawn to scale). There are several Ly49 genes (**red**) in the rodent NKC. Their number is dependent on the haplotype and varies between different strains. There is one human Ly49 gene (pseudogene). Similar to human, only one Ly49 gene has been found in baboon, orang-utan, dog, cat, cow and pig (not shown).
Adapted with permission from Elsevier: Kelley, Walter & Trowsdale (2005), *PLoS Genetics* 1



encoded by murine cytomegalovirus which is recognized by Ly49H [Smith 2002]. Ligand binding of an activating receptor does not necessarily result in killing of the target cell as the stimulatory signal must compete with simulaneous inhibitory signals triggered on the same NK cell. How activating and inhibitory signals are integrated to balance NK activity is not fully understood. This model is further complicated by observations that several receptors can bind to more than one ligand (including allogeneic and xenogenic proteins) and that different NKR, both activating and inhibitory, may share the same ligand [Naper 2005; Kveberg 2011].

NK cells have the ability to reject transplanted hematopoietic cells depending on the MHC-constitution of the donor. Recipient NK cells tolerate injections of genetically identical hematopoietic cells, but destroy allogeneic donor cells which either express allogeneic MHC ligands that engage activating receptors or lack appropriate autologous MHC ligands to inhibitory receptors [Vaage 1994; Ruggeri 2006]. The role of NK cells in allogeneic hematopoietic cell transplantation (HCT), a potent therapeutic procedure for leukemias and other hematological diseases, is becoming more important as the toxicity of this treatment, including T cell alloreactivity, is increasingly well controlled [Velardi 2009].

## *Chapter III. Hematopoietic stem cell transplantation*

HCT is a clinical treatment modality for a variety of malignant diseases such as leukemias (myeloid and lymphoblastic leukemias in acute and chronic forms) and non-malignant diseases such as congenital or acquired hematopoietic disorders (aplastic anemia, myelodysplastic syndrome), immunodeficiency syndromes as well as autoimmune diseases [Storb 2003]. The hematopoietic system of the patient can be replaced by grafted HSC from a healthy allogeneic (*i.e.* genetically different) donor with the prospect of permanent cure of the underlying disease.

HSC are obtained by aspiration of BM or alternatively, by apheresis of PB after the mobilization of HSC from the BM through administration of granulocyte colony-stimulating

factor (G-CSF). Umbilical cord blood (UCB) is an alternative source of HSC at childbirth and can be stored for the long term by cryopreservation. UCB units are becoming more commonly used for the treatment of both malignant and non-malignant diseases [Brunstein and Laughlin 2010] due to a lower risk of adverse immune reactions compared with adult BM grafts [Malgieri 2010].

The MHC is the dominant genetic region that governs mutual tolerance between donor and host. Matching of MHC-I and -II alleles increases the likelihood of successful engraftment of HSC drastically (*cf.* subchapter III.5). However, even in the case of full compatibility of MHC alleles, other polymorphic genes may translate to alloantigens that can be recognized by T cells. These are collectively described as 'minor histocompatibility (H) antigens'. Major and minor H antigens may likewise invoke host-*versus*-graft, graft-*versus*-host (GvH) and graft-*versus*-leukemia (GvL) effects and thus, determine the risk of rejection, recurrence of the original disease (relapse) and graft-*versus*-host disease (GvHD) (described in subchapter III.2).

Qualified donors are either family members (parents, siblings, identical twins) or unrelated volunteers who have variable degrees of MHC compatibility with the patient. Children share one set of MHC genes with each of their parents by inheritance, they are 'haploidentical'. Among siblings, there is a statistical probability of 25 % that two have inherited the same maternal and paternal MHC haplotypes and are thus MHC-identical.

The use of a related or matched unrelated donor (MUD) with a genetic composition that is (in part) different from the HCT recipient raises some important considerations: Firstly, the graft may not be accepted by the host. Secondly, donor-derived immune cells may be activated by immunologic disparities in the host. Thirdly, the host malignancy must be completely eradicated or at least permanently suppressed by the transplanted cells.

## III.1 Allogeneic HCT is an established therapy for diseases of the blood and immune system

In the past year, more than 25,000 allogeneic HCT were performed worldwide, and the total annual number of clinical procedures are predicted to rise in the future [Savani 2011]. This treatment modality is currently performed in patients with relatively severe and advanced diseases for whom few alternative therapeutic options are available.

Allogeneic HCT requires the conditioning of the patient prior to transplantation, either by ionizing radiation (radiotherapy) or pharmacological conditioning (chemotherapy), or both in combination, to deplete hematopoietic host cells. This is important mainly for three reasons: Firstly, to eradicate the malignant cells. Secondly, to destroy competent host immune cells and thereby facilitate the entry of transplanted cells, *i.e.* to avoid host-*versus*-graft reactions and graft rejection. Thirdly, to make available HSC niches in the host BM allowing sustained engraftment by donor progenitor cells. Thus, by successful establishment of donor hematopoiesis, the transplanted patient will develop a new, stable hematopoietic system and permanent immune protection. Whether the host's hematopoietic system will be completely replaced by donor cells (full donor chimerism), or whether mutually tolerant host and donor blood cells will coexist over time (mixed chimerism), can vary between protocols and depends on the type and dose of pretransplant immunosuppression, the genetic constitution of both recipient and donor, the source and composition of the donor graft and unknown factors.

At present, a major complication in allogeneic HCT is GvHD, an immunological condition which is caused by the uncontrolled reactivity of donor leukocytes against host tissues. Allogeneic donor T cells are transferred *via* the hematopoietic graft and become activated by histological disparities encountered in the foreign environment of the transplanted patient (*cf.* subchapter III.2). The host is immunocompromised due to pretransplant conditioning and thus unable to mount an effective immune response against the influx of competent donor T cells. In its severe form, GvHD is associated with a high prevalence of

transplant-related mortality (TRM) and morbidity, resulting in poor prospects of survival of the affected patients.

The main causes of TRM besides GvHD are graft rejection, infections and tumor relapse [Gibbons and Sykes 2008]. The breakdown of the immune defense in immuno-compromised and immunodeficient patients against otherwise harmless opportunistic microorganisms or reactivation of latent viral infections is an impressive, if disastrous, demonstration of the efficient protection provided by a normally functioning immune system.

HCT has made it possible to subject patients to high doses of ionizing radiation or chemotherapy to eliminate leukemic cells (myeloablative treatment) which would otherwise be lethal [Appelbaum 2001]. Sublethal (non-ablative) doses of conditioning represent a feasible alternative to obtain engraftment while reducing TRM. With either alternative, the complete clearance of malignant cells can be achieved by the transfer of competent donor immune cells with graft-*versus*-tumor (GvT) reactivity, *i.e.* GvH reactivity directed against host tumor cells. Although a strong GvT response is required to avoid relapse by killing residual tumor that has escaped the initial cytotoxic treatment, the presence of T cells with GvH-reactivity in the graft is potentially dangerous for the aforementioned reasons.

The toxicities associated with allogeneic HCT may lead to life-threatening complications which can outbalance the potentially curative effects of engraftment, BM reconstitution and beneficial antitumor responses. The strong association of GvH and GvT effects [Appelbaum 2001] mediated by transplanted donor T cells raises the questions whether these two phenomena are separable, and whether GvHD can be avoided without compromising prolonged cure of the underlying disease.

### III.2 Graft-*versus*-host disease is a transplant-related multiorgan system disorder with complex pathophysiology

The physiological events which manifest in the systemic immune syndrome recognized as acute GvHD are relatively well understood (the chronic form of this disease however

remains more elusive, *cf.* subchapter III.3). The principle pathology of GvHD can be divided into sequential stages of disease development (explained below and schematically described in Figure 4).

**IMMUNE PRIMING.** Patients undergo chemotherapy or radiotherapy, or both, before allogeneic HCT. The conditioning regimen causes acute and extensive tissue damage as well as the release of proinflammatory cytokines (*e.g.* IL1, IL6, TNFα, IFNγ) and other endogenous danger signals [Ferrara and Reddy 2006]. Infections and the underlying disease can also contribute to the ongoing inflammation in the patient. This initial 'cytokine storm' [Ferrara 1993] leads to sensitization and activation of tissue-resident APC, resulting in the upregulation of surface MHC and costimulatory molecules as well as increased sequestration of chemokines. LPS, a degradation product of the bacterial cell wall which acts as a potent stimulator of immune functions, may also be released from the gut as a result of intestinal damage.

**T CELL ACTIVATION AND STIMULATION.** Activated APC migrate to SLT, *e.g.* the spleen, lymph nodes or Peyer's Patches, in the inflamed environment of the host. They effect the recruitment of immune cells, including transplanted donor T cells, from the blood stream to SLT. Those donor T cells which possess a TCR specific for a host-derived antigen presented on the APC are primed. The ability of APC to process and present both intra- and extra-cellular antigens opens different possibilities of alloreactive donor T cell activation, *e.g.* through the presentation of host-derived antigens by MHC-I and MHC-II molecules on donor APC established in the HCT recipient. Experimental studies in mice have shown that GvHD may be initiated by donor $CD8^+$ T cells that are activated by host APC [Shlomchik 1999] as well as donor $CD4^+$ T cells that are activated by either host or donor BM-derived APC [Anderson 2005].

**ALLOREACTIVE T CELL EXPANSION.** In the initiation phase of GvHD, activated $CD4^+$ and $CD8^+$ T cells proliferate and differentiate to become alloreactive effector cells which can cause GvHD. Inflammatory cytokines further contribute through bystander effects resulting in unspecific stimulation of $T_E$ cells [Teshima 2002].

**TRAFFICKING.** Subsequently, activated donor T cells go from SLT into circulation and migrate to peripheral tissues in response to chemokine attraction and the enhanced expression of adhesion molecules (integrins and selectins) on endothelial cells in the vasculature [Wysocki 2005]. $T_H$ cells are thought to have less cytotoxic activity, but effect the recruitment of immune cells, including neutrophils, monocytes, NK cells, B cells and $T_C$ cells, to sites of ongoing inflammation and immune activation by the secretion of stimulatory cytokines (*e.g.* IL2, IFNγ, TNFα). Increased local concentrations of these cytokines further enhance antigen presentation as well as cytokine and chemokine secretion by APC, thereby amplifying the activation and recruitment of effector immune cells.

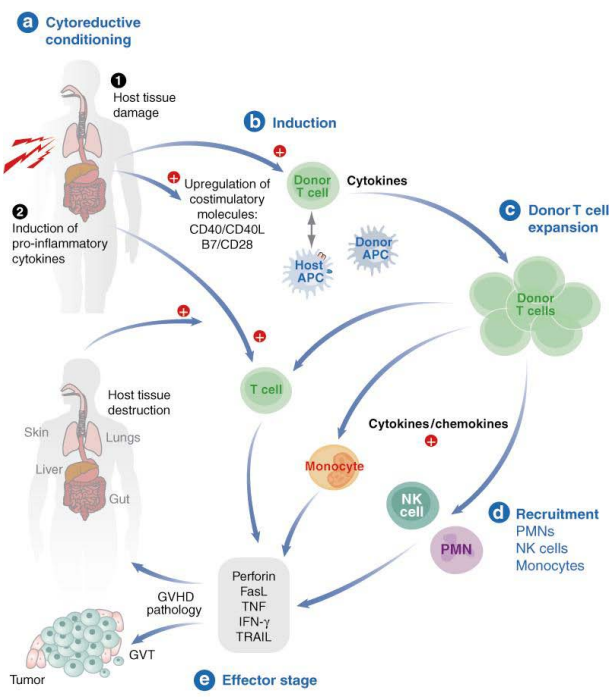



Figure 4 | **Schematic model defining the main pathological stages of GvHD development**
(a) Prior to HCT, the conditioning regimen causes damage of host tissue and substantial secretion of proinflammatory cytokines in the patient. (b) Donor T cells entering the inflammatory milieu of the patient are activated by alloantigens presented on host or donor APC. (c) Activated donor T cells expand, produce inflammatory cytokines and chemokines and (d) recruit innate immune cells (*e.g.* monocytes, NK cells, granulocytes) and other T cells. In the final (e) effector stage, activated immune cells as well as cytokines mediate the destruction of host tissues, leading to the elimination of tumor cells (the GVT effect) and causing multiple organ GvHD.
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**TISSUE INFLAMMATION AND DESTRUCTION.** $T_C$ cells and NK cells are the main effector cell types responsible for tissue destruction through cytotoxicity in the final phase of GvHD. Moreover, soluble inflammatory proteins contribute to host tissue damage either directly or indirectly by stimulating nearby effector cells. IFNγ and TNFα are two major effector molecules which directly mediate damage and necrosis of target tissues causing advanced-stage GvHD pathology [Krenger 1997; Ferrara and Krenger 1998; Brown 2002]. Bacterial products such as LPS may cause additional damage of mucosa and skin in late stages of GvHD as they induce inflammatory pathways through TLR signaling in various innate immune cells, *e.g.* NK cells, monocytes and macrophages [Ferrara 2009].

### III.3 Severe GvHD results in extensive tissue damage, systemic immune failure and possible death of the patient

Acute and chronic GvHD are viewed as two related but different disease syndromes which are distinguished primarily based on the time of disease onset (earlier or later than 100 days, respectively, after transplant) and the clinical disease phenotype. The primary sites of graft-*versus*-host reactions (GvHR) in acute GvHD are the mucosa of skin, liver and the gastrointestinal tract [Ferrara and Reddy 2006]. Lymphoid organs can also be involved, *e.g.* thymus and spleen, while the lungs are a target organ especially in chronic GvHD [Yanik and Cooke 2006]. Recently, Shono *et al.* have identified the BM as a target organ of GvHD [Shono 2010], showing that MHC-mismatched, alloreactive donor T cells can disrupt the hematopoietic niches of host HSC [Shono 2010]. These findings offer a plausible explanation for recognized GvHD-related symptoms such as suppression of hematopoiesis and delayed immune reconstitution.

Box 1 | **Symptoms of acute GvHD**

**Skin**
- maculopapular skin rash

**Upper gastrointestinal tract**
- nausea, anorexia, or both
- positive histological findings

**Lower gastrointestinal tract**
- watery diarrhea
- severe abdominal pain
- bloody diarrhea or ileus

**Liver**
- cholestatic hyperbilirubinaemi

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Ferrara *et al.* (2009), *Lancet* 373

A range of clinical symptoms typical of either form of GvHD has been defined [Ferrara 2009] (Box 1 and Box 2). Transplant recipients may present with heterogeneous syndromes at variable time points and suffer from GvHD of variable degrees of severity. Ongoing alloreactivity and cytotoxicity against host epithelial tissues and hematopoietic cells result in a state of chronic inflammation, target organ damage and a defective immune system of the patient. The necessity to deliver immunosuppressive drugs contributes to the weakened functionality of the host defense and as a consequence, opportunistic infections are frequent causes of complications and death of GvHD patients.

### III.4 Clinical assessment of GvHD

There is a need for objective criteria to clinically assess and grade GvHD in patients to aid a more precise diagnosis and a more reliable prediction of the risk of treatment including the odds of survival. Despite ongoing research efforts in this area (*cf.* subchapter III.6), diagnosis of the disease is mainly based on clinical observations and distinct pathologies of target organs.

Glucksberg and colleagues defined a set of criteria based on defined clinical evaluations of the

Box 2 | **Symptoms of chronic GvHD**

**Skin**
- dyspigmentation
- new-onset alopecia
- poikiloderma
- lichen planus-like eruptions
- sclerotic features

**Nails**
- nail dystrophy or loss

**Mouth**
- xerostomia
- ulcers
- lichen-type features
- restrictions of mouth opening from sclerosis

**Eyes**
- dry eyes
- sicca syndrome
- cicatricial conjunctivitis

**Muscles, Fascia, Joints**
- fasciitis
- myositis
- joint stiff ness from contractures

**Female Genitalia**
- vaginal sclerosis
- ulcerations

**Gastrointestinal Tract**
- anorexia, weight loss
- oesophageal web or strictures

**Liver**
- jaundice
- transaminitis

**Lungs**
- restrictive or obstructive defects on pulmonary function tests
- bronchiolitis obliterans
- pleural effusions

**Kidneys**
- nephrotic syndrome (rare)

**Heart**
- pericarditis

**Marrow**
- thrombocytopenia
- anaemia
- neutropenia

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Ferrara *et al.* (2009), *Lancet* 373

main target organs of acute GvHD (skin, liver and the gastrointestinal tract) as well as subjective assessment of patient performance [Glucksberg 1974]. The clinical parameters are categorized in escalating stages of severity as outlined in Table 1. Patients are assigned grades from I to IV (mild to severe) according to the pattern of observed clinical symptoms as outlined in Table 2. This general grading system is a useful tool for the practical management of GvHD and is widely used by hematologists and HCT specialists.

Table 1 | **Clinical stages of acute GvHD symptoms after Glucksberg *et al.***

| stage | skin | liver | intestinal tract |
|---|---|---|---|
| + | macropopular rash<br>< 25 % of body surface | bilirubin<br>2-3 mg per 100 mL | > 500 mL diarrhea<br>per day |
| ++ | macropopular rash<br>25-50 % of body surface | bilirubin<br>3-6 mg per 100 mL | > 1000 mL diarrhoea<br>per day |
| +++ | generalized erythroderma | bilirubin<br>6-15 mg per 100 mL | > 1500 mL diarrhoea<br>per day |
| ++++ | generalized erythroderma<br>with bullous formation and<br>desquamation | bilirubin<br>> 15 mg per 100 mL | severe abdominal pain with<br>or without ileus |

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Table 2 | **Grading of GvHD severity after Glucksberg *et al.***

| grade | degree of organ involvement |
|---|---|
| I | + to ++ skin rash; no gut involvement; no liver involvement; no decrease in clinical performance; |
| II | + to +++ skin rash; + gut involvement or + liver involvement (or both); mild decrease in clinical performance; |
| III | ++ to +++ skin rash; ++ to +++ gut involvement or ++ to +++ liver involvement (or both); marked decrease in clinical performance; |
| IV | similar to grade III with ++ to ++++ organ involvement and extreme decrease in clinical performance; |

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Table 3 | **Grading of cutaneous GvHD after Lerner *et al.***

| grade | symptoms |
|---|---|
| 0 | normal |
| I | vacuolization of epidermal basal cells |
| II | diffuse vacuolization of basal cells with dyskeratotic bodies |
| III | subepidermal cleft formation |
| IV | complete epidermal separation |

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Clinical assessment may be complemented with histopathological evaluation of biopsies from GvHD target organs. We have applied the grading scheme for cutaneous GvHD introduced by Lerner and colleagues [Lerner 1974] as outlined in Table 3 to measure GvHR of the skin in our studies.

### III.5 The MHC is the primary determinant for the outcome of allogeneic HCT

Matching the donor's with the recipient's HLA-I and -II genes is an essential prerequisite for successful HCT [Lee 2007]. Allele-matching of *HLA-A*, *-B*, *-C, -DRB1* ('8/8 match') and *-DQB1* ('10/10 match') significantly reduces the risk of treatment failure [Petersdorf 2001; Petersdorf 2007]. However, GvHD may occur despite full HLA compatibility, for example after transplantation between HLA-identical siblings due to disparities in minor H antigens [Dickinson 2008]. In the case of unrelated donors, MHC mismatches (permitted or undetected) and the high number of minor H antigen differences result in an increased incidence of GvHD [Petersdorf 2007]. Furthermore, the majority of genes encoded in the MHC do not encode for HLA proteins (*cf.* Figure 2), but polypeptides that function as 'non-HLA' minor H antigens which also contribute to the risk of GvHD.

## III.6 Gene polymorphisms can influence susceptibility to GvHD and serve as biomarkers for disease diagnosis

Robust risk estimates of GvHD incidence and severity would allow clinicians to intervene at the stage of donor selection, patient preconditioning and immunosuppressive treatment in the course of transplantation. Despite the abundance of genetic disparities between most allogeneic HCT recipients and their donors, indicators which can be used as unequivocal markers of the disease or reliable predictors of treatment outcome have yet to be identified [Ferrara 2009; Paczesny 2009b].

Gene polymorphisms, *i.e.* divergent DNA sequences of allelic gene variants, *e.g.* single nucleotide base pairs and microsatellites, between individuals may affect gene expression, translate to alloantigens and predispose to or protect from disease. Several non-HLA genes with immunological roles, *e.g.* genes encoding for cytokines (including IL1 receptor antagonist, IL4, IL6, IL10, IFNγ, TNFα), chemokines (*e.g.* CCL8) and receptor molecules (NOD/CARD15, estrogen and vitamin D receptors), display polymorphic phenotypes and have been implicated as predictors of risk of GvHD [Dickinson 2008; Paczesny 2009a; Dickinson 2010].

For example, while polymorphisms in the gene encoding for TNFα are linked to increased risk of acute GvHD [Holler 1995; Middleton 1998], the natural TNFα antagonist IL10 can protect patients who produce relatively high amounts of this cytokine [Holler 2000]. Blocking of TNFα is used as a clinical treatment of GvHD at present (*cf.* subchapter III.9, *Targeted pharmacological intervention*). IFNγ is another cytokine which has critical importance in GvHD pathology (*cf.* subchapter III.2), exerting immune cell activation and upregulation of MHC gene expression on target cells among other roles. Recipient polymorphisms of this gene have been associated with acute GvHD after HLA-identical sibling HCT [Cavet 2001]. It has been proposed that monitoring TNFα and IFNγ concentrations in

the blood plasma during acute GvHD could be exploited for better disease diagnosis [Hu 1999; Levine 2008].

The recipient's gene for IL6 can predispose for GvHD in the acute or chronic form [Cavet 2001; Socie 2001]. Middleton and coworkers found that family donors with a variable number tandem repeat in the *IL1RN* gene, coding for a IL1 family cytokine, are less likely to cause acute GvHD in HCT recipients [Cullup 2001].

Polymorphisms in genes encoding for innate immune receptors have also been identified as risk factors in experimental animal studies, *e.g.* TLR9 in chronic GvHD [She 2007]. Mutations in the *NOD2*/*CARD15* gene (which encodes a protein sensor of the bacterial cell wall component muramyl-dipeptide expressed in the cytoplasm of intestinal epithelial cells, monocytes and macrophages) could predict GvHD incidence and TRM in recipients of MHC-matched related and unrelated donor BM transplants [Holler 2004; Holler 2008].

Heat-shock proteins (HSP) are encoded by a class of highly conserved genes which maintain fundamental functions in the protection of the cell against exogenous and endogenous stresses (heat, irradiation, inflammation, *et c.*) [reviewed by Srivastava 2002]. Such stress-inducible intracellular chaperones, *e.g.* the human HSP70 family of genes located in the MHC-III region, are implicated as regulatory molecules in adaptive and innate immune responses owing to their ability to bind antigenic peptides and make them available for cross-presentation by APC [Srivastava 2002]. Regulation of GvHD by polymorphic HSP is therefore plausible. Evidence for such an association are anti-HSP70 antibodies present in the blood serum and overexpression of HSP70 in SLT of rats with GvHD [Goral 1995; Goral 1998]. Serum antibodies towards HSP70 and HSP90 were also found increased at an early stage following PB-mobilized HCT in patients with acute GvHD, but not in GvHD-free patients [Goral 2002].

More recently, Paczesny and coworkers have studied HCT patient plasma by high-throughput screening using antibodies and isolated 8 candidate proteins which were associated with the incidence of acquiring acute GvHD [Paczesny 2009c]. Subsequent evaluation of these indicators resulted in a panel of four soluble blood serum molecules, namely IL2 receptor α, IL8, TNF receptor 1 and hepatocyte growth factor, which had superior predictive power over similar risk factor assessments [Paczesny 2009c].

In addition to genetic risk factors, clinical parameters such as age and gender of both donor and patient, history of previous infections, time from diagnosis until transplant and the type of transplant used, among others, may influence the likelihood of developing acute or chronic GvHD and predict posttransplant survival [Gratwohl 1998].

### III.7 *In vitro* models of GvHD

The wish to gain a better understanding of the etiopathology of GvHD and to improve the clinical management of this disease has driven research in developing adequate laboratory methods and animal models. The mixed lymphocyte reaction (MLR), where donor and recipient lymphocytes from the PB of patients or autopsied lymphoid tissues from laboratory animals are coincubated in standard cell culture, is a feasible method to elicit GvHR *in vitro*. Allogeneic donor lymphocytes serve as responder cells, which are sensitized during several days of coculture with recipient lymphocytes that have been irradiated in order to inhibit mitosis and serve as stimulator cells. The proliferative response can be assayed as a measure of T cell activation and expansion.

Other laboratory techniques have been designed to prospectively quantify GvHR for predictive GvHD risk measurement, *e.g.* limiting dilution assays to determine the frequencies of alloreactive $T_C$ and $T_H$ lymphocyte precursors *in vitro* [Kaminski 1989; Theobald 1992]. These models, however, either lacked the sensitivity to predict GvHR reliably or were too impractical to find their way into routine testing.

The skin explant assay is a model which can predict GvHR with high sensitivity and specificity *in vitro*. The method was originally described by Berkman *et al.* and was prospectively tested for the prediction of GvHD incidence and severity in a donor/patient cohort by Vogelsang *et al.* [reviewed by Sviland and Dickinson 1999]. The skin explant assay had a reported correlation of 80-90 % with clinical outcome and was superior to other *in vitro* models for this purpose [Dickinson 1998; Sviland and Dickinson 1999; Dickinson 2001; Hromadnikova 2001]. The assay is performed *ex vivo* in medium culture and comprises a primary MLR to activate T cells from the marrow donor against recipient lymphocytes, followed by a secondary reaction of the primed T cells against a skin biopsy from the same patient and subsequent histological evaluation. The skin explant closely resembles the histopathology of cutaneous GvHD and allows the study of pathophysiological processes related to GvHR [Dickinson 2002]. Thus, histological grading of the skin explant represents a standardized method to evaluate the risk for selected donor-recipient combinations before transplantation. As a scoring table the scheme for pathological grading of GvHD-affected skin (Table 3) may be used.

### III.8 *In vivo* models of GvHD

Experimental work using animal models of HCT, first and foremost in mice and dogs (*Canis lupus*), has contributed substantially to the current knowledge of the etiopathogenesis of GvHD [Welniak 2007; Socie and Blazar 2009]. Animal models have also been instrumental for discovering and testing a variety of therapeutic options for their feasibility and efficacy [Socie and Blazar 2009; Ferrara 2009].

Preclinical models can, for example, yield important insights into the effects and toxicities of prophylactic or therapeutic pharmacological treatments and may help elucidate clinically relevant questions, *e.g.* the factors determining a patient's unresponsiveness to these medications. It has to be taken into account, however, that medical treatment of animals may

deviate significantly from the effects and pharmacodynamics observed in humans [Socie and Blazar 2009]. The limitations to the interpretability of research performed in animal models are dealt with in more detail in the **General Discussion**.

Dog models of allogeneic BM transplantation (BMT) have provided insights into important aspects of transplantation and GvHD prevention in clinical practice (*cf.* subchapter III.9). Non-myeloablative conditioning using reduced intensities to decrease toxicity and morbidity in patients; pharmacological immunosuppression regimens posttransplant; *ex vivo* depletion of T cells from the graft by polyclonal antilymphocyte and antithymocyte globulins for prevention of GvHD; and delayed donor lymphocyte transfusions to induce GvT effects and conversion of mixed to complete donor chimerism are examples of protocols that were developed or improved in canine models [Storb and Thomas 1985; Storb 2003; Socie and Blazar 2009].

The mouse has been the species of choice in biomedical research historically and continues to dominate experimental studies of acute and chronic GvHD to date. Recipient mice are usually conditioned for transplantation with a lethal irradiation regimen. Animal models which are based on preconditioning by chemotherapy are rare [Sadeghi 2008; Socie and Blazar 2009]. In further contrast to humans, the BM graft is conventionally supplemented with spleen or lymph node cells to invoke GvHD in the experimental setting.

An array of murine models designed for mismatch of the MHC, minor H antigens, or both, are available today [Schroeder and DiPersio 2011]. Fully incompatible strain combinations regarding both the MHC and the genetic background, *e.g.* [C57/BL6 (*H-2*$^{b}$) → Balb/C (*H-2*$^{d}$)], are often used. Parent-to-$F_1$ HCT models are employed for haploidentical transplantations. Experimental protocols using non-myeloablative preconditioning are particularly interesting to mimic low-dose conditioning regimens for patients [Schroeder and DiPersio 2011].

In the context of MHC mismatches, differences in minor H antigens are of minor importance in the pathogenesis of GvHD. In MHC-matched models, on the other hand, the contributions of minor H antigens to GvHR can be studied. In order to target individual antigens more specifically, transgenic mouse models which express a single recombinant TCR with defined antigen-specificity are also available.

Transplantation of human cells into mice is made possible by xenogenic transplant models to study 'humanized' GvHD *in vivo*, albeit in immunodeficient individuals of a different species. Recombination activating gene 2-deficient, IL2 receptor γ-deficient and non-obese diabetic, severe combined-immunodeficient mice can be used for this purpose. For a comprehensive summary of often used experimental mouse models of acute and chronic GvHD, the reader is referred to a recent review [Schroeder and DiPersio 2011].

### III.9 State-of-the-art prevention and treatment of GvHD

The available and novel therapeutic approaches for the treatment of GvHD are too diverse for detailed discussion in this thesis. The main lines of treatment presently in clinical use are here briefly mentioned. Their individual advantages and complications are discussed. Where applicable, insights into the efficacies of selected strategies of GvHD prevention and therapy through clinical trials are updated. They include therapeutic approaches which will be dealt with in the following categories: Adaptation of conditioning regimens, Alteration of the graft composition, Targeted pharmacological intervention and Cell-mediated therapy. Intervention can be attempted prior to transfer of the disease-causing agent (donor immune cells) or at different stages of disease progression (host APC activation; donor T cell activation, differentiation and trafficking; effector cell-mediated cytotoxicity).

Generally, positive effects of preventive and therapeutic treatments are often outweighed by adverse effects, such as an increased risk of graft failure, increased frequency of disease relapse and secondary lymphoproliferative disorders, severe side effects of immuno-

suppressive drugs or loss of protection against opportunistic infections. Achieving long-lasting tumor protection through a beneficial GvT effect without triggering the detrimental alloimmunity manifested in GvHD, while establishing full immune reconstitution and maintaining host tolerance to the graft, renders the clinical management of this disease a formidable challenge at present.

### *Adaptation of conditioning regimens*

**RADIATION, CHEMOTHERAPY AND PROPHYLAXIS.** Patients awaiting allogeneic HCT receive chemotherapy or total body irradiation or both, to kill hematopoietic cells and render the host receptive for the donor graft. Calcineurin inhibitors (cyclosporine, tacrolimus, *et c.*) target T cells and prevent their activation. Other metabolic inhibitors (methotrexate, mycophenolate mofetil, *et c.*) are often applied in combination with calcineurin inhibitors to further dampen the host immune response. The corticosteroids used in pretransplant prophylaxis are often administered for continued immunosuppression after HCT or acutely for treatment of symptomatic GvHD. The agents may be applied either systemically, topically (skin GvHD) or by inhalation (lung GvHD). First-line treatment of acute GvHD with steroids results in complete or partial remission (resolution of symptoms) in approximately half the patients [MacMillan 2002]. For those who do not respond and develop steroid-refractory (resistant) GvHD, a number of second-line treatment options are available (see below).

**ANTIBODIES.** Mono- and polyclonal antibodies (*e.g.* antithymocyte and antilymphocyte globulins) may be used to prevent GvHD by depleting donor T cells *in vivo*. As with other treatments targeting T cells directly (such as calcineurin inhibitors), however, GvHD prevention and alleviation is compromised by the loss of beneficial antitumor effects as GvH and GvT reactivities are equally affected, resulting in an increased risk of tumor relapse.

**REDUCED INTENSITY CONDITIONING (RIC).** Lethal doses of irradiation and chemotherapy can be used to deplete host cells in conjunction with subsequent transfer of BM stem cells that

rescue the patient [Appelbaum 2001]. However, these treatment regimens are highly toxic and cause severe detrimental effects in the patient. The use of reduced or minimum intensity conditioning with lower toxicity was designed to deminish, if not avoid, tissue damage and treatment-related complications, but is associated with increased rates of leukemic relapse and bacterial infections [Appelbaum 2010].

### *Alteration of the graft composition*

**T-CELL DEPLETION (TCD).** TCD of the stem cell graft is a non-pharmacological strategy to avoid GvHD, which is especially useful in donor-patient combinations that have partial MHC mismatches, because they activate higher numbers of alloreactive T cells [Kolb 2008]. Depletion is performed *ex vivo* prior to transplantation by use of immunological (T cell-specific antibodies in combination with complement-mediated lysis, immunotoxins, magnetic beads) or physical methods (agglutination, density gradient fractionation) [Ho and Soiffer 2001]. Since the broad removal of donor T cells results in the loss of beneficial GvT activity, TCD results in higher rates of tumor relapse as well as increased risk of graft rejection, delayed immune reconstitution, viral reactivation and, possibly, infection [Ho and Soiffer 2001]. Stronger tumor prophylaxis or prolonged and additional immunosuppressive regimens may therefore be required and can be complemented with additional transfusions of donor lymphocytes (see *Cell-mediated therapy*, p.34) posttransplant to confer GvT-reactivity and avoid transplant-related complications.

**GRAFT ENRICHMENT.** To overcome the obstacle of engraftment failure that is associated with RIC and TCD, graft enrichment of $CD34^+$ HSC and infusion of a large number ('megadose') of BM stem cells are performed with success [Bachar-Lustig 1995; Ringdén 2003].

### *Targeted pharmacological intervention*

**CYTOKINE MODULATION.** Cytokine modulation through pharmacological intervention has become a subject of GvHD therapy. The key importance of IFNγ and TNFα during all stages

of GvHD, from initiation to the late effector phase (*cf.* subchapter III.2) has been recognized. Blocking TNFα by systemic administration of soluble TNFα receptor 2 (etanercept) alleviated GvHD in the clinical setting [Levine 2008]. However, attempts to enhance or neutralize cytokines with putative anti- or proinflammatory activity *in vivo* may have unintended effects. The systemic administration of immunosuppressive cytokines (*e.g.* IL4, IL10) has produced obscure results in experimental and clinical studies [Murphy and Blazar 1999]. Paradoxically, cytokines generally considered as proinflammatory/immunostimulatory (*e.g.* IL2, IL18, IFNγ) can dampen alloreactivity and improve GvHD. For instance, blocking the IL2 signal by calcineurin inhibitors may not only induce T cell anergy, but also adversely influence the maintenance of $T_R$ cells [Zeiser 2006], which may in turn lead to problems regarding long-term tolerance and GvHD prevention. Infusion of recombinant IL2 was shown to alleviate, rather than exacerbate, GvHD [Wang 1995] by increasing the number of $T_R$ cells in patients [Zorn 2006]. In fact, inducing GvHD patients' $T_R$ cells by IL2 administration is now being tested in a clinical phase II trial [http://clinicaltrials.gov/ct2/show/NCT00539695].

G-CSF administration, which is often used to accelerate neutrophil engraftment after total body irradiation and BMT, initiated a complex immune cascade involving the stimulation of host APC and led to increased GvHD in a mouse model [Morris 2009].

Therefore, the deliberate manipulation of complex signaling pathways by administration of cytokines, chemokines or their antagonists can have unexpected effects in ways that may not be apparent, but are potentially dangerous.

### *Cell-mediated therapy*

**DONOR LYMPHOCYTE INFUSION (DLI).** DLI is applicable for the prevention or treatment of relapse of hematological malignancies and is often combined with chemotherapy or other immunosuppression [reviewed by Kolb 2008]. Donor lymphocyte grafts contain CD4 T cells, CD8 T cells and NK cells with GvT potential. DLI has been especially successful in patients

with chronic myelogenic leukemia. The risks of this treatment are GvHD and myelosuppression [Kolb 2008]. The optimal timing and dose of DLI in the treatment of the different malignancies are not known [Tomblyn and Lazarus 2008].

**ADOPTIVE IMMUNOTHERAPY WITH ALLOREACTIVE T CELLS.** Allelic mutations can translate to altered protein structures which may serve as epitopes for allogeneic T cells when the corresponding peptide is presented in the context of HLA, thus forming a minor H antigen. Alloreactive donor T cells with specificity for such antigens can be exploited for the treatment of residual, refractory or relapsing tumors [reviewed by Bleakley and Riddell 2011]. T cell clones which react with tumor-derived antigens can be used to prevent or treat leukemia [Dudley 2002]. By the same token, therapeutic infusions of *ex vivo*-expanded T cells which specifically target common human pathogens (*e.g.* Epstein-Barr virus, herpes simplex virus and cytomegalovirus) can provide persistent posttransplant immunity against infection and viral reactivation [Riddell 1992; Bleakley and Riddell 2011].

Adoptive immunotherapy is particularly promising when T cell clones are selected which can augment GvL effects directed against minor H antigens that are only expressed on recipient hematopoietic cells, including leukemia, but leave non-hematopoietic cells intact [Dickinson 2002; Bleakley and Riddell 2011]. Widely expressed antigens, on the other hand, bear the risk of triggering GvHR [Dickinson 2002]. T cell clones with specificity for recipient minor H antigens have been successfully isolated from GvHD patients, enriched by *ex vivo* expansion and applied to prevent leukemic relapse [Goulmy 1996; Falkenburg 1999; Mutis 1999]. The first phase I clinical trial to use such an approach showed that adoptive transfer of minor H antigen-specific donor $T_C$ cells augmented the GvL effect and led to complete remission in several patients who experienced leukemic relapse after HLA-matched allogeneic HCT [Warren 2010]. Expression of the respective alloantigen on lung epithelia caused pulmonary toxicity in some patients, illustrating the potential danger of this method if

tissue-specific expression patterns of the target antigens are not meticulously mapped. Furthermore, infused antitumor T cells must persist in the host in order to provide durable protection from tumor relapse.

**REGULATORY T CELLS.** Refined strategies which aim to selectively remove GvH-reactive populations from the graft or add back T cells with suppressor function have also been developed. In a clinical pilot study, *ex vivo*-expanded $T_R$ cells from UCB units were administered to a cohort of patients who received partially HLA-matched double UCB transplants [Brunstein 2011]. Infusions were tolerated with no intervention-related toxicity, and adverse effects of the treatment regarding early mortality, risk of infection or relapse were not observed. The incidence of acute GvHD was statistically lower in this patient group relative to comparable historical controls. Future clinical trials will elucidate whether adoptive $T_R$ cell transfer is a feasible and effective therapy, and whether GvHD prevention can be achieved without compromising immune protection from neoplastic relapse, formation of secondary tumors and infections [Riley 2009].

In general, the production of sufficient amounts of the desired T cell subset requires *ex vivo* expansion to achieve satisfactory purity and desirable potency. This approach is currently too labor- and cost-intensive for its general application, especially if the effector cell unit has to be tailored to individual donor-patient pairs [Riley 2009].

**GVH-REACTIVE NK CELLS.** Although putting the patient at a higher risk of GvHD, partial HLA-mismatch of the HSC donor may hold an advantage for the treatment of leukemias in HCT [Velardi 2009]. Transplantation of donor NK cells that are alloreactive against patient hematopoietic and leukemic cells which lack cognate MHC ligands to inhibitory receptors reduces the rate of rejection and relapse [Ruggeri 2002]. Importantly, donor-*versus*-host reactive NK cells do not cause, but protect against GvHD due to cytotoxicity against host

APC [Ruggeri 2002]. Thus, the selection of donors with KIR:HLA mismatches may be beneficial for recipients of haploidentical HCT.

**EXTRACORPOREAL PHOTOPHERESIS (ECP).** ECP represents an adjunct therapy for severe steroid-resistant GvHD. The procedure comprises apheresis of PB followed by loading with 8-methoxypsoralen, a DNA-intercalating agent, to sensitize blood cells to ultraviolet radiation and subsequently induce apoptosis of PB mononuclear cells *ex vivo*. Reinfusion of psoralen and ultraviolet A-treated patient PB led to complete resolution of steroid-refractory or steroid-dependent GvHD in phase I and II studies [Greinix 2000; Messina 2003; Foss 2005; Greinix 2006; Couriel 2006; Flowers 2008] and was particularly efficient in patients suffering from acute or chronic GvHD with skin involvement. Significantly better long-term survival rates were recorded. ECP is generally tolerated well without significant side effects [Knobler 2009]. Complete or partial response to the ECP regimen may allow an earlier reduction or the discontinuation of corticosteroid treatment, thereby improving patient morbidity and mortality. Inhibition of antigen presentation on dendritic cells, increase in the number of donor $T_R$ cells and reduction of alloreactive donor effector cells in experimental GvHD treatment have been implicated, but much of the underlying immunological mechanisms of this approach remains unknown [Knobler 2009].

**MESENCHYMAL STROMAL CELLS.** Transfusion of MSC has emerged as a new treatment modality for GvHD after their immunosuppressive potential has been realized (*cf.* subchapter IV.3). The demonstrated efficacy and safety of single or repeated MSC infusions in early studies [Le Blanc 2004; Ringdén 2006] gave promise to the development of this therapy in subsequent clinical trials [Le Blanc 2008].

## *Chapter IV. Mesenchymal Stromal Cells*

### IV.1 Mesenchymal stromal cells are a heterogeneous population of early progenitor cells in the stroma of various organs

MSC are non-hematopoietic progenitor cells with multilineage differentiation potential. Friedenstein *et al.* were the first to identify and define explanted clonal progenitor cells from the BM that produced fibroblast-like progeny in monolayer cultures (then termed 'colony-forming unit fibroblast', CFU-F) and retained differentiation potential for bone, cartilage and adipose cells [Friedenstein 1968]. Heterotopic transplantation of BM stromal cells generated ectopic skeletal and fibrous tissue *in vivo* [Friedenstein 1968; Friedenstein 1974].

MSC from several species have been characterized, but were most thoroughly studied in humans and mice. An illustrative protocol of isolation, enrichment and single colony-derived generation of rat MSC lines is given in a video article by Zhang and Chan [Zhang and Chan 2010]. MSC have been isolated from the BM and other adult organs [Pittenger 1999; Zuk 2001; da Silva Meirelles 2006; Beltrami 2007; Covas 2008] as well as the female reproductive system [Erices 2000; in 't Anker 2003; in 't Anker 2004; Patki 2010] and fetal tissues [Campagnoli 2001; in 't Anker 2003]. Of these, adipose tissue, amniotic fluid and UCB have lately

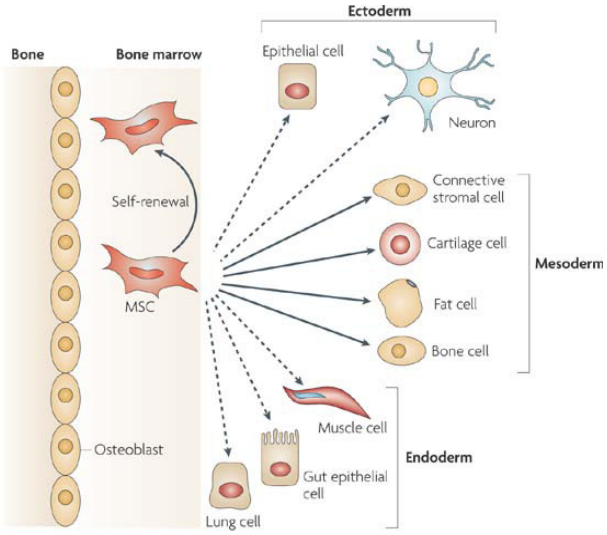


Figure 5 | **Differentiation potential of BM-derived MSC**
BM-resident MSC have the ability self-renew and differentiate into several cell types (*e.g.* bone, cartilage, fat cells) of the mesodermal lineage. The reported ability to transdifferentiate also along other germline lineages (ectodermal and endodermal) *in vivo* is currently controversial.
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gained importance as practically available sources of MSC for clinical use [Malgieri 2010] (*cf.* subchapter IV.4).

The MSC population contains only few self-renewing progenitor cells with multipotency for the mesodermal lineage [Bianco 2008], including stroma, bone, cartilage and fat cells [Horwitz 2005] (Figure 5). Transdifferentiation of MSC into cells of non-mesodermal lineages, *e.g.* epithelial cells, hepatocytes or neurons, has been reported by single studies [Oswald 2004; Arthur 2008; Snykers 2009]. To date, the developmental ontogeny and the *in vivo* identity of the *bona fide* mesenchymal stem cell remains unknown, mainly due to the current lack of specific biomolecular markers and appropriate *in vivo* assays for true 'stemness' of this cell type [da Silva Meirelles 2008; Nombela-Arrieta 2011].

Box 3 | **MSC minimal criteria**

- MSC adhere to plastic surfaces under standard *in vitro* culture conditions.
- MSC have the capacity to differentiate into adipocytes, chondrocytes and osteocytes.
- MSC express CD73, CD90 and CD105 markers, but not the hematopoietic markers CD11b, CD14, CD19, CD34 and CD45 or surface MHC-II molecules.

Adapted from informa healthcare: Dominici *et al.* (2006), *Cytotherapy* 8

Different protocols of isolation and *ex vivo* expansion have led to descriptions of MSC from different organ sources which vary with respect to their phenotype and differentiation potential [Nombela-Arrieta 2011]. At present, a characterization of MSC ought to fulfill certain minimal requirements regarding their morphology, differentiation potential and surface expression phenotype set by The International Society for Cellular Therapy [Dominici 2006] (Box 3).

## IV.2 Stromal cells control cell maintenance and function in various tissues

Stromal cells create a suitable microenvironment for the growth, maturation and survival of other cells and thereby, maintain their correct function in the respective organ. MSC support hematopoiesis in the BM through cytokine cues and cell contact-dependent mechanisms [Méndez-Ferrer 2010]. The constitutive expression of adhesion molecules

including integrins [Stagg 2007] is consistent with the ability of MSC to interact with hematopoietic progenitors in HSC niches [reviewed by Ehninger and Trumpp 2011]. It has been suggested that the heterogeneous MSC population comprises different cell types with distinct *in vivo* functions, *i.e.* they either support HSC survival and hematopoiesis or homing and maintenance of memory immune cells [Tokoyoda 2010].

### IV.3 MSC regulate immune responses

MSC exhibit low densities of MHC-I molecules and do not express MHC-II molecules on the cell surface unless exposed to inflammatory stimuli [Le Blanc 2003; Le Blanc and Pittenger 2005]. They also lack costimulatory molecules such as CD40, CD80 and CD86 [Uccelli 2006]. This has led to speculations that MSC are 'immune privileged', *i.e.* MHC-disparate MSC are not recognized by the immune system, but this hypothesis is presently controversial. MSC survived in allogeneic hosts and mediated immunomodulatory effects such as acceptance of allogeneic skin grafts [Bartholomew 2002; Le Blanc and Pittenger 2005]. MSC were also shown to be inert to $T_C$ cell recognition and lysis [Rasmusson 2007]. Other studies demonstrated that MSC are in rejected in allogeneic recipients [Eliopoulos 2005; Nauta 2006b]. The ability of MSC to elicit immune responses (immunogenicity), or lack thereof, is important for their use in clinical therapy (*cf.* subchapter IV.4) as this will determine the rejection of or tolerance towards non-autologous MSC upon transfer *in vivo*.

In past years, a wealth of studies has reported on the modulation of immune responses by MSC. These cells were shown to inhibit T cell proliferation, cytokine secretion and cytotoxicity [Krampera 2003; Le Blanc 2003; Maitra 2004; Meisel 2004; Aggarwal and Pittenger 2005; Groh 2005; Chabannes 2007; Sato 2007; Nasef 2008; Ren 2008; Ren 2009; Gieseke 2010], induce apoptosis in activated T cells [Plumas 2005], inhibit $T_H17$ cell differentiation [Tatara 2010; Duffy 2011] and induce $T_R$ cell expansion [Nasef 2008; Ghannam 2010a; Mougiakakos 2011], inhibit B cell proliferation and differentiation [Glennie

2005; Corcione 2006], inhibit NK cell proliferation, cytokine secretion and cytotoxicity [Sotiropoulou 2006; Spaggiari 2006], inhibit dendritic cell maturation [Beyth 2005; Jiang 2005; Nauta 2006a; Djouad 2007], inhibit TNFα secretion by macrophages [Ortiz 2007] and protect neutrophils from apoptosis [Raffaghello 2008]. Different explanations for their immunosuppressive properties have been offered, some postulating the need for cell contact while others implicated different soluble factors and signaling molecules [reviewed by Uccelli 2006; Ghannam 2010b]. Inhibitory mechanisms include the secretion of cytokines (*e.g.* IL6, IL10, TGFβ) and other messenger molecules (*e.g.* prostaglandin $E_2$), enzymatic production of small bioactive compounds (*e.g.* nitric oxide, carbon monoxide, L-kynurenine) as well as carbohydrate substrates (lectins) [Di Nicola 2002; Meisel 2004; Aggarwal and Pittenger 2005; Chabannes 2007; Sato 2007; Ren 2008; Gieseke 2010; Mougiakakos 2011].

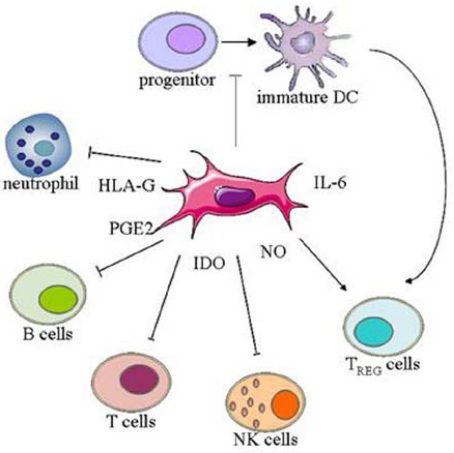


Figure 6 | **Suppression of immune cells by MSC**
MSC use different molecular signals and pathways (*e.g.* IL6, $PGE_2$, IDO, NO HLA-G) to inhibit the proliferation and functions of neutrophilic granulocytes, T cells, B cells, NK cells and dendritic cells, or to induce $T_R$ cells to exert immunosuppression.
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It is noteworthy that MSC utilize a range of different molecular pathways, which may be overlapping and redundant to some degree, to suppress immune responses and modulate the functions of target cells. Variations in the *modus operandi* of human and rodent MSC have been described [Ren 2009].

### IV.4 MSC have therapeutic potential for GvHD and autoimmune diseases

The property to replace damaged cells and form new tissues *in vivo* has earned MSC a long-standing interest in regenerative medicine. MSC transplantation is now being tested for injury repair and replacement strategies of different tissues, *e.g.* cartilage, bone, cardiac muscle, brain and spinal cord, *et c.* [http://clinicaltrials.gov/]. More recently, their potential as inhibitors of immune responses has made this cell type a promising candidate for cell-based treatments of alloimmune (GvHD) and autoimmune (diabetes mellitus type 1, multiple sclerosis, ulcerative colitis, Crohn's disease, oral lichen planus, systemic lupus erythematosus) diseases [Uccelli and Prockop 2010; Dazzi and Krampera 2011; Kebriaei and Robinson 2011; Tolar 2011].

The first report of temporary efficacy of MSC came from a pediatric patient with severe, refractory liver and gut GvHD [Le Blanc 2004]. A subsequent phase I clinical trial [Ringdén 2006] demonstrated the feasibility and safety of MSC adoptive therapy, where most patients responded favorably to the treatment. A non-randomized multicenter trial [Le Blanc 2008] measured an initial response rate of 70 % with improved TRM and longer overall survival at 2 years post transplant, compared with non-responders. Injections of *ex vivo*-expanded MSC were tolerated without side effects and the allotype (HLA-matched and haploidentical donors were used) was not important [Le Blanc 2008]. A number of non-randomized trials [Fang 2007; Müller 2008; von Bonin 2008; Kebriaei 2009; Prasad 2010] also showed positive effects of MSC therapy compared with historical patient data, and recorded no significant adverse effects. These findings, although lacking the proper controls, were promising and led to a sponsored, randomized phase III clinical trial [Martin 2010], where 244 patients received repeated infusions of commercially produced MSC (Prochymal®) from unrelated donors or placebo for four weeks. The results from this study were disappointing [Allison M 2009] as the primary endpoint of durable complete response at 28 days post

treatment initiation was not reached. Response rates for visceral (liver and gastrointestinal) GvHD at 100 days were significantly improved, but a positive effect in cutaneous GvHD was not detected [Martin 2010].

Multipotent adult progenitor cells (MAPC) represent an alternative cell type with greater proliferative and multilineage potential than 'conventional' MSC [Jiang 2002] and proven immunosuppressive capacity *in vivo* [Highfill 2009]. A human MAPC product is commercially available (MultiStem®) and currently the object of a clinical proof-of-safety study for GvHD prevention [http://clinicaltrials.gov/ct2/show/NCT00677859].

MSC-based therapy has entered trials for chronic GvHD as well [reviewed by Kebriaei and Robinson 2011; Tolar 2011] with reports of favorable responses in a number of patients [Ringdén 2006; Müller 2008; Lucchini 2010; Weng 2010; Zhou 2010]. The pilot study conducted by Zhou *et al.* showed significant alleviation of sclerodermal chronic GvHD in all four patients after repeated intra-BM injections of MSC [Zhou 2010]. Transient beneficial effects upon single application of MSC as salvage treatment for severe, resistant chronic GvHD were noted in four out of eleven pediatric patients by Lucchini *et al.* [Lucchini 2010].

Of particular concern regarding this treatment modality is the possible inhibition of beneficial GvL effects by transplanted MSC [Tolar 2011]. One randomized trial reported a decrease in GvHD incidence with a concurrent increase in leukemia relapse in patients who received MSC injections [Ning 2008]. It is presently disputed whether MSC promote or protect against tumor growth *in vivo* [Klopp 2011]. Future studies should address putative adverse effects, such as transfer of infectious agents, attenuation of GvL effects, enhanced neoplastic relapse and carcinogenesis in the presence of MSC or malignant transformation of the transplanted MSC *per se* [Tolar 2011].

## *Chapter V. Mycoplasma infection*

### V.1 Mycoplasma occur as cell-associated or intracellular pathogens in a wide range of species

*Mycoplasma* is a genus of bacteria under the taxonomic order *Mycoplasmatales* in the class of *Mollicutes* [Tree of Life web project 2006] and comprise the smallest known uni-cellular organisms regarding both physical size (300-800 nm in diameter) and genome size (*M. genitalium*, 580,070 base pairs). Their cell surface is composed of a single plasma membrane and lacks a cell wall.

Mycoplasma evolution has led to an obligate parasitic or commensal lifestyle in a wide host of species, including fungi, plants and animals. In mammals, most *Mycoplasma* species infect the respiratory tract (*M. pneumoniae*, *et c.*) where they cause coughing and respiratory impairment, whereas a number of species have their natural habitat in mucous surfaces of the genitourinary tract (*M. genitalium*, *et c.*) and less frequently, in the eyes, joints or mammary glands [Razin 1998].

Mycoplasmas are adapted to the protective environment of their natural host cell and live either attached to the outer cell surface (cytadherence) or inside the cell lumen (intra-cellular residence) [Razin 1998]. However, they may retain the ability to cross tissue and species boundaries and adjust to new host environments [Razin 1998]. The majority of *Mycoplasma* species cause no symptoms or only mild or chronic forms of infection, but rarely kill their hosts [Razin 1998]. However, mycoplasmal diseases do occur, and the pathogenicity of infection ranges from pneumonia to autoimmune manifestations by non-specific immune activation of their host organisms. Furthermore, mycoplasmas are suspected to be a factor in carcinogenesis [Namiki 2009].

### V.2 Mycoplasma infections successfully evade the immune system

Mycoplasma infection can, besides causing specific antimycoplasmal immune responses, either stimulate or suppress the host immune defense non-specifically by

influencing the behavior of immune cells *in vitro* and *in vivo* [reviewed by Razin 1998]. Activation of immunity may result in macrophage and complement activation, increased proliferation and cytotoxicity of effector cells, induction of surface receptor expression and secretion of inflammatory cytokines (*e.g.* IFNγ, TNFα, IL6) as well as non-specific polyclonal T cell and B cell activation [Razin 1998]. For example, it is known that *M. hyorhinis* or cell products derived from this organism are able to induce the secretion of several pro-inflammatory cytokines by monocytes, and may activate the proliferation and maturation of B cells [Proust 1985; Kostyal 1995]. Different molecular components may be responsible for the stimulation of, respectively, mitosis and cytokine production by the same species of *Mycoplasma* [Razin 1998]. On the other hand, infection with *M. hyorhinis* can also result in the mitotic and functional suppression of B and T cells [Teh 1988; Zinöcker 2011b]. Mechanisms of immune suppression by mycoplasma encompass the induction of anergy, substrate depletion and production of cytokines (*e.g.* IL10 and IL13) or compounds with cytocidal effects (*e.g.* nitric oxide) [Razin 1998]. Such effects can be initiated by both viable and inactive mycoplasma cells as well as isolated cell membrane components and soluble factors. Mycoplasmas are furthermore able to actively escape immune recognition by antigenic variation of lipid membrane molecules [Razin 1998].

Thus, the ability to inhibit and modify immune responses and inflammatory processes provides a distinct advantage to these highly adapted parasites in the evasion of the host immune system. Efficient vaccination strategies against mycoplasmal diseases have proven difficult to develop [Nicholas 2002]. The need to control this disease in human and veterinary medicine warrants further investigation to better understand the complex aspects of mycoplasma infection.

## V.3 Mycoplasma are ubiquitous cell culture contaminants in laboratory science

The arrival of cell culture in laboratories around the world has created new artificial habitats for mollicutes. A small number of species account for more than 90 % of the detected contaminations: *M. orale*, *M. hyorhinis*, *M. arginini*, *M. fermentans*, *M. hominis* and *Acholeplasma laidlawii* [Drexler and Uphoff 2002]. The microorganisms found in contaminated cell cultures stem from cross-infection by previously contaminated cell lines, culture media, reagents and additives (*e.g.* bovine serum) or from the human operator [Drexler 2002].

Contamination with mycoplasmas may have a variety of biological effects on eukaryotic and hybridoma cells [Drexler and Uphoff 2002]. However, they do not always cause obvious functional or phenotypical alterations in the original cell lines and are therfore often not detected. Consequently, mycoplasma contaminantion generates substantial losses and increases the costs of managing and monitoring cell culture. Moreover, they may lead to experimental artifacts and misinterpretations of research results [Proust 1985; Ruuth 1985; Sinigaglia and Talmadge 1985; Merkenschlager 1988]. It is therefore critical to prevent and avoid mycoplasma contamination in the experimental setup.

Among the different detection protocols which have been proposed [Drexler and Uphoff 2002], mycoplasma-specific PCR remains the most reliable and widely used method today. To eradicate infections from cell cultures, antimicrobial substances such as tetracyclin and quinolone compounds are commonly used, but may be toxic to the target cells under treatment [Drexler and Uphoff 2002]. Antibiotics which block cell-wall synthesis, such as penicillin, do not obstruct mycoplasma growth and are therefore non-efficient. Effective and robust techniques for the detection and elimination of mycoplasma infections are nowadays commercially available.

## V.4 Mycoplasma as a bacterial model of the 'minimal cell'

The complete genome sequence of *M. genitalium*, the smallest gene complement of a non-viral living organism known to date, has been published [Fraser 1995]. The recent artificial generation of a unicellular organism (*M. laboratorium*) that is capable of self-replication was achieved by the assembly of a synthetic genome based on the sequence of *M. mycoides* and transplanted into a DNA-purged *M. capricolum* cell [Gibson 2010].

The idea that *M. genitalium*, because of its small genome and low total number of genes, embodies the minimal (or 'close-to' minimal) requirements of a self-replicating cell is currently being explored to identify genes that are essential for the maintenance of cell-cycling functions under defined conditions. This research should allow investigators to gain insight also into other important molecular functions of the cell, *e.g.* the modulation or avoidance of immune responses, which are sustained by these organisms and could prove important for the development of effective vaccines against them.

## Aims of the thesis

This work was initiated with an early researcher grant in the research training network "TRANS-NET" under the Sixth Framework Programme (Marie Curie Actions) of the European Commission. Our collaborative efforts were focused on the "[I]dentification of genomic and biological markers as [predictive, diagnostic and therapeutic] tools for use in allogeneic stem cell transplantation" ["TRANS-NET" Technical Annex 2004], with the overall aim to translate our research results into better accessibility, safety and efficacy of this important but hazardous medical procedure. The subordinate milestone of our workpackage ('Models of GvHD') was to develop the skin explant assay in the rat model to study GvHD and GvL responses; this work was carried out in cooperation with our research partners at the University of Newcastle-upon-Tyne and Georg-August-Universität Göttingen.

In a parallel collaborative project, we aimed to test and develop MSC therapy against GvHD in the rat model, together with Gunnar Kvalheim at the Norwegian Radium Hospital.

In summary, the overall research objectives of this thesis were:

1. to characterize molecular and cellular interactions behind the pathogenic mechanisms of GvHR;
2. to develop enhanced diagnostic tools for GvHD in the rat, in particular, to develop the skin explant assay for this species and compare it with the hallmarks of human GvHD;
3. to investigate the roles of MHC genes as well as genes encoding for receptors of the innate immune system on stem cell engraftment, immune reconstitution and GvH responses;
4. to test and improve MSC therapy of GvHD in the experimental allogeneic HCT rat model;
5. to elucidate the molecular mechanisms by which MSC suppress T cell alloreactivity.

# Summary of Results

*Paper I*

**Kinetics of lymphocyte development after allogeneic bone marrow transplantation: Markers of acute graft-versus-host disease.**

This study provides a characterization of the experimental GvHD model which was the subject of investigation throughout this doctoral thesis. We tried to identify reliable markers related to acute GvHD in the rat model, with the long-term goal to translate these findings to clinical allogeneic HCT. To this aim, we applied a twofold approach, where we monitored the frequency of donor- and host-derived leukocyte populations following BMT and during acute GvHD, and at the same time studied the histopathology of selected organs known to be involved in the physiopathological development of the disease.

In this model, recipient BN rats received myeloablative irradiation before transplantation of fully mismatched T cell-depleted BM from donor PVG.7B rats. Severe acute GvHD was induced in the test group by a delayed injection of donor lymphocytes that was lethal in all recipients within 2 to 6 weeks after DLI. GvHD presented with rapid weight loss, apathy, hunching, ruffled fur and hair loss, skin flaking and occasional skin lesions. Histological changes were mainly restricted to the skin. Lymph nodes and spleen showed a marked destruction of the normal architecture, while the gut, liver and lung appeared unaffected.

Host reconstitution with donor NK cells ensued rapidly and completely, and the DLI established stable and full donor chimerism of T cells. We noted a significant decrease in $Ly49s3^+$ NK cells and $CD62L^+$ T cells as well as an extensive loss of $CD4^+CD25^{hi}FoxP3^+$ $T_R$ cells in the PB of animals at the onset of GvHD, in marked contrast to controls. We suggest that these cell populations may serve as accessible cellular markers of acute GvHD.

*Paper II*

**Correlation of Hsp70-1 and Hsp70-2 gene expression with the degree of graft-versus-host reaction in a rat skin explant model**.

*Paper III*

**Expression profiling of major histocompatibility complex genes in skin explant assays reveals new candidates for controlling risk of graft-versus-host disease.**

In these two papers, we developed an *in vitro* model for cutaneous GvHD in the rat in analogy to the human skin explant assay in a collaborative project. We employed the rat skin explant assay to study the pathology of (*Paper II*) and regulation of gene expression (*Paper II* and *Paper III*) in GvHR with the aim to identify genes involved in GvHD pathophysiology. We focused on the MHC and the NKC as the gene regions of prime importance in allogeneic transplantation. The use of inbred, genetically well-defined rat strains with disparity either in the MHC, the non-MHC genetic background, or both, allowed us to study the importance of major and minor H antigens in GvHR.

Rat skin explants displayed similar pathology as has been described previously in the human. The grade of observed GvHR was in correlation with the degree of genetic mismatch (no mismatch < minor < major < minor plus major). *Hsp70* genes *-1* and *-2*, but not *-3*, all located within the MHC class III region, were differentially regulated in GvHR. More elaborate studies of the gene expression profiles in skin GvHR using a custom-designed oligonucleotide microarray revealed the significant and strong regulation of 25 MHC, 6 NKC, and 168 genes encoded in other genomic regions. We observed similar regulation patterns of a panel of selected MHC and NKC genes in skin lesions from rats with GvHD and of their human homologues in skin samples from GvHD patients. Several of the identified genes, including *HLA-DMB*, *C2*, *AIF1*, *SPR1*, *UBD,* and *OLR1*, show polymorphisms and are therefore valid candidates for genetic risk assessment of GvHD in recipients of allogeneic HCT.

*Paper IV*

**Rat bone marrow-derived mesenchymal stromal cells suppress T cell stimulation in vitro through nitric oxide production**

Several molecular pathways by which MSC inhibit lymphocyte activation, expansion and effector functions have been described in the literature. The appropriate physiological conditions and the exact mode of action of MSC inhibition, however, are still not fully understood, in particular for MSC derived from various tissues and from other species than mouse and human. Herein, we demonstrate that MSC generated from rat BM utilized inducible nitric oxide synthase (iNOS) to inhibit T cell stimulation *in vitro*. MSC were primed by soluble immunostimulatory factors, including TNFα and IFNγ, from mitogen-induced or allogeneic mixed lymphocyte cultures to express iNOS, leading to the release of high levels of nitric oxide and suppression of T cell proliferation. Furthermore, MSC blocked the secretion of proinflammatory cytokines in activated lymphocyte cultures, a function that appeared to be regulated *via* an iNOS-independent mechanism.

*Paper V*

**Mycoplasma contamination revisited: Mesenchymal stromal cells harboring *Mycoplasma hyorhinis* potently inhibit lymphocyte proliferation *in vitro***

Our investigation of the suppressive effects of MSC on activated lymphocytes was turned topsy-turvy by the unintended infection of primary rat marrow stromal cell lines with the common contaminant *M. hyorhinis*. Because we observed a pronounced inhibitory effect by both MSC and cell-free MSC-conditioned culture medium in mitogen-activated and mixed lymphocyte cultures, we became interested in mapping a putative effect of the infectious agent in this context. We ascertained that mycoplasma infection increased the suppression of both mitogenic and alloantigen-induced T cell proliferation in cocultures with MSC dramatically. Also, cytokine production by T cells was altered in the presence of infected

MSC. Therefore, *M. hyorhinis* infection had a strong effect on T cell activation assays, highlighting the importance of controlling and monitoring cell cultures for this contaminant.

*Paper VI*

**Mesenchymal stromal cells fail to alleviate graft-versus-host disease in rats transplanted with major histocompatibility complex-mismatched bone marrow**

MSC have immunomodulatory potential which can be exploited in the clinic, *e.g.* to inhibit undesirable auto- or alloimmunity by T cells *in vivo*. We wanted to test the hypothesis that rat BM-derived MSC have a beneficial, suppressive effect on T cell alloreactivity in experimental GvHD, as has been shown both in animal models and in the clinical treatment of GvHD previously. We employed two different donor-recipient combinations of rat strains differing in the degree of genetic compatibility, with full major and minor H mismatch in one setup, and mismatch of the MHC class II and non-classical MHC class I subregions in the other setup. We applied an experimental protocol of three infusions of donor-syngeneic MSC doses starting at the time point of allogeneic donor lymphocyte transfusion and repeated in weekly intervals. This treatment showed a beneficial effect on acute GvHD in 3 out of 10 rats in the partially mismatched transplantation setting, however, improved overall survival was not statistically significant.

# General Discussion

## *Animal HCT models*

Minor H antigens contribute to the genesis of GvHD as HCT between HLA-identical siblings less frequently result in GvHD compared to HLA-MUD [Welniak 2007]. The most severe forms of the disease are seen in recipients of HLA-non-identical transplants from haploidentical family or imperfectly HLA-MUD. Nevertheless, such transplantation scenarios may have certain advantages with respect to the treatment of leukemias because of the presence of donor-*versus*-host-reactive NK cell populations which mediate beneficial GvL effects without GvHD [Velardi 2009]. Fully mismatched donors are not considered for transplantation, because even if the risk of rejection can be successfully managed, the risk of GvHD is exceedingly high. TCD of the graft lowers the risk of acquiring GvHD substantially. Potent conditioning regimens combined with high cell numbers in the graft are necessary in order to achieve stable long-term engraftment.

Our group has developed rat BMT models to study the consequences of MHC disparity on engraftment, GvL effects and GvHR [Engh 2001; Nestvold 2008]. The experimental models we applied were based on a simulated transplantation scenario where, after myelo-ablative radiotherapy and successful engraftment of high-dose, TCD BMT, an additional DLI is administered to counteract relapse of the malignant disease, but resulted in severe acute GvHD [Nestvold 2008]. The experimental protocol of allogeneic HCT with partial MHC-mismatch [PVG.1U ($RT1^{u}$) → PVG.R23 ($RT1^{r^{23}}$)] between donor and host rat strains (*Paper VI*) was designed to resemble the clinical setting of HLA-mismatched BMT more closely than the fully mismatched [PVG.7B ($RT1^{c}$) → BN ($RT1^{n}$)] model.

The HCT protocol described in depth in *Paper I* resulted in GvHD-typical symptoms mainly confined to the skin and lymphoid organs, while the liver and gastrointestinal tract were not significantly affected. Thus, the results from this model are valid primarily with

respect to cutaneous GvHD, and interpretations regarding other GvHD target sites are more limited. Furthermore, we have established and included the *in-vitro* skin explant assay in our investigations (*cf. Paper II* and *Paper III*).

Laboratory animals give us the opportunity to do functional studies which are not ethical and not feasible in humans. Rodent strains are systematically bred, genetically homogeneous and biologically well characterized. This facilitates the study of effects of defined genes and gene regions, characterization of the physiological functions of proteins and mapping associations with observable pathological phenotypes. In addition, genetic engineering represents a viable scientific tool which has been historically restricted to the mouse and has of late become possible also in the rat [reviewed by Huang 2011].

Conditioning regimens vary in the extent of leukemia eradication and tissue damage between patients [Appelbaum 2010] and therefore, influence both the GvHD phenotype and the risk of relapse and consequently, have a substantial impact on transplantation outcome. Patients usually undergo pretransplant chemotherapy which may or may not be combined with irradiation, whereas rodent models typically receive only irradiation before transplantation [Schroeder and DiPersio 2011]. As a consequence, the initial stage of GvHD induction in experimental hosts may not be similar to patients.

Experimental animal models of HCT, such as those employed in our own studies, allow the omission of immunosuppressive treatment with corticosteroids or other T cell-targeted pharmaceuticals to study GvHD *de novo* on the molecular and cellular level. This is an advantage both in determining the individual contributions of selected cell populations and subpopulations and in dissecting the immunological interactions between donor and host biology.

The fact that immune cell types show not only species-specific but also tissue-dependent variations regarding phenotype, antigen expression, relative frequencies, stages and

kinetics of development, regulatory mechanisms, migratory properties, *et c.* in homeostasis as well as after transplantation, has important implications for the selection and design of appropriate experimental models. For instance, conventional experimental protocols of rodent HCT models apply a transfusion of BM cells supplemented with whole spleen or lymph node cell suspensions which contain the GvH-reactive donor T cell populations. In human recipients of HCT, on the other hand, GvHD is caused by passenger donor T lymphocytes from the marrow, peripheral or UCB graft.

Moreover, age, average lifespan, circadian cycles, microbiological environment (housing under specific pathogen-free conditions restricts the natural exposure to microbes and pathogens), spatial conditions and nutrition (limited motion and unlimited access to food favor obesity) are only some of the factors which make up the plethora of biological variations between laboratory animals and human individuals and complicate the translation of research findings from small and large animal models to the human species.

Notwithstanding such considerations which apply to the use of animal models in general, the disease pathology that we observed both in rats which undergo experimental acute GvHD and in the rat skin explant model was very similar to the typical histopathology of cutaneous GvHD in patients (*Paper II*). The rat model seems to share the pathological phenotype of clinical GvHR of the skin and therefore, ought to be useful for translational studies. The concordance of the markers identified in gene expression profiling studies between human and rat samples (*Paper III*) is indicative of common underlying mechanisms in skin GvHD between these two species.

## *Markers of GvHD*

Biological markers which are unambiguously and robustly associated with GvHD could significantly improve the accuracy of diagnosis and influence the choice of treatment alternatives for allogeneic HCT patients. Gene association studies can be performed in human

volunteers to discover genetic determinants of GvHD, but are impeded by the non-random coupling (*i.e.* genetic linkage) of alleles on a given haplotype, resulting in the distribution of allele combinations which deviate from stochastic frequencies (the phenomenon is known as 'linkage disequilibrium'). Effect correlations with certain genetic loci can thus lead to the identification of false associations.

Gene expression profiling studies can be used to sidestep this methodological restriction. The DNA microarray technique has the potential to discover novel gene associations, but depends on complementary methods to confirm the generated hypotheses. Ideally, such findings should be confirmed by demonstrating the functional relevance of the gene association in question.

We identified a number of molecular and cellular markers associated with experimental acute GvHD and discussed their putative use in prospective disease diagnosis [Novota 2008; Novota 2011; Zinöcker 2011a]. L-Selectin (CD62L) is a cell adhesion molecule expressed on naïve T cells that enables their homing to SLT. $T_E$ cells gain access to peripheral tissues after losing this marker upon activation. $CD62L^-$ T cells did not induce GvHD in allogeneic hosts [Anderson 2003]. The reduction in CD62L-expressing T cells associated with GvHD (*Paper I*) may be explained by activation-dependent downregulation of surface CD62L on alloreactive T cells and migration to peripheral sites of allostimulation and inflammation, *e.g.* GvHD-affected skin.

$T_R$ cells also require L-Selectin to alleviate GvHD upon adoptive transfer [Taylor 2004; Ermann 2005] in order to inhibit the activation of alloreactive T cells in lymph nodes and spleen, *i.e.* at the site of allopriming. In accordance with other studies performed in experimental GvHD model systems as well as GvHD patient cohorts [Miura 2004; Zorn 2005; Rieger 2006; Matthews 2009], we identified a specific lack of $T_R$ cells in the PB. Again, this could be due to recruitment of these cells as part of the $CD4^+CD25^{hi}CD62L^+$ T cell population

from the blood, possibly to suppress T cell activation and expansion in SLT or other peripheral tissues. Alternatively, the reconstitution, expansion or induction of $T_R$ cells might be deficient in allotransplanted hosts.

NKT cells of the host can induce the expansion of donor $T_R$ cells and thereby protect against GvHD [Pillai 2009]. Donor NKT cells may also regulate GvHD [Zeng 1999; Pillai 2007; Kohrt 2010]. In our experiments (*Paper I* and unpublished observations), donor NKT cells were reconstituted at negligible numbers in myeloablated recipients after allogeneic HCT. Host NKT cells were not present in the blood of transplanted rats suffering from GvHD. Peripheral $T_R$ cell frequencies may have been influenced by the lack of NKT cells of both donor and host origin, but we did not address this hypothesis experimentally.

In a report by Magenau *et al.*, $T_R$ cell frequency in the PB of patients had prognostic value in acute GvHD [Magenau 2010]. More recently, however, Waldmann and colleagues have illustrated in a set of gene expression experiments the importance of using syngeneic controls in comparative animal models to confirm or exclude the validity of biomarkers unequivocally in the context of transplantation [Cobbold 2011]. Including syngeneic donors, *i.e.* identical twins, as control groups could also improve GvHD biomarker studies in patient cohorts. This finding may to some extent explain why the multitude of biomarkers implicated in preclinical models of various diseases rarely translates to clinically useful diagnostic tools.

Importantly, the study by Cobbold *et al.* also found that $T_R$-associated genes were similarly expressed in both rejected and tolerated allogeneic skin grafts as well as syngeneic transplants [Cobbold 2011], thus arguing against the validity of $T_R$ cells as a cellular biomarker of transplantation tolerance.

For our biomarker studies of GvHD, we applied both healthy and syngeneic skin as controls for comparison with allogeneic skin explants (*cf. Paper II* and *Paper III*). Controls receiving syngeneic donor cell transfusions may have strengthened the validity of cellular

markers discovered in allogeneic transplantation experiments (*Paper I*), and the lack of such a control group is a weakness of this study.

HSP of several gene families have the capacity to channel peptides into MHC:antigen presentation pathways [Srivastava 2002]. Due to their functional roles in antigen presentation and thereby, in the activation of T cells, HSP may be directly involved in the pathophysiological processes of GvHD. Indeed, several studies support the role of HSP70 in GvHD (*cf.* subchapter III.5 of the **Introduction**). In line with previous findings that HSP70 genes were highly expressed in human allogeneic skin explants [Jarvis 2003], we found a close correlation of expression levels of stress-inducible HSP70-1 and HSP70-2 with increasing grades of GvHR, presented in *Paper II* of this thesis. In line with these results, *Olr1*, a gene encoding for a HSP70:peptide complex-binding receptor on APC, was upregulated in both rat and human allogeneic skin explants (*Paper III*).

The MHC is the primary genetic determinant for the outcome of allogeneic HCT. This region contains genes that do not encode for MHC-I/-II molecules but may predispose for GvHD and thus, could be useful as diagnostic biomarkers (*cf.* subchapter III.6, p.26*ff.*). The NKC in the rat encodes receptor proteins that have proven or putative ability to bind MHC ligands and thereby, may inhibit or activate NK cell cytotoxic GvH and GvL effects after MHC-mismatched HCT (*cf.* subchapter III.9, p.36*ff.*).

We designed a customized microarray chip which covered these two regions of the rat genome and included non-MHC/non-NKC genes for reference and data normalization. The oligonucleotide microarray contained specific probes for 224 MHC genes, 43 NKC genes as well as 6342 non-MHC/non-NKC genes (*Paper III*). Of these, 4.9 % MHC genes and 14.0 % NKC genes were significantly regulated in GvHR, compared with 2.6 % of genes located outside these genomic regions. Thus, predictive genes were more frequent in the NKC and MHC than in the rest of the genome. A number of the GvHR-associated MHC genes that were

identified have known functions in the processing and presentation of antigen to T cells, are involved in cell signaling and cell adhesion or are related to apoptosis. Several classical (*RT1-A*) and non-classical (*RT1-CE/N/M*) class I MHC genes were significantly upregulated and may function as ligands for Ly49 and other receptors expressed on NK cells [Naper 2002a; Naper 2002b; Naper 2005 and unpublished observations by our group]. Six of the NKC genes which were also upregulated in skin GvHR were inhibitory receptors of the Ly49 gene family.

The majority of MHC and NKC gene associations were confirmed by quantitative real-time PCR. We also showed an overall concordance of the gene expression patterns in the skin explant assay and skin biopsies of GvH-diseased rats. Exceptions were the genes encoding for the Ly49si1 and Ly49i9 receptors which were not completely consistent in skin GvHD samples. Putative ligands of these receptors have not been isolated, and their exact functions are therefore not known. Moreover, the peripheral Ly49s3$^+$ NK cell subset was associated with GvHD development (*Paper I*), which could be due to receptor downregulation in the BN host, or alternatively, preferential recruitment from the circulation to other sites during GvHD. However, other NKR markers, including the DAR13 antibody which also binds Ly49s3, tested on PB cells did not confirm this association. The microarray analysis showed no significant change in Ly49s3 expression in skin GvHR. The possible infiltration of NK cells in GvHD skin or regulation of NKR expression *in vivo* may not be authentically reflected in GvHR occurring in the simplified skin explant assay. Thus, it is not straightforward to interpret the findings regarding the expression of Ly49 receptors in rat skin GvHR and GvHD.

We validated the gene associations identified in rat skin explant assays also in human skin explants. This analysis revealed a high overall concordance with the human homologues tested (*e.g. TAP1*, *C2*, *AIF1*, *UBD*, *OLR1*; these genes were upregulated both in human and rat skin explants) and indicates that GvHR are similarly regulated in humans and rats. For a number of these genes polymorphic human homologues exist (*e.g. HLA-DMB*, *C2*, *AIF1*,

*SPR1*, *OLR1*) making them interesting candidates for predictive biomarkers in the clinical setting. These findings could potentially improve the prognostic value of grading GvHR in the skin explant or GvHD in patients for better prediction of transplantation outcomes in the future.

The expression profile of cutaneous GvHD has previously been assessed in a mouse model of minor H antigen-mismatched HCT [Sugerman 2004]. Several of the gene associations identified (*e.g.* upregulation of MHC-I genes, *Tap1*, *Psmb8*) overlapped and the general gene expression patterns compared well with our study in the rat. The significance of other genes with known involvement in GvHD pathological processes (*e.g.* TNFα, IFNγ and HSP70 genes), however, was not confirmed by our experiments. The possibility that important candidate genes with true disease associations are missed by this type of study should be underlined here.

Recent studies compared the gene expression profiles of PB cells from patients with acute and chronic GvHD, respectively, to transplant recipients who did not develop GvHD [Buzzeo 2008; Poloni 2011]. They found that proinflammatory cytokines (*e.g.* IFNγ and TNF superfamily genes) were upregulated in GvHD. Baron *et al.* tested whether the gene expression profile of PB T cells from the donor could predict the risk of acute and chronic GvHD prior to transplantation [Baron 2007]. They showed that gene expression patterns in donor T lymphocytes were relatively stable over time and were predictive of GvHD. The dangerous 'strong alloresponder' trait was under multigenic control. Therefore, the use of genetic signatures might help to select 'low alloresponders' as donors and allow tailoring of immunosuppressive regimens for HCT recipients who are at risk of GvHD or rejection [Baron 2007].

Extending the numbers of genes used in diagnostic tests could lead to more specific and more sensitive prediction of GvHD incidence, onset and progression [Baron 2007; Paczesny

2009c]. Therefore, rather than relying on single biomarkers, the analysis of a composite set of disease-associated genes or global gene expression patterns of the recipient, the donor, or both in combination, might result in clinically more relevant diagnostic tools in the future [Baron 2007; Cobbold 2011].

## *MSC pathways of immunosuppression*

MSC have an important role in the regulation of immune responses. They exert immuno-suppressive effects on different cell types involved in the immune defense and employ several, possibly overlapping, pathways to facilitate this (*cf.* **Introduction**, subchapter IV.3). Observations that infusions of MSC dampened allo- and autoimmune reactions *in vivo* under clinical as well as experimental conditions [reviewed by Le Blanc and Pittenger 2005; Uccelli and Prockop 2010; Dazzi and Krampera 2011; Kebriaei and Robinson 2011; Tolar 2011] substantiated these findings. It is worthwhile to further explore how these cells serve their physiological roles in the complex immune response network of different species.

We described a molecular pathway which was critical for the inhibitory properties of rat MSC. Mouse and rat MSC express iNOS, an enzyme which catalyzes the conversion of L-arginine to nitric oxide, in response to lymphokine activation signals and in turn, suppress lymphocyte stimulation with respect to cell proliferation and cytokine secretion [Oh 2007; Sato 2007; Ren 2008; Ren 2009; and *Paper IV* of this thesis] this inhibitory pathway with macrophages [Niedbala 2006]. However, the possibility that rat MSC suppress T cell proliferation through, *e.g.*, heme oxygenase-1 [Chabannes 2007] and other molecular pathways should not be dismissed. Notably, human MSC utilize distinct inhibitory mechanisms, *e.g.* secretion of indoleamine 2,3-dioxygenase and prostaglandin $E_2$, but not iNOS [Ren 2009], to suppress T cells.

Our own results suggest that even closely related species such as the mouse and rat may display mechanistic divergence in the regulation of molecular targets (different cytokine

stimuli induced iNOS expression in mouse and rat MSC) that have the same function (suppression of T cell activation).

### *MSC therapy*

MSC transfusion as a therapy against acute and chronic GvHD has produced beneficial effects [Le Blanc 2004; Ringdén 2006; Le Blanc 2008; Weng 2010; Zhou 2010] especially in patients with severe and rapidly progressing, steroid-resistant GvHD, although results from a recent randomized clinical trial raised doubts on the effectiveness of this treatment [Martin 2010]. The study reported an improvement of intestinal and liver GvHD, but MSC therapy lacked efficiency in the cutaneous GvHD patient cohort [Martin 2010]. One may therefore speculate that MSC are more competent to attenuate hepatic and intestinal GvHD than cutaneous GvHD.

In our hands, repeated infusions of MSC did not rescue lethally irradiated, transplanted rats from severe acute GvHD following partially or fully MHC-mismatched DLI and death due to rapid wasting and weight loss (*Paper V* and *Paper VI*). MSC therapy applied in similar preclinical animal models have produced conflicting results, with some protocols that cured GvHD successfully [Yanez 2006; Tisato 2007; Polchert 2008; Joo 2010] while other studies failed to show beneficial effects [Sudres 2006; Badillo 2008; Prigozhina 2008; Mielcarek 2011].

Several factors may explain why MSC immunosuppression was not effective in HCT models. It is possible that GvH-reactivity after fully or partially MHC-mismatched allogeneic HCT and DLI was too strong to be controlled by MSC *in vivo*. MSC infusions may have beneficial effects in a less challenging allogeneic setting, *e.g.* in a model of minor H-mismatched HCT, or in haploidentical HCT where rat MAPC were recently shown to alleviate GvHD [Kovacsovics-Bankowski 2009].

Furthermore, the source of MSC, optimal cell passage, transfusion dose, time points of intervention, as well as the route of administration (*e.g.* systemic, site-directed or local) may all be crucial factors in transplantation protocols [Wang 2011]. The lack of standardized culture conditions for the production of MSC *ex vivo* represents another parameter which may contribute to variations in outcome. The aforementioned aspects may require optimization depending on the type of underlying disease and other practical considerations for the future use of MSC as a potent therapy.

**SOURCE OF MSC.** MSC are routinely isolated from BM aspirates for clinical administration, although a variety of fetal and adult tissue types have become more readily available as alternative sources (*cf.* Chapter IV). In our studies, we applied a protocol of *in vitro* cell adhesion and expansion to generate BM-derived MSC lines [Lennon and Caplan 2006]. Tissue-dependent variability regarding the *in vivo* efficacy of MSC immunosuppression is currently elusive.

**TIMING AND DOSAGE OF MSC INFUSIONS.** The timing and dosage of single or repeated MSC injections vary considerably between different studies, as standardized treatment protocols remain to be established. The MSC transfusion protocols applied in our own experiments were designed to directly intervene at three main stages of GvHD development: prior to donor T cell activation (concurrent with the DLI); after the onset of donor T cell expansion and migration to peripheral sites of GvHR; and acutely during the late effector stage of GvHD.

***IN VITRO* CULTURE CONDITIONS.** Changes in MSC biology induced through prolonged *in vitro* culture offers another explanation for lack of *in vivo* effectiveness. The possible influences of *in vitro* culture (*e.g.* culture medium composition, atmospheric constitution, plating density, number of passages, *et c.*) under artificial laboratory conditions are incompletely understood. This obstacle is difficult to overcome methodologically, because expansion of MSC *ex vivo* is a prerequisite to obtain sufficient cell numbers for therapeutic

use. It seems preferable to generate MSC under conditions resembling their natural niche as closely as possible, however, this goal is hardly achievable at present even in complex three-dimensional culture.

Bovine serum components (fetal calf serum is commonly used as a physiological source of growth factors) incorporated during *in vitro* culture can lead to immune reactions directed against serum-derived antigens *in vivo* [Horwitz 2002; Spees 2004] and bear the risk of transfer of infectious agents. Newer protocols substitute xenogenic serum with growth factors derived from human platelet lysate as a medium supplement for clinical grade MSC expansion [von Bonin 2008] to circumvent these problems. The possibility of immune recognition of transfused MSC *in vivo* following bovine serum-supplemented culture cannot be ruled out in our studies, even though we did not register T cell activation or proliferation responses to syngeneic or allogeneic MSC *in vitro*. Replacement of fetal bovine with rat serum might improve our culture protocols in order to exclude this possibility.

**IMMUNOGENICITY OF MSC.** Transfused MSC might be recognized and extinguished by the recipient's immune system if the MSC donor is allogeneic to the host or the HSC donor. Large-scale production of HLA-disparate MSC for clinical application is nonetheless desirable for reasons of convenience (*e.g.* long-term storage). Third-party MSC were safely applied in several clinical trials where these cells were equally effective as were HLA-identical MSC [Ringdén 2006; Le Blanc 2008].

The topic of MSC immunogenicity is disputed (*cf.* Chapter IV). Early reports have shown that MSC express low levels of MHC-I molecules and lack MHC-II as well as co-stimulatory molecules on the cell surface [Le Blanc 2003]. MSC evaded immune recognition even when MHC-I and -II expression was upregulated in an inflammatory milieu [Le Blanc and Pittenger 2005]. El Haddad *et al.* demonstrated that murine MSC employ specific serine protease inhibitors to evade host immune responses through inactivation of granzyme B by $T_C$

cells [El Haddad 2011]. Other studies have demonstrated, however, that allogeneic MSC were rejected by NK cells *in vitro* [Spaggiari 2006] and by T cells *in vivo* [Eliopoulos 2005; Nauta 2006b].

Patients who suffer from GvHD are under immunosuppressive treatment, which makes the rejection of allogeneic cells less likely [Nauta 2006b]. In our BMT experiments, we tested donor-syngeneic (*i.e.* fully histocompatible with the donor graft) MSC in high intensity radiation-conditioned recipient rats which showed rapid donor hematopoietic reconstitution and full donor chimerism, thereby minimizing the risk of allorejection in the host-*versus*-graft direction.

***IN VIVO* MIGRATION OF MSC.** The route of administration may play a crucial role in MSC trafficking and homing to anatomical locations of ongoing GvHR. This may ultimately determine the effectiveness or failure of the treatment if colocalization is required for the efficient suppression of alloreactive immune cells [Sato 2007; Ren 2008]. Support for the importance of homing to the site of T cell priming *in vivo* came from a murine model showing a reduction of GvHD symptoms when MAPC were administered intrasplenically, but not after systemic injection [Highfill 2009].

Furthermore, Rombouts *et al.* showed that *in vitro* passage can negatively affect MSC homing ability to the BM and spleen in syngeneic hosts [Rombouts and Ploemacher 2003]. Failure to migrate to sites of GvHR may explain why repeated MSC injections did not cure diseased rats in our models (*Paper VI*).

Trapping of MSC in the capillaries of visceral organs, *e.g.* lungs, liver and spleen, after systemic administration is known from animal studies [Sudres 2006; Schrepfer 2007]. We and others have found that transplanted animals are at risk of potentially fatal pulmonary embolisms after intravenous injection and aggregation of MSC in the lungs [Schrepfer 2007; Prigozhina 2008; Zinöcker 2011b]. In addition, we observed by *in vivo* imaging that

transgenic MSC expressing enhanced green fluorescence protein accumulated in large numbers in the lungs shortly (within minutes) after injection, but were detectable neither at this site nor in other organs after several days *in vivo* and *post mortem* (unpublished observations). We were unable to ascertain whether MSC migrate further after the initial aggregation in the pulmonary vasculature. It has been shown that MSC are indeed able to migrate to sites of tissue injury after systemic injection in various animal experimental contexts [reviewed by Chamberlain 2007].

**LICENSING OF MSC.** Lately, it has been proposed that MSC require a 'license' to become fully competent immunosuppressor cells by activation through certain physiological signals [Dazzi and Krampera 2011]. Priming of MSC by incubation with IFNγ [Polchert 2008] or a TLR ligand [Waterman 2010] improved the inhibitory potential of mouse and human MSC both *in vivo* and *in vitro*. We showed that combinations of TNFα, IFNγ and the TLR4 agonist LPS induced iNOS expression and production of nitric oxide by rat MSC *in vitro*, resulting in potent inhibition of proliferation of stimulated T cells (*cf. Paper IV*).

## *Mycoplasma immune modulation*

The vertebrate immune system has developed ways by which it is able to discover and fight infections, and microorganisms have likewise adapted to these strategies. The capacity of *Mycoplasma ssp.* to either suppress or stimulate immune functions [Razin 1998] is intriguing and could, at least in part, explain how these bacteria are able to evade immune recognition.

In our hands, *M. hyorhinis* infection multiplied and spread quickly in lymphocyte cultures, starting at low numbers below the detection threshold of PCR to high titers (*cf. Paper V*). Mycoplasma completely blocked the proliferation of T lymphocytes in the presence of potent mitogenic or allogeneic stimuli and caused increased cell death in infected cultures.

We made use of the intracellular fluorescent dye CFSE to confirm mycoplasma-dependent inhibition of the lymphocyte stimulation assays. We could exclude the possibility

that *M. hyorhinis* degraded CFSE, in analogy to its ability to cleave thymidine substrates [Sinigaglia and Talmadge 1985; Merkenschlager 1988]: suppressed, non-proliferating lymphocytes that did not divide showed full CFSE fluorescence intensity in mycoplasma-infected cell cultures in our assays.

It is theoretically conceivable that live, non-adherent mycoplasma were present in the transferred MSC solution, and that they infected and suppressed lymphocyte cultures independently of the cell vector. Experiments that could verify the presence of precisely one contaminated cell per well (possibly by radiolabeling of MSC prior to incubation or expansion of the cell clone following readout) might help to validate the observed 'single cell' effect.

Because mycoplasma infection potently suppressed allostimulated lymphocytes *in vitro*, one might expect a strong immunosuppressive effect of infected MSC also in GvHD animals. On the other hand, we do not know whether mycoplasma infection had the opposite effect *in vivo* and stimulated, rather than suppressed, alloreactive donor cells. We did not observe a beneficial effect of mycoplasma-infected MSC on GvHD mortality and morbidity *in vivo* (*Paper V*). Mycoplasma-free MSC were also ineffective as GvHD therapy in the same experimental setting (*Paper VI*). Thus, *M. hyorhinis* infection did not improve the inhibitory potency of MSC *in vivo* as we have seen *in vitro*. On the contrary, it is possible that intra-cellular mycoplasma marked infected MSC for elimination and elicited an immune response in transplanted and immune reconstituted hosts.

## Conclusions

1. In a rat model of fully mismatched BMT [PVG.7B ($RT1^c$) → BN ($RT1^n$)], acute GvHD symptoms were mainly confined to the skin and correlated with pathological findings. Also, the frequencies of $T_R$ cells and $CD62L^+$ T cells in the PB were decreased in acute GvHD and thus could potentially serve as accessible diagnostic biomarkers.

2. Rat skin explants exhibited GvHR with similar histopathology as is known from human skin explants and was observed also in rat skin GvHD. The assay may be used complementary with the human skin explant assay as a model of skin GvHD to study disease-related tissue pathology and underlying mechanisms of GvHR. Furthermore, the rat skin explant assay is an alternative to the use of live animals.

3. The degree of mismatch of MHC and non-MHC genes predicted the average grade of GvHR in rat skin explants. The rat skin explant assay was a valuable research tool for gene association studies and helped identify a number of genes that were differentially regulated in GvHR. *Hspa1b* and *Hspa1a* genes, respectively, encoding for HSP70-1 and HSP70-2 of the rat, were significantly upregulated in allogeneic rat skin explants and correlated with the grade of GvHR. A number of genes of the MHC and NKC were significantly up- or downregulated in allogeneic skin explants compared to both syngeneic skin explants and healthy skin, and their expression patterns were in line with GvHD-affected rat skin. Comparative studies in human skin explants were largely in agreement with the observed expression patterns of homologous genes. In addition, a significant proportion of the non-MHC/non-NKC genes tested was also differentially regulated. Therefore, gene expression profiling performed on rat skin explants identified putative biomarkers which may become useful for clinical GvHD diagnosis and prognosis.

4. MSC lines generated from rat BM expressed iNOS in response to soluble inflammatory factors such as TNFα and IFNγ produced by mitogen- or alloantigen-activated lymphocytes. iNOS expression resulted in an increased output of nitric oxide and the efficient suppression of T cell proliferation in lymphocyte cocultures with MSC. Furthermore, MSC inhibited the expression of proinflammatory cytokines by lymphocytes in the presence of mitogen by an iNOS-independent mechanism.

5. MSC had potent immunosuppressive potential *in vitro*, but failed to alleviate acute GvHD after fully or partially MHC-mismatched experimental BMT.

6. *M. hyorhinis* rapidly infected lymphocyte cultures *in vitro* and efficiently blocked both mitogen- and alloantigen-induced T cell proliferation independently of its cell vector. Also, mycoplasma infection further increased the potential of MSC to inhibit IFNγ secretion.

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# I

# II

III

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# Expression Profiling of Major Histocompatibility and Natural Killer Complex Genes Reveals Candidates for Controlling Risk of Graft versus Host Disease

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## Abstract

***Background:*** The major histocompatibility complex (MHC) is the most important genomic region that contributes to the risk of graft versus host disease (GVHD) after haematopoietic stem cell transplantation. Matching of MHC class I and II genes is essential for the success of transplantation. However, the MHC contains additional genes that also contribute to the risk of developing acute GVHD. It is difficult to identify these genes by genetic association studies alone due to linkage disequilibrium in this region. Therefore, we aimed to identify MHC genes and other genes involved in the pathophysiology of GVHD by mRNA expression profiling.

***Methodology/Principal Findings:*** To reduce the complexity of the task, we used genetically well-defined rat inbred strains and a rat skin explant assay, an *in-vitro*-model of the graft versus host reaction (GVHR), to analyze the expression of MHC, natural killer complex (NKC), and other genes in cutaneous GVHR. We observed a statistically significant and strong up or down regulation of 11 MHC, 6 NKC, and 168 genes encoded in other genomic regions, i.e. 4.9%, 14.0%, and 2.6% of the tested genes respectively. The regulation of 7 selected MHC and 3 NKC genes was confirmed by quantitative real-time PCR and in independent skin explant assays. In addition, similar regulations of most of the selected genes were observed in GVHD-affected skin lesions of transplanted rats and in human skin explant assays.

***Conclusions/Significance:*** We identified rat and human MHC and NKC genes that are regulated during GVHR in skin explant assays and could therefore serve as biomarkers for GVHD. Several of the respective human genes, including *HLA-DMB*, *C2*, *AIF1*, *SPR1*, *UBD*, and *OLR1*, are polymorphic. These candidates may therefore contribute to the genetic risk of GVHD in patients.

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## Introduction

Haematopoietic stem cell transplantation (HSCT) is currently the only potentially curative treatment for many malignant and non-malignant haematological diseases. However, the overall survival rate after transplantation is still only 40% to 60% due to severe posttransplant complications [1], which include graft versus host disease (GVHD), relapse, and infection. Human leukocyte antigen (HLA) matching is essential to reduce the risk of graft rejection and GVHD [2]. However, non-HLA genes also impact on transplant outcome [3] and acute GVHD can be fatal even in patients receiving transplants from HLA-identical matched sibling donors (MSD). The cumulative incidence of grade II to IV GVHD was 35% in a recent study evaluating 1960 MSD transplants [4]. MSDs are currently available for about one third of the patients and, therefore, alternative donors are needed. HLA-matched unrelated donors (MUD) are more readily accepted than cord blood or mismatched related donors.

The level of HLA matching used for selection of MUDs has changed over time and usually includes now *HLA-A*, *B*, *C*, and *DRB1* loci (8/8 match). In some studies matching has been extended to the *HLA-DQB1* locus (10/10 match). A large recent

study has compared MSD and 8/8 matched MUD transplants in a homogenous cohort of patients with chronic myeloid leukemia and found a 2.44 times higher risk of grade II to IV acute GVHD in 8/8 matched MUD compared to MSD transplants [5]. In another study, the incidence of grade II to IV acute GVHD was still higher in 10/10 matched MUD compared to MSD transplants [6]. The higher risk of GVHD after MUD compared to MSD transplants could be due to a higher degree of similarity in non-HLA genes for siblings, who share 50% of their genome with the respective recipient. However, also the HLA region itself could contribute to this difference since it harbors, in addition to the classical HLA class I and II genes, more than 200 other genes [7], many with immunological functions. In accordance with this hypothesis, mismatching of microsatellite markers in HLA class I, class II, and class III regions was associated with an increased risk of death in 10/10 matched MUD transplants [8]. The HLA complex, as is the whole human genome, is organized into segments of closely linked genetic variants that are inherited as haplotypes on the same DNA strand [9]. HLA haplotypes can be defined by HLA class I and II alleles and they are in strong linkage disequilibrium with defined genetic variants of non-class I/non-class II genes within the haplotype blocks within this region [10,11]. However, a given HLA allele can occur in the context of different HLA haplotypes in MUDs. Interestingly, HLA haplotype mismatching in 10/10 matched MUD transplants was associated with an increased risk of severe acute GVHD [12]. This finding demonstrates that the HLA complex encodes in addition to *HLA-A*, *B*, *C*, *DRB1*, and *DQB1* further unidentified genes that contribute significantly to the risk of developing acute GVHD. In case of disparity between donor and recipient alleles these genes may function as minor histocompatibility antigens. Alternatively, specific allelic variants may also increase the risk of GVHD, e.g., *TNFA*, a gene located within the class III region of the MHC encoding the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α.) Several *TNFA* polymorphisms have been associated with an increased risk of GVHD and some of them are associated with increased TNF-α levels [13]. The strong linkage disequilibrium within the HLA complex makes it very difficult to identify further non-class I/non-class II HLA genes involved in the pathophysiology of GVHD by genetic association studies alone.

HLA gene expression profiling may be an alternative strategy to identify HLA genes that are involved in the pathophysiology of GVHD. We assumed that at least some non-class I/non-class II HLA genes that contribute to the risk of GVHD change their expression levels during disease progression. However, the genetic variation between clinical samples complicates the situation because allelic variation of gene expression could interfere with expression change in the pathophysiological process. Therefore, we decided to analyze a rat model of GVHD making use of genetically well-defined inbred strains. Importantly, the non-class I/non-class II genes of human (HLA) and rat (RT1) MHCs are highly conserved [14,15,16]. However, the size and organization of MHC class I encoding regions are considerably variable and the rat possesses a significant number of MHC class Ib genes for which no human homologues exist [15]. At least some of these genes have already been proven to encode ligands for inhibitory or activating natural killer (NK) receptors [17,18]. In the rat, in contrast to human, NK receptors of the Ly49 killer cell lectin-like receptor type predominate over killer cell Ig-like receptor genes [19]. Therefore, we also included the natural killer complex (NKC) in the expression profiling which harbors the Ly49 genes and additional natural cytotoxicity receptor genes.

To reduce the complexity of the experimental approach, we used an *in-vitro*-model of the graft versus host reaction (GVHR) – the skin explant assay. This assay has been shown to be a sensitive predictor of GVHD in patients [20]. It was also used to study the pathophysiology of GVHR [21]. Recently, we developed a rat skin explant assay [22]. This standardized *in-vitro*-model allows the study of gene expression during GVHR in a setting that is not influenced by undefined genetic differences between tissue samples which is unavoidable in human studies. In the present study we used this model to analyze the MHC and NKC gene expression profiles of GVHR.

## Results

### GVHR in rat skin explant assays

To obtain skin explant samples for an expression profiling experiment, we used BN rats ($RT1^n$) as recipients and PVG rats ($RT1^c$) as donors. This combination is mismatched for minor and major histocompatibility antigens, which gives rise to GVHR grades I to IV [22]. PVG splenocytes were stimulated for 7 days in a mixed lymphocyte reaction (MLR) with irradiated BN splenocytes. Syngeneic co-cultures (BN plus irradiated BN splenocytes) were performed as control experiments. The stimulation index indicated a specific proliferation of PVG lymphocytes in response to irradiated BN lymphocytes in contrast to syngeneic cultures of BN lymphocytes ($p<0.0001$, U test; $n = 12$ responder animals per strain, data not shown). After 7 days the PVG and BN lymphocytes were harvested, added to fresh BN skin samples from 12 individual animals and cultured for 3 further days. For further controls, additional BN skin samples from the same animals were cultured in medium only. On day 3 the skin samples were harvested and snap frozen for RNA preparation. Parallel samples were fixed and assayed for histological evidence of GVHR (**Fig. 1**). Co-culture of BN skin explants with pre-stimulated allogeneic PVG lymphocytes resulted in higher grade GVHR than co-culture with BN lymphocytes ($p = 0.0336$; U test). As in a previous experimental series [22], the syngeneic lymphocyte co-culture more frequently resulted in GVHR-like pathology of grade II or higher than culture of the skin explants in medium only.

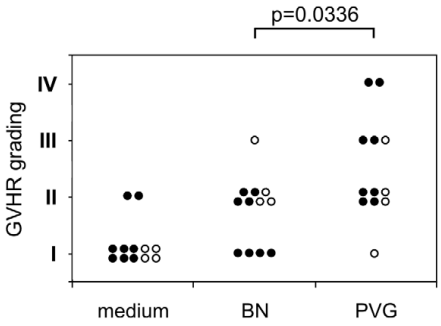


**Figure 1. Induction of a GVHR in BN rat skin explants exposed to PVG lymphocytes.** A summary of the histological GVHR grading of BN skin samples cultured in medium alone, together with syngeneic BN lymphocytes, and together with pre-stimulated allogeneic PVG lymphocytes ($n = 12$ in each group) is given. The samples represented by closed circles were used for both gene expression profiling and qRT-PCR experiments, whereas the other samples were only used for gene expression profiling. The pair-wise comparison (U test) indicated a significant difference between skin explant cultures with BN and PVG lymphocytes.
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## Expression profiling of GVHR in rat skin explants

RNA was prepared from the 24 BN skin explants exposed either to syngeneic (BN; n = 12) or to allogeneic (PVG, n = 12) lymphocytes and used for MHC gene expression profiling. We designed a custom microarray which contained specific probes for the 224 MHC genes. For this purpose the annotated sequence of the MHC of the BN strain was used [16]. For 88 of these genes, i.e. 39.3%, we had to design custom probes. A list of the MHC genes in the chromosomal order with all results obtained in the expression profiling experiment is given in the **Table S1a**. For 42 of the 224 MHC genes, a probe on the array indicated a significant regulation ($p<0.05$) in the allogeneic skin explant assays (n = 12) compared to the syngeneic controls (n = 12) (**Table S1b**). Eleven of these MHC genes showed on average at least a 2-fold up-regulation (log2-fold change $\geq 1$) or 50% reduction (log2-fold change $\leq -1$) of mRNA levels (**Fig. 2*A***, **Table S1c**). This amplitude of change is conventionally considered to be biologically relevant. Of these genes one was down-regulated (*Ly6g6e*) while 10 were up-regulated (**Fig. 2*A***). Fourteen further MHC genes were regulated significantly ($p<0.05$) but with smaller amplitude (**Table S1c**). The regulation of 17 MHC genes appeared to be more doubtful because less than 50% of the probes for that gene indicated a significant regulation. Thus, we considered 25 MHC genes to be significantly regulated in the expression profiling

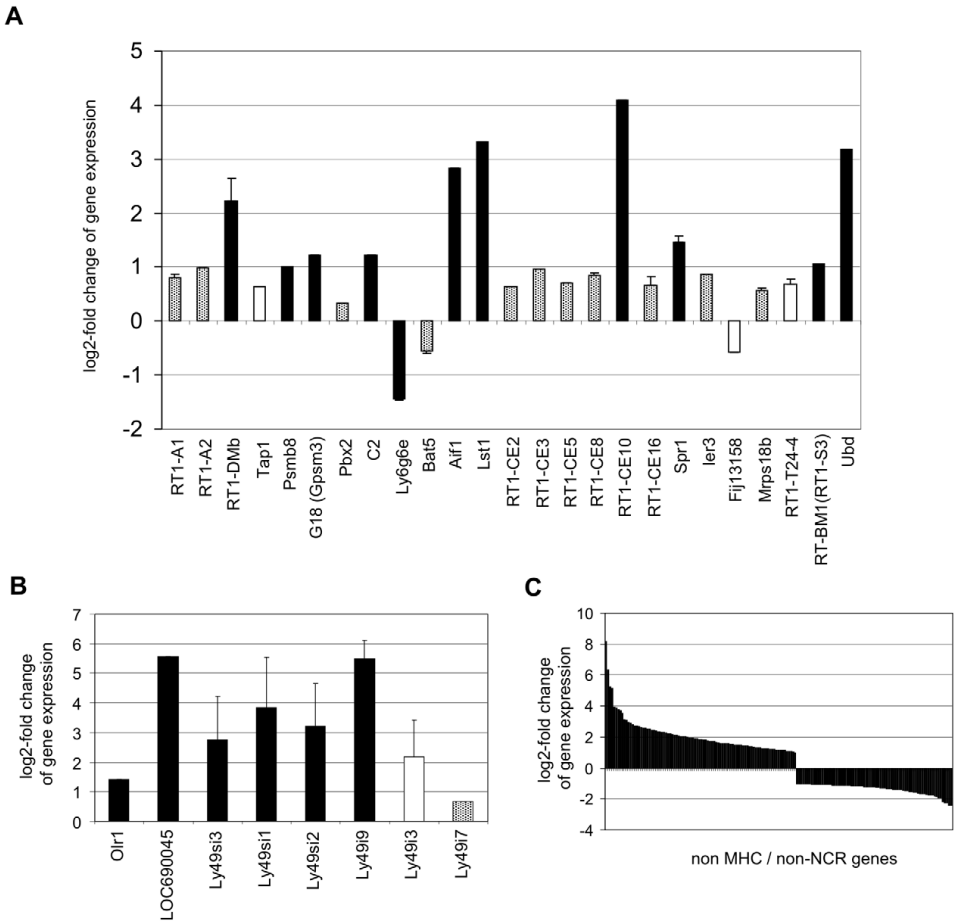


**Figure 2. Expression profiling of BN skin explant samples exposed to allogeneic (PVG) lymphocytes in comparison to those exposed to syngeneic (BN) lymphocytes.** (*A*) The log2-fold changes in gene expression of significantly regulated MHC genes ($p<0.05$) are shown. (*B*) The log2-fold changes in gene expression of significantly regulated NKC genes ($p<0.05$) are shown. (*C*) The log2-fold changes in gene expression of 168 significantly ($p<0.05$) and strongly (log2-fold change $\geq 1$ or $\leq -1$) regulated non-MHC and non-NKC genes indicate the range of observed alterations in gene expression levels among the 6342 tested genes. In panels *A* and *B*, black bars indicate a strong change (log2-fold change $\geq 1$ or $\leq -1$), dotted bars alterations below this amplitude, and white bars expression changes that were not detected at a significant level with all, but at least with 50% of the probes present on the array for that gene. When more than one probe indicated a significant change of gene expression the means and standard deviations of the log2-fold changes are shown (see Tables S1, S2, and S3 for further details).
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experiment (**Fig. 2*A***). These included the classical class Ia genes *RT1-A1* and *RT1-A2*, 8 non-classical class Ib genes (*RT1-CE2*, *RT1-CE3*, *RT1-CE5*, *RT1-CE8*, *RT1-CE10*, *RT1-CE16*, *RT1-T24-4*, *RT-BM1*) and 3 genes involved in antigen presentation (*RT1-DMb*, *Tap1*, *Psmb8*).

Furthermore, 43 genes of the NKC region, as a second important immune gene cluster, were represented on the array including all *Ly49* genes in this region (**Table S2a**). For 8 of the 43 NKC genes represented on the array, a probe indicated a significant regulation ($p<0.05$) in the allogeneic skin explant assays compared to the syngeneic controls (**Table S2b, S2c**). In addition to the *Olr1* gene, 6 *Ly49* genes appeared to be up-regulated in the allogeneic skin explant assays (**Fig. 2*B***). Not all probes for the *Ly49i3* gene indicated a significant up-regulation. However, all significant results for this gene indicated a strong regulation (log2-fold change $>2$). A statistically significant ($p<0.05$) but only moderate up-regulation (log2-fold change $<1$) was detected for the *Ly49i7* gene.

Probes for 6342 additional genes from all chromosomes were included mainly to allow for data normalization. For 168 of the non-MHC/non-NKC genes, a probe on the array indicated a significant ($p<0.05$) and strong (log2-fold change $\geq 1$ or $\leq -1$) regulation in the allogeneic skin explant assays compared to the syngeneic controls (**Fig. 3*C***, **Table S3**). The 20 genes showing the strongest change in expression levels are shown in **Table 1**. All 20 genes were up-regulated and they included several genes with functions clearly associated with the immune response such as genes encoding chemokines (*Ccl9*, *Ccl6*), Fc receptors (*Fcgr3a*, *Fcgr2b*), the proteases cathepsin S (*Ctss*) and granzyme C (*Gzmc*), and the inflammatory triggering receptor on myeloid cells 2 (*Trem2*).

The percentage of significantly ($p<0.05$) and strongly (log2-fold change $\geq 1$ or $\leq -1$) up- or down-regulated genes was higher in the NKC region (14.0%) compared to MHC region (4.9%) and the genes encoded in other regions of the genome (2.6%). This difference was even more pronounced for up-regulated genes. 14.0% of the NKC, but only 4.5% of the MHC and 1.5% of the other genes were up-regulated (**Table 2**).

For a general analysis of the gene expression data the PANTHER system [23] was used. With this tool we found a significant up-regulation of genes taking part in "immunity and defence" ($p<0.0001$, binominal test). More specifically, genes involved in "T cell-mediated immunity" ($p<0.0001$), "NK cell-mediated immunity" ($p<0.0001$), "cytokine and chemokine-mediated signaling" ($p = 0.0032$), and "B cell and antibody-mediated immunity" ($p = 0.0235$) were up-regulated. Genes involved in "complement-mediated immunity" ($p = 0.0336$) and "cell adhesion" ($p = 0.0003$) were down-regulated (data not shown).

## Confirmation of microarray results by quantitative real-time polymerase chain reaction (qRT-PCR)

To determine the reliability of the microarray results, we analyzed the expression of 13 selected genes from the MHC and NKC regions by qRT-PCR experiments in 8 of the sample pairs that had been used for the microarrays (see **Fig. 1**). For 12 genes the regulation that was observed in the microarray experiment was confirmed by qRT-PCR as indicated by a regulation into the same direction when the allogeneic and syngeneic skin explant assays were compared using the ΔΔ cycle threshold (ct) method for relative quantification of gene expression (**Fig. 3**). Only one gene, *RT1-CE10*, was found to be strongly up-regulated in allogeneic skin explants in the microarray experiment but slightly down-regulated in qRT-PCR. In the qRT-PCR experiments, we also included parallel skin explants that were cultured in medium only. Eight genes (*RT1-DMb*, *Aif1*, *Lst1*, *RT1-CE3*, *Ubd*, *Olr1*, *Ly49si1*,

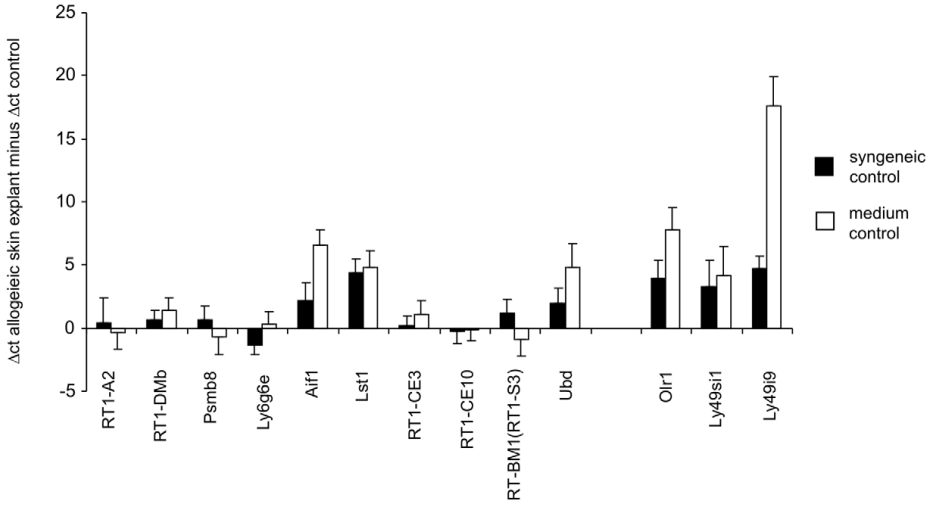


**Figure 3. Verification of the regulation in gene expression observed in the microarray experiment by qRT-PCR.** A subgroup of 8 samples used for the microarray experiment (see **Fig. 1**) was analyzed by qRT-PCR for the expression of 10 MHC and 3 NKC genes. The ΔΔct value was calculated, i.e. the Δct (*Gapdh* – gene of interest) of the allogeneic skin explant samples minus Δct (*Gapdh* – gene of interest) of the corresponding control sample. The control sample was either a parallel skin explant exposed to syngeneic lymphocytes as in the microarray experiment (syngeneic control, black bars) or a parallel skin explant sample cultured in medium only (medium control, white bars). The means of the ΔΔct values plus standard errors of the mean (SEM) are shown. A positive value indicates an up-regulation of gene expression in the allogeneic samples.
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**Table 1.** The 20 most strongly regulated non-MHC/non-NKC genes in allogeneic skin explants compared to syngeneic controls as revealed by the microarray experiment.

| gene | log2-fold change | adjusted p-value | gene description |
|---|---|---|---|
| LOC685020 | 8.18 | 0.0100 | paired immunoglobin-like type 2 receptor alpha |
| Ptpns1l3 | 6.36 | 0.0100 | protein tyrosine phosphatase, non-receptor type substrate 1-like 3 |
| Fcgr3a | 5.24 | 0.0100 | Fc fragment of IgG, low affinity IIIa, receptor |
| Nat8 | 5.14 | 0.0100 | Rattus norvegicus endogenous retrovirus mRNA, partial sequence [AY212271] |
| Ccl9 | 4.16 | 0.0100 | chemokine (C-C motif) ligand 9 |
| XM_226926 | 3.92 | 0.0149 | Rattus norvegicus similar to protein tyrosine phosphatase, non-receptor type substrate; brain immunological-like with tyrosine-based motifs (LOC310212) |
| Hck | 3.87 | 0.0100 | hemopoietic cell kinase |
| Trem2 | 3.78 | 0.0100 | triggering receptor expressed on myeloid cells 2 |
| Ccl6 | 3.71 | 0.0100 | Rattus norvegicus chemokine (C-C motif) ligand 6 |
| Cd36 | 3.57 | 0.0100 | CD36 antigen |
| Igf1 | 3.23 | 0.0100 | insulin-like growth factor 1 |
| Ctss | 3.15 | 0.0100 | cathepsin S |
| Gzmc | 3.11 | 0.0373 | granzyme C |
| LOC100048479 | 2.97 | 0.0373 | one cut domain, family member 1 |
| Plscr1 | 2.83 | 0.0100 | phospholipid scramblase 1 |
| Nfe2 | 2.74 | 0.0149 | nuclear factor, erythroid derived 2 |
| Prg4 | 2.74 | 0.0149 | proteoglycan 4 |
| Spic | 2.68 | 0.0278 | Spi-C transcription factor |
| Fcgr2b | 2.62 | 0.0100 | Fc receptor, IgG, low affinity IIb |
| LOC498277 | 2.61 | 0.0100 | similar to Low affinity immunoglobulin gamma Fc region receptor III precursor |

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and *Ly49i9*) showed an up-regulation in the allogeneic skin explant assay also in this comparison (**Fig. 3**). Six of these genes (*Aif1*, *Lst1*, *Ubd*, *Olr1*, *Ly49si1*, and *Ly49i9*) were clearly found to be up-regulated in both comparisons.

The up-regulation of genes in skin explants could be due to the change of gene expression in cells of the skin or due to infiltration of donor lymphocytes. Non-infiltrating or non-attaching donor lymphocytes were washed off before freezing of the skin explants and therefore would not contribute significantly to the results. Infiltrating lymphocytes were rarely seen in skin explants by histological analysis (data not shown). To further determine T cell infiltration at the RNA level, we analyzed the expression of the CD3 zeta chain in qRT-PCR. *Cd3z* expression was found to be up-regulated in comparison to syngeneic controls and medium controls (**Fig. 4*A***). The expression of most tested genes showed no correlation with *Cd3z* mRNA levels (**Fig. 4*B***). Only two of the genes analyzed in qRT-PCR (*Ly6g6e* and *Olr1*) showed a moderately positive correlation ($r>0.50$) with the *Cd3z* expression level (**Fig. 4*B***). Importantly, *Ly6g6e* was down- and not up-regulated in allogeneic skin explants. The expression levels of three up-regulated genes (*Psmb8*, *Aif1*, and *Lst1*) were even negatively associated with *Cd3z* expression (**Fig. 4*B***). Thus, of the tested genes only the increase of *Olr1* expression may be formally explained by infiltrating T cells. However, *Olr1* has not been described to be expressed in T cells. Therefore, infiltration of skin explants with T cells is unlikely to explain the observed gene expression changes.

**Table 2.** Proportion of regulated genes as indicated by the gene expression profiling experiment.

| region | analyzed genes | regulated[1] | up-regulated | down-regulated |
|---|---|---|---|---|
| **MHC** | 224 | 11 (4.9%) | 10 (4.5%) | 1 (0.4%) |
| **NKC** | 43 | 6 (14.0%) | 6 (14.0%) | 0 (0%) |
| **others** | 6342 | 168 (2.6%) | 93 (1.5%) | 75 (1.2%) |

[1]Only those genes that were both significantly ($p<0.05$) and strongly (log2-fold change $\geq 1$ or $\leq -1$) regulated were taken into account for this comparison.

doi:10.1371/journal.pone.0016582.t002

## Analysis of microarray results by qRT-PCR in independent skin explant samples

Next we determined the expression of 10 selected genes in an independent set of skin explant assays. Skin explants derived from BN ($RT1^n$) and LEW.1N ($RT1^n$) rats were co-cultured with pre-stimulated allogeneic lymphocytes from rats with minor (BN lymphocytes and LEW.1N skin), major (LEW.1A [$RT1^a$] or LEW.1AV1 [$RT1^{av1}$] lymphocytes and LEW.1N skin), or minor and major histoincompatibility (PVG lymphocytes [$RT1^c$] and BN skin or LOU/C [$RT1^u$] lymphocytes and LEW.1N skin). Skin samples cultured with syngeneic lymphocytes (BN or LEW.1N) or cultured in medium only served as controls. The GVHR grading obtained in these experiments is shown in **Figure 5**. The general regulation of the selected genes during GVHR was reproduced in this second experimental set when compared to skin explants exposed to syngeneic lymphocytes and also to samples cultured in medium only (**Fig. 6**). *Aif1* and *Lst1* were the most consistently up-regulated genes in skin explants with minor, major, and minor plus major histoincompatibility. The samples with minor plus major histoincompatibility showed the highest variation in gene regulation (**Fig. 6**). However, these samples were also most heterogeneous in the GVHR grading (**Fig. 5**). Therefore, we analyzed

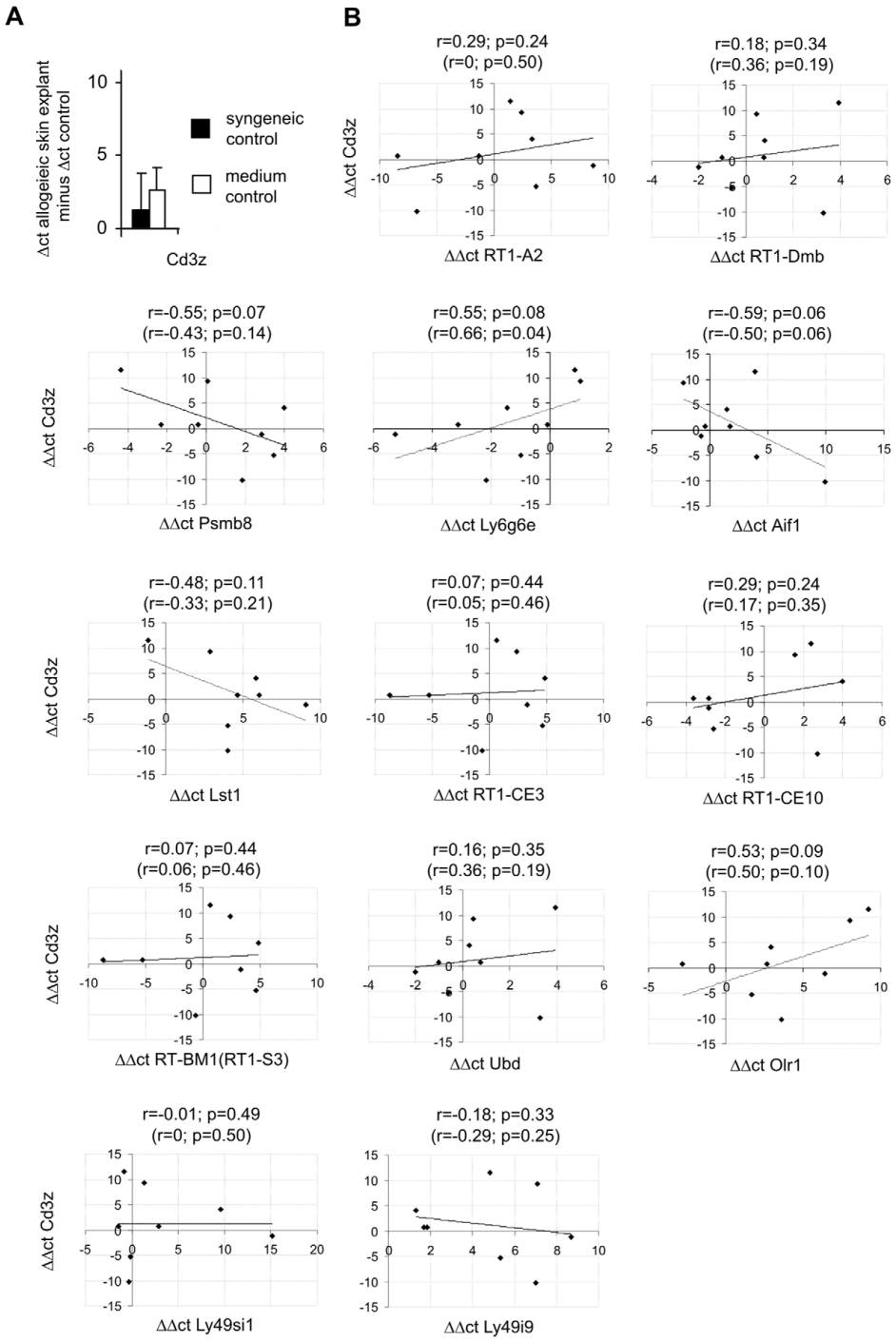
A
Δct allogeieic skin explant minus Δct control
10
5
0
syngeneic control
medium control
Cd3z
B
r=0.29; p=0.24
(r=0; p=0.50)
ΔΔct Cd3z
ΔΔct RT1-A2
r=0.18; p=0.34
(r=0.36; p=0.19)
ΔΔct RT1-Dmb
r=-0.55; p=0.07
(r=-0.43; p=0.14)
ΔΔct Psmb8
r=0.55; p=0.08
(r=0.66; p=0.04)
ΔΔct Ly6g6e
r=-0.59; p=0.06
(r=-0.50; p=0.06)
ΔΔct Aif1
r=-0.48; p=0.11
(r=-0.33; p=0.21)
ΔΔct Lst1
r=0.07; p=0.44
(r=0.05; p=0.46)
ΔΔct RT1-CE3
r=0.29; p=0.24
(r=0.17; p=0.35)
ΔΔct RT1-CE10
r=0.07; p=0.44
(r=0.06; p=0.46)
ΔΔct RT-BM1(RT1-S3)
r=0.16; p=0.35
(r=0.36; p=0.19)
ΔΔct Ubd
r=0.53; p=0.09
(r=0.50; p=0.10)
ΔΔct Olr1
r=-0.01; p=0.49
(r=0; p=0.50)
ΔΔct Ly49si1
r=-0.18; p=0.33
(r=-0.29; p=0.25)
ΔΔct Ly49i9

**Figure 4. Analysis of T cell infiltration in skin explants.** (*A*) Analysis of *Cd3z* gene expression in the same samples as shown in **Fig. 3**. (*B*) Correlation of *Cd3z* and other gene expression levels (ΔΔct values for allogeneic skin explants minus syngeneic controls) in these samples. Pearson's correlation coefficients (r) and the p-values for the corresponding tests are given above the diagrams. In brackets Spearman's correlation coefficients (r) and the p-values for the corresponding tests are shown.
doi:10.1371/journal.pone.0016582.g004

the gene regulation dependent from the GVHR grading in samples from both experimental sets.

## Regulation of selected MHC and NKC genes during GVHR

The expression of 7 MHC and 3 NKC genes was evaluated in the skin explant samples showing grade I, II, III or IV GVHR (**Fig. 7**). To provide an even more accurate comparison of the different genes in this evaluation of the data, the relative changes of gene expression levels were calculated using a mathematical model for relative quantification of real-time PCR data which takes into account variations in the amplification efficiencies of different primer pairs [24]. When compared to skin explants exposed to syngeneic lymphocytes or to medium controls, the genes *Aif1*, *Lst1*, *Olr1*, and *Ly49i9* were consistently up-regulated. *Ly6g6e* was down-regulated in some but not all comparisons. The expression of *Aif1*, *Lst1* and *Ly49i9* was found to be increased in all GVHR grades. The extremely high up-regulation of *Ly49i9* encoding an NK receptor in comparison to medium controls might be explained by complete absence of NK cells in normal skin biopsies and infiltration of few NK cells during GVHR. When the gene expression was compared to freshly frozen healthy skin, the principal findings were confirmed. Interestingly, *Olr1* was up-regulated mainly in grade II and III GVHR samples when compared to syngeneic control skin explants and healthy skin. Thus, this gene could be a marker of intermediate grade GVHR.

## Regulation of selected MHC and NKC genes during GVHD

Next, we wanted to know whether the genes found to be differentially expressed in GVHR in skin explant assays were also regulated *in vivo* in GVHD. For this purpose we analyzed skin samples from BN rats that were transplanted with bone marrow from PVG rats and developed acute GVHD. The analyzed skin samples showed in histology a grade I or grade II GVHD. The results of qRT-PCR for 7 MHC genes and 3 NKC genes are shown in **Figure 8**. The strongest up-regulation in GVHD-affected skin was observed for *RT1-DMb*, *Aif1*, *Lst1*, and *Olr1*. Thus, most genes that were found to be regulated in GVHR in skin explants were also regulated in GVHD-affected skin. However, the *Ly49si1* gene that was up-regulated consistently in allogeneic skin explants showing GVHR of grade II and above appeared to be down-regulated in GVHD. Compared to the skin explant samples, also the *Ly49i9* gene was only moderately up-regulated in grade II GVHD samples from transplanted rats.

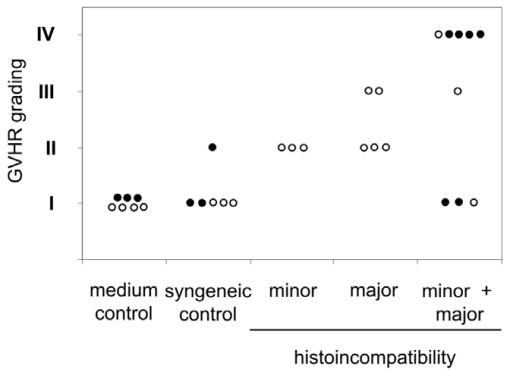


**Figure 5. Induction of a GVHR in a second series of BN (filled circles) and LEW.1N (open circles) rat skin explants.** Skin explants were co-cultured with pre-stimulated allogeneic lymphocytes from rats with a minor (BN lymphocytes and LEW.1N skin), major (LEW.1A ($RT1^a$) or LEW.1AV1 ($RT1^{av1}$) lymphocytes and LEW.1N skin), or a minor and major histoincompatibility (PVG lymphocytes ($RT1^c$) and BN skin or LOU/C ($RT1^u$) lymphocytes and LEW.1N skin). A summary of the histological GVHR grading of skin samples cultured in medium alone, together with syngeneic BN or LEW.1N lymphocytes, and together with allogeneic lymphocytes is given.
doi:10.1371/journal.pone.0016582.g005

## Regulation of selected MHC and NKC genes during GVHR in human skin explant assays

Finally, we explored the regulation of the identified genes during GVHR in human skin explant assays. We determined the expression of those genes for which human homologues exist. At 1, 2 and 3 days of co-culture with alloreactive lymphocytes skin samples of one donor were taken and analyzed in comparison to parallel samples cultured in medium only. At day 1 a GVHR of grade I was observed that increased to grade II at day 2 and grade III at day 3. We determined the expression of 15 MHC and 1 NKC gene by qRT-PCR (**Table 3**). Of these 16 genes 12 (75%) were regulated at least in one skin explant sample in the way predicted by the results of the rat expression profiling experiments (**Table 4**). Three genes *TAP1*, *PSMB8*, and *UBD* were up-regulated in all 3 human skin explant samples. The genes *C2*, *FLI13158*, and *OLR1* were regulated in 2 of the 3 samples as predicted by the rat experiments. In addition, we determined the expression of 153 non-MHC/non-NCR genes that were identified to be regulated in rat skin explant assays. Also of these genes 105 (69%) were regulated in at least one of the human skin explant samples in accordance with the results obtained in the rat model (**Table 4**). These results suggest that the rat model of the skin explant assay can reliably predict gene expression changes that occur also in human skin explant assays during GVHR.

# Discussion

We aimed to identify genes that are regulated during GVHR in the skin explant assay because these genes could be involved in the pathophysiology of GVHR and contribute to the genetic risk of GVHD. Special attention was given to genes encoded within the MHC region for the following reasons: Firstly, evidence has been presented that further risk genes for GVHD in addition to MHC class I and class II genes are present in this region [12]. Secondly, those genes cannot easily be identified by genetic linkage analysis alone due to the strong linkage disequilibrium with MHC class I and class II genes so that expression profiling could be a worthwhile alternative approach. Thirdly, we wanted to focus in this initial study on a fully characterized genomic region of special immunological importance rather than to follow a whole genome expression profiling approach. Importantly, 39% of the BN rat MHC genes ($RT1^n$ haplotype) annotated by Hurt and colleagues [16] were at the time point of array construction not represented

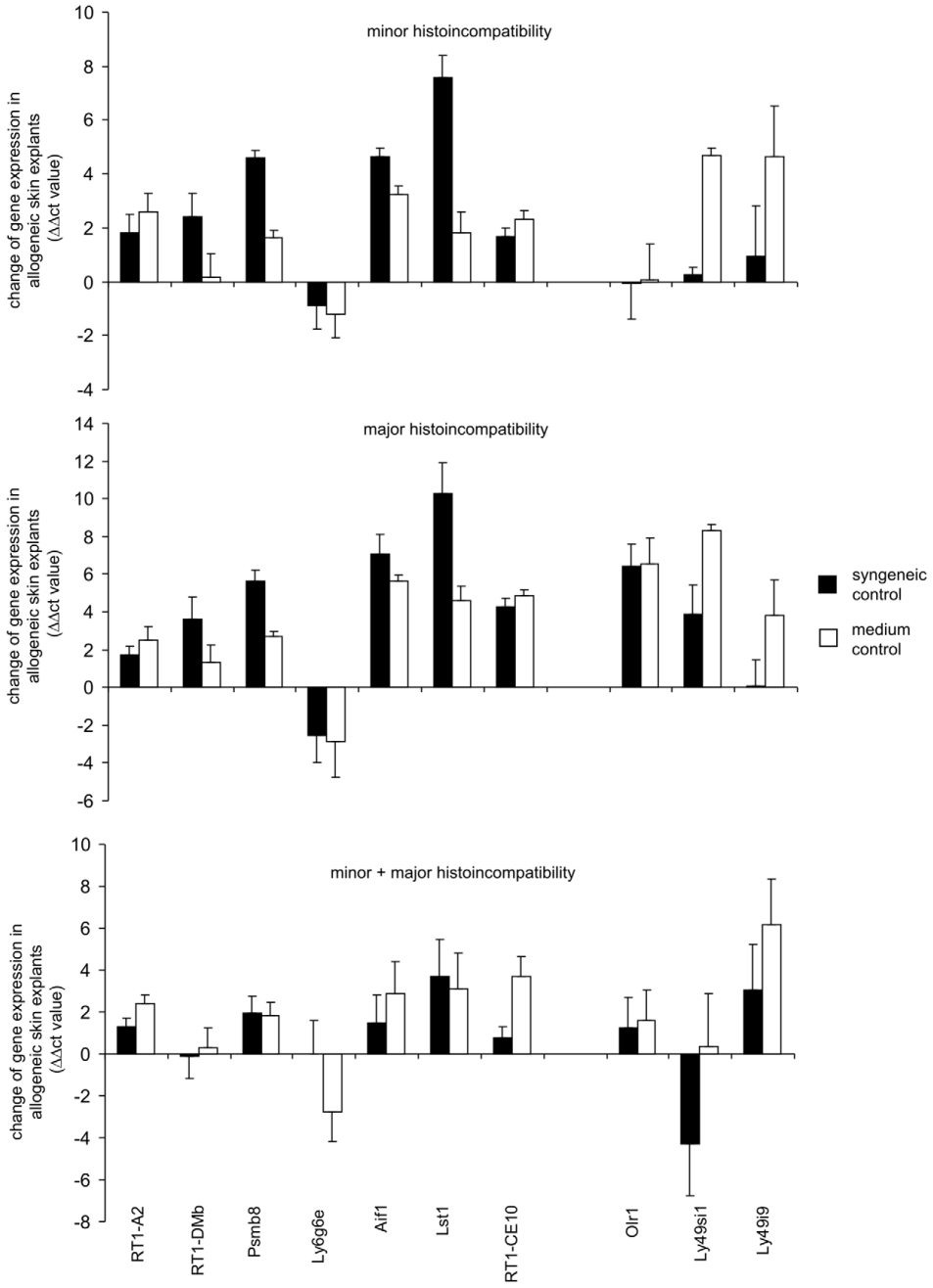


**Figure 6. Verification of gene regulations observed in the microarray experiment by qRT-PCR in an independent set of 17 skin explant assays.** Three samples were derived from skin explant assays with minor (upper panel), 5 with major (middle panel), and 9 with minor and major histoincompatibility (lower panel). The GVHR grading for these samples is shown in **Fig. 5**. The expression of 7 MHC and 3 NKC was analyzed by qRT-PCR. The ΔΔct value, i.e. Δct (*Gapdh* – gene of interest) of the allogeneic skin explant samples minus mean of Δct (*Gapdh* – gene of interest) of

the corresponding control samples (BN or LEW.1N, respectively), was calculated. The control samples were either skin explant samples exposed to syngeneic lymphocytes (syngeneic control) or skin explant samples cultured in medium only without added lymphocytes (medium control) and their GVHR grading is also shown in **Fig. 5**. The means of the ΔΔct values plus SEM are shown. A positive value indicates an up-regulation of gene expression in the allogeneic samples.
doi:10.1371/journal.pone.0016582.g006

in the Agilent database and therefore not represented on the Agilent whole rat genome array. In addition to the MHC region, genes of the NKC region were included because this region encodes *Ly49* genes and their products can function as receptors for the numerous MHC class Ia and Ib gene products encoded in the MHC [17].

A higher percentage of MHC genes and NKC genes than genes in other regions of the genome were found to be regulated in the allogeneic skin explants compared to skin samples co-cultured with syngeneic lymphocytes. Of the 25 MHC genes found to be significantly regulated ($p<0.05$), 5 are known to be involved in antigen processing and presentation. Besides two of three MHC class Ia genes in the BN strain (*RT1-A1* and *RT1-A2*) that present peptides to cytotoxic T lymphocytes (CTL), the genes *Tap1* and *Psmb8*, encoding a subunit of the antigen transporter and a subunit of the immunoproteasome (also known as LMP7), were found to be up-regulated. *RT1-DMb* encodes a homologue of *HLA-DMB*, a chaperone in the MHC class II presentation pathway. Furthermore, non-classical MHC class Ib genes (*RT1-CE2*, *RT1-CE3*, *RT1-CE5*, *RT1-CE8*, *RT1-CE10*, *RT1-CE16*, *RT1-T24-4*, *RT-BM1*) were up-regulated during GVHR in the skin explants. The function of the RT1-C/E/M class I genes is not well defined. It is known that they can become targets of CTL [25] and function as ligands for activating or inhibitory NK receptors [17,18]. RT1-C/E/M incompatibility has been shown to induce skin and pancreas graft rejection [26] and to modulate the fate of MHC class II-mismatched heart grafts [27]. The *RT1-T24-4* gene belongs to a family of genes that was originally identified as pseudogenes in the haplotype *r21* [28]. In the $RT1^n$ haplotype all four family members are presumably functional [16]. However, their actual function has not been experimentally demonstrated so far. The *RT-BM1* (*RT1-S3*) gene is assumed to be orthologous to the mouse *H2-T23* gene [29,30], which encodes the Qa-1 molecule. This is a functional homologue of HLA-E, which presents leader peptides of MHC class I molecules to the inhibitory NK receptor CD94/NKG2A [31]. Interestingly, its expression can vary substantially depending on the RT1 haplotype [14]. It has to be noticed that no human/rat orthology can be established for the class I genes in the various class I clusters. Therefore, with respect to class I genes, the rat cannot serve as a model for the HLA complex. However, the non-class I genes are clearly orthologous [15,16].

In addition to *Tap1*, *Psmb8*, and *RT1-DMb*, 12 further non-class I MHC genes were found to be regulated in the rat skin explant assays, some of them also involved in the immune response, such as the complement component *C2*, while such a role is strongly assumed for other genes. The allograft inflammatory factor 1 (*Aif1*), was cloned from chronically rejecting rat cardiac allografts [32] and it was also found in transplanted human hearts [33]. Persistent expression of AIF-1 is associated with the development of a cardiac allograft vasculopathy [34]. The expression of AIF-1 is mostly limited to the monocyte/macrophage lineage, and can be augmented by interferon (IFN)-γ. The specific function of the leukocyte specific transcript 1 (*Lst1*) gene is not known, although its strong expression in dendritic cells and functional data suggest an immunomodulatory role [35]. The expression of human *LST1*, specifically of splice variants encoding soluble isoforms, was increased in rheumatoid arthritis-affected blood and synovium and was up-regulated in response to IFN-γ [36]. The immediate early response 3 (*Ier3*) gene is stress-inducible and is involved in the regulation of cell death and oncogenesis [37]. The protein (also known as IEX-1 or IEX-1L) functions in the protection of cells from Fas or TNF-α-induced apoptosis [38]. However, it increases the rate of apoptosis in ultraviolet B irradiated keratinocytes [39]. Distinct domains of the proteins were described to be responsible for pro and anti-apoptotic activities of the protein [40]. The diubiquitin gene (*Ubd*) has been shown to be expressed in rat lymphoblasts, thymus, and testis [41]. In the mouse it is expressed in dendritic cells and B cells, is inducible by IFN-γ, and can cause apoptosis [42]. The protein (also known as FAT10) provides an ubiquitin-independent signal for proteasomal degradation [43]. It has been suggested to participate in antigen processing [44], but its expression did not affect MHC class I expression or antigen presentation [42]. In view of the reported roles of these genes in the immune response, a direct involvement in GVHD is conceivable.

For the other regulated MHC genes an involvement in immune functions has not been established so far. *Spr1* (or *Psors1c2*) is the psoriasis susceptibility 1 candidate 2 gene and was found to be expressed in the thymus of rats [41]. Its human homologue is expressed in normal and psoriatic skin and has been suggested to confer susceptibility to psoriasis [45]. The function of the gene product is not known so far. *G18* (*Gpsm3*) is an activator of G-protein signaling [46]. *Pbx2* encodes an ubiquitously expressed transcriptional activator [47]. The *Ly6g6e* gene belongs to the lymphocyte antigen 6 (Ly-6) superfamily that encodes proteins attached to the cell surface by a glycosylphosphatidylinositol (GPI) anchor that is directly involved in signal transduction [48]. Mouse Ly6g6e was found to be highly expressed at the leading edges of cells, on filopodia, which are normally involved in cell adhesion and migration [49]. The mitochondrial ribosomal protein S18B (*Mrsps18b*) gene encodes a 28S subunit protein that belongs to the ribosomal protein S18P family. The functions of the HLA-B associated transcript 5 (*Bat5*) and *Fij13158* (or *RGD1303066*) genes have not been characterized so far. Some MHC genes, such as *Tnf* encoding the tumor necrosis factor alpha or the heat shock protein 70 genes *Hspa1b* and *Hspa1a*, which were expected to be up-regulated [22,50], were actually not found to be significantly regulated in the rat skin explants. Thus, it is possible that some MHC genes that can be regulated during GVHR were not identified in our microarray experiments.

Many of the up-regulated MHC genes are inducible by IFN-γ a type II cytokine that is primarily secreted by activated T and NK cells. Several studies have demonstrated an increased level of IFN-γ in the early phase of GVHD [51,52]. Therefore, this cytokine might be highly important for the regulation of the expression of MHC genes during GVHR.

We also included the NKC region in the expression profiling which harbors the Ly49 genes that encode NK receptors of the killer cell lectin-like receptor type [19] and some of these have been shown to interact with both MHC class Ia and Ib molecules [17,18]. In contrast to the MHC region, no reference sequence has been published for the NKC region of the rat. Therefore, 20 genes that were recently assigned to this region in the assembly RGSC v3.4 [53] were not represented on the array. However, for most of them no function associated with the immune system has been reported. Interestingly, only *Ly49* receptor genes which have an

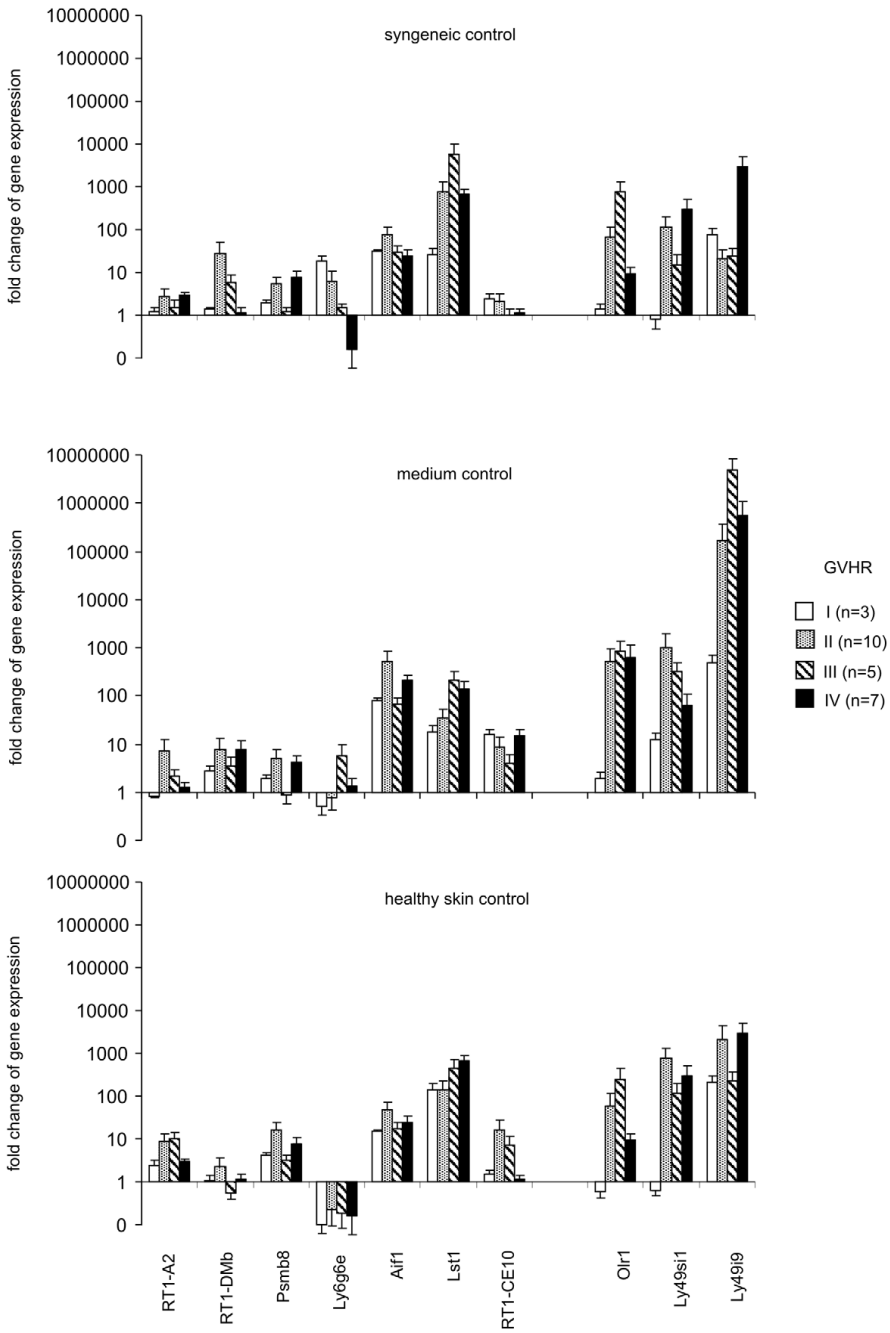


**Figure 7. Analysis of MHC and NKC gene regulation in skin explants exposed to pre-stimulated allogeneic lymphocytes depending on GVHR grading.** The expression of 7 MHC and 3 NKC was analyzed by qRT-PCR. The relative changes of gene expression levels were calculated using a mathematical model for relative quantification of real-time PCR data which also takes into account variations of the amplification efficiencies

of different primer pairs [24]. The means plus SEM are shown. A value >1 indicates an up-regulation of gene expression in the allogeneic samples. The control samples were either skin explant samples exposed to syngeneic lymphocytes (syngeneic control, upper panel), skin explant samples cultured in medium only (medium control, mean panel), or freshly frozen healthy skin samples (healthy skin control, lower panel).
doi:10.1371/journal.pone.0016582.g007

ITIM motif in their cytoplasmic region were up-regulated in the allogeneic skin explant assays. This includes also the *LOC690045* gene which encodes an immunoreceptor similar to *Ly49si1*. It is not clear whether one of these gene products interacts with the MHC class Ib molecules that we found to be up-regulated. Ly49 receptors are normally present mainly on NK cells and the skin explants harbored few leukocytes. However, skin resident lymphocytes can become activated in human skin explant assays [54]. Although few NK cells infiltrating a tissue that normally does not contain these cells might cause a drastic relative change in the presence of *Ly49* transcripts, the possibility should not be dismissed that other cells may express the receptors under pathological conditions. The role of NK cells for GVHR in skin explants needs to be further explored. In general NK cells are assumed to prevent GVHR, improve engraftment and to exert strong graft-versus-leukemia effects without causing GVHD [55].

In the NKC region we found one non-*Ly49* gene to be regulated. The *Olr1* gene encodes a receptor protein which belongs to the C-type lectin superfamily. The protein (also known as LOX-1) binds, internalizes and degrades oxidized low-density lipoprotein, which induces vascular endothelial cell activation and dysfunction, resulting in pro-inflammatory responses, pro-oxidative conditions and apoptosis [56]. In addition, it acts as a receptor for extracellular heat shock protein 70 on dendritic cells. Binding and internalization of heat shock protein 70/peptide complexes channels peptides into the MHC class I presentation pathway [57]. Thus, the protein is involved in antigen cross-presentation to naive T cells.

In addition to the MHC and NKC region genes, 168 further genes were significantly regulated in allogeneic skin explants. Many of them also have immunological functions and need to be analyzed in more detail in subsequent studies.

The results obtained in the MHC and NKC gene expression profiling experiment were confirmed in most tested cases by qRT-PCR on the skin explant samples. Some genes, e.g. *Aif1* and *Ly49i9*, appeared to be up-regulated even in grade I GVHR. *Olr1*, in contrast, was up-regulated predominantly in grade II and III GVHR in all comparisons. Importantly, several of the MHC and NKC genes that were identified to be regulated in the skin explant assays, including *Aif1*, *Lst1*, and *Olr1*, were also regulated in the GVHD affected skin of transplanted animals. Thus, the skin explant assay can model GVHD not only histologically but also with respect to gene regulation. However, the up-regulation of the tested *Ly49* genes (*Ly49si1* and *Ly49i9*) that were observed in the skin explant was not clearly confirmed in the GVHD-affected skin of transplanted rats. Skin lesions from transplanted animals are likely to be more heterogeneous with respect to the dynamics of the pathophysiological process, including infiltration of NK cells, than skin explant samples, and this may contribute to the variation in results. In addition, variability between microarray and qRT-PCR results might be partly attributed to the fact that in qRT-PCR experiments the results were normalized to only one control gene. In contrast, many genes were used in the microarray experiments for data normalization. More control genes could be employed for data normalization in qRT-PCR experiments in further validation studies to reduce this source of variability [58].

In an exploratory experiment, we analyzed the expression of 169 genes with human homologues, including the respective MHC and NKC region genes, identified in the rat in human skin explant samples. These human skin explants were cultured for 1, 2, or 3 days resulting in GVHR of grades I, II, and III, respectively. Notably, 69% of all tested human genes were found to be regulated in at least one of these human samples as predicted by the results of the rat expression profiling experiments. 21%, i. e. 36

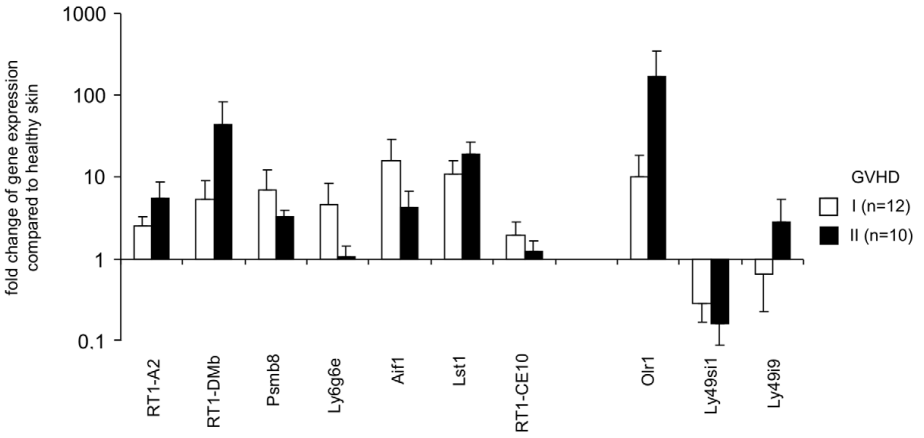


**Figure 8. Analysis of MHC and NKC gene regulation in GVHD skin lesions from transplanted animals.** BN (*RT1*$^{n}$) rats were transplanted with bone marrow of PVG (*RT1*$^{c}$) rats. Rats that developed acute GVHD were scarified and skin lesions with signs of GVHD were obtained for RNA preparation and histology. The expression of 7 MHC and 3 NKC was analyzed by qRT-PCR using the *B2m* gene as reference. The relative changes of gene expression levels were calculated [24]. The means plus SEM are shown for skin lesion with grade I and grade II GVHD. A value >1 indicates an up-regulation of gene expression in the allogeneic samples. The control samples were freshly frozen skin samples from healthy BN rats (n = 7).
doi:10.1371/journal.pone.0016582.g008

**Table 3.** Regulation of MHC and NCR candidate genes in human skin explants.

| | regulation in rat skin explant assays (expression profiling) | regulation in human skin explant assay | | | concordance rate |
|---|---|---|---|---|---|
| | | day 1 (GVHR I) | day 2 (GVHR II) | day 3 (GVHR III) | |
| **MHC region** | | | | | |
| HLA-DMB | ↑[1] | - | ↑ | - | 1/3 |
| TAP1 | (↑) | ↑ | ↑ | ↑ | 3/3 |
| PSMB8 | ↑ | ↑ | ↑ | ↑ | 3/3 |
| G18 (GPSM3) | ↑ | n.d. | n.d. | n.d. | |
| PBX2 | (↑) | ↓ | n.d. | ↑ | 1/3 |
| C2 | ↑ | ↑ | ↑ | ↓ | 2/3 |
| LY6G6E | ↓ | n.d. | ↑ | n.d. | 0/3 |
| BAT5 | ↓ | - | - | - | 0/3 |
| AIF1 | ↑ | ↓ | ↑ | ↓ | 1/3 |
| LST1 | ↑ | - | ↑ | n.d. | 1/3 |
| SPR1 (PSORS1C2) | ↑ | - | - | ↑ | 1/3 |
| IER3 | ↑ | ↓ | ↑ | - | 1/3 |
| FLI13158 | (↓) | ↓ | ↓ | - | 2/3 |
| MRPS18B | (↑) | ↓ | ↓ | ↓ | 0/3 |
| UBD | ↑ | ↑ | ↑ | ↑ | 3/3 |
| **NCR region** | | | | | |
| OLR1 | ↑ | ↑ | ↑ | n.d. | 2/3 |

[1]Explanation of symbols:
↑ up-regulated mRNA expression level (log2-fold change $\geq 1$).
↓ down-regulated mRNA level (log2-fold change $\leq -1$).
- unchanged mRNA expression level (log2-fold change $>$-1 and $<1$).
(↑) significant ($p<0.05$) but moderate up-regulation (log2-fold change $<1$) of mRNA expression level in the rat expression profiling experiment.
(↓) significant ($p<0.05$) but moderate down-regulation (log2-fold change $>-1$) of mRNA expression level.
n.d. no mRNA detected.
doi:10.1371/journal.pone.0016582.t003

of the tested genes, were regulated in all 3 human skin explant samples in accordance with the rat model, but this regulation varied depending on the GVHR grade and the time course of the skin explant assay. Although we only validated these genes on 3 samples, the unexpectedly high concordance rate between the results of rat and human skin explant assays strongly suggests that the rat skin explant assay is an informative model for human GVHR and possibly GVHD. However, the variability of results in these few human skin explant samples also indicates that it is unlikely to find a single gene that can serve as universal marker for GVHR. It is more conceivable that patterns of gene expression could contribute to an improved diagnosis and classification of GVHR.

Interestingly, for some of the genes that we found to be regulated in GVHR and GVHD in the rat, the human homologues are polymorphic and disease associations of gene polymorphisms have been described. These include *HLA-DMB* [59], *C2* [60], *AIF1* [61], *SPR1* [45], and possibly *UBD* [62]. Therefore, these genes are especially interesting candidates of further non-class I/class II HLA genes that might confer an increased genetic risk of GVHD after HSCT depending on the genotype. In addition, the *OLR1* gene in the NKC is polymorphic and polymorphisms of this gene have been associated with atherosclerosis, myocardial infarction [63], and Alzheimer's disease [64].

**Table 4.** Proportion of concordantly regulated in MHC, NKC, and genes encoded in other regions in human skin explant assays in comparison to rat skin explant assays.

| region | analyzed human genes | mRNA not detected | concordantly regulated in human skin explant assays in comparison to rat skin explant assays | | | not concordantly regulated |
|---|---|---|---|---|---|---|
| | | | 3/3 | 2/3 | 1/3 | 0/3 |
| **MHC** | 15 | 1 (7%) | 3 (20%) | 2 (13%) | 6 (40%) | 3 (20%) |
| **NKC** | 1 | 0 (0%) | 0 (0%) | 1 (100%) | 0 (0%) | 0 (0%) |
| **others** | 153 | 18 (12%) | 33 (22%) | 31 (20%) | 41 (27%) | 30 (20%) |

doi:10.1371/journal.pone.0016582.t004

Several laboratory tests have been assessed for their ability to predict the risk of GVHD in patients. The skin explant assay has a predictive value of about 80% when cyclosporine alone is used for GVHD prophylaxis [65]. A gene expression analysis of selected genes may help to further improve the predictive value of the assay. Pre-transplant gene expression profiling of donor peripheral blood mononuclear cells (PBMC) has recently been shown to be a useful tool to predict the risk of GVHD [66]. Post transplant differences in the gene expression profile of PBMC of patients with acute [67,68] and chronic GVHD [69] compared to non-GVHD samples have been described. The general gene expression profile of target tissues of GVHD has been previously analyzed only in mouse models of cutaneous and hepatic GVHD [70,71]. Some of the genes identified in these studies overlap with our results. In cutaneous GVHD the MHC genes *Tap1*, *Psmb8*, and *Ubd* were also found to be up-regulated [71]. In hepatic GVHD the expression of *Tap1*, *Psmb8* (*Lmp7*), *H2-DMb*, *Aif1*, and *Ubd* (*Fat10*) was increased [70].

In conclusion, the MHC gene expression profiling approach in the rat skin explant assay identified a number of non-class I/class II genes that might contribute to the MHC-associated risk of GVHD following HSCT. These genes could be directly involved in the pathophysiology of GVHD or serve as molecular markers for GVHD and GVHR. The possibility should not be dismissed, however, that these marker genes could indicate that protective pathways are induced which modulate tissue damage during inflammation. Moreover, their human homologues may be useful for risk assessment, diagnosis, and as potential targets for therapy of GVHD in patients.

## Materials and Methods

### Rat strains

For the skin explant assays, rats of the inbred strains LEW.1N ($RT1^n$), LEW.1A ($RT1^a$), LEW.1AV1 ($RT1^{av1}$), LOU/C ($RT1^n$), and BUF ($RT1^b$) were bred in the central animal facility of the Medical Faculty of the University of Göttingen. Rats of the strains PVG/OlaHsd ($RT1^c$) and BN/RijHsd ($RT1^n$) were purchased from Harlan Winkelmann (Borchen, Germany). Animals between 10 and 20 weeks of age were used for the experiments. For transplantation experiments, PVG rats of the RT7.2 allotype (allelic variant $RT7^b$), originally obtained from Harlan OLAC, UK), were bred at the animal facility of the University of Oslo and BN rats were purchased from Harlan.

### Rat skin explant assays

Rat skin explant assays were performed as previously described in detail [22]. Briefly, mononuclear cells were obtained from rat spleens. Responder and irradiated (25 Gy) stimulator splenocytes were co-cultured in a MLR and the proliferation of responder lymphocytes was tested by [methyl-$^3$H]-thymidine (Amersham, Braunschweig, Germany) incorporation. The stimulation index was calculated as described [22]. After 7 days $10^6$ responder lymphocytes were added to freshly obtained skin samples from the stimulator strain that were cultured in 200 µl $NaHCO_3$-buffered Dulbecco's modified Eagle's medium (DMEM; Biochrom) supplemented with 3% normal rat serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and antibiotics in round-bottomed microtitre plates (Sarstedt, Nümbrecht, Germany). The skin samples were excised from the paws of rats after washing with 70% ethanol. The subcutaneous fat tissue was removed and the samples were trimmed to a size of approximately 1.5×1.5 mm. Skin samples cultured in medium only and samples co-cultured with lymphocytes from a "syngeneic MLR" were used as controls. After 3 days, the skin explants were washed with *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES)-buffered DMEM and snap frozen in liquid nitrogen and stored at −80°C for RNA preparation. Parallel samples were fixed in 10% neutral-buffered formalin, sectioned, and stained with hematoxylin and eosin (H&E). The histological evaluation of the skin explants was performed blind by an expert histopathologist (L.S.) based on the grading system described by Lerner [72].

### Human skin explant assays

PBMC and skin samples were obtained from healthy volunteers or autologous HSCT patients following written informed consent and approval from the North Tyneside Research Ethics Committee. Buffy coat from normal blood donations were obtained from Newcastle National Blood Service with consent. Skin explant assays were performed as previously described with slight variations [73]. In brief, $1\times10^7$ responder PBMC from a healthy volunteer were cultured with an equal number of irradiated PBMC from a bone marrow transplant patient, in 10 ml complete medium (RPMI 1640 supplemented with antibiotics, 2 mM L-glutamine and 10% heat inactivated human AB serum) in a 25 $cm^2$ flask. After 7 days of culture, MLR primed lymphocytes were washed and resuspended in complete medium supplemented with 20% heat inactivated autologous (patient) serum and co-cultured with patient skin explants at a cell concentration of $1\times10^6$ cells/well in a volume of 200 µl/well. Standard 4-mm punch skin biopsy specimens were obtained pre-transplant from the patients. Under sterile conditions the skin biopsies were trimmed of excess dermis and divided into 8 to 10 sections of equal size. Each section was cultured separately with either MLR-primed responder cells or culture medium in 96-well round-bottomed microtitre plates. After 1, 2 or 3 days of co-culture skin explants were fixed in 10% buffered formalin, sectioned and stained with H&E. The histopathological evaluation [72] of the skin explants was performed blindly and independently by at least 2 assessors. Grade I histopathological damage in skin biopsies was regarded as background which would be observed in medium control or autologous cell/autologous MLR controls. All biopsies presenting histopathological damage of grade II or above were regarded as positive.

### Bone marrow transplantation

Transplantation experiments were approved by the Experimental Animal Board under the Ministry of Agriculture of Norway (ID 09.1514, 09.1515 and VIT 09.1512). Male PVG ($RT7^b$) rats served as bone marrow and lymph node donors. Mononuclear bone marrow cells were purified by density gradient centrifugation in Nycoprep 1.077A (Medinor ASA, Norway). The cells were depleted of T cells by magnetic separation using anti-CD5 (Ox19) and anti-αβ T cell receptor (R73) antibodies conjugated to pan-mouse IgG coated Dynabeads (Dynal Biotech ASA, Norway). This procedure reduced the $CD3^+$ T cell content in the bone marrow from 3% to less than 0.3%. Male BN rats were used as recipients. They were irradiated (9 Gy) and subsequently received an i.v. injection of $30\times10^6$ PVG.7b T cell-depleted bone marrow cells. 14 days post transplantation, $1.5\times10^6$ lymph node cells were injected i.v. to evoke GVHD. The rats were regularly monitored for GVHD symptoms. Rats suffering from irreversible GVHD were sacrificed and skin samples were processed for RNA preparation and histology in parallel.

### RNA preparation

RNA extraction was carried out using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's

recommendations. Afterwards, the RNA samples were treated with RQ1 RNase free DNase (Promega, Madison, WI, USA) for 20 min at 37°C in order to remove genomic DNA contaminations. The RNA was then purified as described previously [22]. Quantity and quality of extracted RNA were controlled by capillary electrophoresis using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA).

### Microarray experiment

For the expression profiling, a custom-designed oligo DNA microarray (Agilent) was used. The 15K microarray covered 224 MHC genes by 649 oligonucleotide probes and 43 NKC genes by 101 probes. These probes were spotted in triplicates. Further probes representing 6342 genes were added mainly to allow for data normalization. A two-color 12×2 paired swap design [74] using 24 arrays was applied, comparing RNA samples from 12 independent allogeneic and 12 independent syngeneic skin explant assays. Aliquots of total RNA (200 ng) were used as starting material. The "Low RNA Input linear Amplification Kit Plus, two color" (Agilent, 5188-5340) and the "RNA Spike-In Kit" (Agilent, 5188-5279) were used for cDNA synthesis and *in-vitro* transcription according to the manufacturer's recommendations. Quantity and dye incorporation rates of the amplified cRNAs were determined using the NanoDrop ND-1000 UV-VIS Spectrophotometer version 3.2.1 (NanoDrop Technologies, Wilmington, DE, USA). Afterwards, 300 ng aliquots of Cy3 and Cy5-labeled cRNAs from syngeneic and allogeneic skin explant assays, respectively, were mixed and hybridized to the microarrays. The hybridization was performed for 17 hours at 10 rpm and 65°C. After washing, Cy3 and Cy5 intensities were detected by two-color scanning using a DNA microarray scanner (Agilent, G2505B) at 5 micron resolution. Scanned image files were visually inspected for artifacts. The generated raw data were extracted using the Feature Extraction 9.1 software (Agilent). The normalization of the raw microarray data was done with a non-linear loess regression [75]. Differentially expressed genes were identified by an analysis of variance (ANOVA) mixed effects model [74] using SAS PROC MIXED. The resulting p-values were adjusted with the Benjamini-Hochberg method to control the false discovery rate [76]. The microarray data were generated conforming to the MIAME guidelines and have been deposited in NCBI's Gene Expression Omnibus (accessible through GEO series accession number GSE17928). For a general analysis of the gene expression data the PANTHER (Protein ANalysis THrough Evolutionary Relationships) system [23] was used, which classifies genes by their functions (www.pantherdb.org/tools/genexAnalysis.jsp). The microarray data were mapped to PANTHER molecular function and biological process categories, as well as to biological pathways [77].

### Validation of rat candidate genes by quantitative real-time PCR

To validate the expression change of candidate genes, qRT-PCR assays were used. Specific primers for 10 MHC and 3 NKC genes were designed (**Table S4**). To generate external standard curves and to calculate the amplification efficiency of each primer pair, a pool of 20 random cDNAs was amplified in serial 10-fold dilutions [24]. The amplification reactions were carried out as described previously [22] using an ABI 7500 Real-Time PCR System. The data were analyzed with the ABI 7500 SDS software (Applied Biosystems). As internal control, mRNA expression of housekeeping genes *Gapdh* (Rn_Gapd_1_SG QuantiTect Primer Assay QT00199633, Qiagen, Hilden, Germany) or *B2m* (**Table S4**) were monitored. To normalize variations in the RNA concentration in different samples, the ct values obtained in real-time PCR for the genes were corrected by the ct-value obtained for the housekeeping gene in the same sample ($\Delta$ct = ct housekeeping gene - ct gene of interest). For direct comparison with microarray data, the relative changes of mRNA expression were calculated using the $\Delta\Delta$ct method ($\Delta\Delta$ct = $\Delta$ct sample of interest – $\Delta$ct control sample) [78]. For additional analyses, the relative changes of gene expression levels were calculated using a mathematical model for relative quantification of real-time PCR data which takes into account variations of the amplification efficiencies of different primer pairs [24].

### Validation candidate genes in human skin explant assays by quantitative real-time PCR

Validation of the rat candidate genes with human homologues in the human skin explant assay was also done by qRT-PCR. For this we used relative quantification using custom designed Taqman low density array (TLDA) cards (Applied Biosystems), each card contained 4 replicates of 95 unique genes and a control gene, *18S*. The qRT-PCR reactions were set up using Taqman x2 gene expression mastermix (Applied Biosystems), 50 ng RNA equivalent of cDNA and the total volume adjusted to 200 µl with nuclease free water (Quiagen). The TLDA cards were run on a 7900 qRT-PCR system (Applied Biosystems) using the TLDA block and analysed using the RQ manager 1.2 software (Applied Biosystems). The relative changes in RNA expression were also calculated using the $\Delta\Delta$ct method described above.

### Statistical analyses not related to microarray experiments

Paired comparisons between experimental groups were performed using the nonparametric Mann-Whitney U test. Pearson's and Spearman's correlation coefficients were calculated to determine the correlation between mRNA expression levels of two genes. The statistical analyses were performed using WinSTAT® software.[79]

## Supporting Information

**Table S1 Expression profiling results of MHC genes.** In **Table S1a**, results for all 224 MHC genes are shown in their chromosomal order [16]. The expression profiling results of BN skin explant samples exposed to pre-stimulated allogeneic (PVG) lymphocytes in comparison to those exposed to syngeneic (BN) lymphocytes are given. The log2-fold changes and the fold changes in gene expression are shown for every oligonucleotide probe used. The adjusted p-values are indicated. Significant ($p<0.05$) and strong (log2-fold change $\geq 1$ or $\leq -1$; i.e. fold change $\geq 2$ or $\leq 0.5$) results are indicated in bold font. In addition, the identification numbers of the probes on the arrays are given (probe ID) together with the information whether these probes were taken from the Agilent database or custom designed. **Table S1b** contains the same information for all MHC genes for which at least one probe indicated a significant alteration of gene expression. In **Table S1c**, the data for those genes are summarized that are considered to be regulated significantly because either at least a single probe indicated a significant ($p<0.05$) and strong (log2-fold change $\geq 1$ or $\leq -1$) regulation or at least 50% of the gene probes indicated a significant ($p<0.05$) regulation of gene expression.
(XLS)

**Table S2 Expression profiling results of NKC genes.** In **Table S2a**, results for all 43 NKC genes investigated are indicated in their chromosomal order (*Klrg; Pzp* to *Csda*). The

expression profiling results of BN skin explant samples exposed to pre-stimulated allogeneic (PVG) lymphocytes in comparison to those exposed to syngeneic (BN) lymphocytes are given. The log2-fold changes and the fold changes in gene expression are shown for every oligonucleotide probe used. The adjusted p-values are indicated. Significant ($p<0.05$) and strong (log2-fold change $\geq 1$ or $\leq -1$; i.e. fold change $\geq 2$ or $\leq 0.5$) results are indicated in bold font. In addition, the identification numbers of the probes on the arrays are given (probe ID) together with the information whether these probes were taken from the Agilent database or custom designed. **Table S2b** contains the information for all NKC genes for which at least one probe indicted a significant alteration of gene expression. In **Table S2c**, the data for those genes are summarized that are considered to be regulated significantly because either at least a single probe indicated a significant ($p<0.05$) and strong (log2-fold change $\geq 1$ or $\leq -1$) regulation or at least 50% of the probes indicated a significant ($p<0.05$) regulation of gene expression.
(XLS)

**Table S3 Regulated non-MHC non-NKC genes.** The expression profiling results of non-MHC non-NKC genes are given for those genes that were both significantly ($p<0.05$) and strongly (log2-fold change $\geq 1$ or $\leq -1$; i.e. fold change $\geq 2$ or $\leq 0.5$) regulated. The log2-fold changes and the fold changes in gene expression are shown. The adjusted p-values are indicated. For 20 of these genes at least two different probes were present on the array. In 8 cases (indicated by gene symbols in bold) the second probe indicated the same strong and significant regulation and in 7 further cases (indicated by gene symbols in italics) the second probe indicated a regulation with borderline amplitude or significance. In 5 cases (indicated by gene symbols in blue font) the results of the two probes for a gene did not confirm each other. Furthermore, the identification numbers of the probes on the arrays are given (probe ID) together with the information whether these probes were taken from the Agilent database or custom designed.
(XLS)

**Table S4 Primer sequences used for mRNA expression analysis.**
(DOC)

## Author Contributions

Conceived and designed the experiments: PN SZ JN XNW BR AMD LW RD. Performed the experiments: PN SZ JN XNW. Analyzed the data: PN SZ JN XNW LS GSR BR AMD LW RD. Wrote the paper: RD. Wrote the manuscript in part: PN SZ LO GSR BR AMD LW.

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**Table S4. Primer sequences used for mRNA expression analysis**

| | Primer sequence 5`- 3`[1] | Amplicon (bp) | Proximity to poly-A (bp) | Efficiency coefficient ($E$)[2] |
|---|---|---|---|---|
| *RT1-A2* | F: TCCCTCCCTGCTACCCTGAG<br>R: GCCATCCACACTTGGGTCAA | 103 | 105 | 1.93 |
| *RT1-DMb* | F: TCAAATCTGCCTCGGGTGTTT<br>R: GACAAGGTGGGGCTTTCAGG | 80 | 53 | 1.87 |
| *Psmb8* | F: CACTGCTGGGCAGACATCCT<br>R: GCTTTGTCTCCAGCCCAGGT | 109 | 91 | 1.92 |
| *Ly6g6e* | F: CCCAGGCAAAGGGACAGAAG<br>R: TGAGACCCTCAGGCACCAAG | 87 | 151 | 1.97 |
| *Aif1* | F: TCCCCCAGCCAAGAAAGCTA<br>R: TCTTTTCCCATGCTGCTGTCA | 99 | 51 | 1.86 |
| *Lst1* | F: GGGCAGGAGCTCCACTACG<br>R: CGATGCAGGCATAGTCAGTGC | 118 | 20 | 1.89 |
| *RT1-CE3* | F: TGTCGTCCTTGGAGCCATCT<br>R : TCCTCACAACAGGCACCAGA | 62 | 106 | 1.91 |
| *RT1-CE10* | F: ACACAGGTGGGGAAGGAGGA<br>R : CAATCTGGGAGGGACACATCAG | 82 | 10 | 1.94 |
| *RT-BM1 (RT1-S3)* | F: GCAGCTATGCTCATGTTCTAGGC<br>R: TGCCTTCTGAGGCCAGTCAG | 62 | 7 | 1.89 |
| *Ubd* | F: TGGGGTGATGAGAAGCTCAAAA<br>R: CCCCACCTCAAATCTTTATTTCATTC | 105 | 7 | 1.92 |
| *Olr1* | F: GGAAGTCAGAAGAGGGCATGG<br>R: TCCTGGGTTCAATTTCCAGAGT | 89 | 271 | 1.90 |
| *Ly49si1* | F: TGGCCAATCTGAATTTTCCTTG<br>R : ACATGGGAAGGGGTTCATGC | 115 | 36 | 1.84 |
| *Ly49i9* | F: GGGACTTGGCAACCTCAGGA<br>R: TTGGAACATCTGCACAATGGAA | 110 | 179 | 1.88 |
| *Cd3z* | F: AGTGCCTGCTGGGATTTAGC<br>R: CATCCATGGTCACAGGCACTT | 118 | 50 | 1.93 |
| *B2m* | F: GAGCAGGTTGCTCCACAGGT<br>R: CAAGCTTTGAGTGCAAGAGATTGA | 128 | 246 | 1.94 |

[1] F: forward primer, R: reverse primer

[2] The real-time PCR efficiency coefficient ($E$) of one cycle in the exponential phase was calculated according to the equation: $E = 10^{[-1/\text{slope of standard curve}]}$

# IV

V

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# Mycoplasma Contamination Revisited: Mesenchymal Stromal Cells Harboring *Mycoplasma hyorhinis* Potently Inhibit Lymphocyte Proliferation *In Vitro*

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## Abstract

***Background:*** Mesenchymal stromal cells (MSC) have important immunomodulatory effects that can be exploited in the clinical setting, *e.g.* in patients suffering from graft-*versus*-host disease after allogeneic stem cell transplantation. In an experimental animal model, cultures of rat T lymphocytes were stimulated *in vitro* either with the mitogen Concanavalin A or with irradiated allogeneic cells in mixed lymphocyte reactions, the latter to simulate allo-immunogenic activation of transplanted T cells *in vivo*. This study investigated the inhibitory effects of rat bone marrow-derived MSC subsequently found to be infected with a common mycoplasma species (*Mycoplasma hyorhinis*) on T cell activation *in vitro* and experimental graft-*versus*-host disease *in vivo*.

***Principal Findings:*** We found that *M. hyorhinis* infection increased the anti-proliferative effect of MSC dramatically, as measured by both radiometric and fluorimetric methods. Inhibition could not be explained solely by the well-known ability of mycoplasmas to degrade tritiated thymidine, but likely was the result of rapid dissemination of *M. hyorhinis* in the lymphocyte culture.

***Conclusions:*** This study demonstrates the potent inhibitory effect exerted by *M. hyorhinis* in standard lymphocyte proliferation assays *in vitro*. MSC are efficient vectors of mycoplasma infection, emphasizing the importance of monitoring cell cultures for contamination.

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## Introduction

Mesenchymal stromal cells (MSC) comprise a heterogeneous population of progenitor cells that can differentiate along mesodermal lineages [1]. As characteristic markers are lacking, MSC are currently defined by a set of minimal criteria based on their morphology, phenotype and multipotency [2]. Multipotent stromal progenitor cells were originally isolated from the bone marrow (BM) [3], but are readily available from many adult and fetal tissues [4–7]. In recent years, a number of studies have shown that MSC have important immunomodulatory potential [8–12]. MSC can suppress the activation and proliferation of T and B cells, inhibit proliferation and cytotoxicity of NK cells, block the activation and maturation of dendritic cells, and induce expansion of regulatory T cells [13]. Although a variety of soluble mediator molecules have been implicated [14], the molecular mechanisms by which MSC exert their immunomodulatory effects are presently not well understood.

Graft-*versus*-host disease (GvHD) is caused by activation of donor T cells due to disparities of major histocompatibility complex (MHC) and minor histocompatibility antigens with the recipient. MSC have emerged as a promising treatment modality for GvHD after allogeneic stem cell transplantation [15,16]. A clinical phase II study showed that a majority of patients suffering from acute, steroid-refractory GvHD responded to treatment with one or several MSC transfusions [17]. This effect was independent of the MHC constitution of the MSC donor. Conversely, results from experimental animal models have been conflicting. While some attempts to treat GvHD with MSC have been successful in murine models of allogeneic BM transplantation (BMT) [18,19], other studies have failed to show a protective effect [20–22]. The influence of source of MSC, experimental protocols and treatment strategies on the efficiency of MSC remain unclear.

Mycoplasmas are parasitic bacteria that lack a cell wall and colonize animals and plants. In humans and other mammals, they have been associated with a variety of maladies in the respiratory tract and the genito-urinary tract. Interactions of mycoplasma with the host immune system are currently not fully understood. Efforts to prevent mycoplasma infection have proven difficult, partly due

to the lack of successful vaccination strategies [23]. Several strains of mycoplasma frequently occur as latent contaminants of human and animal cell lines in research laboratories [24]. *Mycoplasma hyorhinis* is a pathogen of the porcine respiratory system and one of the most common cell culture contaminants [24]. Infections can remain undetected unless methods such as polymerase chain reaction (PCR) using mycoplasma-specific primer sequences are employed. Recently, *M. hyorhinis* has been implicated in the transformation of human prostate cells [25] and thus may pose a significant health risk related to carcinogenesis.

The aim of this work was to evaluate the immunosuppressive effects of rat MSC on mixed lymphocyte reactions (MLR) *in vitro* and GvHD *in vivo* using an experimental animal model of MHC-mismatched BMT. In the course of this work, we noted that our MSC lines were unusually potent inhibitors of MLR *in vitro* and discovered that this was caused by accidental contamination of the primary MSC cultures with *M. hyorhinis*.

## Results

### Mycoplasma-infected MSC strongly inhibit MLR and mitogen-induced T cell proliferation *in vitro*

A MSC line generated from the BM of PVG strain rats (expressing the *RT1*$^c$ MHC haplotype) exhibited a potent inhibitory effect on lymphocyte proliferation *in vitro* (Figure 1). Tenfold dilutions of MSC were added to a MLR of PVG.7B (*RT1*$^c$; used interchangeably with PVG) lymph node cells (LNC) and allogeneic irradiated BN (*RT1*$^n$) stimulator cells. Incorporation of tritiated thymidine ([$^3$H]TTP) was inhibited at ratios up to 1 MSC per 10 000 responder cells ($10^{-4}$) (Figure 1A). MSC were equally effective inhibitors of proliferation induced by Concanavalin A (Con A; data not shown). Irradiation of MSC (20 Gy) did not change their inhibitory capacity (data not shown). MSC showed a marked inhibitory effect when introduced at delayed time points during MLR or mitogen-induced stimulation, but required at least 3 d of co-incubation with the responder cells (Figure 1B and data not shown).

MSC exerted a potent inhibitory effect in transwell co-cultures with LNC using 0.4 μm pore size membranes (data not shown). Addition of cell-free MSC culture medium also resulted in strong inhibition of MLR, but this effect was reversed by filtering through 0.22 μm membranes or repeated centrifugation at 100 000 *g* (Figure 2A,B). Inhibition was furthermore mediated by the pellet fraction sedimented at 100 000 *g*, but reversed by heat-inactivation for 30 min at 60°C (Figure 2B,C). These inhibitory effects were striking compared with several previous reports of MSC from different species [8,9,11,12,20,21], and it was therefore with some disappointment that we discovered that the cells were infected with *M. hyorhinis*, a common cell culture contaminant (cf. Materials and Methods).

### A single mycoplasma-infected MSC can inhibit allogeneic MLR

A second MSC line derived from another MHC-congenic strain on the PVG background (PVG.1U expressing the *RT1*$^u$ MHC haplotype), which was also subsequently found to be infected with *M. hyorhinis*, displayed an even stronger inhibition of lymphocyte proliferation (Figure 3). [$^3$H]TTP incorporation was fully abrogated (less than 15% of the positive control) by addition of only 2 MSC per 200 000 LNC responder cells ($10^{-5}$) at the start of the MLR culture. At even lower MSC:LNC ratios ($10^{-6}$), we observed an "all-or-none" phenomenon, with either full inhibition or normal proliferation in individual wells. To evaluate whether inhibition was mediated by single infected cells, we added serial threefold dilutions in replicate wells as depicted schematically in Figure 3A. Complete block of proliferation was observed in mycoplasma-contaminated wells as detected by PCR, while normal responses were recorded in mycoplasma-negative wells (Figure 3B,C). Analogous results were obtained for MSC/LNC co-cultures stimulated with Con A (data not shown). These data

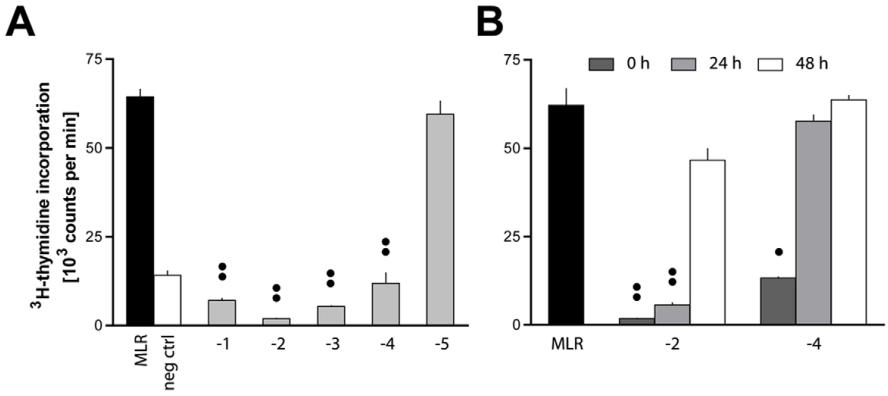


**Figure 1. Mycoplasma-infected MSC effectively inhibit MLR.** (**A**) MSC generated from the BM of PVG rats, subsequently found to be infected with *M. hyorhinis,* were added in tenfold dilutions at the start of co-cultures of $2\times10^5$ PVG.7B LNC and $2\times10^5$ irradiated BN LNC (gray bars). No MSC were added to the positive control (MLR, black bar). The basal proliferation of PVG.7B LNC in the absence of stimulus is also shown as negative control (neg ctrl, white bar). Values on the *x*-axis denote the common logarithm (log) of MSC:LNC ratios, *i.e.* 1:10 (−1) through 1:100 000 (−5). [$^3$H]TTP incorporation was abrogated depending on the number of mycoplasma-infected MSC added. (**B**) MSC were added either at the start of MLR (dark gray bars), after 24 h (light gray) or 48 h (white), respectively, at 1:100 (−2) or 1:10 000 (−4) MSC:LNC ratios, and co-cultured for a total of 4 d. No MSC were added to the positive control (MLR). Mycoplasma-infected MSC fully inhibited the assay with at least 3 d of co-incubation at the 1:100 MSC:LNC ratio. Representative data from at least three independent experiments are shown as the mean plus the standard error of the mean of triplicates. Statistical difference to the respective positive controls, ● $P<.05$, ●● $P<.01$.
doi:10.1371/journal.pone.0016005.g001

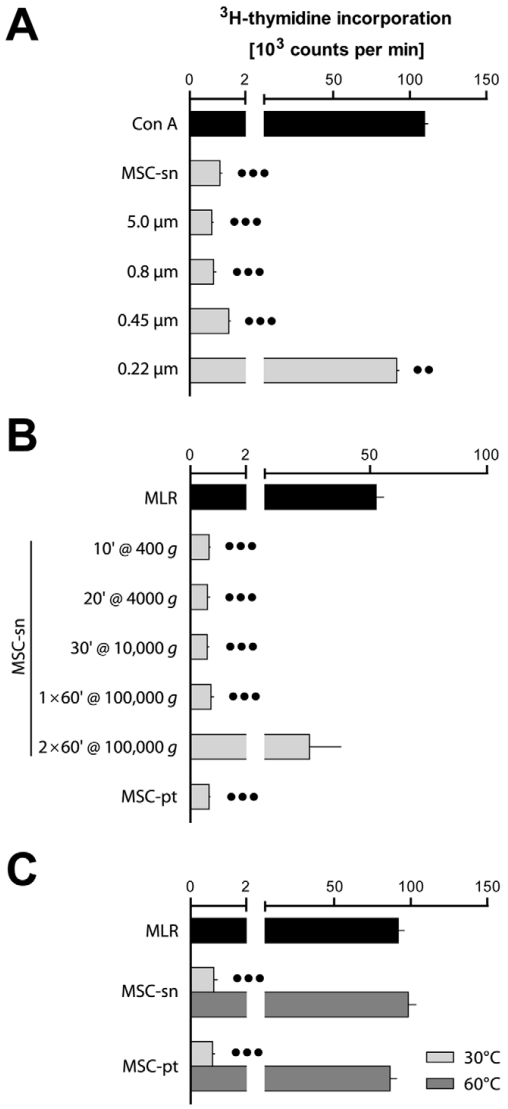


**Figure 2. MLR is inhibited by MSC culture supernatant.** Inhibition of lymphocyte proliferation was reversed either by (**A**) filtration at 0.22 µm, (**B**) serial centrifugation at 100 000 *g*, or (**C**) heat-treatment at 60°C. (**A**) MSC-conditioned supernatant (MSC-sn; gray bars) was added at 1:10 (v/v) dilution (22 µL added to 200 µL per well) at the start of mitogenic stimulation of $2\times10^5$ PVG.7B LNC with Con A (positive control with no MSC-sn added, black bar). MSC-sn was added either unfiltered or filtered with the indicated cut-off sizes. (**B**) MSC-sn was sedimented with the indicated centrifugal forces and durations (min) and added at 1:10 (v/v) dilution at the start of allogeneic MLR (cf. Figure 1). The pellet fraction (MSC-pt) was obtained after centrifugation twice for 60 min at 100 000 *g*, resuspended and added at 1:10 (v/v) dilution to the MLR. (**C**) Unprocessed MSC-sn or the resuspended pellet fraction described in panel B were treated at 30°C (light gray bars) and 60°C (dark gray) for 30 min, respectively, before adding to MLR (dilution 1:10). Representative data of at least two independent experiments are shown as mean values plus the standard error of the mean of quadruplicates. Statistical difference to the respective positive controls, ●● $P<.01$, ●●● $P<.001$.
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suggest that *M. hyorhinis* has an extremely potent inhibitory effect on lymphocyte proliferation *in vitro*.

## The suppressive effect of mycoplasma infection is removed by anti-mycoplasma treatment and restored by re-infection

The PVG.1U MSC line was treated with Mynox (cf. Materials and Methods) to resolve the mycoplasma infection. As a result of the treatment, the cells were consistently free from mycoplasma as confirmed by PCR, and most of the observed inhibitory capacity was concomitantly lost (Figure 4A). MLR stimulation was prevented only at a relatively high MSC:LNC cell ratio of 1:10, in line with several previous MSC studies [9,20,21]. Similar results were obtained with Con A-induced proliferation (unpublished observations). Intentional contamination of a previously uninfected MSC line with mycoplasma-containing supernatant increased its suppressive potential dramatically (Figure 4B), showing the same phenomenon of either full inhibition or a normal proliferative response in individual wells at limiting dilutions.

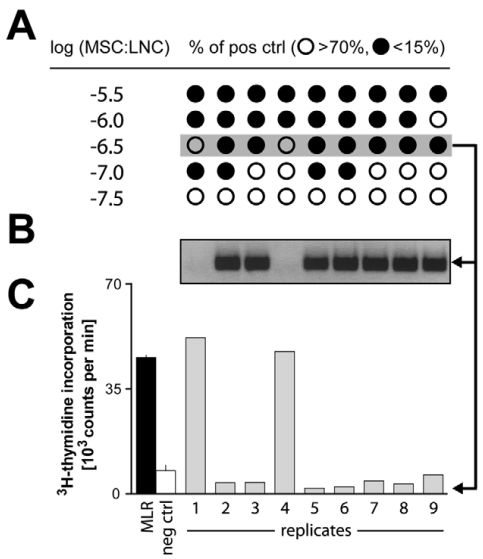


**Figure 3. A single mycoplasma-infected MSC can completely block the MLR.** (**A**) Mycoplasma-infected MSC were added at limiting dilution conditions at the start of MLR cultures with $2\times10^5$ responder cells. Proliferation was assessed in 9 replicate wells for each dilution. Wells with more than 70% or less than 15% of the [$^3$H]TTP incorporation observed in the positive control (MLR without MSC) are shown as open (○) and filled circles (●), respectively. The log of MSC:LNC ratios are denoted. (**B**) Mycoplasma was detected in the supernatant of seven out of nine wells at the −6.5 dilution shown in panel A, and in the same wells (**C**) complete inhibition of MLR was observed. Positive (MLR) and negative (neg ctrl) controls are also shown (mean of triplicates). Results are representative of three independent experiments.
doi:10.1371/journal.pone.0016005.g003

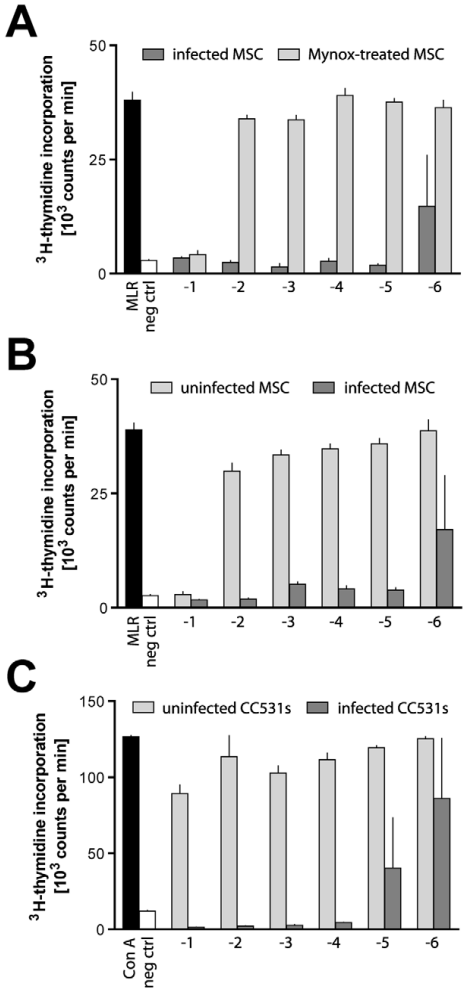


**Figure 4. Mycoplasma-treated or previously uninfected MSC show a markedly reduced ability to inhibit lymphocyte proliferation.** (**A**) Infected PVG.1U MSC were treated with Mynox reagent to eradicate the mycoplasma infection. Tenfold dilutions of untreated (dark gray bars) and treated MSC (light gray) were tested in allogeneic MLR. Mycoplasma-treated MSC, which tested negative for mycoplasma by PCR, inhibited MLR at 1:10 (−1) but not at higher dilutions. Mycoplasma-infected MSC, on the other hand, effectively inhibited up to a cell ratio of 1:10$^6$ (−6). Previously uninfected PVG.7B MSC (**B**) or the rat colon carcinoma cell line CC531s (**C**) were intentionally infected with *M. hyorhinis* by transfer of cell culture supernatant from the infected PVG.1U MSC line shown in panel A. Infection was verified by PCR after passage. MSC were added at the start of MLR, and CC531s cells were irradiated to prevent spontaneous proliferation and added at the start of lymphocyte culture with Con A. Proliferation was effectively inhibited by addition of *M. hyorhinis*-infected cells, but not by uninfected CC531s cells. Values on the *x*-axis denote the log of MSC:LNC and CC531s:LNC ratios, respectively. When infected cells were added at the highest dilutions, individual wells showed either full inhibition or a normal proliferative response (cf. Figure 3); *e.g.* when infected CC531s cells were added to the Con A culture, proliferation was detected in one of three (log dilution −5) and two of three (−6) replicates. Representative data from at least three independent experiments are shown as the mean plus the standard error of the mean of (**A, B**) quadruplicates or (**C**) triplicates.
doi:10.1371/journal.pone.0016005.g004

Similar inhibitory potential was observed after deliberate infection of a rat colon carcinoma (CC531s) cell line (Figure 4C). These data were firm evidence that the strong inhibitory effect was mediated by the contaminant and was not dependent on a specific cellular vehicle.

## *M. hyorhinis* effectively inhibits T cell proliferation as evaluated by Carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assay

Mycoplasmas are known to interfere with the read-out of [$^3$H]TTP incorporation assays commonly used to measure DNA synthesis during lymphocyte proliferation, due to their endogenous pyrimidine-nucleosidase activity [26]. We therefore opted to use the CFSE dilution assay as an alternative method to measure cell division and cell proliferation [27]. Infected and mycoplasma-treated MSC were added to Con A cultures of CFSE-labeled LNC (Figure 5). The presence of mycoplasma-infected MSC led to increased cell division arrest of both CD4$^+$ and CD8$^+$ T cells compared to mycoplasma-free MSC. The levels of inhibition of cell division correlated with [$^3$H]TTP incorporation of LNC performed in parallel, and similar results were obtained for allogeneic MLR (data not shown).

We also determined the relative numbers of natural T regulatory ($T_{reg}$) cells in our assays. The frequency of CD4$^+$CD25$^{hi}$FoxP3$^+$ $T_{reg}$ cells markedly decreased as a result of addition of MSC to Con A cultures (Figure S1A), indicating that inhibition was not mediated by $T_{reg}$ cells. In line with this observation, cell death was increased in mycoplasma-contaminated LNC cultures (Figure S1B). Taken together, these data show that *M. hyorhinis* has a high capacity to arrest lymphocyte proliferation *in vitro*. The extent to which *M. hyorhinis* interfered with the [$^3$H]TTP incorporation assay by substrate degradation is not known, however, the results from CFSE dilution assays suggest that this effect is of less importance.

## Mycoplasma is rapidly disseminated in lymphocyte cultures

Because cultivation of *M. hyorhinis* in cell-free anaerobic agar medium for microbiological assays is rarely feasible [24], we performed semi-quantitative measurements of bacterial load by PCR analysis of sequentially collected culture supernatants (Figure 6). Initially, mycoplasma was detected only at high MSC:LNC ratios, but bacterial titers increased more rapidly in co-culture with LNC as compared to cultures of MSC alone, and mycoplasma infection manifested even in the highest dilutions where only single infected MSC were added to the MLR. Similar results were obtained for PCR-testing of supernatants from Con A-stimulated LNC co-cultures with MSC (data not shown).

## Mycoplasma-infected MSC retain their cell phenotype, differentiation potential and cytokine expression profile in mixed lymphocyte cultures

Mycoplasma-contaminated MSC adhered to plastic surface *in vitro* and had the cell morphology of spindle-shaped colony-forming unit fibroblasts (Figure S2A). The infection did not seem to inhibit *in vitro* growth or the differentiation potential of MSC (Figure S2B,C,E-H and unpublished observations). MSC expressed surface markers CD59, CD71, CD90, and CXCR4, but

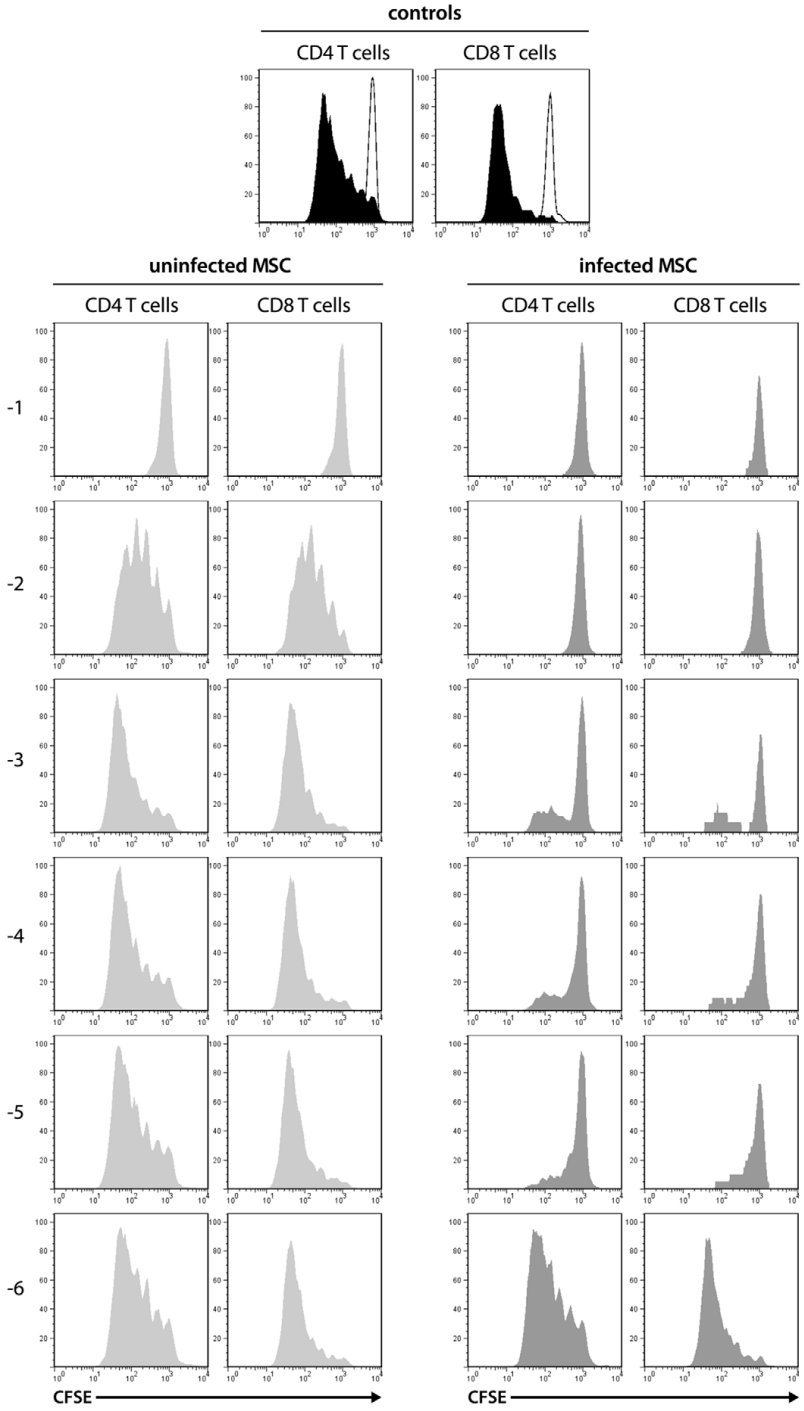
controls
CD4 T cells
CD8 T cells
uninfected MSC
infected MSC
CD4 T cells
CD8 T cells
CD4 T cells
CD8 T cells
-1
-2
-3
-4
-5
-6
CFSE
CFSE

**Figure 5. Mycoplasma-infected MSC inhibit T lymphocyte proliferation in vitro as measured by CFSE dilution.** Previously uninfected (light gray histograms) and intentionally infected PVG.7B MSC (dark gray; cf. Figure 4B) were added to CFSE-labeled, Con A-stimulated PVG.7B LNC (values to the left denote the log of MSC:LNC ratios). LNC alone were cultured either with (black histogram) or without (white) Con A as positive and negative controls, respectively. CFSE dilution indicating cell divisions was measured by flow cytometry after 3 d of incubation. Histogram plots (percent of maximum count) are representative of triplicates and show the fluorescence intensity of lymphocyte populations gated on $CD3^+CD4^+CD8^-$ (CD4 T cells) and $CD3^+CD4^-CD8^+$ (CD8 T cells). The potent inhibition of lymphocyte proliferation by infected MSC measured by CFSE dilution cannot be explained by degradation of [$^3$H]TTP. For the highest dilution (−6) of mycoplasma-infected MSC, individual cultures displayed either full inhibition or full proliferation (cf. Figure 3); one replicate in which full lymphocyte proliferation was detected is shown. Data are representative of three independent experiments.
doi:10.1371/journal.pone.0016005.g005

lacked CD31 and CD45 (Figure S2D). Class I MHC molecules (RT1-A) were also expressed while class II MHC molecules (RT1-B/D) were not detected by flow cytometric staining. Based on these parameters, the cells are in accordance with the current definition of MSC [2].

Culture supernatants from mycoplasma-positive MSC alone contained considerable amounts of interleukin (IL)-6, but not other cytokines tested (Figure S3A), in agreement with previous observations of uninfected MSC [28]. Adding increasing numbers of mycoplasma-positive MSC to MLR resulted in no significant changes of the concentrations of IL-1β, IL-2 and IL-10. The levels of IL-1α, IL-6, and granulocyte macrophage colony-stimulating factor, however, were markedly increased, while IL-4, interferon-γ (IFNγ) and tumor necrosis factor-α were clearly diminished after 6 d of co-incubation (Figure 7A and data not shown). These results were in agreement with several previous observations on the effects of murine and human MSC on cytokine secretion by stimulated lymphocytes [29–31], but failed to reproduce an increase of IL-10 in co-cultures with human MSC [29,32].

Next, we studied the capacity of *M. hyorhinis* to suppress the production of IFNγ as an important function of activated T cells. A significant fraction of T cells produce intracellular IFNγ after 24 h of Con A stimulation (Figure 7B,C). Addition of mycoplasma-infected MSC at ratios up to 1:1000 reduced the relative frequency of $IFN\gamma^+$ T cells. This effect can explain the concentration-dependent decrease of IFNγ observed in MLR/MSC co-cultures (Figure 7A).

Blocking the IL-6 signal by addition of IL-6 specific antibody did not counteract the suppressive effect of infected MSC. Also, addition of recombinant IFNγ, either alone or in combination with anti-IL-6, was without effect (Figure S3B). These data suggest that the changes in IL-6 or IFNγ were not important for the suppressive effect mediated by mycoplasma-infected MSC.

### Intravenous injections of mycoplasma-positive MSC fail to reduce GvHD severity in BM-transplanted rats

In a model of experimental allogeneic BMT, we used the same combination of PVG.7B donor rats and fully MHC-mismatched BN recipient rats as for MLR *in vitro* experiments. Lethally irradiated (9.0 Gy total body irradiation) BN rats were injected with $30\times10^6$ T cell-depleted PVG.7B BM cells. Two weeks later, at a time point when the transplanted recipients had regained their initial body weight, donor lymphocyte infusions (DLI) of graded minimal doses of $1.5–2.5\times10^6$ $CD3^+$ T cells from PVG.7B lymph node donors reproducibly invoked lethal GvHD in 90% of the recipients. Two groups were treated with intravenous injections of high doses of MSC that later proved to be infected with *M. hyorhinis*.

Transplanted rats received either single or repeated injections of $1\times10^6$ MSC 14 d after DLI before or after rats had developed symptoms of ongoing acute GvHD, but with no improvements of GvHD morbidity and mortality. All recipients were moribund between 14 and 26 d after DLI regardless of either repeated prophylactic (3× MSC) or acutely curative (1× MSC) cell therapy (for details refer to Figure S4).

## Discussion

We here report a potent inhibitory effect of *M. hyorhinis* infection of MSC on *in vitro* lymphocyte proliferation assays based on [$^3$H]TTP incorporation and CFSE dilution. Addition of single infected MSC led to rapid dissemination of mycoplasma in mixed lymphocyte cultures. *Mycoplasma* spp. have been shown to reach titers in cell cultures sufficient to degrade pyrimidine substrates with more than 90% efficiency within 3–5 d [34]. In our study, high titers of mycoplasma were reached already after 2 d which may explain the observed potent inhibition of lymphocyte proliferation as a result of direct cytopathic/cytolytic effects. Studies testing for proliferative responses should therefore rigorously control their cell cultures for mycoplasma contamination.

Many mycoplasma strains, including *M. hyorhinis*, produce nucleoside phosphorylases which rapidly convert [$^3$H]TTP to its thymine-derivative *in vitro* [26,35]. Caution has therefore been advised in the interpretation of *in vitro* proliferation assays based on quantification of such compounds [26,34]. Due to its potent enzymatic activity, it has been suggested that [$^3$H]TTP degradation can be utilized as a sensitive detection method for mycoplasma infection [36], however, specific PCR and other readily available

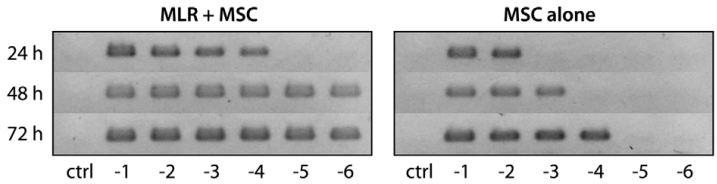


**Figure 6. Mycoplasma infection spreads rapidly in MLR cultures.** Mycoplasma-positive MSC were cultured either alone (right panel) or together with PVG responder cells and irradiated BN cells (left panel) for 3 d. Supernatants were collected at the indicated time points and tested by PCR. The log dilutions of MSC are denoted; ctrl, no MSC added. *Mycoplasma* was detectable at considerably lower MSC concentrations when co-cultured with lymphocytes.
doi:10.1371/journal.pone.0016005.g006

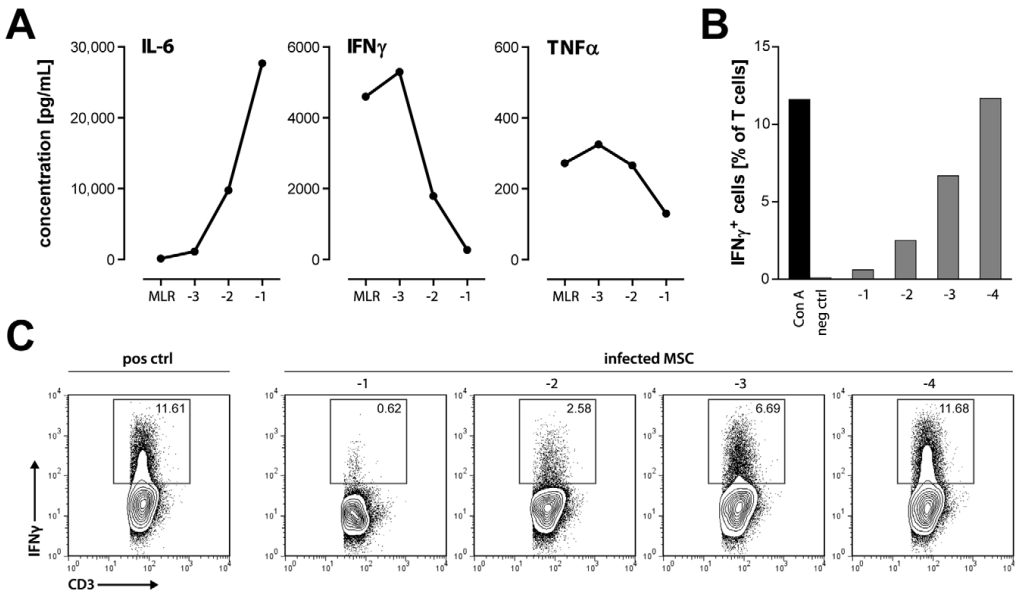


**Figure 7. Mycoplasma-infected MSC alter the cytokine profile of MLR and inhibit IFNγ production by T cells.** (**A**) Mycoplasma-positive MSC (PVG) were added at the start of MLR of PVG.7B and irradiated BN LNC ($1\times10^6$ cells each) at the indicated dilutions (log [MSC:LNC]). The concentration of various cytokines was determined in the supernatant at the end of 6 d co-culture. IL-6 was markedly increased, while interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) were reduced by addition of MSC compared to the control MLR. Representative data from three independent experiments are shown as the average of duplicates. (**B**) Mycoplasma-infected MSC (PVG.7B) were added at the start of Con A-stimulated culture of PVG.7B LNC at the indicated tenfold dilutions. The fraction of IFNγ-expressing $CD3^+$ T cells was determined after 24 h by intracellular staining (triplicates were pooled) and flow cytometry, as shown in (**C**). Cells were gated on $CD3^+$ lymphocytes, numbers represent the percentage of gated cells. Data are representative of three independent experiments.
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detection methods today have advantages of ease of use and relatively low cost. We did not directly assess to which extent our results reflect degradation of pyrimidine substrate, but this mechanism could not explain the observed effect as proliferation was similarly inhibited when measured by CFSE dilution.

These experiments also showed that proliferation of both $CD4^+$ and $CD8^+$ T cells was equally abrogated, and were in agreement with the finding that ovine Con A-activated lymphoblast cultures were inhibited by a different mycoplasma species (*M. ovipneumoniae)* in a recent study [37]. $CD4^+$ T cells are critically involved in clearing persisting bacterial infection. We speculate that the ability to directly infect proliferating T lymphocytes inducing cell cycle arrest and cell death can be an important mechanism for mycoplasmas to evade and counteract an immune response. Other plausible mechanisms such as inhibition through mycoplasma membrane proteins have been suggested [38,39].

The inhibitory properties of rat MSC were dramatically reduced by removal of *M. hyorhinis* by anti-mycoplasma treatment. Mycoplasma-negative MSC had a weaker, but significant inhibitory effect on T cell proliferation. Their suppressive potential is in agreement with several previous findings where MSC inhibition of responder cells were measured at ratios of 1:1 to 1:100 [8,9,11,12,20,21], while some studies have reported exceptionally high immunosuppressive potential of MSC [10,40,41].

In contrast to their substantial suppressive effects on lymphocytes *in vitro*, mycoplasma-infected MSC did not have a beneficial effect upon preemptive or curative treatment of BM-transplanted rats suffering from acute GvHD. The applied dosage of $1–2\times10^6$ MSC (approximately $4–10\times10^6$ per kg body weight) is comparable to what is typically administered in patients [16,17]. Increasing the dose above $2\times10^6$ MSC per injection was associated with a high risk of pulmonary embolism and sudden death of the animals (unpublished observations). Injected MSC may be susceptible to immune responses in the host against mycoplasma infection, which could explain their lack of efficiency in alleviating GvHD. We cannot rule out, however, that the MSC lines applied in the present study are ineffective in suppressing alloreactive donor lymphocytes *in vivo*, as has been reported for other animal studies [20–22,42]. The extent to which *M. hyorhinis* infection can affect the inhibitory properties of MSC *in vivo* is therefore not clear.

In our hands, mycoplasma infection did not deprive MSC of their characteristic differentiation potential, nor their capacity to proliferate or to secrete cytokines. Infection did not result in an upregulation of class I or class II MHC surface expression. Thus, *M. hyorhinis* infection of MSC may pass unnoticed. The host-pathogen relationship between *M. hyorhinis* and MSC remains elusive. The two important pro-inflammatory cytokines IFNγ and tumor necrosis factor-α were strongly reduced by the addition of infected MSC to mixed lymphocyte cultures [29–31]. It is conceivable that *M. hyorhinis* can alter the inherent immunosuppressive potential of MSC [43], e.g. by changing the expression of cytokines and other important secreted proteins [44,45]. In order

to dissect such mechanisms, it will be necessary to gain a better understanding of the immunomodulatory properties of BM stromal progenitor cells as well as the pathobiology of mycoplasma infection on MSC and immune cells. This research may have important implications for the design of vaccination strategies against mycoplasma species which continue to cause disease requiring extensive use of antibiotics in veterinary and human medicine.

## Materials and Methods

### Ethics statement

Our Department of Comparative Medicine at the Institute of Basic Medical Sciences led by our institutional veterinarian has established the rules for feeding, monitoring and handling of laboratory animals in compliance with regulations set by the Ministry of Agriculture of Norway. The institutional veterinarian has delegated authority from the Norwegian Animal Research Authority (NARA) and approved the protocols (license numbers: VIT02.02, VIT09.1512, 05.07), which were conducted in compliance with "The Norwegian Regulations on Animal Experimentation" and "The European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes". Intravenous injections were performed under neuroleptanalgesia with fentanyl citrate and fluanisone (Hypnorm®; VetaPharma, UK). All animals used in this study were euthanized with $CO_2$, and every effort was made to minimize their suffering.

### Animals

The PVG-*RT7*$^b$ (PVG.7B) strain expresses an allotype of rat CD45 (RT7.2) and was used interchangeably with the standard PVG strain (RT7.1) as both strains express the *RT1*$^c$ haplotype of the rat MHC. PVG.7B and PVG-*RT1*$^u$ (PVG.1U; *RT1*$^u$) rats were bred at the Institute of Basic Medical Sciences, University of Oslo. PVG and BN (*RT1*$^n$) rats were purchased from Harlan, The Netherlands. The animals were housed on location under a 12:12 h light/dark cycle with access to food and filtered drinking water *ad libitum*, and were routinely screened for common pathogens following FELASA recommendations [46].

### MSC isolation, *ex vivo* culture and differentiation assays

MSC lines were obtained from 8–11 w-old PVG, PVG.7B and PVG.1U strain rats as previously described [47]. In short, BM cells were aspirated from femurs and tibias, filtered through nylon cell strainers (70 μm mesh; BD Biosciences, MA), and cultured in MSC medium comprising α-modified minimal essential medium supplemented with 20% fetal bovine serum and 100 U $mL^{-1}$ penicillin, 100 μg $mL^{-1}$ streptomycin, 250 ng $mL^{-1}$ amphotericin B (all from Invitrogen, UK) and 2 mM L-glutamine (Millipore, MA) in 175 $cm^2$ culture flasks (Nunc, Denmark) at 37°C in a humidified atmosphere of 5% $CO_2$. Non-adherent cells were removed after approximately 24 h by replacement of the MSC medium. Adherent cells were allowed to expand to near confluence, detached using 500 μg $mL^{-1}$ trypsin and 200 μg $mL^{-1}$ EDTA•4Na (Invitrogen) and reseeded at a density of approximately 500 cells per $cm^2$. Cells were used in experiments after the third passage.

The differentiation potential of MSC was tested using adipogenic and osteogenic assays. MSC were cultured for 3 w in MSC medium supplemented with adipogenic (StemCell Technologies, BC) or osteogenic (10 nM dexamethasone, 50 μg $mL^{-1}$ ascorbic acid, and 5 mM β-glycerophosphate; StemCell Technologies) stimulatory supplements. Neutral lipids in fat vacuoles were visualized by staining with Oil Red O (Sigma Aldrich, CO) and mineralization by staining with 40 mM Alizarin Red (Sigma Aldrich).

### Mycoplasma detection, treatment and infection

Supernatants from confluent cultures were tested for mycoplasmas by nested PCR [48] using the following primer sequences: Mike-O-Plasma-F1 5′-ACACCATGGGAGCTGCTAAT-3′; Mike-O-Plasma-R1 5′-CTTCWTCGACTTYCAGACCCAAGGCAT-3′; Mike-O-Plasma-F2 5′-GTTCTTTGAAAACTGAAT-3′; Mike-O-Plasma-R2 5′-GCATCCACCAWAWACTCT-3′. Mycoplasma-positive PCR test results were confirmed using a detection kit (MycoAlert®; Lonza Rockland, ME) based on enzymatic ATP-conversion combined with luminescence measurement. MSC that tested positive for mycoplasma were cultured in a quarantine laboratory using MSC medium without antibiotics.

The mycoplasma strain was detected and identified as *M. hyorhinis* from cell cultures by means of 16S ribosomal DNA PCR and sequencing. DNA extracted from the cell cultures was amplified and sequenced (ABI Prism 3730 DNA analyzer; Applied Biosystems, CA) using two primers within the 16S rRNA gene. The obtained sequences were compared to sequences in the NCBI database using BLAST version 2.210.

In some experiments, non-infected MSC lines (PVG.1U or PVG.7B) and a rat colon carcinoma cell line (CC531s, a kind gift from Peter Kuppen, Leiden, The Netherlands) were deliberately infected with *M. hyorhinis* by transferring membrane-filtered (0.45 μm Filtropur S; Sarstedt, Germany) culture supernatant from nearly confluent cultures of mycoplasma-positive MSC and incubated for 1–2 d before replacement of fresh culture medium. Infected cells were confirmed as mycoplasma-positive by PCR. CC531s cells were maintained in culture medium comprising Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 20% fetal bovine serum (Invitrogen) without antibiotics in a quarantine laboratory. In another experiment, the original infected MSC line was cleared of mycoplasma using Mynox® reagent (Minerva Biolabs, Germany) according to the manufacturer's protocol, and was confirmed negative by PCR. The treated cell line continued to grow normally and no recurrence of mycoplasma was observed in several passages after the treatment.

### Mixed lymphocyte cultures and radionucleotide incorporation assays

Mononuclear cells from mesenteric and cervical lymph nodes of 8–14 w-old PVG, PVG.7B and BN rats were obtained by filtering through nylon cell strainers (BD Biosciences) and density gradient centrifugation (Lymphoprep™; Medinor, Norway) following the manufacturer's protocol. Stimulator cells were irradiated with a dose of 20.0 Gy using a $^{137}$Cs source (Gammacell® 3000; MDS Nordion, Canada). MLR were performed in RPMI medium 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U $mL^{-1}$ penicillin, 100 μg $mL^{-1}$ streptomycin, 2 mM L-glutamine and 25 μM 2-mercaptoethanol (MLR medium; all from Invitrogen) with responder (PVG or PVG.7B) and stimulator cells (BN; each $2\times10^5$ unless otherwise specified) in 200 μL per well using round-bottom, 96-well cell culture clusters (Corning, NY). In the same format, Con A (Sigma Aldrich, MO) was used at a final concentration of 5 μg $mL^{-1}$ for mitogenic stimulation of responder cells. Polyclonal rabbit anti-rat IL-6 antibody (PeproTech, UK) at 2 μg $mL^{-1}$ or recombinant rat IFNγ at 500 U $mL^{-1}$ was added where specified.

CC531s and MSC were harvested using trypsin and EDTA (Invitrogen), washed twice, diluted in MLR medium, and in some experiments irradiated (20 Gy) to inhibit mitosis, before adding to

MLR cultures at the indicated cell ratios. Medium supernatants were removed from MSC cultures prior to cell harvest and sedimented at 400 *g* for 10 min before adding to MLR cultures at the indicated dilutions. They were further processed using syringe filter units (Millipore, Ireland) of different mesh size (indicated) or serial sedimentation applying increasing centrifugal force and durations (indicated). The pellet fraction of 100 000 *g* sedimentation (Optima$^{TM}$ LE-80K Ultracentrifuge; Beckman Coulter Inc., CA) was resuspended in a volume of MLR medium equal to the original amount of supernatant.

DNA synthesis was assessed after 3 d of mitogen stimulation or 4 d of mixed lymphocyte culture by pulsing with 1 µCi of [$^3$H]TTP (diluted in 20 µL MLR medium; Hartmann Analytic, Germany) 18–20 h before the termination of the culture. Cells were then harvested on glass fiber filters using a cell harvester (Filtermate 196; Packard Bioscience Co., CT) and radioactivity was measured with MicroScint$^{TM}$ O solution (PerkinElmer, MA) using a Top Count® NXT$^{TM}$ (Packard Bioscience) or Wallac 1450 MicroBeta® TriLux (PerkinElmer) microplate scintillation counter.

## CFSE dilution assays

In some experiments, responder cells were stained with CFSE (Sigma Aldrich) prior to *in vitro* culture. Briefly, cells were resuspended in OPTI-MEM (Invitrogen) at a concentration of $2\times10^6$ mL$^{-1}$ and incubated with 500 nM CFSE for 10 min at 37°C before adding MLR medium. The cells were then washed in MLR medium twice, incubated for 5 min at 37°C, washed again and resuspended in MLR medium. At the termination of MLR and Con A cultures, cells were harvested and washed in phosphate buffered saline before labeling with monoclonal antibodies (mAb) and flow cytometric analysis.

## Intracellular staining and flow cytometry

The following mouse anti-rat mAb (conjugated in our own lab unless stated otherwise; W3/25 and OX antibodies were a kind gift from A. Neil Barclay, Oxford, UK) were used for flow cytometric analysis: FITC-conjugated W3/25 (anti-CD4), PE-conjugated G4.18 (anti-CD3; BD Biosciences), Alexa Fluor® 647-conjugated OX-38 (anti-CD4), biotin-conjugated OX-8 (anti-CD8) or OX-39 (anti-CD25) followed by secondary staining with PerCP-conjugated Streptavidin (BD Biosciences). In some experiments, 50 µM propidium iodide (Sigma Aldrich) was added to stained cells before analysis.

For intracellular FoxP3 staining, stimulated LNC were harvested after 3 d of culture, stained with mAb, fixed with fixation/permeabilization buffers (eBioscience, CA) and stained with APC-conjugated rat anti-mouse/rat FJK-16s mAb (anti-FoxP3; eBioscience) according to the manufacturer's guidelines. For intracellular IFNγ staining, LNC were stimulated with Con A (5 µg mL$^{-1}$; Sigma Aldrich) for 24 h, and Brefeldin A (10 µg mL$^{-1}$; Sigma Aldrich) was added 4 h before termination of the culture. Triplicate wells were pooled and cells stained with FITC-conjugated G4.18 (anti-CD3; BD Biosciences), fixed with 4% paraformaldehyde, permeabilized with 0.5% saponin, and stained with PE-conjugated mouse anti-rat IFNγ mAb (BD Biosciences).

For phenotypic characterization of MSC, cells were labeled with the following conjugated mouse anti-rat mAb: PE-conjugated TLD-3A12 (anti-CD31) or OX-7 (anti-CD90); FITC-conjugated TH9 (anti-CD59); PE-Cy5 conjugated OX-1 (anti-CD45; all from BD Biosciences). For two-step immunostaining, cells were first incubated with supernatant of OX-26 (anti-CD71), or purified OX-18 (anti-RT1-A, pan-MHC class I) or OX-6 (anti-RT1-B/D, pan-MHC class II; all our own), followed by secondary PE-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, UK); or with TP-503 (polyclonal rabbit anti-rat CXCR4; Torrey Pines Biolabs, NJ) followed by secondary FITC-conjugated anti-rabbit IgG (BD Biosciences). PE-conjugated mouse $IgG_1$ (BD Biosciences) and FITC-conjugated mouse $IgG_{2a}$ (DAKO, Denmark) were used as isotype controls. Cells were analyzed on a FACSCalibur$^{TM}$ or FACSCanto$^{TM}$ flow cytometer (BD Biosciences) using CellQuest$^{TM}$ software (BD Biosciences). FACS data were further analyzed using FlowJo$^{TM}$ software (TreeStar Inc., OR).

## Cytokine assays

For cytokine measurements, $10\times10^6$ responder and $10\times10^6$ stimulator cells were co-cultured for 6–7 d in MLR medium together with specified numbers of MSC in upright 25 cm$^2$ culture flasks (Nunc). Cytokine levels were analyzed using the Bio-Plex$^{TM}$ Rat Cytokine 9-Plex A Panel (Bio-Rad, CA) according to the manufacturer's protocol. Concentrations were determined on the basis of a standard curve using defined reference samples which were assayed (Luminex xMAP® Technology; Bio-Rad) in parallel.

## Experimental BMT and GvHD monitoring

Allogeneic stem cell transplantations were adapted from a previously described protocol [49]. BM cells were aspirated from femurs and tibias of 8-12 w-old male and female PVG.7B rats, filtered through cell strainers (BD Biosciences) and purified by density gradient centrifugation (Nycoprep$^{TM}$ 1.077A; Medinor). T cells were removed using magnetic pan-mouse IgG-coated Dynabeads® (Invitrogen Dynal, Norway) coated with OX-19 (anti-CD5) and R73 (anti-αβ T cell receptor) mAb. The number of CD3$^+$ cells was thus reduced at least fivefold to less than 0.25% of total BM cells (data not shown). For DLI, LNC (of which approximately 60% were CD3$^+$ T cells, data not shown) were obtained from mesenteric and cervical lymph nodes of 10-12 w-old male and female PVG.7B rats by filtering through cell strainers (BD Biosciences). The CD3$^+$ T cell contents of the BM graft and DLI were controlled by flow cytometric analysis (not shown) after staining with PE-conjugated G4.18 (anti-CD3; BD Biosciences) mAb.

Male BN rats were transplanted at 9 w of age with $30\times10^6$ T cell-depleted PVG.7B BM cells by intravenous injection shortly after receiving 9.0 Gy total body irradiation (Gammacell® 3000; MDS Nordion) under anesthesia. 14 days post transplantation, GvHD was invoked by DLI of the specified doses of PVG.7B LNC. MSC were harvested, washed and resuspended in phosphate buffered saline at a cell density of $1–2\times10^6$ per mL for injection at the specified time points. Transplanted animals were frequently and carefully monitored, and were scored weekly for disease symptoms using a protocol by Cooke *et alia* [33]. In this model, cachexia, severe kyphosis, ruffled fur, skin flaking and lesions and alopecia were predictive of GvHD. Rats suffering from lethal GvHD were euthanized with $CO_2$ at defined humane end points (GvHD score exceeding 8; body weight below 150 g). Donor BM engraftment was evaluated both by clinical observation (weight gain, anemia) and flow cytometry using HIS41 (anti-rat CD45) mAb (a kind gift from Jaap Kampinga, Groningen, The Netherlands) specific for the RT7.2 allele to identify PVG.7B donor leukocytes. 7 rats (8.9%) of a total of 79 recipients failed to engraft and were excluded from the analysis.

## Statistical analysis

Normal distribution of data was assumed and tested using Shapiro-Wilk's test. Student's *t* test (paired, two-tailed) was used to evaluate the probability of differences between samples. For *in vivo* experiments, overall survival was compared by the log-rank test and GvHD scores by Student's *t* test (unpaired, two-tailed) for group differences. Analysis was performed using SPSS® software version 17.0 (SPSS Inc., IL) with a *P* value below 0.05 deemed as significant.

## Supporting Information

**Figure S1 Mycoplasma-infected MSC reduce the numbers of T regulatory and live cells in mitogen-stimulated lymphocyte cultures.** Mycoplasma-infected MSC (PVG.7B; gray bars) were added at the start of a Con A stimulated culture of PVG.7B LNC at the indicated dilutions (log [MSC:LNC]). The relative frequency of (**A**) $CD4^+CD25^{hi}FoxP3^+$ T regulatory ($T_{reg}$) cells and (**B**) live cells (propidium $iodide^{low}$) were determined by flow cytometry after 3 d of co-culture. Cell death was increased when mycoplasma-infected MSC were present. In panel A, the mean values plus the standard error of the mean of triplicates as well as the test statistics for cell frequencies compared to the positive control are shown. Data are representative of three independent experiments.
(TIF)

**Figure S2 Morphology, differentiation potential and phenotype of mycoplasma-infected rat BM-derived MSC.** Light microscopy of cell culture, differentiation assays and flow cytometric staining of MSC infected with *M. hyorhinis*. (**A**) MSC from PVG BM appear as fibroblast-like spindle-shaped cells that adhere to plastic *in vitro* (third passage). MSC have the capacity to differentiate into adipocytes (**B**) as shown by staining of neutral lipids in fat vacuoles with Oil Red O and osteocytes (**C**) by staining areas of calcification with Alizarin Red. (**D**) Surface expression of CD31 (PECAM-1), CD45 (CLA), CD59 (MAC inhibitor), CD71 (transferrin receptor), CD90 (Thy-1) and CXCR4 as well as MHC-I (RT1-A) and MHC-II (RT1-B/D) on MSC. Histograms show the relative intensity of surface antigen (solid lines) compared to isotype controls (filled) by flow cytometric staining. Mycoplasma-infected PVG.1U MSC form adipocytes (**E**) and osteocytes (**F**) under culture conditions inducing differentiation (cf. Materials and Methods) and remain multipotent (**G**, **H**) after clearing the infection with Mynox reagent. (**A**) Original magnification 100×, (**B**, **C**) 40×, (**E–H**) 200×.
(TIF)

**Figure S3 MSC inhibition of MLR is not reversed by addition of exogenous IFNγ nor by anti-IL-6 antibody.** (**A**) A panel of cytokines was measured in the medium supernatant of a confluent culture of infected MSC (PVG) 20–24 h after medium was replaced. MSC constitutively secrete IL-6, while other cytokines were not detectable. (**B**) Inhibition of proliferation in allogeneic MLR at 1:1000 MSC:LNC ratio could not be reverted by addition of anti-rat IL-6 mAb (αIL-6; 2 μg $mL^{-1}$) or recombinant rat IFNγ (500 U $mL^{-1}$) at the start of co-culture. Representative data from two independent experiments are shown as mean values plus one standard error of the mean of (**A**) triplicate and (**B**) quadruplicates tests.
(TIF)

**Figure S4 Rats suffering from GvHD are not rescued by repeated injections of mycoplasma-infected MSC.** MSC from two different cell lines subsequently found to be infected with *M. hyorhinis* were injected in rats suffering from experimental acute GvHD. Irradiated BN recipients were transplanted with $30\times10^6$ T cell-depleted donor PVG.7B BM cells (△) and received a DLI 14 d later (▲) of graded doses of either (**A**, **B**) $2.5\times10^6$ or (**C**, **D**) $1.5\times10^6$ donor PVG.7B LNC. Two rats that received BM cells only (no DLI) and did not develop GvHD are also shown in panel C. $0.5–1\times10^6$ MSC from PVG.1U (**A**, **B**) or $1–2\times10^6$ MSC from PVG (**C**, **D**) were injected either repeatedly on 0, 7, and 14 d (bold line, 3× MSC) or once on 14 d (solid line, 1× MSC) after DLI. Control rats received no MSC (dashed line, no MSC). (**A**, **C**) Cumulative survival is depicted as Kaplan-Meier plots. (**B**, **D**) GvHD symptoms, including relative change in body weight, were assigned discrete values using a semi-quantitative scoring table adapted from Cooke *et alia* [33]. GvHD scores are shown together with the median (horizontal line) at 7 d and 14 d after DLI, respectively. The respective MSC treatment protocols had no statistically significant effect on overall survival nor the GvHD score of rats treated with 3× MSC (●) or 1× MSC (●) compared to the controls (○). Data were pooled from (**A**, **B**) two and (**C**, **D**) four individual experiments.
(TIF)

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## Author Contributions

Conceived and designed the experiments: SZ MYW GK BR JTV. Performed the experiments: SZ MYW. Analyzed the data: SZ BR JTV. Contributed reagents/materials/analysis tools: PG GK. Wrote the paper: SZ JTV.

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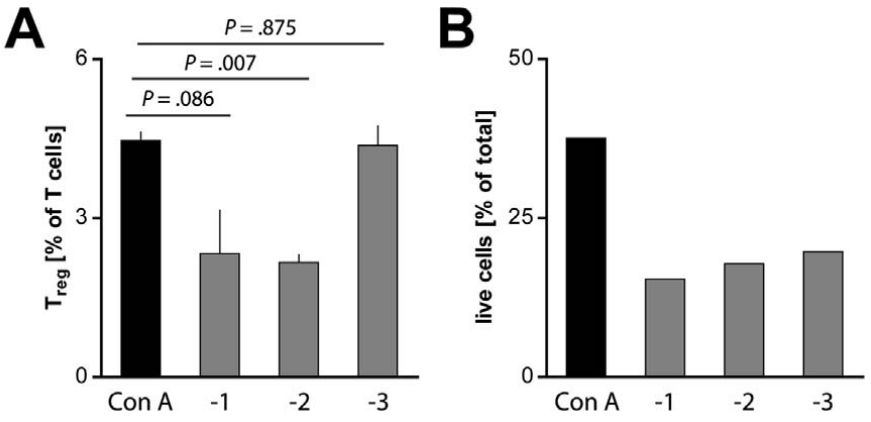
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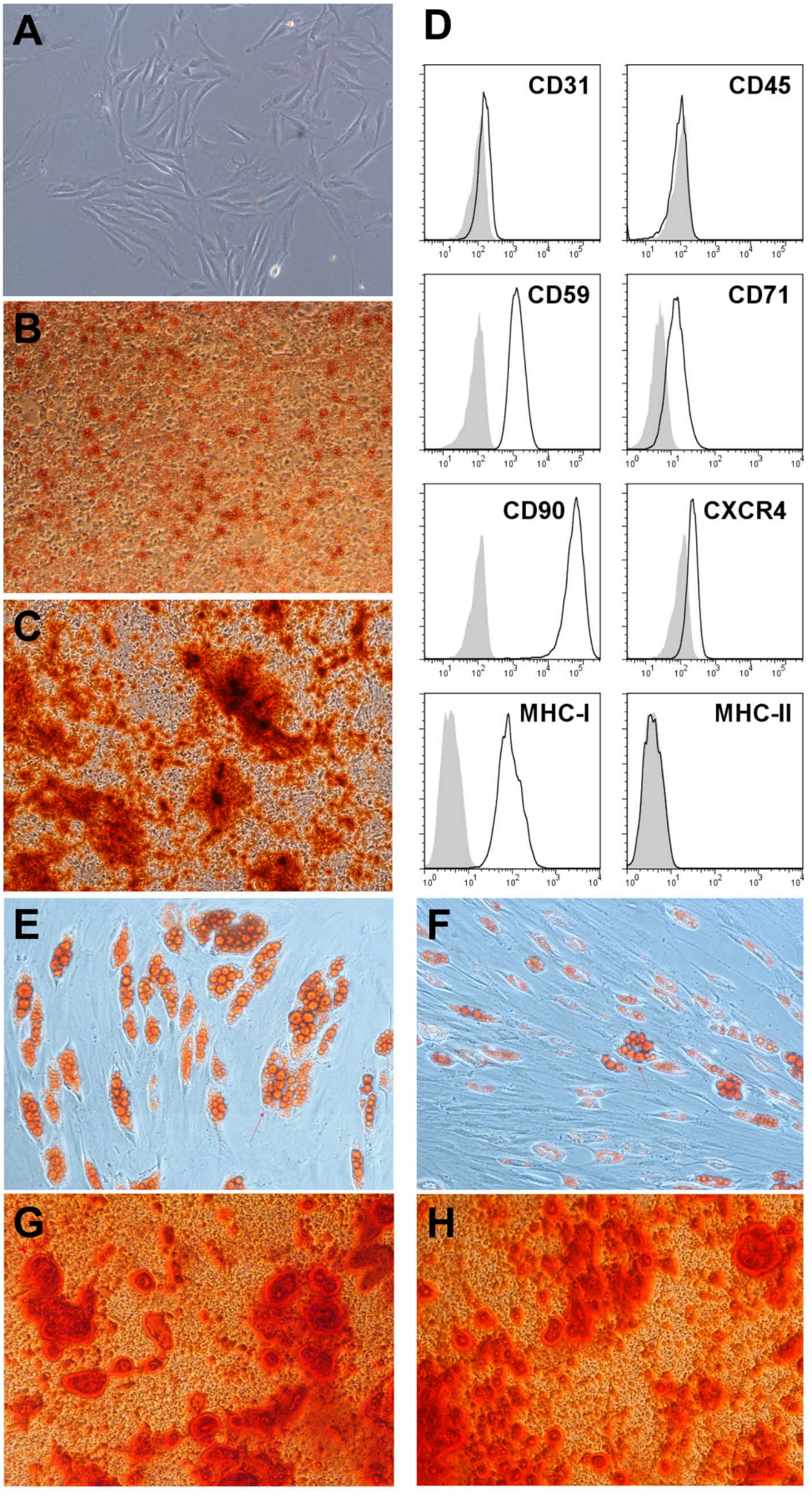
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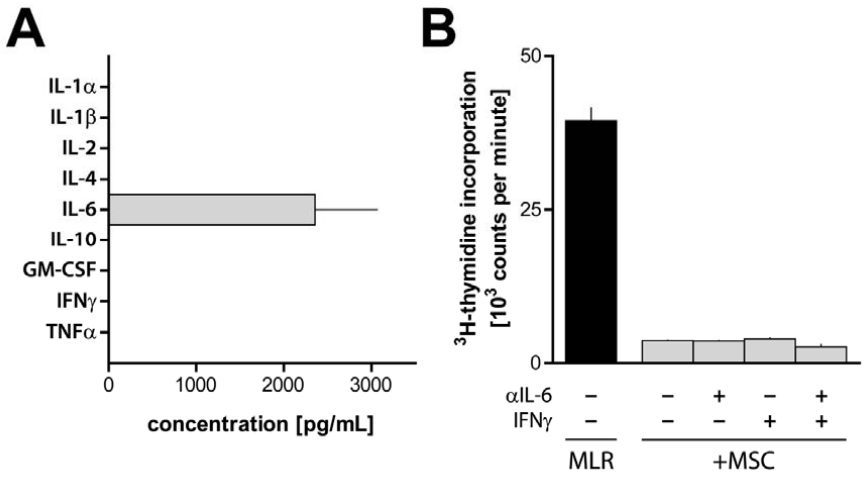
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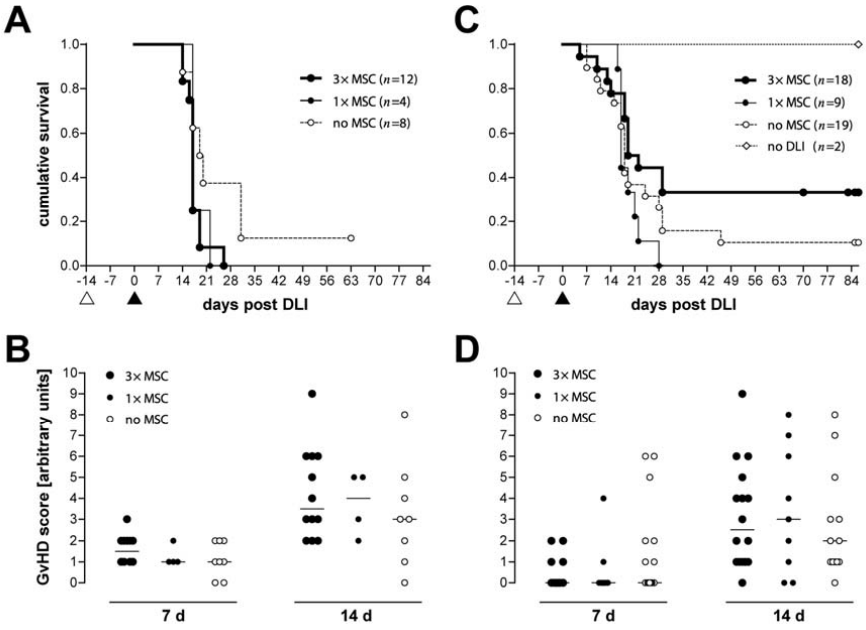
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