

Aus dem Institut für Transplantationsimmunologie
der Medizinischen Hochschule Hannover

**Phenotypical and functional
characterization of donor NK and T
cells in the peripheral blood of lung
transplant recipients**

Dissertation

zur Erlangung des Doktorgrades der Medizin
in der Medizinischen Hochschule Hannover

vorgelegt von
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aus Münster

Hannover 2022

Angenommen vom Senat der Medizinischen Hochschule Hannover am 01.11.2022

Gedruckt mit Genehmigung der Medizinischen Hochschule Hannover

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1. Abbreviations

ACR	acute cellular rejection
ADCC	antibody-dependent cell-mediated cytotoxicity
APC	antigen presenting cells
aKIR	activating killer cell immunoglobulin-like receptors
BOS	bronchiolitis obliterans syndrome
CD	cluster of differentiation
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CIT	cold ischemic time
CLAD	chronic lung allograft dysfunction
COPD	chronic obstructive pulmonary disease
CO₂	carbon dioxide
DAMP	damage-associated molecular patterns
DLTx	double lung transplantation
DMSO	Dimethylsulfoxide
DSA	donor-specific antibodies
DSO	Deutsche Stiftung Organspende
EDTA	Ethylenediaminetetraacetic acid
EVLP	<i>ex vivo</i> lung perfusion system
FBS	fetal bovine serum
FEV₁	forced expiratory volume in one second
FiO₂	fractional inspired oxygen
HLA	human leukocyte antigen
Ig	immunoglobulin
iKIR	inhibitory killer cell immunoglobulin-like receptors
IL	interleukin

ILC	innate lymphoid cell
IPF	idiopathic pulmonary fibrosis
IRI	ischemia reperfusion injury
ISHLT	International Society for Heart and Lung Transplantation
ITAM	immunoreceptor tyrosine-based activating motifs
ITIM	immunoreceptor tyrosine-based inhibitory motifs
KIR	killer cell immunoglobulin-like receptors
LTx	lung transplantation
MHC	major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B
NCR	natural cytotoxicity receptors
NK	natural killer
SLTx	single lung transplantation
O₂	oxygen
OCS™	Organ Care System™
PAMP	pathogen-associated molecular patterns
PaO₂	arterial oxygen partial pressure
PBMC	peripheral blood mononuclear cells
Pen/Strep	Penicillin/Streptomycin
PF	pulmonary fibrosis
PGD	primary graft dysfunction
PRR	pattern recognition receptors
PIPH	Primary idiopathic pulmonary hypertension
RAS	restrictive allograft syndrome
ROS	reactive oxygen species
SCS	static cold storage
TCR	T cell receptor

TLR	toll-like receptor
TRM	tissue-resident memory T cells

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3. Introduction

3.1. Lung transplantation

For end-stage organ failure, solid organ transplantation is the only curative treatment option. In order to handle those severe diseases 2667 solid organ transplantations have been performed in 2019 in Germany, among these are kidney, liver, lung, heart, pancreas and gut transplantations (Deutsche Stiftung Organspende Jahresbericht 2019). Lung transplantations (LTx) account for a rather rare proportion of all solid organ transplantations especially compared to kidney and liver transplantations (Deutsche Stiftung Organspende Jahresbericht 2019). 361 LTx were executed in 2019 in Germany, 103 at Hannover Medical School, making it Germany's most important and largest lung transplantation center (Deutsche Stiftung Organspende Jahresbericht 2019).

Worldwide 4554 patients were lung transplanted in 2016, among these double lung transplantations (DLTx), in which right and left lungs are transplanted, make up the majority compared to single lung transplantations (SLTx) (Chambers et al., 2019).

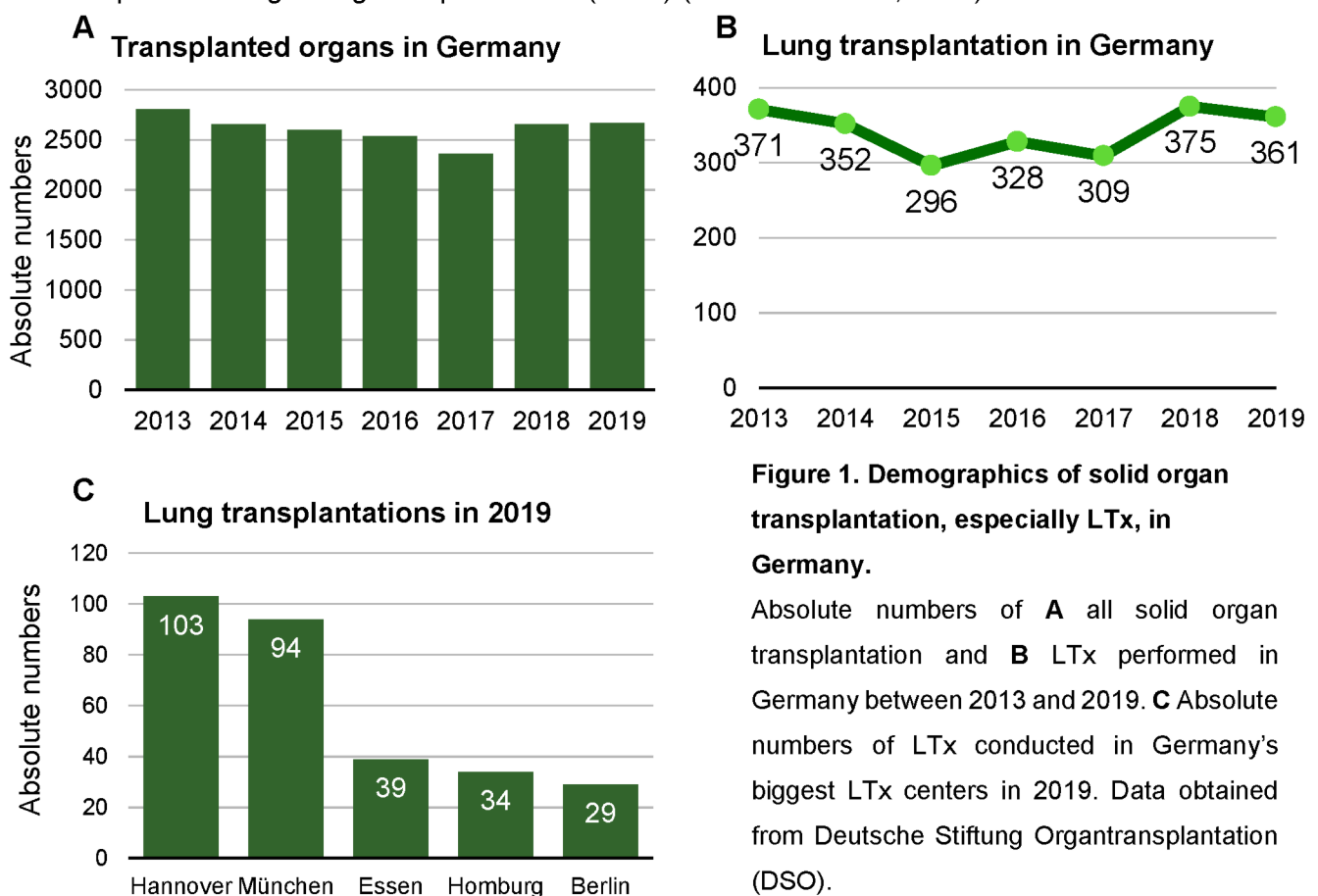


Figure 1. Demographics of solid organ transplantation, especially LTx, in Germany.

Absolute numbers of **A** all solid organ transplantation and **B** LTx performed in Germany between 2013 and 2019. **C** Absolute numbers of LTx conducted in Germany's biggest LTx centers in 2019. Data obtained from Deutsche Stiftung Organtransplantation (DSO).

The human lung represents the main organ regarding respiration and acid-base balance. Its most important function is gas exchange between air and blood, in which blood is enriched with oxygen and waste carbon dioxide is removed.

The human lung consists of a right and a left lung located in the thorax. Whereas the right lung can be divided into three lobes, the left lung only comprises two lobes. Furthermore, these lobes can be subdivided into functional bronchopulmonary segments, ten on the right and nine on the left side.

Inhaled air flows into the nose and via throat, larynx and trachea into the bronchial system, which main function is the air conduction. From the bifurcation of the trachea, the bronchial system splits up twenty times dichotomously and finally ends with the bronchioles and alveoli. With its surface of about 100 square meters the alveoli represent the location of gas exchange. The microscopic structure of the alveoli is perfectly adjusted for gas exchange. Alveoli are mainly build by type I pneumocytes, accounting for 95% of the surface of the alveoli and presenting the main component of the air-blood barrier. Type I pneumocytes are flat epithelial cells in contact with capillaries carrying blood that is low in oxygen. Via diffusion, possible only because of the flat layer, the blood is enriched with oxygen. Besides, type II pneumocytes are present in the alveoli. These cells produce a substance called surfactant that lowers surface tension of the alveoli reducing breathing work and therefore allowing a normal and easy respiration.

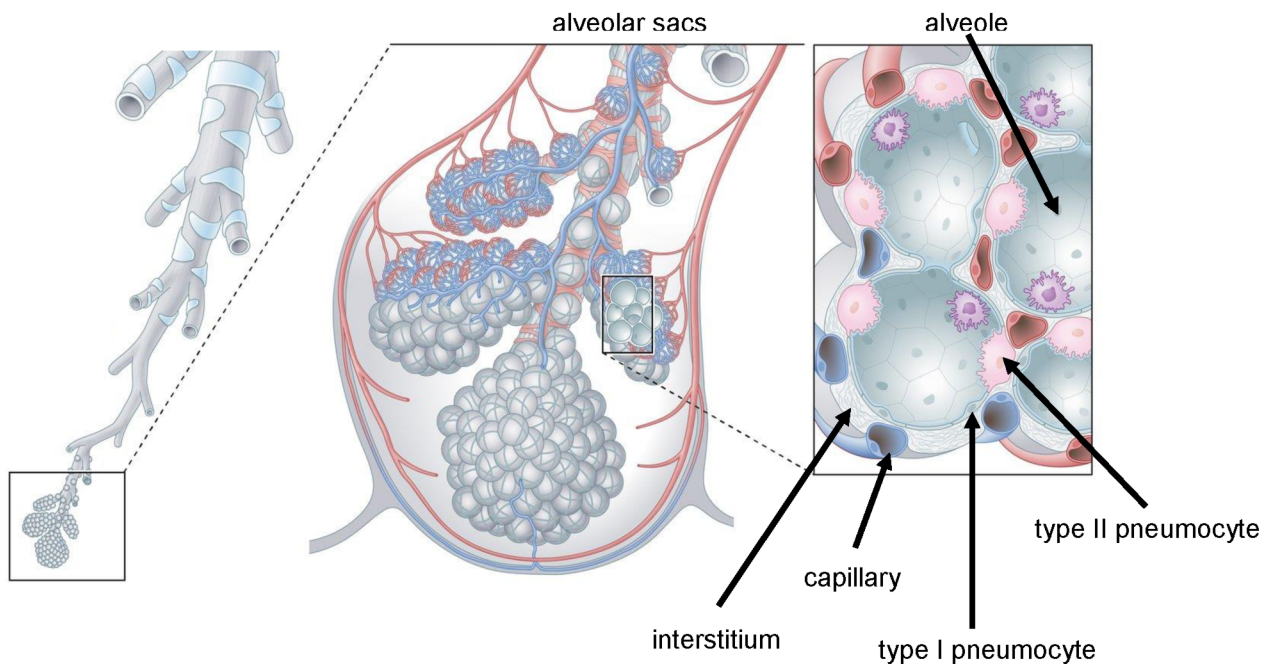


Figure 2. Anatomical structure of the sites of gas exchange. On the left side, the splitting of the bronchial tree is illustrated with a detailed structure of the alveolar sacs, where gas exchange takes place, in the middle. On the right, the microscopic structure of the alveoli with its air-blood barrier is shown. Modified from amboss.com

(<https://next.amboss.com/de/article/6o0j1S?q=lunge#Z301082c0f1f91662815fe3a03ec6320f>) retrieved on 30th of June 2021 at 10:00.

3.1.1. History of lung transplantation

The origin of the word transplantation lays in the Latin word 'transplantatio' meaning a medical procedure where organs or tissue are removed from a healthy donor into a diseased recipient. In human transplantation, autografts, in which donor and recipient are the same, and allografts, where donor and recipient are different individuals but from the same species, can be distinguished (Definition organ transplantation). A suitable example for autografts is skin transplantation whereas all types of solid organ transplantation in humans are allografts.

Over the past decades, LTx has become a well-established treatment option for end-stage respiratory diseases, though this took longer than for other solid organ transplantations. After some noticeable attempts of LTx in animals in the early and mid-20th century, James D. Hardy performed the first human LTx on the 15th of April 1963 at the University of Mississippi Hospital. He executed a left lung transplantation and the patient's survival was 18 days (Venuta

and Van Raemdonck, 2017). Due to very poor outcomes, nearly no LTx was performed in the 1970s. However, because of an increasing experience regarding immunosuppression, improvements in surgical technique and choosing appropriate donor–recipient combinations more lungs with a better long-term survival were transplanted in the 1980s, for example a SLTx 1983, in which the patient survived for more than seven years (Venuta and Van Raemdonck, 2017).

In 1986, the first successful DLTx was conducted at the University of Toronto. Surgeons achieved this by a modification of the operation procedure where two SLTx were performed instead of an en-bloc DLTx, which was common before then, but causing an insufficiency of tracheal anastomosis (Patterson et al., 1988). Nowadays DLTx is the preferred operating technique.

Since then the number of realized LTx has increased consistently worldwide. Today it is even possible to perform living-donor LTx e.g. at Hannover Medical School in 2012.

3.1.2. Indications for lung transplantation

The main function of the lung is gas exchange (O_2 and CO_2) between blood and the environment. Oxygen (O_2) is one of the central molecules keeping the metabolism of every body cell working; therefore, severe respiratory failure is an acute life-threatening situation. Due to the underlying pathomechanism or damaged anatomic structures of the lung, the major indications leading to LTx are obstructive, fibrotic, septic and vascular lung diseases (Yeung and Keshavjee, 2014).

Among these in Germany, principally chronic obstructive pulmonary disease (COPD), interstitial lung diseases like idiopathic pulmonary fibrosis (IPF) and cystic fibrosis (CF) represent major indications accounting for up to 70% of all indications for LTx. Whereas, primary idiopathic pulmonary hypertension (PIPH), a form of vascular lung disease plays a subordinated role (Deutsche Stiftung Organspende Jahresbericht 2019).

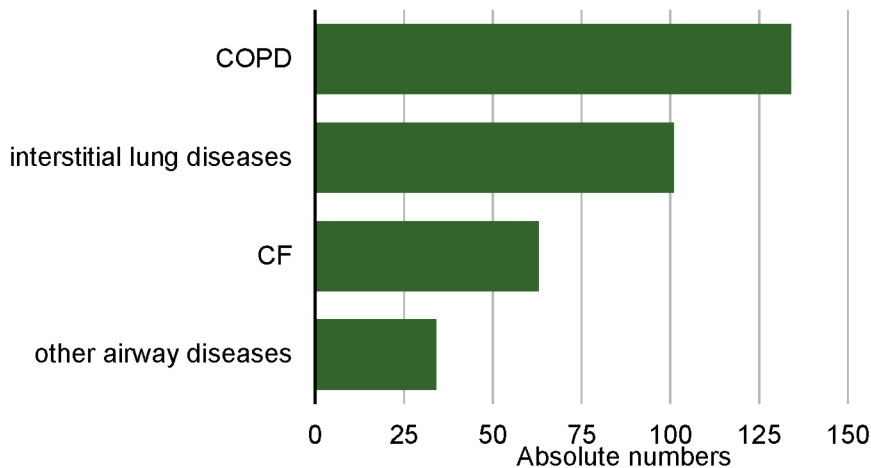


Figure 3. Indications for LTx in Germany.

Absolute numbers of the four main end-stage lung diseases leading to LTx in 2019. Data obtained from Deutsche Stiftung Organtransplantation (DSO).

COPD is a chronic and progressive lung disease leading to an almost irreversible restriction of airflow especially during expiration. Particularly exposure to inhaled noxious agents like tobacco smoke and particulate matter but also endogenous factors like Alpha-1 antitrypsin deficiency result in a chronic inflammatory response of the lung causing fibrotic reconstruction and bronchial instability. Hence, during expiration bronchial collapse occurs arising chronic obstruction, air trapping and finally pulmonary emphysema. Main symptoms of patients suffering from COPD are dyspnea, chronic coughing, sputum and ultimately leading to respiratory insufficiency (Barnes et al., 2015). As COPD is the third most common reason for death worldwide, this explains COPD being the most common cause for LTx in Germany (Deutsche Stiftung Organspende Jahresbericht 2019).

The second biggest group of diseases leading to LTx in Germany are interstitial lung diseases resulting in pulmonary fibrosis (PF), which can be described as irreversible reconstruction and growth of connective tissue in the lung inducing loss of function and poor prognosis (Martinez et al., 2017). Interstitial lung diseases can be divided into two groups. On the one hand interstitial lung develop in the context of other (especially autoimmune) diseases like sarcoidosis or as a reaction to noxious agents like asbestos and cytotoxic drugs, on the other hand interstitial lung diseases develop idiopathically, meaning no certain cause can be found. Among these, IPF represents the greatest group. Pathophysiological relevant aspects are the 'genetic architecture affecting epithelial cell integrity, environmental factors and aging-

associated changes' (Martinez et al., 2017) resulting in an abnormal epithelial cell activation.

Most relevant symptoms are exertional dyspnea and dry coughing.

CF is the third most relevant illness causing LTx. CF is an autosomal recessive inherited disorder induced by a malfunction of the *cystic fibrosis transmembrane conductance regulator* (CFTR) gene. About 2000 different gene mutations have been detected (Ratjen et al., 2015). CFTR is a chloride channel in membranes of excretory cells such as goblet cells in the whole body. Due to a malfunction of this channel, a more viscous secret than normal is produced blocking the excretory duct of those cells. Over time, this obstruction provokes chronic inflammation and eventually organ failure. As this chloride channel does not only exist in the respiratory system but also in pancreas and the gastrointestinal tract, these organs are affected as well as the liver and the bile system. In northern Europe, one of every 3000 newborns is born with this genetic defect. Furthermore CF implies a reduction in life expectancy; the median survival is limited to the early thirties (Ratjen et al., 2015).

Lastly, another indication for LTx is re-transplantation (Re-LTx) due to graft failure of the primary transplant, representing the endpoint of complications in the field of LTx.

3.1.3. Complications after lung transplantation

The median survival rate worldwide in primary LTx between 2010 and June 2017 was 6.7 years (Chambers et al., 2019). Even though this rate improved over the last decades, long-term outcomes in LTx are the poorest as compared to other solid organ transplantations (Hachem, 2019).

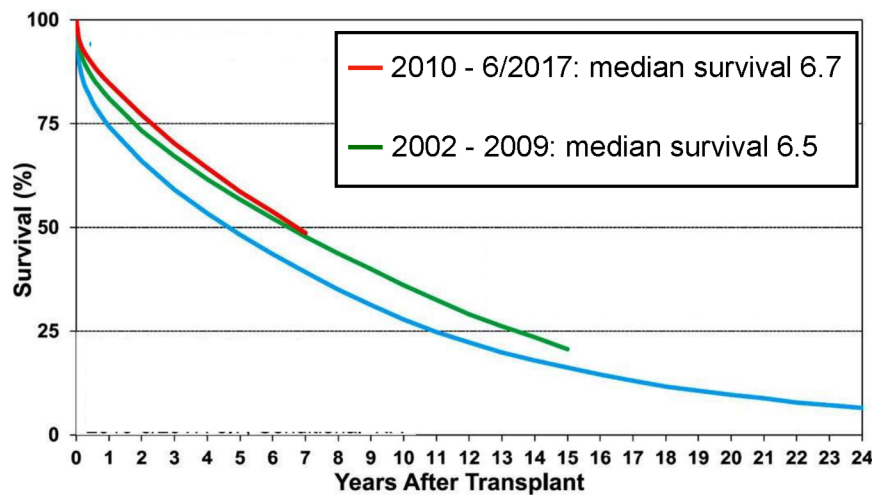


Figure 4. Median survival rates of lung transplant recipients according to decade. Median survival in years of lung transplanted patients between 1992 and 2001 (blue), 2002 and 2009 (green) and 2010 and June 2017 (red). Graph adopted from the 36th adult lung and heart-lung transplantation report — 2019 of the International Society for Heart and Lung Transplantation (ISHLT) (Chambers et al. 2019).

The reasons for this rather short survival after LTx are diverse. Early after LTx primary graft dysfunction (PGD) and infections play a relevant role, whereas the main cause of graft failure after the first year of LTx is chronic lung allograft dysfunction (CLAD) (Fig. 5) (Hachem, 2019).

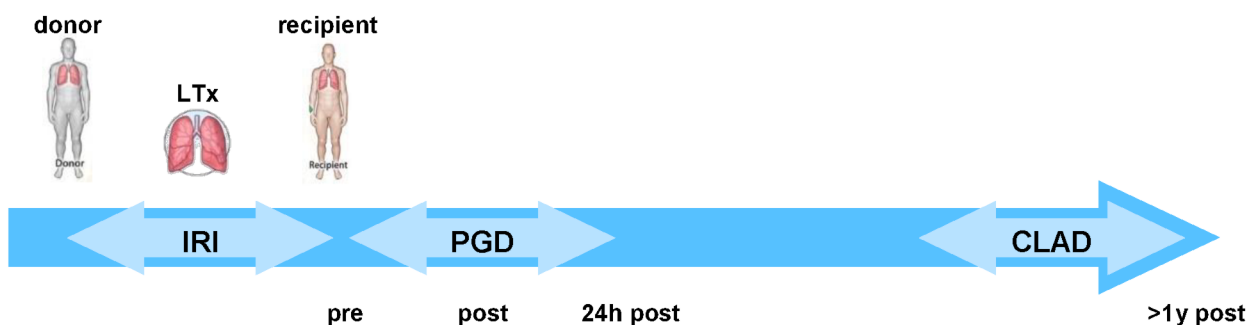


Figure 5. Chronology of IRI, PGD and CLAD in LTx.

Besides these two major complications, several other forms of acute rejection can be discriminated. Hyperacute rejection occurs within the first 24 hours after LTx, where pre-existing donor-specific antibodies (DSA) bind human leukocyte antigen (HLA) molecules on endothelial cells resulting in an activation of the complement system causing damage of the transplanted lung (Hachem, 2019). Due to enormous improvements in the detection of harmful

DSA hyperacute rejection nowadays plays only a minor role. Another relevant type of acute rejection — particularly in LTx — is acute cellular rejection. About 30% of all lung-transplanted patients experience at least one episode of acute cellular rejection within the first year after LTx requiring treatment. Due to the HLA mismatch between donor and recipients LTx, recipient T cells may recognize donor alloantigen triggering immune responses such as CD8⁺ T cell-mediated cytotoxicity (Koutsokera et al., 2018). Furthermore, antibody-mediated rejection in the context of LTx is mainly caused by B cells.

3.1.4. Primary graft dysfunction

PGD describes an acute lung injury evolving within the first 72 hours after LTx (Lee et al., 2010) and represents one of the main reasons for early morbidity and mortality. 10-25% of all LTx patients suffer from PGD. In 2005, the International Society for Heart and Lung Transplantation (ISHLT) developed a grading system for PGD severity grouping. As shown in Table 1, four grades can be distinguished according to present infiltrates in chest x-rays and the Horovitz Index, which is a marker for oxygenation impairment. These parameters are measured at least every 24 hours within the first 72 hours after LTx (Christie et al., 2005).

Table 1. Severity grades of PGD in LTx.

PGD grade	PaO ₂ /FiO ₂ ratio = Horovitz Index in mmHg	Radiological infiltrates in chest X-ray indicating edema
0	>300	Absent
1	>300	Existent
2	200-300	Existent
3	<200	Existent

Grading of both indices is carried out within the first 6 hours and every 24 hours for 3 days after LTx according to the guidelines of ISHLT. Table adopted from Christie et al., 2005. PaO₂: arterial oxygen partial pressure, FiO₂: fractional inspired oxygen.

The pathogenesis of PGD is multifactorial and its severity depends among other factors on the condition of both donor and recipient during the transplantation procedure regarding e.g. size-matching, selection and cause of brain death but also on genetic predisposition of the organ recipient (Suzuki et al., 2013). Furthermore, one of the main causes for PGD is ischemia reperfusion injury (IRI). Ischemia in general describes a status in which an organ is due to the lack of circulating blood not sufficiently oxygenated leading to severe tissue damage and necrosis. In LTx, ischemia occurs during organ transport and reperfusion after implantation of the donor organ into the recipient. Due to the an undersupply in O₂ during hypothermic aerobic preservation and reperfusion, reactive oxygen species (ROS) are released in particular by donor alveolar macrophages and lung endothelial cells initiating an inflammatory cascade with cytokine release and activation of the immune system resulting in immigration of immune cells such as recipient neutrophils and lymphocytes into the lung.

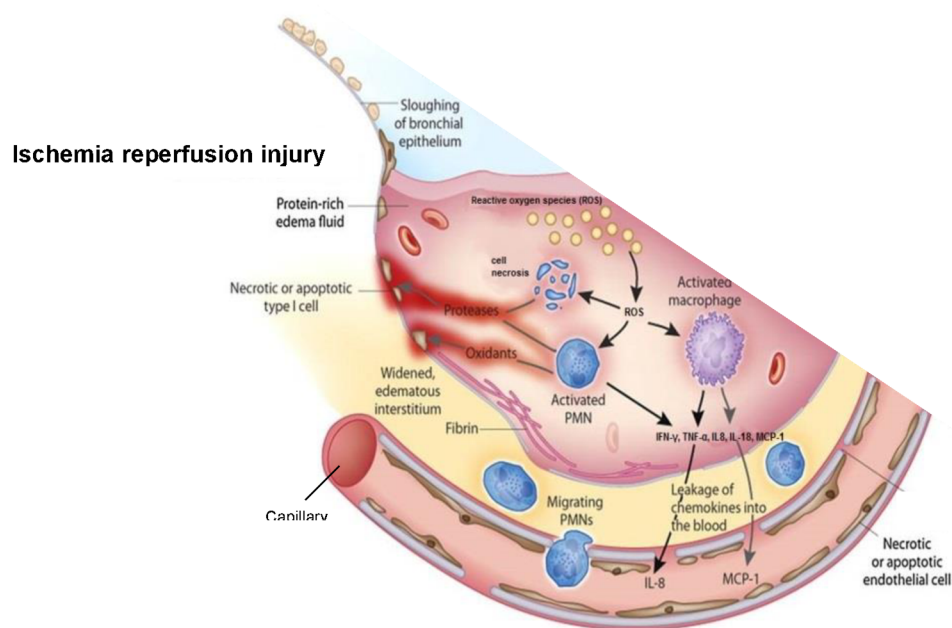


Figure 6. Mechanism of ischemia reperfusion injury in LTx. The figure illustrates the main mechanisms in an alveoli occurring during IRI with cell necrosis and apoptosis, interstitial edema, leakage of capillaries and severe activation of the immune system leading to impairment of gas exchange and inflammation. Modified from Diep et al., 2010.

Especially those neutrophils and lymphocytes maintain a sterile lung inflammation leading to diffuse alveolar damage and pulmonary edema concluding in poor oxygenation at an end-stage (Laubach and Sharma, 2016; Lee et al., 2010; Suzuki et al., 2013).

Regarding PGD only supportive but no causal therapy is available e.g. lung protective ventilation, early mobilization and as an *ultima ratio* extracorporeal membrane oxygenation. Furthermore, a reduction of risk factors in relation to IRI is helpful e.g. improvements in preservation of explanted organs during transport (Shah and Diamond, 2018).

In addition, complications within the first days after LTx, concrete PGD, affect the long-term outcome, as there is a direct connection between the severity of PGD and the development of CLAD (Royer et al., 2016).

3.1.5. Chronic lung allograft dysfunction

CLAD represents one of the major causes of death at a later stage (Verleden et al., 2019) and is defined as an irreversible reduction of forced expiratory volume in one second (FEV₁) of more than 20% compared to baseline FEV₁ levels over more than three month, where no other causes for this reduction like e.g. the normal aging process, infection or surgical factors can be found (Verleden et al., 2019). 50% of all LTx patients may develop CLAD within five years after LTx (Royer et al., 2016). Depending on the seriousness of FEV₁ reduction CLAD can be divided into five degrees: CLAD 0 to CLAD 4 (Verleden et al., 2019).

In the clinical setting three typical types of CLAD exist. The most common type is the *bronchiolitis obliterans* syndrome (BOS) comprising the already mentioned reduction of FEV₁ of more than 20% plus a diminished *Tiffeneau Index* and a lack of radiological correlate. As the *Tiffeneau Index* is an indicator for obstruction this is the leading symptom concerning BOS (Verleden et al., 2019). In contrast the restrictive allograft syndrome (RAS) is a more seldom manifestation of CLAD but is associated with a worse outcome. RAS can be specified as a combination of reduced FEV₁ of more than 20%, a decreased total lung capacity of under 90%

of the normal baseline, indicating restriction, and present infiltrates in chest X-ray (Verleden et al., 2019). Furthermore, a combination of restriction and obstruction can be present in patients. Among the mechanisms leading to CLAD the immune system is one of the driving factors reacting to endogenous and exogenous patterns causing inflammation and damage in the transplanted lung as it responds to this with wound-healing and fibrosis (Royer et al., 2016). Therapeutic options are very low and mostly experimental. Common options are a change of the chemotherapeutic regime, reduction of gastroenteral reflux and for certain patient groups even re-transplantation.

As chronic rejection and infection are responsible for about 70% of deaths within the first year after LTx, this shows the importance of the immune system with regard to patient survival. Therefore, a better understanding of the contribution of immune responses in the field is critically needed (Hachem, 2019).

3.1.6. Organ preservation

Besides improvements in aspects like surgical procedure also more specified organ preservation techniques and the ischemic time play a crucial role on the outcome after LTx as the highest quality of donor organs leads to best survival rates (Jing et al., 2018). Due to the shortage of donor organs, organs with extended donor criteria become more and more relevant and organ preservation might be a possibility to improve organ function.

In the clinical setting two preservation systems are commonly used. On the one hand there is the static cold storage (SCS), which has been the gold standard for several decades. In this preservation type, the donor organ is flushed with a hypothermic perfusion solution, mimicking the human blood, and stored cold (4°C) until transplantation. Therefore this period is called cold ischemic time (CIT). The main aim of SCS is the reduction of metabolism and therefore, cause less harm to the organ (Jing et al., 2018). On the other hand, the *ex vivo* lung perfusion system (EVLP) is used in clinical studies. EVLP systems provide O₂ and metabolites to maintain an almost physiological environment for the donor organ while being out of the human

body. Furthermore, EVLP systems provide an opportunity to assess and even improve lung function outside the human body prior to transplantation (Jing et al., 2018). In general, three different EVLP systems are available on the market (Nakajima and Date, 2021). One of them is the Organ Care SystemTM (OCSTM) developed by TransMedics, Andover, USA. It is the only portable EVLP system worldwide and was used during the INSPIRE trial at Hannover Medical School. The INSPIRE trial is a multi-center phase 3 trial comparing SCS and OCS concerning the endpoints absence of PGD grade 3 after 72 hours after LTx and 30-day survival. Concluding marks of this trial are a reduction of PGD grade 3 after 72 hours, but no benefit in short-term survival (Warnecke et al., 2018).

3.2. Immune system

The human being lives in an environment with other species that appear as potential pathogens as there is a consistent exchange of substances between human being and environment e.g. while breathing or eating. Over millions of years, the human organism has developed several mechanisms and strategies to defend itself against possible life-threatening pathogens. Those mechanisms and strategies can be summarized as the immune system.

In general the innate immune system can be distinguished from the adaptive immune system both deeply interwoven with each other.

In the context of LTx, the immune system plays a crucial role, as the implanted graft is considered as foreign and therefore, the immune system is activated causing acute or chronic rejection as well as infection. Both aspects are leading reasons for death after LTx (Hachem, 2019). To diminish the risk of rejection lung transplanted patients need to take immunosuppressive drugs lifelong.

3.2.1. Innate immune system

The innate immune system is the first line defense against pathogens. As it recognizes evolutionally highly conserved molecular patterns, present on many different microorganisms, it reacts rather unspecific but in a fast manner after activation. Besides permanently existing mechanisms, like physical or chemical barriers, e.g. the skin, and antimicrobial peptides, e.g. granzymes, most mechanisms of the innate immune system are inducible by infection.

Cells of the innate immune system are predominantly myeloid and lymphoid cells developing from hematopoietic stem cells.

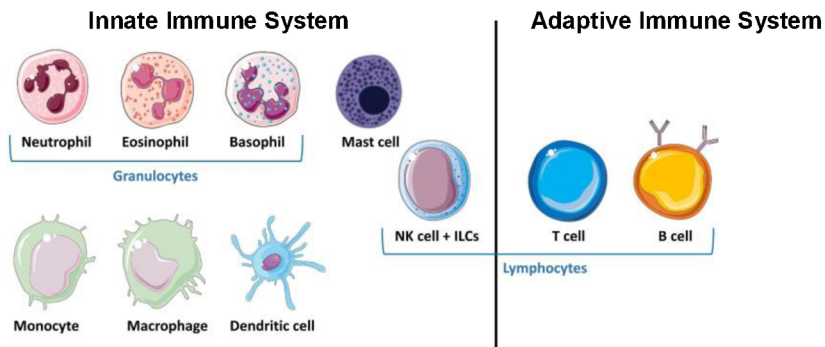


Figure 7. Overview of the key cells of the innate and adaptive immune system. The innate immune system (left side) is the first line defense against pathogens, whereas the adaptive immune system (right side) obtains a specialized and very effective immune response to certain pathogens, lasting hours to days until full efficiency and even generates memory.

Special tissue-resident and blood cells express pattern recognition receptors (PRR) like e.g. toll-like receptors (TLR) on their surface. Those PRR are a group of germline-encoded proteins binding to pathogen-associated molecular patterns (PAMP) e.g. lipopolysaccharides that are only present on pathogens but not on human cells and are crucial to survival of those pathogens. Similarly to PAMP, so-called damage-associated molecular patterns (DAMP), where loss of function caused by infection is detected, have been described. Upon binding of PRR and PAMP, cytokines and chemokines such as IL-8 are released causing migration of innate immune cells to the site of pathogens. Firstly, neutrophils move to the site of infection on the one hand to phagocytose present pathogens, on the other hand to liberate factors of the complement system starting the complement cascade, and furthermore to release granule inducing apoptosis and necrosis of the pathogens. Secondly, monocytes, especially macrophages, and dendritic cells travel to the site of infection. The main function of macrophages in this setting is phagocytosis and antigen presentation, whereas dendritic cells only perform antigen presentation (Gasteiger et al., 2017; Medzhitov and Janeway, 2000; Turvey and Broide, 2010).

Besides the above-described mechanisms of recognizing microbial pathogens, other methods of the innate immune system are identifying 'altered self' and 'missing self'. Especially in the

context of missing self, as this also may be crucial in LTx, natural killer (NK) cells are of great importance.

3.2.1.1. NK cells

NK cells account for about 5-15% of all lymphocytes in peripheral blood of healthy donors (Yu et al., 2013), but they can also be found in tissue and organs. NK cells were recently integrated into the innate lymphoid cell (ILC) system as one of the two ILC1 subsets (Eberl et al., 2015). Originating from lymphatic hematopoietic stem cells, NK cell maturation takes place in the bone marrow. Generally, NK cells are larger in size than B and T cells, containing granules, and they do not express antigen-specific receptors on their surface (Yu et al., 2013).

The functions of NK cells are diverse. On the one hand, they are able to kill virus-infected or malignant cells, on the other hand they regulate various crucial steps in immune responses for example via cytokine release (Carrega and Ferlazzo, 2012).

NK cells can be described according to their surface receptors as CD56⁺NKp46⁺CD3⁻CD19⁻CD14⁻ and can be further subdivided regarding their level of expression of CD56 and CD16. CD56 is an adhesion molecule of leukocytes and endothelial cells conveying migration. CD56^{bright}CD16^{low} NK cells, so called 'brights', predominantly present in secondary lymphoid tissue, produce high amounts of cytokines, such as γ -interferon, and chemokines and therefore migrate into tissue e.g. of liver and tonsils but have a low killing capability. Furthermore, CD56^{bright} NK cells were shown to be the precursor cells to CD56^{dim}CD16^{high} NK cells, also known as 'dims', CD56^{dim} NK cells are the main cohort of NK cells in the peripheral blood and the lung. In contrast to CD56^{bright} NK cells, they are very efficient killers, due to the fact that they produce high amounts of granzyme and perforin, but only produce low amounts of cytokines and chemokines (Yu et al., 2013).

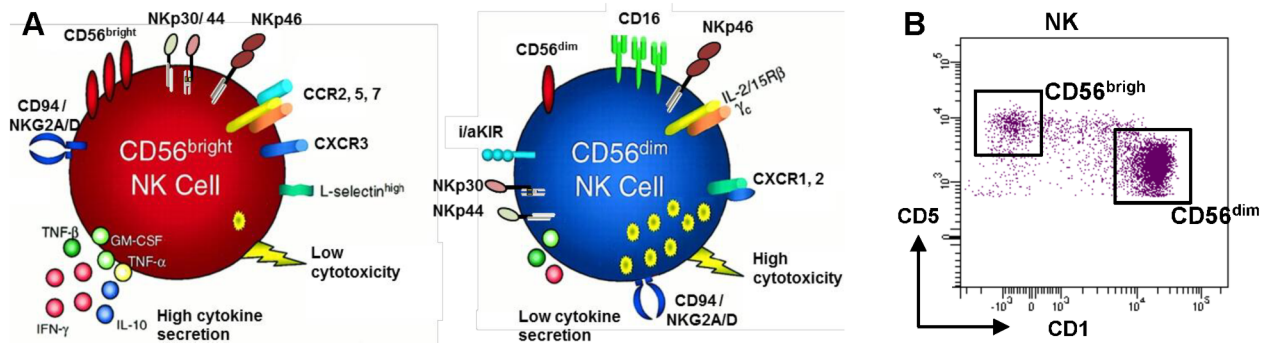


Figure 8. The main subsets among human NK cells — DC56^{dim} and CD56^{bright}. **A.** Typically expressed surface receptors on DC56^{dim} versus CD56^{bright}. Modified from Cooper et al., 2001. **B.** Representative flow cytometry plot regarding CD56 and CD16 expression on DC56^{dim} and CD56^{bright}.

NK cells are regulated by a close interplay of their activating and inhibitory surface receptors. Crucial for their final function is the integration of those different signals.

Typically virus-infected cells as well as tumorous cells show certain signals that can be detected by NK cells, among these are major histocompatibility complex (MHC) class I molecules.

Under normal, healthy circumstances MHC class I molecules, also known as HLA class I molecules, bind to inhibitory receptors on NK cells leading to inhibition of NK cells and therefore no killing of target cells. Malignant or virus-infected cells are lacking MHC class I molecule expression to escape killing by cytotoxic T cells. As NK cells bind to target cells with reduced or missing MHC class I molecules on their surface, they are not sufficiently inhibited but more activated causing degranulation of NK cells and killing of target cells. In literature, this is also known as the ‘missing-self hypothesis’ (Ljunggren and Karre, 1990; Raulet, 2006).

Other signals of target cells that can be detected by NK cells are natural cytotoxicity receptors (NCR) that are activated due to binding e.g. special virus epitopes or MICA and MICB, up regulated upon stress and recognized by the activating receptor NKG2D.

As mentioned above NK cells express CD16, also known as Fc γ RIIIa. As aberrant cells are often already marked by the immune system, Fc γ RIIIa binds the Fc-region of a specific IgG

bound the target cell leading to activation and so called antibody-dependent cell-mediated cytotoxicity (ADCC) (Topham and Hewitt, 2009; Vivier et al., 2011).

3.2.1.2 Killer cell immunoglobulin-like receptors (KIR)

In general, three groups of NK cell receptors can be distinguished. Among these are KIR, NCR and C-type lectin receptors.

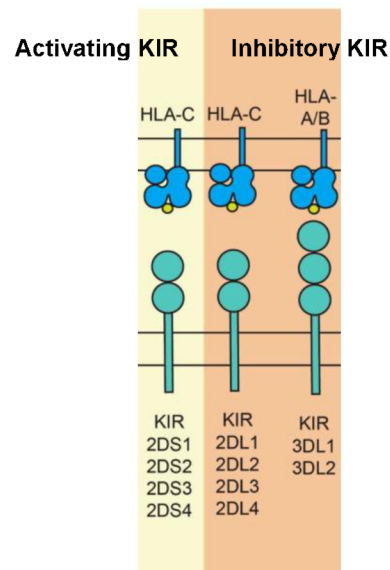
KIR are coded by 15 genes on chromosome 19 (Marsh et al., 2003) and two standard haplotypes are known A, summarizing rather inhibitory KIR, and B comprised of more activating KIR.

KIR have their origin in the immunoglobulin (Ig) superfamily, therefore they are glycoprotein receptors located in membrane of NK cells. Their extracellular site consists of two or three Ig-like domains KIR2D or KIR3D. Within the cytoplasm, long and short tails of the KIR can be distinguished. A long tail always indicates for an inhibitory KIR (iKIR) due to comprising immunoreceptor tyrosine-based inhibitory motifs (ITIM) which is phosphorylated upon binding to a target cell, whereas a short tail specifies a activating KIR (aKIR) due to immunoreceptor tyrosine-based activating motifs (ITAM) (Lanier, 2005).

In my thesis KIR2DL/S1, KIR2DL/S2/3 and KIR3DL/S1 were analyzed, as they are the most relevant.

The typical ligands of KIR are HLA-A, HLA-B and HLA-C. KIR are more likely to be expressed by CD56^{dim} than CD56^{bright} NK cells.

Figure 9. Simplified illustration of aKIR and iKIR on NK (and T cells) and their ligands on target cells. KIR with two extracellular domains always bind to HLA-C, whereas KIR with three extracellular domains link to HLA-A/B. Images modified from Calabrese et al., 2019.



3.2.1.3. Human leukocyte antigen (HLA)

The human HLA gene complex, also known as major histocompatibility complex (MHC) in animals like e.g. mice can be found on chromosome 6 and is one of the most polymorphic gene loci in human (Ju et al., 2019). In order to summarize HLA's function it can be named the 'identity card of cells', therefore its functions are the differentiation between self and non-self, antigen presentation to T cells and regulation of immune responses (Lin and Yan, 2019). To handle the high variability of microorganisms, the high polymorphism rate of the HLA gene locus is crucial.

In general, HLA class I molecules can be differentiated from HLA class II molecules (DR, DQ, DM and DP). HLA class I molecules can be further subdivided to HLA class Ia, comprising HLA-A, HLA-B and HLA-C, and HLA class Ib, formed by HLA-E, HLA-F and HLA-G (Crux and Elahi, 2017). Moreover, there exist two alleles, on maternal and one paternal, of each HLA molecule.

HLA class I molecules are expressed on every cell with a nucleus except for erythrocytes. They take up endogenous peptides and therefore, present self, viral and tumor peptides to their ligands on the one hand KIR on NK cells and on the other hand T cell receptors on CD8⁺ T cells. Both ways lead to apoptosis of the target cell.

By contrast, HLA class II molecules are only present on professionally antigen presenting cells (APC) like dendritic cells, B cells and macrophages. They take up exogenous peptides, mostly of bacteria, and present it to their ligand, that is the T cell receptor on CD4⁺ cells initiating the adaptive immune system.

3.2.1.4. Role of NK cells in LTx

In comparison to other solid organ transplantations like e.g. kidney transplantation, in LTx there is no HLA matching performed organ donor and recipient, therefore immunosuppression is essential to prevent alloimmunization. As KIR experience selection in order to avoid reaction to endogenous HLA class I molecules within HLA mismatched LTx, NK cells may react to foreign or missing HLA class I on transplanted cells and may cause — depending on which target cell is depleted — rejection or graft tolerance (Calabrese et al., 2019; Spahn et al., 2014). Donor APC show foreign HLA class I molecules on their surface leading to activation of alloreactive T cells in the recipient causing damage in the transplanted lung. By killing of that donor APC by NK cells graft tolerance and improved survival might be achieved (Jungraithmayr et al., 2013). Furthermore, NK cells are able to kill alloreactive T cells, thus itself inducing graft tolerance.

Contrarily to the above mentioned, ADCC, in which NK cells play a crucial role, is a driving aspect for antibody-mediated rejection. Moreover, the higher expression levels of stress signals on transplanted cells activate NK cells via NKG2D. In conclusion, these factors may cause cytotoxicity and therefore, not induce tolerance but harm to the transplanted lung (Kawakami et al., 2017).

Overall, the amount, timing and context of NK-cell activation critically influence whether NK cells are protective or causing damage to the transplanted lung.

NK cells may be considered as the connection between innate and adaptive immune systems in the field of LTx (Hadad et al., 2014) as e.g. γ -interferon stimulates T cells and NK cells also express MHC class II molecules enabling them for antigen presentation (Fildes et al., 2008).

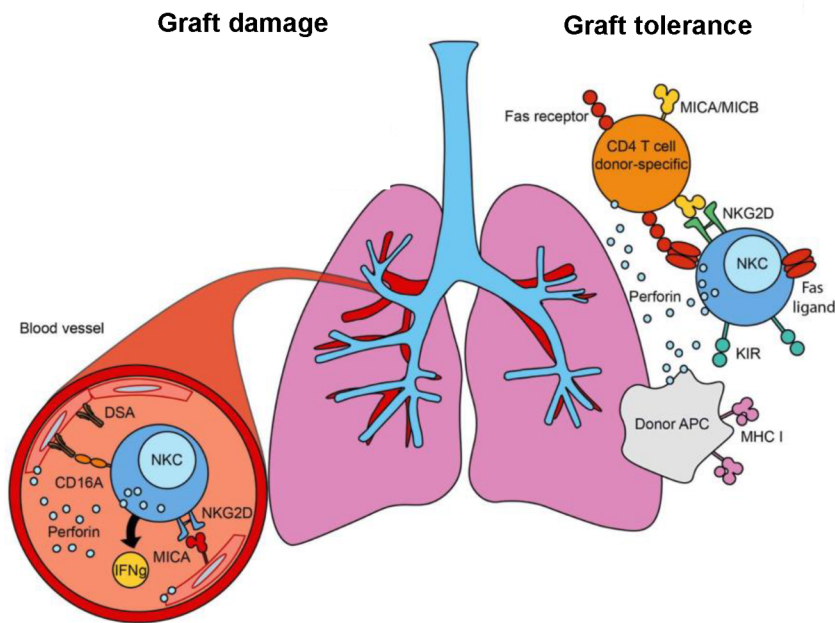


Figure 10. Mechanisms of NK cells in LTx involved in graft damage and tolerance. On the left side, as shown, ADCC and stress signals, detected by NK cells and their surface receptors, play a key role in impairment of the transplanted organ. Contrarily, on the right side NK cells inducing apoptosis of donor APC and T cells leads to graft tolerance. Figure adapted from Calabrese et al., 2019. NKC: NK cell, APC: antigen presenting cell, DSA: donor specific antibody.

3.2.2. Adaptive immune system

In addition to the innate immune system, the adaptive immune system elicits a specialized and very effective immune response to certain pathogens, lasting hours to days until full efficiency. Furthermore, the adaptive immune system can develop immunological memory indicating a faster and more potent immune response against already familiar pathogens. The main players of the adaptive immune system are lymphocytes. Firstly, there are B lymphocytes developing in the bone marrow and producing antigen-specific antibodies after differentiating into mature plasma cells. Secondly, T lymphocytes can be distinguished evolving within bone marrow and thymus.

In contrast, because of the limited ability of the innate immune system to recognize the huge variability of all pathogens, T and B cell receptors are highly variable due to somatic recombination of gene segments encoding for those receptors. Therefore, each lymphocyte expresses a unique and antigen-specific receptor on its surface. After clonal expansion, a large number of antigen-specific lymphocytes is able to eliminate the respective pathogens (Bonilla and Oettgen, 2010; Chaplin, 2010).

As T lymphocytes, besides NK cells, are in the focus of my thesis they are described more detailed in the following.

3.2.1.1. T lymphocytes

T lymphocytes are a rather small cell type, which have their origin in a lymphatic progenitor cell, developing from a pluripotent hematopoietic stem cell in bone marrow. In general, $CD4^+$ T cells, also known as T helper cells, can be distinguished from $CD8^+$ T cells, also known as cytotoxic T cells. According to their maturation status and functionality naive T cells, responding to new, unknown antigens, can be discriminated from memory T cells, crucial in long-term immune reaction to already familiar antigens, and regulatory T cells (Kumar et al., 2018) using surface receptors such as CCR7, CD45RO.

T cell maturation occurs primary within the thymus. Immature progenitor T cells leave the bone marrow into the thymus to proliferate. Afterwards antigen-independent somatic recombination of gene loci, in particular V, D and J segments coding for the T cell receptor (TCR) occurs. This genetic rearrangement leads to an enormous amount of potentially detectable antigens by the TCR (Krangel, 2009). This step also implies the maturation of the surface markers CD4 and CD8 creating double-positive T cells.

Further differentiation takes place via so-called positive and negative selection in the thymus. Positive selection indicates that after binding of the TCR with low affinity to MHC on thymic epithelial cells, presenting self-peptides, the appropriate T cell survives selection, whereas otherwise apoptosis is induced. During negative selection, T cells are killed as their TCR binds to endogenous antigens presented by APC otherwise, negative selection is followed by survival of the tested T cell. Both mechanisms are crucial to achieve self-tolerance (Bonilla and Oettgen, 2010). CD4⁺ T cells develop upon connection to thymic MHC class II molecules, while CD8⁺ T cells evolve upon connection to thymic MHC class I molecules. Afterwards these single-positive and antigen-naïve T cells are released into the blood traveling to secondary lymphoid organs, where they potentially meet their matching antigen and are activated, described in the following.

APC present phagocytosed antigens on MHC class I and II molecules, a matching TCR on CD8⁺ T cells binds to MHC class I whereas a CD4⁺ T cell binds to MHC class II. Furthermore, complexes are built with CD3, the lineage surface marker of T cells. For final activation and the survival of the antigen-specific T cell, an interaction between CD28 on T cells and B7.1/B7.2 (CD80/CD86) on APC is necessary. Finally, APC secrete cytokines and IL-2 leading to proliferation of the activated T cell to fight pathogens (Pennock et al., 2013).

Upon stimulation via different cytokines T lymphocytes differentiate into effector and memory T cells. Among CD4⁺ T helper cells, various types with specialized functions are known depending on the kind of cytokine secreted such as IL-17 and T helper 17 cell. CD4⁺ T cells' main functions are interaction and stimulation of B cells and production of cytokines (Luckheeram et al., 2012). In contrast, CD8⁺ cytotoxic T cells are specialized killer cells. Upon

contact between CD8⁺ T cells and target cell, as described above, apoptosis of the target cell is induced as well as via granzyme and perforin release. In addition, secretion of cytokines maintains the described killing cycle (Andersen et al., 2006).

Besides the TCR, T cells also express other receptors such as KIR. Especially T cells with an effector or memory phenotype express KIR on their surface, usually being CD8⁺ T cells (Lanier, 2005). Similar to NK cells, KIR on T cells can also be distinguished in inhibitory and activating and thus, modulate effector function. As a ligand binds to iKIRs on CD8⁺ T cells simultaneously with the TCR, the T cell response is down-regulated (Vivier and Anfossi, 2004).

Therefore, KIRs on T cells may be relevant in the context of LTx, as especially CD8⁺ T cells are crucial in T cell mediated rejection.

Via direct allorecognition, where CD8⁺ T cells detect foreign peptides on foreign MHC class I molecules, or indirect allorecognition, where CD8⁺ T cells detect foreign peptide on self-MHC class I molecules, CD8⁺ T cells are activated, thus, releasing cytotoxic granules damaging the transplanted organ.

3.2.3. Donor passenger lymphocytes

In the 1990s, Richter et al. were the first to describe a transient lymphocyte chimerism in the peripheral blood of patients after LTx, meaning that after LTx procedure lymphocytes migrate out of the donor organ into the recipient's periphery (Richter et al., 1994; Richter et al., 1995). Immunologists make use of the HLA mismatch in LTx to detect donor passenger lymphocytes in the organ recipient.

Since then it is also known that this chimerism exists in liver and small bowel, too (Iwaki et al., 1991; Schlitt et al., 1993) but not after heart and kidney transplantation.

In LTx, donor passenger lymphocytes were detectable between two and four weeks after LTx, among these NK and T cells were the leading subsets (Richter et al., 1994; Richter et al., 1995). Therefore, these cell types are investigated in this thesis as well as due to their capacity of allorecognition (Richter et al., 1995).

The clinical relevance of this transient lymphocyte chimerism has been debated since then. On the one hand, donor passenger lymphocytes within the recipient seem to be well fitting target cells of the recipients' immune system due to allorecognition (Madariaga et al., 2015). On the other hand, recent research points out that tissue-resident memory T cells (TRM), a subgroup among donor-derived T cells, in bronchioalveolar lavage may lead to a reduction in PGD appearance (Snyder et al., 2019).

4. Aim of the thesis and working hypothesis

The major aims of the thesis can be summarized as follows:

- Characterization of NK and T cell subsets in the peripheral blood of lung transplant recipients regarding their kinetics and phenotype early after lung transplantation (pre LTx and directly, 24 hours and 3 weeks after LTx)
- Identification of donor-derived passenger leukocytes, especially donor NK and T cells, in the peripheral blood of lung transplant recipients and their kinetics early after lung transplantation (pre LTx and directly, 24 hours and 3 weeks after LTx)
- Comparison of the activation levels of donor versus recipient NK and T cells
- Analysis of the KIR repertoire (KIR2DL/S1, KIR2DL/S2/3 and KIR3DL/S1) on donor NK and T cells compared to recipient NK and T cells
- Determination of donor NK and T cell phenotypes in perfusion solution, obtained during lung transplantation procedure
- Correlation to clinical data like CIT and PGD to identify potential targets for immunotherapy

5. Materials and Methods

5.1. Materials

5.1.1. Equipment

List of used equipments

Equipment	Manufacturer
Centrifuge 5415 D	Eppendorf
Centrifuge 5415 R	Eppendorf
Centrifuge 5410 R	Eppendorf
Flow Cytometer LSRII	Becton Dickinson (BD) Biosciences
Freezer (-80°C)	SANYO
Freezer (-20°C)	Liebherr
Ice machine	Ziegra Eismaschinen
Incubator Galaxy 170 R	New Brunswick
Microscope	Olympus
Milli-Q water purification system	Millipore
Neubauer counting chamber	Brand
Nitrogen tank	Chart
Pipettes (0,5-20 µl, 10-100 µl, 20-200 µl, 50-200 µl, 100-1000 µl)	Eppendorf, Gilson
Pipetus accu-jet	KnF LAB LABOPORT
Refrigerator (4°C)	Liebherr
Sterile working bench	BDK Luft- und Klimatechnik
Vortex VF2	Scientific Industries
Water bath	GFL

5.1.2. Software

List of used softwares

Software	Company
FACSDiva, version 8.0.1	BD Bioscience
GraphPad Prism, versions 7 and 8	GraphPad Software Inc.
Microsoft Word, version 2016	Microsoft
Pages, version 10.1	Apple
Refworks	Proquest

5.1.3. Consumption items

List of used consumption items

Consumption item	Company
Cryotubes (1,8ml)	Nunc
Disposable plastic pipettes (2ml, 5ml, 10ml, 25ml)	Falcon
Eppendorf tubes (1,5ml and 2ml)	Falcon
FACS Microtubes (1,2ml)	Ritter
Falcon tubes (15 ml and 50ml)	Falcon
Glass Pasteur Pipettes	Brand
Pipette tips	Sarstedt

5.1.4. Reagents

List of used reagents

Reagent	Company
Biocoll separation solution	Biochrom AG
Dimethylsulfoxide (DMSO)	Sigma
DMEM medium	Gibco
Ethanol	Th. Geyer
Ethylenediaminetetraacetic acid (EDTA)	Gibco
FACS Clean	BD Bioscience

Reagent	Company
FACS Flow	BD Bioscience
FACS Lysing Solution	BD Bioscience
FACS Rinse	BD Bioscience
Fetal Bovine Serum (FBS)	Gibco
Ionomycin	Sigma
L-Glutamine	Gibco
Paraformaldehyde	Sigma
PBS	Invitrogen
Penicillin/Streptomycin (Pen/Strep)	Gibco
RPMI 1640 medium	Gibco
Sodium Pyruvate	Gibco
Trypan Blue	Dr. K. Holborn und Söhne

5.1.5. Media and Solutions

List of utilized media and solutions

Medium/Solution name	Composition
RPMI-III	L-Glutamine 2mM Pen/Strep 100U/ml Sodium Pyruvate 1mM In RPMI 1640
Freezing medium	DMSO 10% FBS 50% In RPMI-III
Thawing medium	FBS 50% In RPMI-III
FACS buffer	0,1% NaN ₃ 2,5% FBS In PBS
Fixation buffer	1% PFA In PBS
Stopping buffer	EDTA 2mM 1% FBS In PBS

5.1.6. Antibodies for flow cytometry

List of antibodies for flow cytometry

Primary antibodies for HLA staining			
Target	Fluochrome	Clone	Company
HLA-A2	unconjugated	HB-82 (PA2.1)	Self-production
HLA-A2	unconjugated	HB 117 (BB7.2)	Self-production
HLA-A3	unconjugated	Bulk monoclonal	OneLambda
HLA-A1, A11, A26	unconjugated	Bulk monoclonal	OneLambda
HLA-A23, A24	unconjugated	Bulk monoclonal	OneLambda
Secondary antibodies for HLA staining			
Target	Fluochrome	Clone	Company
Goat anti-Mouse IgG (H+L)	PB	polyclonal	Thermo Fisher
F(ab') ₂ Fragment goat anti-Mouse IgG + IgM (H+L)	PE	polyclonal	Dianova
Goat anti-Mouse IgG + IgM (H+L)	FITC	polyclonal	Dianova
Antibodies for NK and T cell staining			
Target	Fluochrome	Clone	Company
CD3	PerCP	Sk7	BD Biosciences
CD4	PE	RPA-T4	BD Biosciences
CD4	PerCP	SK3	BD Biosciences
CD4	FITC	RPT-T4	BD Biosciences
CD4	APC	13B.82	Beckman Coulter
CD8	Pacific Blue	RPA-T8	BD Biosciences
CD16	APC	B73.1	BD Biosciences
CD16	BV605/Qdot605	3G8	Biolegend
CD16	FITC	3G8	Beckman Coulter
CD16	Pacific Blue	3G8	BD Biosciences
CD25	FITC	B1.49.9	Beckman Coulter
CD45	Alexa Fluor 700	HI30	Biolegend
CD45	APC-H7	2D1	BD Biosciences

CD45	AmCyan	SK7	BD Biosciences
CD56	APC	N901 (NKH-1)	Beckman Coulter
CD56	PE	B159	BD Biosciences
CD56	ECD	N901 (NKH-1)	Beckman Coulter
CD69	PE	TP1.55.3	Beckman Coulter
CD107a	FITC	H4A3	BD Biosciences
KIR2DL1/p58.1	FITC	HP-3E4	BD Biosciences
KIR2DL2/3/p58.2	PE	GL183	Beckman Coulter
KIR3DL/S1/CD158e	ECD	Z27.3.7	Beckman Coulter
Antibodies for isotype control			
Target	Fluochrome	Clone	Company
Mouse IgG1, κ	unconjugated	MOPC-21	Sigma-Aldrich
Mouse IgG2a, κ	unconjugated	UPC-10	Sigma-Aldrich
Mouse IgM	unconjugated	MOPC-104E	Sigma-Aldrich
IgG2a	FITC	7T4-1F5	Beckman Coulter
IgG2a	PerCP	X39	BD Biosciences
mIgG2a	APC	X39	BD Biosciences
mIgG2a	Pacific Blue	G155-178	BD Biosciences
IgG1	ECD	679.1Mc7	Beckman Coulter
Life/Dead dye			
Product			Company
LIFE/DEAD Yellow fluorescent reactive dye			Thermo Fisher

5.2. Patients and sample collection

Blood samples and clinical data were collected of n=51 patients that underwent LTx at the Department of Cardiothoracic, Transplantation and Vascular Surgery at Hannover Medical School in Germany between 2011 and 2019. Patients' characteristics of the entire study group as well as subgroups are shown in Table 2.

Table 2. Demographic characterization of lung transplanted patients.

Characteristics	study cohort (n=51)	HLA panel (n=39)	NK/T panel (n=33)	KIR panel (n=14)	degranulation assay (n=10)
<u>Donor</u>					
Age at donation, y±SEM	47.37±2.21	48.72±2.56	45.36±2.7	51.71±4.53	40.6±5.3
Sex, n (% male)	27 (53%)	20 (51%)	15 (45%)	4 (29%)	4 (40%)
<u>Recipient</u>					
Age at LTx, y±SEM	48.8±2.01	48.46±2.18	49.48±2.58	52.5±2.87	42.6±5.91
Sex, n (% male)	26 (51%)	19 (49%)	17 (52%)	6 (43%)	4 (40%)
Transplant indication, n (%)					
COPD	15 (29%)	11 (28%)	10 (30%)	5 (36%)	1 (10%)
IPF	17 (33%)	13 (33%)	11 (33%)	5 (36%)	4 (40%)
CF	10 (20%)	8 (21%)	6 (18%)	1 (7%)	3 (30%)
PIPH	3 (6%)	2 (5%)	2 (6%)	0 (0%)	1 (10%)
Others	6 (12%)	5 (13%)	4 (13%)	3 (21%)	1 (10%)
CCT, min±SEM	561.77±17.63	569.65±20.43	561.94±23.79	609.82±38.84	536.8±41.47
CIT, min±SEM	446.86±18.77	435.38±22.12	442.39±22.64	351.64±41.32 *	463.2±34.54
PGD (2-3), n (%)	12 (24%)	12 (31%)	6 (18%)	4 (29%)	3 (30%)
T24	10 (20%)	10 (26%)	6 (18%)	4 (29%)	3 (30%)
T48	10 (20%)	10 (26%)	5 (15%)	3 (21%)	3 (30%)
T72	3 (6%)	3 (8%)	1 (3%)	1 (7%)	1 (10%)
CLAD at end of follow-up, n (%)	9 (18%)	7 (18%)	5 (15%)	2 (14%)	2 (20%)
BOS	6 (12%)	4 (10%)	5 (15%)	2 (14%)	1 (10%)
RAS	3 (6%)	3 (8%)	0 (0%)	0 (0%)	1 (10%)

Treatment group, n (%)					
SOC	39 (76%)	27 (69%)	27 (82%)	7 (50%)	9 (90%)
EVLP	12 (24%)	12 (31%)	6 (18%)	7 (50%)	1 (10%)

Data are mean values $\pm$ standard error of mean (SEM). Asterisks show significant ($p < 0.05$) differences between HLA/BD/KIR/degranulation panels and study cohort (unpaired t-test, Mann-Whitney test). BOS, bronchiolitis obliterans syndrome; CCT, cross clamp time; CF, cystic fibrosis; CIT, cold ischemic time; CLAD, chronic lung allograft dysfunction; COPD, chronic obstructive pulmonary disease; EVLP, ex-vivo lung perfusion; IPF, idiopathic pulmonary fibrosis; PGD, primary graft dysfunction; PIPH, primary idiopathic pulmonary hypertonia; RAS, restrictive allograft syndrome; SOC, standard of care; y, years

Venous whole blood samples of each patient were provided at four different time points prior to and after LTx: before transplantation (pre), within the first 2-4 hours (T0), 24 hours (T24) and three weeks (3wk) after LTx (Figure 11). Furthermore, perfusion solutions, in which the explanted organs were stored during transplantation procedure, of nine patients were taken at the end of CIT.

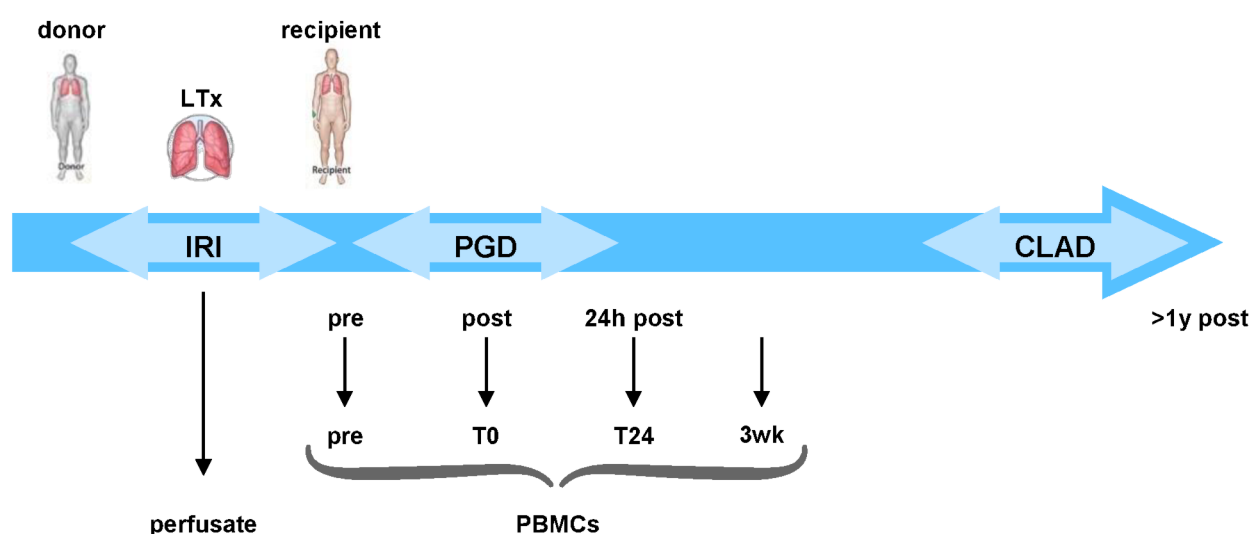


Figure 11. Experimental design. Blood samples of 51 patients were collected at indicated time points and afterwards analyzed. Furthermore, perfusates of nine patients were taken during transplantation procedure and analyzed.

5.3. Ethics

From all included patients, who underwent LTx, written informed consent was provided before sample collection. The Hannover Medical School Ethics Committee (no. 122- 2007, 2500/2014) approved this study.

5.4. PBMC isolation from peripheral blood samples

In general, 15 ml whole venous blood of each patient was diluted with 20 ml PBS and afterwards piled onto 15 ml Biocoll in a 50 ml Falcon tube. Via density, centrifugation blood cells were separated while centrifuging at 2200 rpm for 20 min, without using the break. Subsequently, peripheral blood mononuclear cells (PBMC) were collected from the resulting interphase and transferred to a second Falcon tube for washing using 20 ml PBS. PBMC again were centrifuged at 1800 rpm for 5 min, resuspended in 1 ml PBS and counted in a Neubauer counting chamber. Therefore, 10 µl of cell suspension were diluted in trypan blue and from this dilution 10 µl were moved into a Neubauer counting chamber. Only living cells were counted and then multiplied by dilution factor. Finally, resulting PBMC were either directly used for further experiments or cryopreserved.

5.5. Cell isolation from lung perfusion solution

Circa 100 ml to 200 ml perfusion solution was obtained during the transplantation procedure, was shifted into Falcon tubes and centrifuged at 2100 rpm for 15 min. Thereafter resuspension of the cell pellet with 5 ml Pharm Lyse Lysing Buffer was performed, followed by an incubation time of 15 min. After termination of the lysing with 30 ml stopping solution, the gained cells were centrifuged again and counted, as described in 5.4., afterwards. Finally, cells were either directly used for further experiments or cryopreserved.

5.6. Freezing and thawing of cells

After cell isolation out of whole blood and counting, as described in 5.4. and 5.5., cells were prepared for cold storage, if not used directly for experiments. A maximum of 1×10^7 cells in 1 ml DMSO-containing freezing medium were cryopreserved in each tube. The obtained cells were centrifuged again, resuspended in 500 μ l FBS and 500 μ l RPMI-III with 20 % DMSO was added. Finally, cells were transferred into a pre-cooled cryotube. To allow a constant down-cooling cryotubes were wrapped into three layers of paper towels, stored in a -80°C freezer and shifted into a liquid nitrogen tank (-196°C) after 24 hours.

Alternatively, the thawing process starts with fetching long stored cells from the liquid nitrogen tank, warming them up in a water bath at 37°C and directly suspending them in 5 ml thawing medium. After centrifugation, cells were resuspended in PBS, counted and processed.

5.7. Detection of passenger leukocytes and their characterization via flow cytometry

In general, 2 to 5×10^5 cells were suspended in 100 μ l PBS in FACS microtubes and used for staining.

Firstly, PBMC were incubated with live/dead yellow fluorescent reactive dye (5.1.6.) for 15 min at room temperature in the dark, washed and resuspended in FACS buffer. Secondly, cells were stained with unconjugated donor HLA allele-specific or isotype control antibodies (5.1.6.), stored for 30 min on ice and then washed twice. Thirdly, staining with PB-, PE- and FITC-labeled goat anti-Mouse IgG oder IgM antibodies (5.1.6.) was performed for 30 min on ice. Cells were once more washed and suspended in FACS buffer. To discriminate NK and T cells from other leukocytes, as well as to characterize their KIR repertoire, PBMC were, as final step, incubated with fluorochrome-conjugated lineage marker monoclonal antibodies (5.1.6.) and finally washed.

Of note, inhibitory and activating KIR genes cannot be distinguished by these mAb due to the identical extracellular domains of long inhibitory and short activating KIR.

If not otherwise indicated, staining steps were incubated for 30 min at 4°C and PBMC were washed with FACS buffer.

Multi-color FACS analyses were performed using LSR II flow cytometer and results were analyzed via the Diva software (Version 8.0.1.).

5.8. Gating strategy

To identify the different types of immune cells flow cytometry analyses were performed as described in Figure 12.

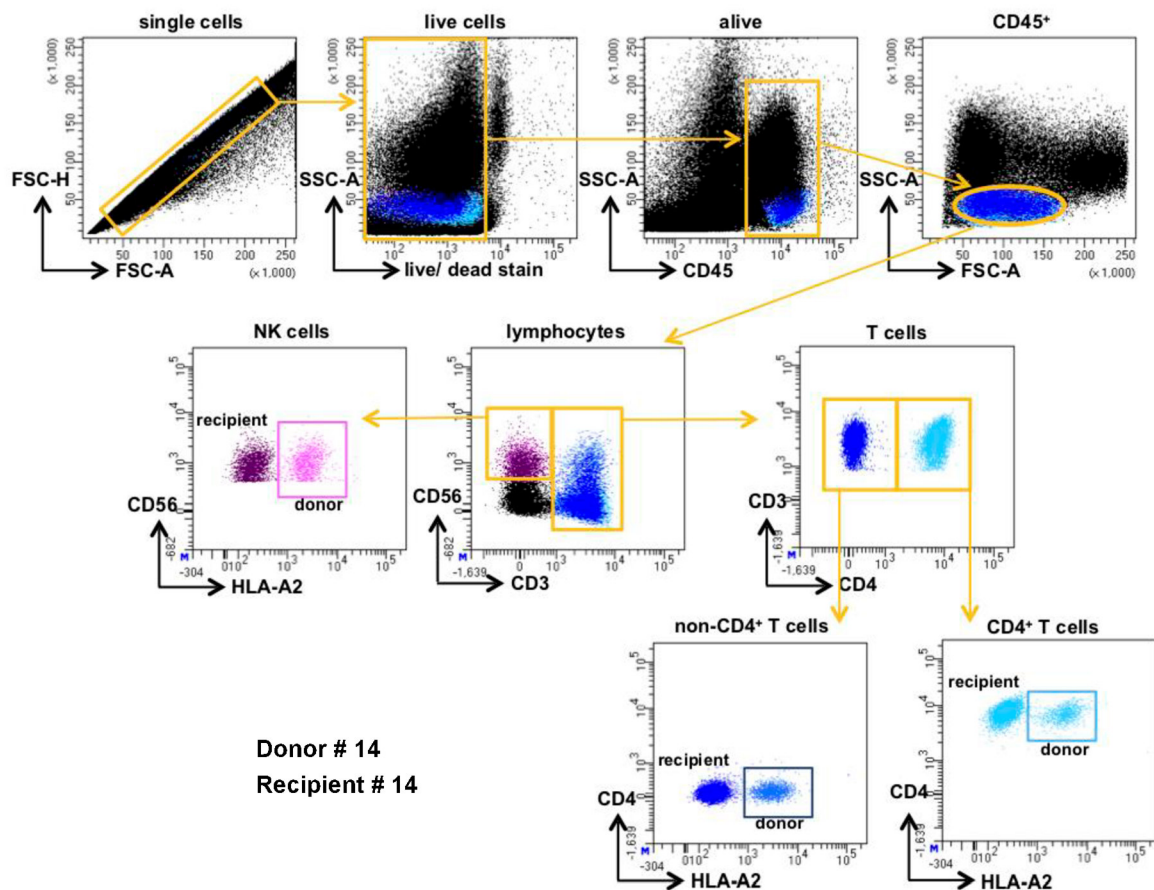


Figure 12. Gating strategy used to identify immune cells and to discriminate between donor and recipient cells. Exemplarily, one representative patient (#14) is shown, in which an HLA-A2-positive donor lung was transferred into an HLA-A2-negative recipient. NK cells are presented in purple, while CD4⁺ T cells are displayed in light blue and non-CD4⁺ T cells in dark blue. The referring HLA-A2-positive donor cells are demonstrated in a lighter tone of the according color compared to the HLA-A2-negative recipient cells. FSC-A: forward scatter area; FSC-H: forward scatter height; SSC: sideward scatter

After excluding doublets via area/height ratio, singlets were subdivided into alive and dead cells. Afterwards alive singlets were gated for CD45⁺ cells and lastly the immune cells were determined depending on their granularity (SSC-A).

To distinguish the different lymphocyte subsets, lymphocytes were gated for CD56, as lineage marker for NK cells (purple), and for CD3, as lineage marker for T cells. T cells were further subdivided according to gating for CD4 in CD4⁺ (light blue) and non-CD4⁺ (dark blue) T cells. Donor cells among NK, CD4⁺ and non-CD4⁺ T cells we defined by gating on the accordingly donor HLA allele-specific antibody. In the above shown representative FACS plots of one example patient HLA-A2-positive donor NK cells are displayed in pink, whereas HLA-A2-negative recipient NK cells are purple. Regarding T cells HLA-A2-positive donor CD4⁺ and non-CD4⁺ T cells are shown in lighter blue compared to HLA-A2-negative recipient CD4⁺ and non-CD4⁺ T cells.

2.9. Statistical analysis

GraphPad Prism software (versions 7 and 8) was used for all statistical analyses. D'Agostino-Pearson omnibus normality test and Kolmogorov-Smirnov normality test were applied to assess for normally distributed data. Parametric tests were performed for normally distributed data, whereas non-parametric tests were used for not normally distributed data. Depending on the experimental design two-way ANOVA with Sidak's multiple comparison test and Tukey's multiple comparison test, one-sample t-test, paired t-test, Wilcoxon test and Pearson correlation were performed as indicated in the figure legends. P-values less than 0.05 were

considered significant ($p < 0.05$). P-values are defined as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

2.10. Cooperation partners

Prof. Dr. Gregor Warnecke	Department of Cardiothoracic, Transplantation and Visceral Surgery (HTTG) Hannover Medical School (until 12/2019) Universitätsklinikum Heidelberg (from 01/2020)
Dr. Bettina Wiegmann	Department of Cardiothoracic, Transplantation and Visceral Surgery (HTTG) Hannover Medical School
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6. Original publication

6.1. Manuscript

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Front. Immunol., 13 December 2021 <https://doi.org/10.3389/fimmu.2021.778885>



Donor NK and T Cells in the Periphery of Lung Transplant Recipients Contain High Frequencies of Killer Cell Immunoglobulin-Like Receptor-Positive Subsets

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Edited by:

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Specialty section:

This article was submitted to
Immunological Tolerance
and Regulation,
a section of the journal
Frontiers in Immunology

Received: 17 September 2021

Accepted: 04 November 2021

Published: 13 December 2021

Citation:

Hitz A-M, Bläsing K-A, Wiegmann B,
Bellmàs-Sanz R, Chichelnitskiy E,
Wandrer F, Horn L-M, Neudörfl C,
Keil J, Beushausen K, Ius F,
Sommer W, Avsar M, Kühn C,
Tudorache I, Salman J, Siemeni T,
Haverich A, Warnecke G, Falk CS and
Kühne JF (2021) Donor NK and T Cells
in the Periphery of Lung Transplant
Recipients Contain High Frequencies
of Killer Cell Immunoglobulin-Like
Receptor-Positive Subsets.
Front. Immunol. 12:778885.
doi: 10.3389/fimmu.2021.778885

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Introduction: For end-stage lung diseases, double lung transplantation (DLTx) is the ultimate curative treatment option. However, acute and chronic rejection and chronic dysfunction are major limitations in thoracic transplantation medicine. Thus, a better understanding of the contribution of immune responses early after DLTx is urgently needed. Passenger cells, derived from donor lungs and migrating into the recipient periphery, are comprised primarily by NK and T cells. Here, we aimed at characterizing the expression of killer cell immunoglobulin-like receptors (KIR) on donor and recipient NK and T cells in recipient blood after DLTx. Furthermore, we investigated the functional status and capacity of donor vs. recipient NK cells.

Methods: Peripheral blood samples of 51 DLTx recipients were analyzed pre Tx and at T0, T24 and 3wk post Tx for the presence of HLA-mismatched donor NK and T cells, their KIR repertoire as well as activation status using flow cytometry.

Results: Within the first 3 weeks after DLTx, donor NK and T cells were detected in all patients with a peak at T0. An increase of the KIR2DL/S1-positive subset was found within the donor NK cell repertoire. Moreover, donor NK cells showed significantly higher frequencies of KIR2DL/S1-positive cells ($p < 0.01$) 3wk post DLTx compared to recipient NK cells. This effect was also observed in donor KIR⁺ T cells 3wk after DLTx with higher proportions of KIR2DL/S1 ($p < 0.05$) and KIR3DL/S1 ($p < 0.01$) positive T cells. Higher activation levels of donor NK and T cells ($p < 0.001$) were detected compared to recipient cells via CD25 expression as well as a higher degranulation capacity upon activation by K562 target cells.

Conclusion: Higher frequencies of donor NK and T cells expressing KIR compared to recipient NK and T cells argue for their origin in the lung as a part of a highly specialized immunocompetent compartment. Despite KIR expression, higher activation levels of donor NK and T cells in the periphery of recipients suggest their pre-activation during the *ex situ* phase. Taken together, donor NK and T cells are likely to have a regulatory effect in the balance between tolerance and rejection and, hence, graft survival after DLTx.

Keywords: lung transplantation, passenger leukocytes, NK cells, T cells, killer cell immunoglobulin-like receptor, primary graft dysfunction, cold ischemic time

INTRODUCTION

Double lung transplantation (DLTx) remains the only curative treatment option for end-stage lung diseases (1). Despite continuous progress in the optimization of the transplantation procedure, clinical outcome of patients undergoing DLTx still is poorer compared to other solid organ transplantations (2). Survival rates after DLTx are limited early due to primary graft dysfunction (PGD), occurring in the first 72 hours after transplantation. Later, survival is impaired by chronic lung allograft dysfunction (CLAD), comprising bronchiolitis obliterans syndrome (BOS) and restrictive allograft syndrome (RAS), which develop typically later than two years after DLTx. PGD grade 2 and 3 have been associated with significantly higher short-term as well as a negative effect on long-term outcome. While PGD has been extensively examined in terms of risk factors, epidemiology and treatment options (3), the underlying immunological mechanisms are still not completely understood. Since T cells represent the main effector cells of the adaptive immunity, they are the preferred subset in studies addressing allograft including lung rejection. In contrast, the role of natural killer (NK) cells in the context of solid organ transplantation is still discussed controversially as they have been shown to be involved in both graft rejection and tolerance induction in different models (4). Recently, the missing-self genetics of KIR-ligand mismatches has been discussed to contribute to microvascular rejection (5, 6). NK cells act as first defense line of the innate immune system against pathogens, capable of producing cytokines and possessing cytotoxic activity. Moreover, they have the ability to discriminate between cells of self and non-self origin, using an inhibitory recognition system of self-human leukocyte antigen (HLA) class I molecules (7, 8). The 'missing self-hypothesis' proposes that recognition of self HLA class I molecules inhibits their lytic activity, while the absence or downregulation of self HLA class I, i.e. missing self, leads to direct recognition and lysis of target cells (8, 9). The lytic activity of NK cells is thoroughly regulated by a multidirectional interaction of inhibitory and activating receptors, such as killer

cell immunoglobulin-like receptors (KIR), C-type lectin and natural cytotoxicity receptors (NCR) (10). Activating KIR-S and inhibitory KIR-L genes encode surface receptors that recognize primarily two groups of HLA-C alleles and HLA-B supratypes, respectively. In addition to NK cell regulation, KIR can also be expressed by certain CD8⁺ T cell subsets and act as inhibitors of TCR signaling. Hence, both NK and T cell subsets expressing these KIR receptors may play a crucial role in the field of organ, especially lung transplantation due to its unique tissue-resident immune repertoire (11). In genetic analyses, a negative association of inhibitory KIR genes within haplotype A was shown for long-term outcome after DLTx suggesting that the KIR-ligand mismatch system may have clinical relevance for lung Tx (12).

Due to this capacity of NK and T cells for allorecognition, these regulatory mechanisms are of critical importance especially in the context of HLA mismatched settings like lung transplantation. Although the appearance of donor passenger leukocytes migrating from the implanted lung into the periphery of the recipient has been described decades ago, the respective NK cell subsets have not been characterized so far. This transient lymphocyte chimerism in the blood of lung recipients has first been described in the 1990s and especially donor NK and T cells were detected for up to four weeks after transplantation (13). The clinical impact of donor cells on lung allograft survival has been discussed ever since, with recent findings suggesting that special subsets of donor-derived T cells, i.e. tissue-resident memory T (TRM) cells, in bronchioalveolar lavage may be associated with lower incidence of primary graft dysfunction (14). Since NK and T cells were shown to account for the major passenger leukocyte populations (13) comprising important subsets of the innate and adaptive immune system, we focused on these two major effector cell populations.

In our study, 51 lung transplant recipients were analyzed for the kinetics of donor passenger NK and T cells in recipient blood directly after lung transplantation and at three weeks post Tx. We could demonstrate the existence of high frequencies of donor NK and T cells directly after transplantation in all recipients, which generated a transient lymphocyte chimerism within the first three weeks after DLTx. Unexpectedly, NK cells represented higher relative proportions of donor cells compared to T cells. In addition, the characterization of donor and recipient NK and T cell subsets revealed high frequencies of KIR-positive NK and T cell subsets, particularly with respect to KIR2DL/S1⁺ subsets. Moreover, donor NK cells displayed higher cytotoxic activity at

Abbreviations: ACR, acute cellular rejection; BOS, bronchiolitis obliterans syndrome; CIT, cold ischemic time; CLAD, chronic lung allograft dysfunction; DC, dendritic cells; DLTx, double-lung transplantation; HLA, human leukocyte antigen; KIR, killer cell immunoglobulin-like receptor; mAb, monoclonal antibody; NK cells, natural killer cells; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PGD, primary graft dysfunction; RAS, restrictive allograft syndrome; SOC, standard of care.

early stages compared to recipient NK cells. Correlation analyses were performed with clinical parameters but did not show a significant impact on primary graft dysfunction. However, higher KIR⁺ NK cell frequencies were observed in lung recipients with longer cold-ischemic times, suggesting an impact of the ischemia/reperfusion injury. Taken together, this early NK and T cell chimerism may rather be involved in the long-term balance between rejection and tolerance in the lung transplant setting.

MATERIALS AND METHODS

Patients and Sample Collection

Blood samples and clinical data of 51 patients undergoing DLTx were collected and preserved as peripheral blood mononuclear cells (PBMC). PBMC of patients were isolated using Biocoll Separating Solution (Biochrom/Merck, Darmstadt, Germany) as described. The study was approved by the ethics committee at the Hannover Medical School (no. 122-2007, 2500/2014) and all patients provided written informed consent. Venous whole blood samples were collected at the following time points: before (pre), 4-5 hours (T0), 24 hours (T24) and three weeks after DLTx (3wk). Donor and recipient demographics of the entire cohort (n=51) and the respective subgroups for the various analyses are summarized in **Table 1**. No induction therapy is applied to recipients and immunosuppression by calcineurin-inhibitors (CNI), steroids and mycophenolate mofetil (MMF) is

administered intraoperatively. PGD scores were ranked according to oxygenation index and the presence of pulmonary radiographic infiltrates (15). Donor lungs were perfused with 2-3 L of Perfadex/Celsior perfusion solution and kept on cold storage in the same solution (perfusate). Perfusates of donor lungs were collected at the end of cold ischemic phase, cold ischemic times (CIT) and cross-clamp times (CCT) were documented. For collection of immune cells migrating or washed out of the allograft, perfusates were centrifuged at 453 x g for 15 min, cells were analyzed by flow cytometry and cell-free perfusate was stored at -20°C.

Detection of Donor-Derived Passenger Leukocytes in Peripheral Recipient Blood by Flow Cytometry

PBMC were thawed and incubated with live/dead yellow fluorescent reactive dye (ThermoFisher Scientific, Waltham, MA, USA), washed and stained with unconjugated donor HLA allele-specific or isotype control antibodies (**Supplementary Table 1**) for 30 min on ice, washed twice, followed by staining with secondary PB-, PE- and FITC-labeled goat-anti mouse IgG or IgM (GaM) mAb (**Supplementary Table 1**). T and NK cell subsets were identified with fluorochrome-conjugated lineage marker mAb (summarized in **Supplementary Table 1**), including mAb specific for KIR2D and 3D receptors. Of note, inhibitory and activating KIR genes cannot be distinguished by these mAb due to the identical extracellular domains of long inhibitory and short activating KIR. All staining steps were

TABLE 1 | Demographic characterization of lung transplanted patients.

Characteristics	Study cohort (n = 51)	HLA panel (n = 39)	NK/T panel (n = 33)	KIR panel (n = 14)	Degranulation assay (n = 10)
Donor					
Age at donation, y±SEM	47.37±2.21	48.72±2.56	45.36±2.7	51.71±4.53	40.6±5.3
sex, n (% male)	27 (53%)	20 (51%)	15 (45%)	4 (29%)	4 (40%)
Recipient					
Age at LTx, y±SEM	48.8±2.01	48.46±2.18	49.48±2.58	52.5±2.87	42.6±5.91
Sex, n (% male)	26 (51%)	19 (49%)	17 (52%)	6 (43%)	4 (40%)
Transplant indication, n (%)					
COPD	15 (29%)	11 (28%)	10 (30%)	5 (36%)	1 (10%)
IPF	17 (33%)	13 (33%)	11 (33%)	5 (36%)	4 (40%)
CF	10 (20%)	8 (21%)	6 (18%)	1 (7%)	3 (30%)
PIPH	3 (6%)	2 (5%)	2 (6%)	0 (0%)	1 (10%)
Others	6 (12%)	5 (13%)	4 (13%)	3 (21%)	1 (10%)
CCT, min±SEM	561.77±17.63	569.65±20.43	561.94±23.79	609.82±38.84	536.8±41.47
CIT, min±SEM	446.86±18.77	435.38±22.12	442.39±22.64	351.64±41.32*	463.2±34.54
PGD (2-3), n (%)	12 (24%)	12 (31%)	6 (18%)	4 (29%)	3 (30%)
T24	10 (20%)	10 (26%)	6 (18%)	4 (29%)	3 (30%)
T48	10 (20%)	10 (26%)	5 (15%)	3 (21%)	3 (30%)
T72	3 (6%)	3 (8%)	1 (3%)	1 (7%)	1 (10%)
CLAD at end of follow-up, n (%)	9 (18%)	7 (18%)	5 (15%)	2 (14%)	2 (20%)
BOS	6 (12%)	4 (10%)	5 (15%)	2 (14%)	1 (10%)
RAS	3 (6%)	3 (8%)	0 (0%)	0 (0%)	1 (10%)
Treatment group, n (%)					
SOC	39 (76%)	27 (69%)	27 (82%)	7 (50%)	9 (90%)
EVLP	12 (24%)	12 (31%)	6 (18%)	7 (50%)	1 (10%)

BOS, bronchiolitis obliterans syndrome; CCT, cross clamp time; CF, cystic fibrosis; CIT, cold ischemic time; CLAD, chronic lung allograft dysfunction; COPD, chronic obstructive pulmonary disease; EVLP, ex-vivo lung perfusion; IPF, idiopathic pulmonary fibrosis; PGD, primary graft dysfunction; PIPH, primary idiopathic pulmonary hypertension; RAS, restrictive allograft syndrome; SOC, standard of care; y, years.

Data are mean values ± standard error of mean (SEM). Asterisks show significant ($p < 0.05$) differences between HLA/BD/KIR/degranulation panels and study cohort (unpaired t-test, Mann-Whitney test).

performed for 30 min at 4°C and PBMC were washed with FACS buffer (PBS plus 0.1% sodium azide, Sigma-Aldrich, Munich, Germany, 1% FBS ThermoFisher Scientific, Waltham, MA, USA). Multi-color FACS analyses were performed using LSR II flow cytometer and the Diva software (8.0.1, BD Biosciences, San Diego, CA, USA).

Functional NK Cell Degranulation Assay

Degranulation assays were performed using recipient PBMC as previously described (16). PBMC were consecutively stained with unconjugated donor HLA allele-specific and secondary PE-labeled GaM mAb at room temperature for 30 minutes respectively, washed and incubated with FITC-labeled CD107a mAb and K562 target cells (16, 17) (1:1) for 4 h at 37°C/5% CO₂ with addition of 50 µM monensin (Sigma-Aldrich, St. Louis, MO; USA) after the 1st h. After two washing steps, cells were stained with CD56, CD16 for NK cells and CD3 as exclusion of T cells for 30 minutes at 4°C, washed twice and resuspended with FACS buffer. Cells were acquired using LSR II flow cytometer and the Diva software.

Statistical Analysis

All statistical analyses were generated using GraphPad Prism (Version 8, San Diego, CA, USA). D'Agostino-Pearson omnibus normality test and Kolmogorov-Smirnov normality test were applied to assess data distribution. Statistical analyses (two-way ANOVA with Sidak's multiple comparison test and Tukey's multiple comparison test, one-sample t-test, paired t-test, Wilcoxon test and Pearson correlation) were performed as indicated in the Figure legends. P values <0.05 were considered significant.

RESULTS

The Proportion of NK Cells Increases While the Frequency of T Cells Decreases in the Periphery of DLTx Recipients Directly After Transplantation

In order to define the kinetics of lymphocyte subsets in lung transplant recipients within the first three weeks after DLTx, PBMC of DLTx patients (n=33 patients, **Table 1**) were analyzed for their NK and T cell subsets using multicolor flow cytometry (**Figures 1A, B**). Directly after DLTx (T0), a trend towards an increase in the proportion of NK cells within CD45⁺ leukocytes (p<0.12) could be observed before decreasing significantly (p<0.001) at T24 and three weeks to an even lower level compared to baseline pre Tx (pre 12.78% vs. 3wk 7.2%; p<0.05; **Figure 1A**). This peak at T0 resulted primarily from CD56^{dim}CD16^{hi} NK cells, with a relative increase compared to stable CD56^{dim}CD16^{lo} and CD56^{bri} NK subsets. In parallel, the proportion of T cells displayed a substantial decrease at T0, which, however, did not reach statistical significance (**Figure 1B**) with primarily CD4⁺ T cells disappearing post DLTx, returning to baseline levels after 3wk post DLTx. CD8⁺ T cells seemed to disappear less intensely, which resulted in transiently significant

changes in the CD4⁺/CD8⁺ ratio. This dynamic change in the NK and T cell subset composition within the first 24h after DLTx indicates a regulated process in recipient blood, with differential effects on these lymphocyte subsets.

Donor-Derived Lymphocytes Influence NK and T Cell Frequencies in the Periphery of DLTx Recipients

The existence of donor-derived 'passenger' lymphocytes migrating from the implanted lung into the periphery of the recipient has been known for a long time, although subset composition and fate of these donor cells remain rather unclear. Therefore, we investigated the kinetics of donor lymphocytes focusing on NK and T cell subsets (**Figures 1C, E**). To test our hypothesis that donor passenger lymphocytes contribute to these postoperative changes, NK and T cells were stained for donor HLA class I alleles at the same time points. Due to the HLA mismatch in DLTx, donor and recipient cells can easily be distinguished using HLA allele-specific Ab, which were available for 39 of the 51 patients (**Tables 1, 2**). Donor and recipient NK and T cell subsets were then defined by their respective phenotype (**Figure 1C**). Directly post DLTx (T0), the highest frequencies of donor cells could be detected in general, with NK cells as predominant lymphocyte subset (**Figures 1C, D**), followed by CD8⁺ T cells. Control stainings for donor cells pre DLTx showed 0-0.8% background staining, which defined the detection limit of >1% (**Supplementary Figures A, B**). The majority of donor NK cells displayed a CD56^{dim}CD16^{hi} phenotype at T0 with a downregulation of CD16 over the course of three weeks (**Figures 1C, E**). At three weeks post DLTx, all donor lymphocyte subsets decreased significantly compared to T0 (p<0.0001). Nevertheless, donor cells, primarily NK cells, were still detectable three weeks after DLTx. Thus, our results demonstrate a transient chimerism by donor NK and T cells during the first three weeks after lung transplantation in peripheral blood of recipients that contributes to the observed changes in NK and T cell frequencies.

Higher Frequencies of KIR⁺ Subsets in Donor NK and T Cells Are Present Within the First Three Weeks After DLTx Compared to Recipient NK and T Cells

To better understand the composition of donor cells after DLTx, NK and T cells in recipient blood were analyzed regarding their KIR repertoire in 14 of the 51 patients (**Table 1**). NK cell activity is among other factors, regulated by the KIR expression at the clonal level. Furthermore, KIR can also be expressed by CD8⁺ T cells and influence their activity, including TCR signaling. Therefore, KIR2DL/S1, KIR2DL/S2/3 and KIR3DL/S1 expression in NK and T cells was assessed during the first three weeks after lung transplantation (**Figure 2**). Among all donor NK cells, the frequency of KIR2DL/S1 was increased significantly (p<0.05) at 3wk post DLTx compared to T0 and T24 (**Figures 2A, B**). Moreover, on donor cells, KIR2DL/S1 was mainly expressed by CD56^{dim}CD16^{lo} NK cells (**Figures 2C, D**). Regarding KIR2DL/S2/3⁺ donor NK cells, a trend towards a

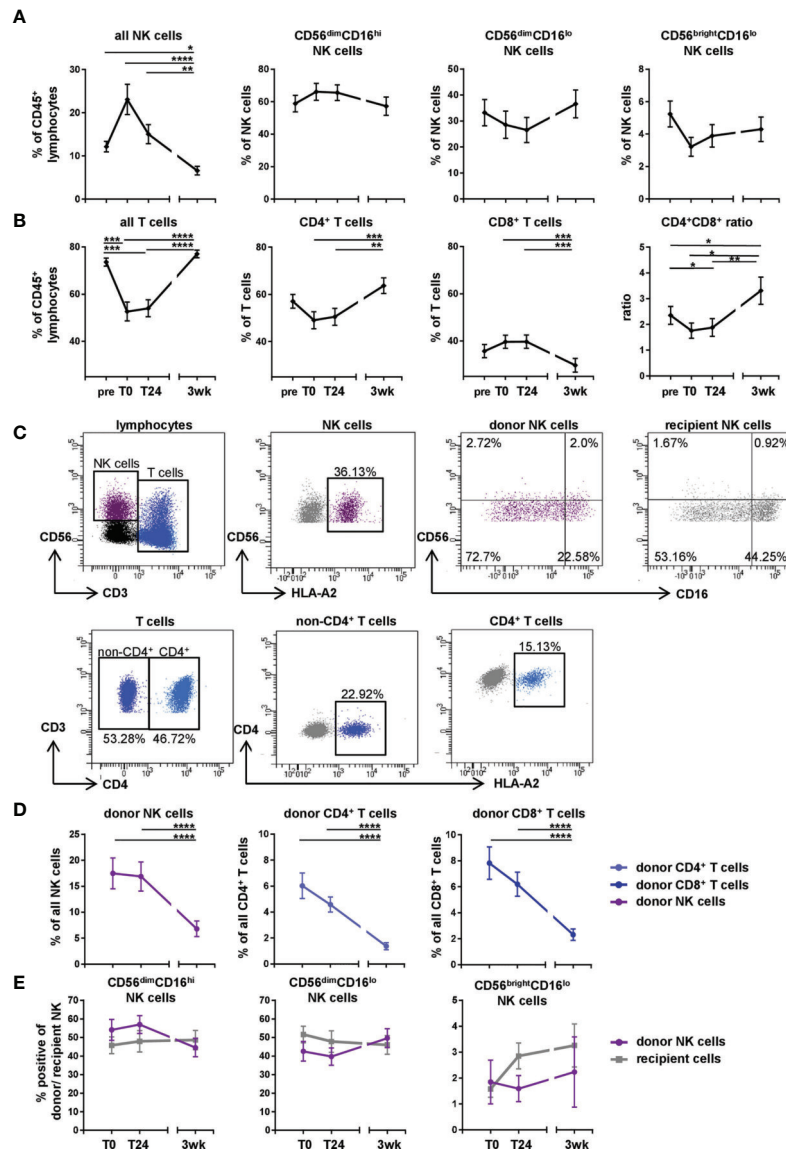


FIGURE 1 | Changes in NK and T cell subsets in first three weeks after DLTx are also mediated by donor cells. **(A, B)** Frequencies of NK and T cell subsets ($n = 33$ of the 51 patients, **Table 1**) in peripheral blood of DLTx recipients were analyzed pre, directly post (T0), 24 hours (T24) and three weeks after transplantation (3wk). **(A)** NK cells were gated as $CD45^{+}CD3^{+}CD56^{+}$ ('all T cells'), $CD3^{+}CD4^{+}$ (' $CD4^{+}$ T cells') and $CD3^{+}CD8^{+}$ (' $CD8^{+}$ T cells'). **(B)** T cells were identified as $CD45^{+}CD3^{+}CD56^{+/+}$ ('all T cells'), $CD3^{+}CD4^{+}$ (' $CD4^{+}$ T cells') and $CD3^{+}CD8^{+}$ (' $CD8^{+}$ T cells'). **(C)** Gating strategy for donor NK and T cells in peripheral blood of one representative patient #14 directly post DLTx is shown. Donor and recipient cells were discriminated via HLA class I mismatch using anti-HLA-A2 specific Ab (**Supplementary Table 1**). Here, $CD3^{+}CD4^{+}$ T cell and $CD3^{+}$ non- $CD4^{+}$ T cell subsets were defined. **(D)** Frequencies of donor NK and T cells ($n = 39$, **Table 1**) in peripheral blood of DLTx patients directly post, 24 hours post and three weeks after transplantation are shown. **(E)** Frequencies of donor and recipient NK cells ($n = 14$) regarding their CD56 and CD16 expression were analyzed. Cells were gated as described in panel **(C)** Statistical analysis: one-way ANOVA with Dunn's multiple comparison test for **(A, B, D)** and two-way ANOVA with Sidak's multiple comparison test and Tukey's multiple comparison test for **(E)**. Data are shown as mean $\pm$ SEM, asterisks indicate p-values with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

TABLE 2 | HLA mismatches of transplanted recipients and donated lungs.

Number of HLA class I mismatches						Number of HLA class II mismatches					
0	1	2	3	4	5	6	0	1	2	3	4
0	0	2	2	13	14	8	1	1	15	13	9

HLA, human leukocyte antigen.

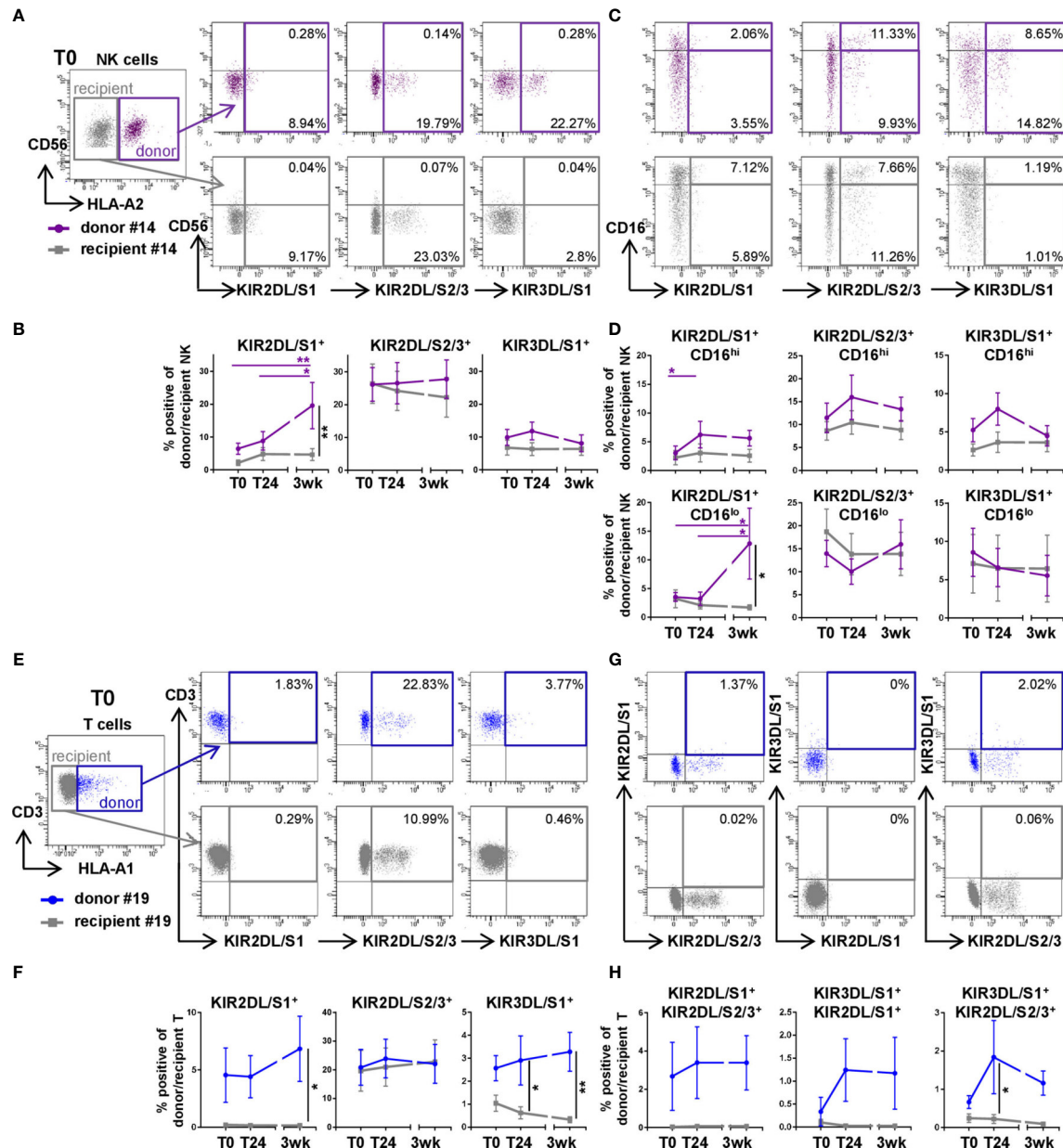


FIGURE 2 | The proportion of killer cell immunoglobulin-like receptors (KIR) on donor NK and T cells is higher compared to recipient cells within the first three weeks after DLTx. **(A–D)** KIR2DL/S1, KIR2DL/S2/3 and KIR3DL/S1 surface expression on NK cells ($n=14$) was analyzed in peripheral blood of double-lung transplant recipients directly post (T0), 24 hours (T24) and three weeks after transplantation (3wk). Donor and recipient cells were distinguished by HLA mismatch: NK cells of the representative donor #14 were identified by HLA-A2 staining **(A, C)** T cells of the representative donor #19 were identified by HLA-A1 staining **(E, G)**, the gating strategies are shown in **Figures 1C** and **Supplementary Figure 1**. **(A)** Representative FACS plots (T0) and **(B)** frequencies of donor (purple colored squares) and recipient (grey colored squares) KIR on NK cells. **(C)** Representative FACS plots (T0) and **(D)** frequencies showing KIR expression on CD16^{hi} and CD16^{lo} NK cells. **(E–H)** The same KIR repertoire was assessed for T cells ($n = 14$) in peripheral blood of DLTx patients for the indicated time points. T cells of donor #19 were stained using anti-HLA-A1-specific Ab. **(E)** Representative FACS plots and **(F)** frequencies of KIR on donor (blue colored squares) and recipient (grey colored squares) T cells are displayed. **(G)** Representative FACS plots and **(H)** frequencies describing double positive KIR surface expression on T cells. Statistical analysis: two-way ANOVA with Sidak's multiple comparison test and Tukey's multiple comparison test. Data are shown as mean $\pm$ SEM, asterisks indicate p-values with * $p < 0.05$, ** $p < 0.01$.

switch from CD16^{hi} to CD16^{lo} donor NK cells could be observed within the first 3 weeks after DLTx. The frequency of KIR3DL/S1⁺ donor NK cells was stably low over time (**Figures 2B, D** and **Supplementary Figure 2A**). This differential dynamic of KIR⁺

donor NK subsets was independent from their HLA-C typing (data not shown). During three weeks after DLTx, the frequencies of KIR2DL/S1⁺ and KIR3DL/S1⁺ donor T cells were significantly higher compared to recipient T cells (both

$p < 0.05$; **Figures 2E, F and Supplementary Figure 2B**), while frequencies of KIR2DL/S2/3⁺ T cells showed no difference between donor and recipient T cells. Due to the individual variability, no significant changes could be seen for single KIR⁺ T cells over time. Only very few KIR double-positive donor T cells were detectable after transplantation (**Figures 2G, H**) indicating a rather single KIR⁺ subset composition. Recipient NK and T cells revealed low and stable KIR⁺ proportions during the first three weeks after DLTx, except for KIR2DL/S2/3. To uncover a potential age-related increase of KIR expression levels on NK and T cells, donor age was correlated to the KIR⁺ NK and T cells 24 hours after transplantation (T24) (**Supplementary Figure 3**). Neither a correlation between donor age nor the frequency of donor cells nor the percentage of KIR⁺ donor NK and T cells could be found, indicating no influence of donor age on the KIR repertoire on donor NK and T cells. Therefore, our results demonstrate a chimerism between donor and recipient NK and T cells in recipient blood during the first three weeks after DLTx with a substantial contribution of donor KIR⁺ NK cell subsets and a minor fraction of donor KIR⁺ T cells.

Increased Activity and Functional Capacity of Donor NK and T Cells in the Periphery of the Recipient During the First Three Weeks Post DLTx

To assess activation levels of donor NK and T cells in comparison to recipient cells in peripheral blood, we analyzed the surface expression of the activation marker CD25, IL-2 receptor alpha chain, pre, directly, 24 hours and three weeks post DLTx by flow cytometry (**Figure 3**). Over time, the proportion of CD25⁺ donor NK and T cells increased up to 20% ($p < 0.001$), whereas recipient CD25⁺ NK and T cells remained stable at a rather low level of approximately 1% ($p < 0.0001$ for donor vs. recipient at 3wk post DLTx) (**Figures 3A, B**). Three weeks post DLTx, activated donor NK cells were characterized as primarily CD56^{dim} (data not shown) CD16^{lo} NK cells (**Figure 3A**). We further analyzed donor and recipient NK cells for their functional capability, i.e. degranulation, by measuring surface expression of CD107a upon exposure to HLA-deficient target cells (K562). Comparing donor and recipient, significantly higher levels of CD107a surface expression could be detected in donor NK cells, already in the absence of K562 target cells (**Figures 3C, D** upper panel). In the presence of K562 target cells, donor NK cells displayed higher degranulation compared to recipient NK cells at all time points (all $p < 0.5$; **Figures 3C, D** lower panel). However, during the first 3 weeks after DLTx a trend towards increased degranulation capacity could be observed for both donor and recipient cells. Further characterization of NK cell subsets revealed that the main proportion of degranulating donor NK cells displayed a CD56^{dim}CD16^{lo} phenotype. Exposure to K562 showed an additional effect neither on recipient nor on donor NK cell degranulation at any time point indicating a suppressive effect *via* immunosuppression also for donor NK cells. Gating on CD16^{bri} NK cells showed a rather poor degranulation potential, as expected (16). Regarding the degranulation capacity of T cells, again donor CD4⁺ and CD8⁺ T cells

displayed significantly higher proportions of CD107a⁺ cells over the course of three weeks without K562 ($p < 0.01$ compared to T0). CD8⁺ donor T cells also showed overall higher levels of degranulation compared to CD8⁺ T cells of the recipient ($p < 0.0001$ at 3wks post DLTx; **Figure 3E**). Since K562 does not stimulate T cells, these data are not shown. The presented results indicate that donor NK and T cells, predominantly CD56^{dim}CD16^{lo} NK and CD8⁺ T cells, reached *per se* an activated and functional state in the periphery of the recipient, expressing higher levels of activation markers compared to the recipients own NK and T cell repertoire.

Predominantly KIR2DL/S1- and KIR2DL/S2/3-Positive T Cells and CD56^{dim}CD16^{lo} NK Cells From the Lung Allograft Are Found in the Recipient Periphery Early Post DLTx

We next compared donor NK and T cell subsets for their phenotypes including KIR expression in recipient blood vs. perfusion solution, i.e. the storage solution of the lung during the *ex situ* phase (**Figure 4**). In perfusion solution, the major NK cell phenotype was CD56^{dim}CD16^{hi}, while donor NK cells in recipient blood at T0 demonstrated a CD56^{dim}CD16^{lo} phenotype indicating previous activation. The frequency of CD56^{bri}CD16^{lo} NK cells was significantly higher ($p < 0.05$) in perfusion solution compared to recipient periphery (**Figure 4A**). Comparing the KIR repertoire on donor NK cells in perfusion solution with recipient blood, high frequencies were observed without significant differences except for KIR2DL/S1 with higher proportions on donor cells in recipient blood (**Figure 4B**). In donor T cells, higher KIR2DL/S1 and KIR2DL/S2/3 (both $p = 0.05$) surface expression in recipient blood compared to perfusion solution was detectable (**Figure 4C**). To reveal a potential age-related influence on the KIR repertoire of NK and T cells in the perfusion solution, we correlated donor age to KIR⁺ donor NK and T cells in perfusion solution. Our results could not reveal an impact of donor age on the KIR repertoire of donor NK and T cells in perfusion solution (**Supplementary Figure 3**). Our data suggest that predominantly CD56^{dim}CD16^{hi} NK cells leave the donated lung during preservation into the perfusion solution, whereas CD56^{dim}CD16^{lo} NK cells may migrate primarily into recipient blood directly after DLTx. KIR expression on donor T cells was higher in the recipient blood compared to perfusion solution while this did not affect KIR⁺ subsets substantially.

The KIR Repertoire on Donor NK and T Cells in DLTx Recipient Blood Does Not Correlate With Primary Graft Dysfunction

Next, we wanted to analyze the clinical impact of donor NK and T cells in the recipient periphery and their KIR repertoire on primary graft dysfunction (PGD), a major cause of early graft failure and poor transplant outcome. Therefore, lung transplant recipients were divided into two groups according to their PGD scores. PGD was assessed 24 hours post DLTx and graded in PGD 0-1 (no and low degree of severity) and PGD 2-3 (high

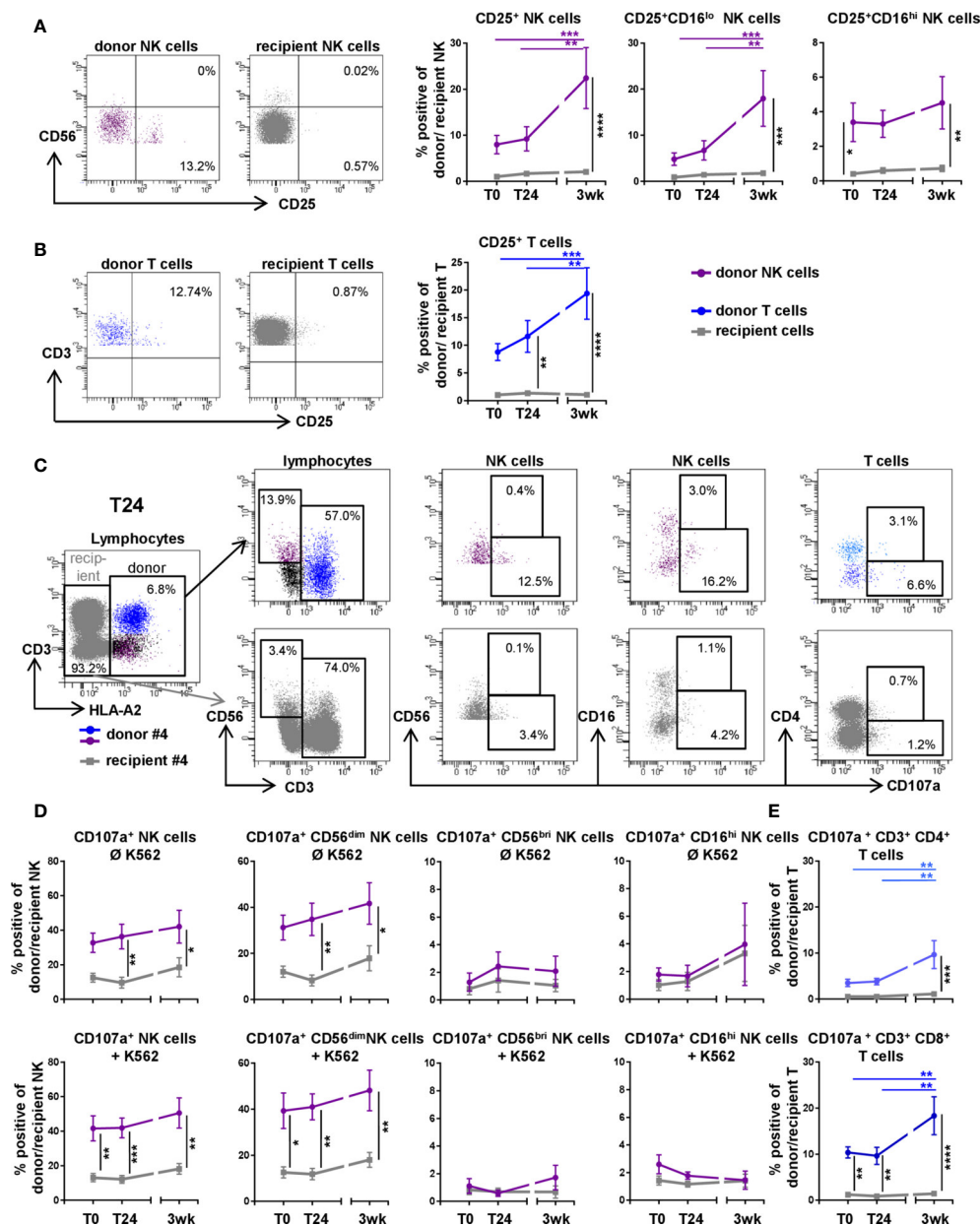


FIGURE 3 | Donor NK and T cells show a higher activation level and degranulation capability three weeks after double-lung transplantation (DLTx) in comparison to recipient cells. **(A, B)** CD25 surface expression was analyzed on NK ($n = 14$) and T cells ($n = 14$) in peripheral blood of lung transplant recipients post (T0), 24 hours post (T24) and three weeks after transplantation (3wk). Underlying gating strategy is displayed in **Figure 2**. Representative FACS plots (3wk) and frequencies of **(A)** NK cells and **(B)** T cells are shown. **(C–E)** CD107a surface expression was investigated on donor and recipient NK ($n = 10$) and T cells ($n = 10$) at indicated time points with and without exposure to K562, respectively. **(C)** Gating strategy for analysis of CD107a⁺ NK and T cells in peripheral blood at T24 without K562 is shown. Events were gated on singlets. Lymphocytes were defined through FSC/SSC. Donor and recipient cells were discriminated via mismatch for HLA-A2. Cells were gated on CD3⁺CD56^{dim/bri} for NK cells and CD3⁺CD56⁺ for T cells. **(D)** Frequencies of CD107-expressing donor and recipient NK as well as NK cell subsets with and without exposure to K562 and **(E)** CD107-expressing donor and recipient T cells without contact to K562. Statistical analysis: two-way ANOVA with Sidak's multiple comparison test and Tukey's multiple comparison test. Data are shown as mean $\pm$ SEM, asterisks indicate p-values with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

degree of severity). Despite a very small sample size, both groups were compared with respect to their donor NK, T cell frequencies, and the KIR repertoire on donor and recipient cells. In general, no differences could be detected for the

frequencies of donor NK and CD4⁺ or CD8⁺ donor T cells between both PGD groups (**Figure 5A**). Furthermore, the data revealed no significant distinction between PGD0-1 and PGD2-3 focusing on KIR repertoire on donor and recipient NK and T

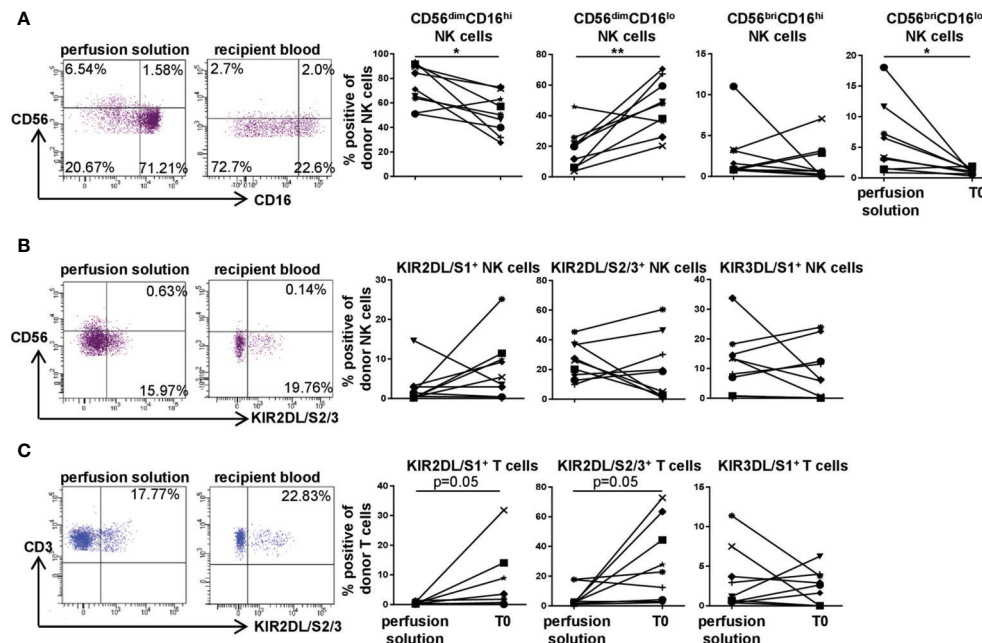


FIGURE 4 | KIR repertoire on donor NK and T cells in perfusion solution distinguishes from the one in recipient blood directly post DLTx (T0). KIR2DL/S1, KIR2DL/S2/3 and KIR3DL/S1 surface expression by donor NK and T in perfusion solution and recipient blood of 9 DLTx patients was determined. Cells were gated for CD56^{dim} and CD56^{bri} NK cells as described in **Figure 1C** and for KIR as illustrated in **Figures 2A, E**. **(A)** Representative FACS plots and frequencies of donor CD56^{dim} or CD56^{bri} and CD16^{hi} or CD16^{lo} NK cells in perfusion solution and recipient blood are shown. **(B)** Representative FACS plots of KIR2DL/S2/3 and frequencies of KIR2DL/S1, KIR2DL/S2/3 and KIR3DL/S1 surface expression on donor NK cells are demonstrated. **(C)** Representative FACS plots of KIR2DL/S1 and frequencies of KIR2DL/S1, KIR2DL/S2/3 and KIR3DL/S1 expression on donor T cells. Statistical analysis: paired t-test was calculated for normally distributed data, otherwise Wilcoxon test. Asterisks indicate p-values with *p < 0.05, **p < 0.01.

cells, although the frequency of KIR⁺ NK and T cells was slightly lower in DLTx patients suffering PGD2-3 (**Figures 5B, C**). The association of PGD and the activation marker CD25 was also analyzed. No differences in the CD25 surface expression on donor NK cells comparing PGD 0-1 and PGD2-3 could be shown (**Figure 5D**). In contrast, an increase in the frequency of CD25⁺ donor T cells (p<0.05) in patients with PGD2-3 over time could be demonstrated (**Figure 5E**). The proportion of CD25⁺ donor T cells in PGD-01 patients also slightly increased without reaching statistical significance. The difference in CD25⁺ donor T cells between PGD2-3 and PGD0-1 patients at three weeks was also not significant. Taken together, our results indicate that neither frequency nor KIR repertoire of donor NK and T cells did affect the early clinical outcome, i.e. PDG, after lung transplantation.

Frequencies of KIR⁺ Donor NK Cells Directly After DLTx Tend to Increase With Longer Cold Ischemic Times (CIT)

Little is known about the impact of the cold static preservation on the explanted lungs in terms of NK and T cell kinetics in recipient blood and clinical outcome. Therefore, we correlated cold ischemic times (CIT) with frequencies of donor NK and T cells at T0 (**Figure 6**). Our data mirrored a positive correlation between the duration of CIT and frequencies of donor NK as well as CD4⁺ and CD8⁺ T cell subsets in recipient blood directly post

DLTx (**Figure 6A**). Especially the proportion of CD4⁺ donor T cells increased with longer CIT (r=0.4; p=0.01). Regarding the influence of CIT on the KIR repertoire of donor NK cells at T0 (**Figure 6B**), a trend for a correlation was found between CIT and all analyzed KIR on donor NK cells (KIR2DL/S1⁺ r=0.48, p=0.09; KIR2DL/S2/3⁺ r=0.5; p=0.07 and KIR3DL/S1⁺ r=0.52; p=0.06). Particularly the KIR2DL/S2/3⁺CD56⁺ NK cells showed a positive correlation to CIT. In contrast, we could not show any correlation between CIT and the KIR repertoire on donor T cells (**Figure 6C, Supplementary Figure 4**). In summary, these results imply that both donor NK and T cells generally are influenced by CIT. Furthermore CIT has an impact on KIR⁺ NK cell subsets whereas the duration of the *ex situ* time of the lung has no influence on the KIR repertoire on donor T cells. These results may have a so far underestimated impact on the donor/recipient distribution in lung transplantation.

DISCUSSION

Recently, the impact of NK cells and the genetics associated with the KIR ligand mismatch has been shown for kidney transplantation (5), which raises also the question for other organs, especially those with tissue-resident cells like the lung. Passenger lymphocytes can be found in the peripheral blood of lung transplant recipients (13, 18), but the knowledge on their

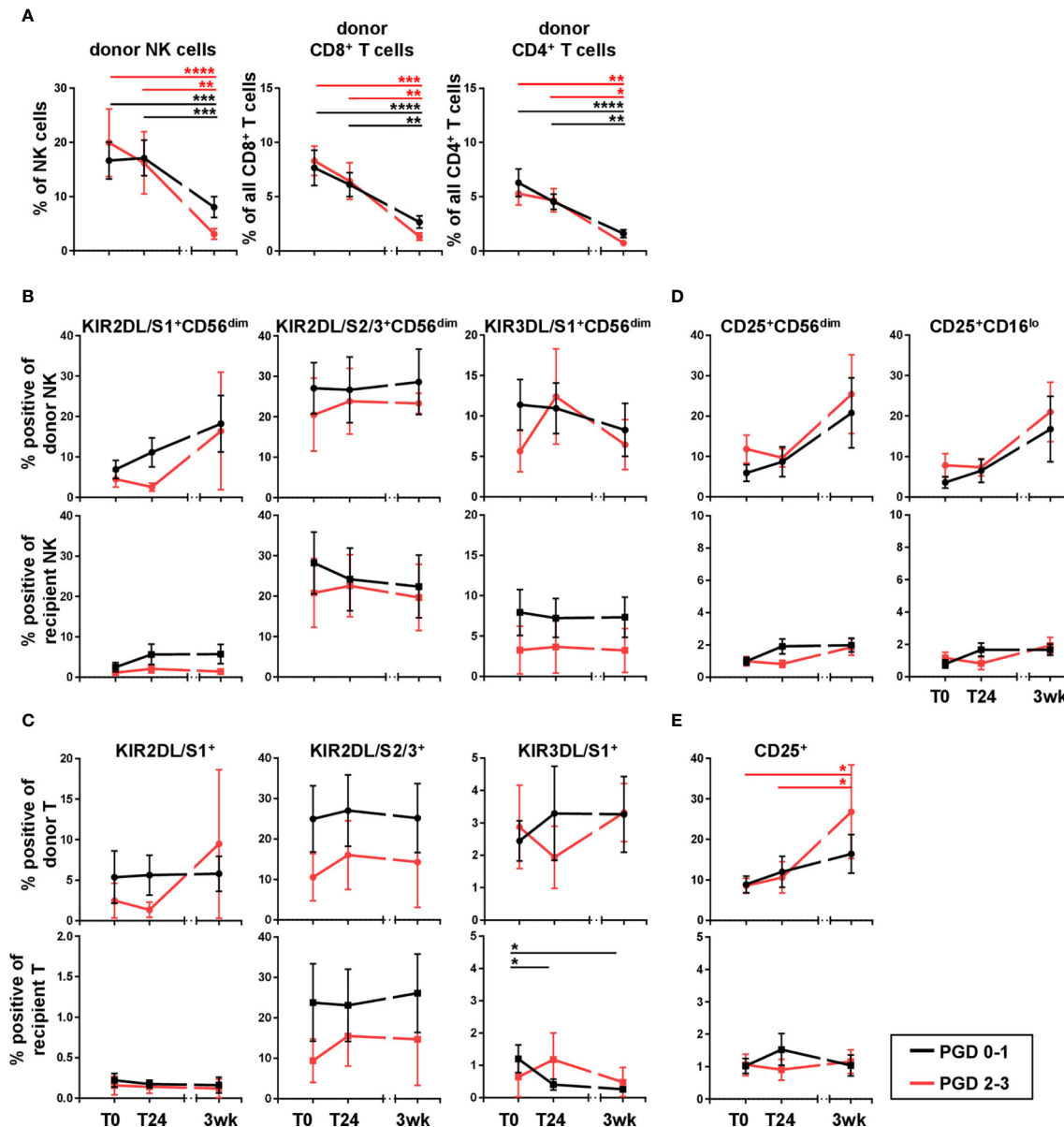


FIGURE 5 | KIR repertoire of donor and recipient NK and T cells does not influence primary graft dysfunction (PGD). **(B, D)** KIR2DL/S1, KIR2DL/S2/3, KIR3DL/S1 and **(C, E)** CD25 expression on NK and T cells was analyzed in peripheral blood of lung transplant recipients post (T0), 24 hours (T24) and three weeks after transplantation (3wk). Donor and recipient cells were distinguished by HLA mismatch using anti-HLA-A2/A1 specific Ab. Cells were gated as described in **Figure 1C**. DLTx recipients were divided into two groups, PGD0-1 [$n = 29$ for **(A)**; $n = 10$ for **(B–E)**] and PGD2-3 [$n = 10$ for **(A)**; $n = 4$ for **(B–E)**], concerning their severity of PGD at 24 hours post DLTx. **(A)** Frequencies of donor NK and donor T cell subsets are shown. **(B, D)** Illustration of frequencies of KIR positive NK and T cells and **(C, E)** their CD25 expression. Statistical analysis: two-way ANOVA with Sidak's multiple comparison test and Tukey's multiple comparison test. Data are shown as mean $\pm$ SEM, asterisks indicate p-values with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

kinetics, phenotype and relevance in the lung transplant setting is still rather scarce. Here, we aimed to elucidate donor NK and T cell characteristics in recipient blood during the first three weeks after lung transplantation with particular focus on their KIR repertoire and their impact on clinical outcome. To the best of our knowledge, these dynamics within the first three weeks after DLTx have not been studied in detail before. In the current study, we unexpectedly observed substantial changes in the

composition of lymphocyte subsets in the recipient periphery immediately after DLTx. In general, our analyses revealed higher frequencies of NK cells, dominated by CD56^{dim}CD16^{hi} NK cells at T0, and a lower proportion of T cells, primarily CD4⁺ T cells, directly post DLTx compared to baseline pre DLTx. Furthermore, a significant decrease in the CD4⁺/CD8⁺ T cell ratio directly post DLTx was detected. In order to identify a potential link between donor passenger cells and the dynamics in

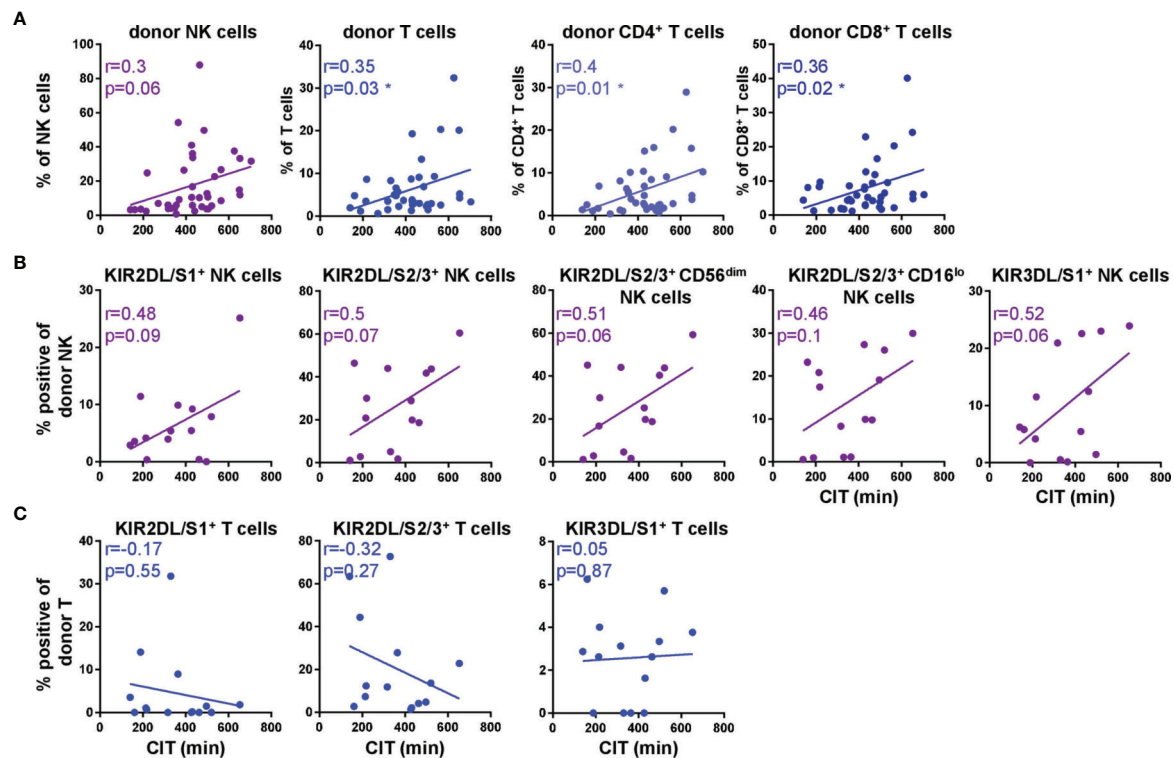


FIGURE 6 | KIR⁺ donor NK cell subsets increase with longer cold ischemic time (CIT). **(A)** Scatter plots show linear regression of CIT to donor NK and T cell subsets ($n = 39$) and **(B)** KIR surface expression on donor NK cells ($n = 14$) and **(C)** T cells ($n = 14$). Each dot represents one patient. Gating strategy for donor NK and T cells is shown in **Figure 1C** and for KIR in **(Figures 2A, E)**. Statistical analysis: linear regression and correlation analysis by Pearson correlation. Asterisks indicate p -values with $*p < 0.05$.

lymphocyte subsets, we focused our analyses on the first three weeks, especially the first 24 hours. This time frame was also supported by previous studies detecting donor passenger leukocytes in recipient peripheral blood during the first four weeks following lung transplantation (14).

With the HLA-based discrimination between donor and recipient leukocytes, we could prove that a transient lymphocyte chimerism, primarily by NK and T cells but not B and myeloid cells (data not shown) is involved in the dynamic changes in lymphocyte subsets after DLTx. Within the NK and T cell subsets, donor NK cells represented the highest proportion at all three time points, followed by CD8⁺ and CD4⁺ T cells. The frequency of donor CD8⁺ T cells among all CD8⁺ T cells was elevated compared to CD4⁺ T cells and, therefore, can partially explain the generally decreased CD4⁺/CD8⁺ T cell ratio directly post DLTx, which was also mediated by substantially decreased recipient CD4⁺ T cells. The reduced frequency of all T cells and the simultaneously increased proportion of NK cells directly post DLTx cannot be explained only by the augmented frequency of donor NK cells leaving the transplanted lung into the recipient periphery. Hence, it is conceivable that T cells leave the periphery, and may potentially migrate into the transplanted lung, as an immune response to the transplanted organ. This scenario has been proposed for NK cells (19). Alternatively, T cells may migrate to the lymphatic system, i.e. lymph nodes and

spleen, which can also be observed during infection. Bidirectional movements of donor and recipient T and NK cells between peripheral blood and the lung allograft are capable to evoke profound changes in leukocyte subsets directly post DLTx.

Next, we aimed for a more detailed characterization of the phenotype of donor NK cells with respect to the major peripheral subsets, i.e. CD56^{dim}CD16^{hi} and CD56^{bri}CD16^{neg} NK cells. We focused on KIR expression, since activating and inhibitory KIR are major surface receptors regulating NK and T cell activity and consequently, might play a crucial role in the context of lung transplantation (11). To get further insights into the KIR expressing donor NK and T cell subsets, we analyzed the surface expression of the most relevant KIR on donor as well as recipient NK and T cells after DLTx. We found higher proportions of KIR2DL/S1, KIR2DL/S2/3 and KIR3DL/S1 expressing subsets in donor NK and T cells compared to recipient cells. Our results furthermore demonstrated a significant increase in frequency of KIR2DL/S1⁺ donor NK cells during the first three weeks post DLTx, whereas the frequency of KIR2DL/S1⁺ recipient NK cells remained stable at low levels over time. In addition, we studied the functionality of these donor NK and T cells in recipient blood and found donor NK cells to exhibit a higher degranulation capability compared to recipient NK cells. The poor capability of both donor and

recipient NK cells to respond to HLA class I-deficient K562 cells in terms of *in vitro* degranulation is likely caused by the onset of immunosuppression in the patient intraoperatively (20, 21). In addition, KIR surface expression increases with progressing maturation of NK and T cells (22, 23). Both aspects, enhanced degranulation capability and increased KIR expression on donor cells, suggests donor NK and T cells to have a more functional phenotype compared to recipient cells after DLTx.

In order to investigate the potential origin of donor NK cells in more detail, we also used perfusion solutions to identify the donor NK cell composition and to compare it to the passenger cells in the recipient periphery directly after DLTx (data not shown). NK cells in perfusion solutions were predominantly composed of CD56^{dim}CD16^{hi} NK cells. Since donor NK cells in recipient blood were mainly represented by CD56^{dim}CD16^{lo} NK cells, this change in CD16 surface expression argues for at least some degree of activation in this CD56^{dim} NK cells subset. This CD56^{dim} NK subset has also been identified as lung-resident subset in human lung parenchyma by Marquardt et al. (24). Since we primarily detected the CD56^{dim}CD16^{hi} NK cell subset in perfusion solution, we propose that this subset leaves the donor lung early during preservation, whereas directly after DLTx, the CD56^{dim}CD16^{lo} NK cell subset is migrating out of transplanted lung into the recipient periphery. In addition, the low CD16 expression can be an indicator for a partial activation, which may also explain their spontaneously high CD107a expression level even in the absence of K562 target cells.

Our group has previously demonstrated that downregulation of CD16 surface expression on NK cells in patients after kidney transplantation was associated with activation and induction of interferon- γ (20). Here, we detected a downregulation of CD16 surface expression on NK cells up to three weeks after DLTx, thereby showing a similar CD16 modulation also in lung transplantation. In the DLTx cohort, CD16 downregulation was even more pronounced in donor compared to recipient NK cells. In parallel, the activation marker CD25 was significantly higher expressed on both donor NK and T cells, which argues for a higher activation level in donor vs. recipient cells. This higher activation status of donor NK and T cells was observed continuously within the first three weeks after DLTx and may be explained by a permanent recognition of the recipient, which may be driven by the HLA mismatch, especially in KIR ligand mismatch constellations between donor and recipient, according to the 'missing self' hypothesis. This is rather likely to be the case since in our cohort, approximately 90% of recipients show more than three HLA class I and three HLA class II mismatches to the respective donor HLA alleles. In conclusion, we characterized donor NK and T cells to exhibit a more activated and functional phenotype compared to recipient cells in peripheral blood of lung recipients during the first three weeks following DLTx. Thus, future studies are required to further elucidate whether activated NK cells may be associated with rejection also in lung transplantation as it was shown for CD69⁺CD56^{dim} NK cells in AMR biopsies of kidney transplant recipients (25).

The clinical outcome after DLTx is still limited, predominantly due to early, i.e. PGD, and late chronic allograft

dysfunction (CLAD). Since the impact of a transient chimerism has not been addressed clinically in DLTx, we investigated the relevance of donor NK and T cells in terms of PGD and CIT. In BAL of lung recipients, it has been recently shown that certain proportions of donor-derived T cells with a tissue-resident memory phenotype are associated with a lower incidence of primary graft dysfunction (14). In contrast to BAL, donor T cells were not detectable in recipient blood in these analyses, which started 4 weeks after transplantation and, hence, did not cover the very early phase directly after DLTx. Therefore, we can assume that the chimerism detected in our setting is likely to be transient and may wane after several weeks, which is also the case by own observations (data not shown).

To elucidate the function and relevance of the KIR family in the lung transplant setting, we compared the KIR repertoires on donor vs. recipient NK and T cells and correlated these to PGD severity, representing a major complication early after DLTx, as well as CIT. In this small cohort, we could not show a correlation between the proportions of KIR⁺ donor NK or T cells and PGD, since patients suffering from PGD2-3 did not display higher KIR frequencies. Hence, the KIR NK and T cell repertoire does not seem to have a direct effect on clinical outcome, i.e. PGD during the first 72 hours after DLTx. However, the preservation period may impinge on the frequency of donor NK cells since we observed a positive correlation between CIT and KIR⁺ NK subsets. Interestingly, the frequencies of KIR-expressing donor T cells did not increase with longer CIT, further underlining the important role of NK cells. This unexpected finding points towards a physiological impact of ischemia reperfusion injury on the mobilization of NK and T cells in lung transplantation, which is likely influenced by the inflammatory milieu of the lung during the *ex situ* phase. In addition to the known ischemic mechanisms (26), extended preservation times may also be critical for the transient chimerism and, rather indirectly, maybe also for early clinical outcome. Our findings are also supporting a previous study demonstrating a crucial role of NK cells on clinical outcome within the early phase after kidney transplantation (27). However, the mechanisms may be unrelated since in kidney transplantation, no passenger cells have been identified (28).

Acute cellular rejection (ACR), driven mainly by alloreactive cytotoxic T cells, can lead to acute graft failure, and with regards to the long-term outcome, can also result in CLAD development. Furthermore, besides NK cells, inhibitory KIRs are expressed by CD8⁺ T cells and, therefore, regulate their functions and survival in i.e. viral infections (22, 29, 30). In our DLTx cohort, we detected a significantly higher KIR3DL1/S1 expression on donor compared to recipient T cells. It will be interesting to validate these findings in a larger patient cohort and to relate them to the long-term-survival of the allograft. Still, iKIR are not able to downregulate all aspects of T cell activation, as demonstrated by elegant experiments in a transgenic mouse system (31). Therefore, inhibitory KIR may be important for the fine-tuning and adjustment of T cell functions. In addition, it has been shown that NK and T cells share many functional and phenotypical properties (32). Thus, it might be possible to transfer – at least in part – conclusions for donor NK cells onto donor T cells. Yet, future studies will be needed to further elucidate the functional consequences of iKIR expression by

(donor) CD8⁺ T cells in the context of lung transplantation with a focus on ACR development.

Based on the known impact of age on NK and T cell subset distributions, we correlated the KIR⁺ subsets to donor age with the aim to show whether, increasing KIR proportions may be elevated with progressing age indicating enhanced maturation, as many groups have demonstrated (22, 23). Surprisingly, no correlations between KIR expression and donor age were found indicating that the donor age only has a weak effect on the KIR repertoire on donor NK and T cells. Furthermore, due to limitations of donor lung availability these results imply that even older people could serve as potential donors in the lung transplant setting.

In conclusion, our findings reveal donor NK and T cells in the periphery of lung transplant recipients as highly activated and functional subsets and therefore might be crucial in the lung transplant setting. Further studies investigating regulatory receptors on donor NK and T cells are necessary to better understand their contribution to graft tolerance thereby improving patient survival after lung transplantation.

Limitations of the Study

The results presented here are derived from a single transplant center with a limited sample size. Thus, expanding the patient number is desirable to substantiate our findings. Due to the incomplete HLA-C typing, we were unable to define the HLA-C KIR ligand mismatches in detail. Moreover, based on the limited availability of allele-specific α -HLA monoclonal antibodies, only selected patient combinations with donor-specific HLA class I alleles could be analyzed for the discrimination of donor vs. recipient cells. Moreover, it would be interesting to identify the KIR-expressing NK and T cells subsets in BAL samples representing airway compartment. The clinical correlations were limited to the early events like PGD and CIT but in the future, we plan to investigate also a possible impact on intermediate outcome like acute rejection as well as long-term outcome, i.e. CLAD. These aspects are part of ongoing studies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethics committee at the Hannover Medical School (no. 122-2007, 2500/2014). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AMH and KAB performed experiments, analyzed data and wrote the manuscript. BW coordinated sample collection and

contributed to writing of the manuscript. RBS and FW helped with statistics and writing of the manuscript. EC revised statistics, figures, tables and text of the manuscript. JK, KB, LMH and CN performed and supported key experiments. FI, WS, MA, CK, IT, JS, TS, AH and GW performed DLTx and were instrumental for sample and clinical data collection. CSF and JFK supervised the work, designed experiments, analyzed data and wrote the manuscript.

ACKNOWLEDGMENTS

This project was supported by the German Research Foundation DFG, SFB738 project B3, and FA-483/1-1, the German Center for Infection Research DZIF TTU-IIICH, and the MHH Transplant Center project 19_09.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2021.778885/full#supplementary-material>

Supplementary Figure 1 | Flow cytometry gating strategy for the discrimination of donor vs. recipient cells, exemplary shown for an HLA-A2-positive donor (lung) transferred into an HLA-A2-negative recipient. **(A)** Cell doublets were excluded based on the area/height ratio. In the next step, dead cells were excluded based on the life/dead staining. CD45⁺ cells were then gated and finally lymphocytes were gated based on their CD45 expression and granularity (SSC). Subsequently, CD56⁺CD3⁻ NK cells as well as CD3⁺CD56⁺ T cells were defined. NK cells were further discriminated into either donor or recipient origin based on HLA-A2 expression. In this example, the donor is HLA-A2⁺ and donor NK cells (CD56⁺HLA-A2⁺) are displayed in pink, whereas the recipient is HLA-A2⁻ and recipient NK cells (CD56⁺HLA-A2⁻) are shown in purple. T cells were further divided into CD3⁺CD4⁺ T cells and CD3⁺non-CD4⁺ ("non-CD4⁺") T cells. Finally, donor as well as recipient origin for both CD4⁺ and non-CD4⁺ T cells was determined based on HLA-A2 expression (donor T cells HLA-A2⁺, recipient T cells HLA-A2⁻) and are displayed in different blue shades. **(B)** High proportions of donor NK and T cells can be detected in recipient blood directly post DLTx. Frequencies of donor NK and T cell subsets in peripheral blood of DLTx recipients were analyzed pre, directly post (T0), 24 hours (T24) and three weeks after transplantation (3wk). Donor and recipient cells were discriminated via HLA mismatch using anti-HLA-A2 specific Ab. Gating strategy is shown in **Figure 1C**. Statistical analysis: one-way ANOVA with Dunn's multiple comparison test, (n = 39). Data are shown as mean $\pm$ SEM, asterisks indicate p-values with ****p < 0.0001.

Supplementary Figure 2 | Exemplary flow cytometric analyses for KIR2DL/S1, KIR2DL/S2/3 and KIR3DL/S1 surface expression on donor NK and T cells in peripheral blood of double-lung transplant recipients directly post (T0), 24 hours (T24) and three weeks after transplantation (3wk). Donor and recipient cells were distinguished by HLA mismatch: NK cells of the representative donor #14 were identified by HLA-A2 staining **(A)**; T cells of the representative donor #19 were identified by HLA-A1 staining **(B)**, the gating strategies are shown in **Figure 1C** and **S1**. **(A)** Representative FACS plots of one patient of donor#14 (HLA-A2⁺; purple) and recipient#14 (HLA-A2⁻; grey) KIR on NK cells directly after DLTx (T0), 24 hours (T24) and 3 weeks following DLTx. **(B)** Representative FACS plots of one patient of donor#19 (HLA-A1⁺; blue) and recipient#19 (HLA-A1⁻; grey) KIR on T cells.

Supplementary Figure 3 | Donor age might have no impact on the KIR repertoire on donor NK and T cells directly post DLTx (T0). Linear regression of donor age to **(A)** donor NK and T cell subsets (n = 39) directly post DLTx (T0) and to KIR on **(B)** donor NK cells (n = 14) and **(C)** donor T cells (n = 14) directly post DLTx (T0) is shown. Each dot represents one patient. Gating strategy for donor NK and T cells is

shown in **Figure 1C** and for KIR in **Figures 2A, E**. Statistical analysis: linear regression and correlation analysis by Pearson correlation.

Supplementary Figure 4 | Donor age may have no impact on the KIR repertoire on donor NK and T cells in perfusion solution. Linear regression of donor age to KIR on **(A)** donor NK cells ($n = 9$) and **(B)** donor T cells ($n = 9$) in perfusion solution is illustrated. Gating strategy for KIR in **Figures 2A, E**. Each dot represents

one patient. Statistical analysis: linear regression and correlation analysis by Pearson correlation.

Supplementary Table 1 | Antibodies for flow cytometry. APC, allophycocyanine; BV, brilliant violet; ECD, energy coupled dye (phycoerythrin-texas red conjugate); FITC, fluorescein isothiocyanate; PB, pacific blue; PE, phycoerythrin; PerCP, peridinin-chlorophyll-protein complex.

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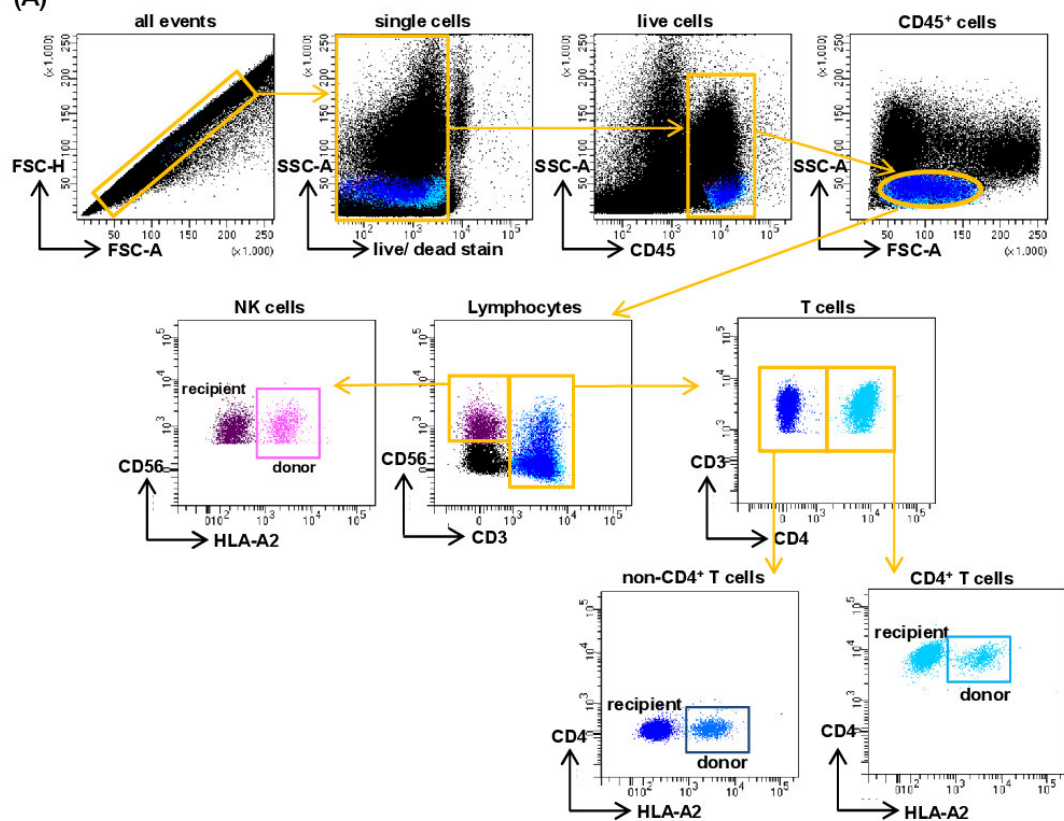
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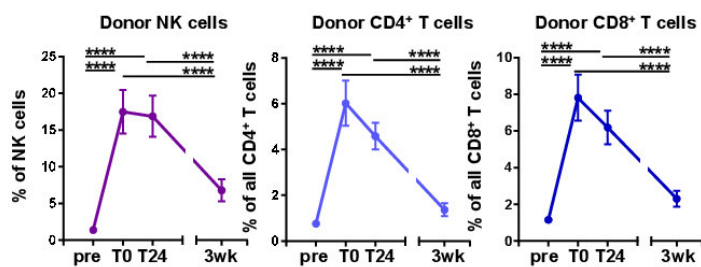
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Suppl. Figure S1: HLA staining together with T cell and NK cell markers

(A)

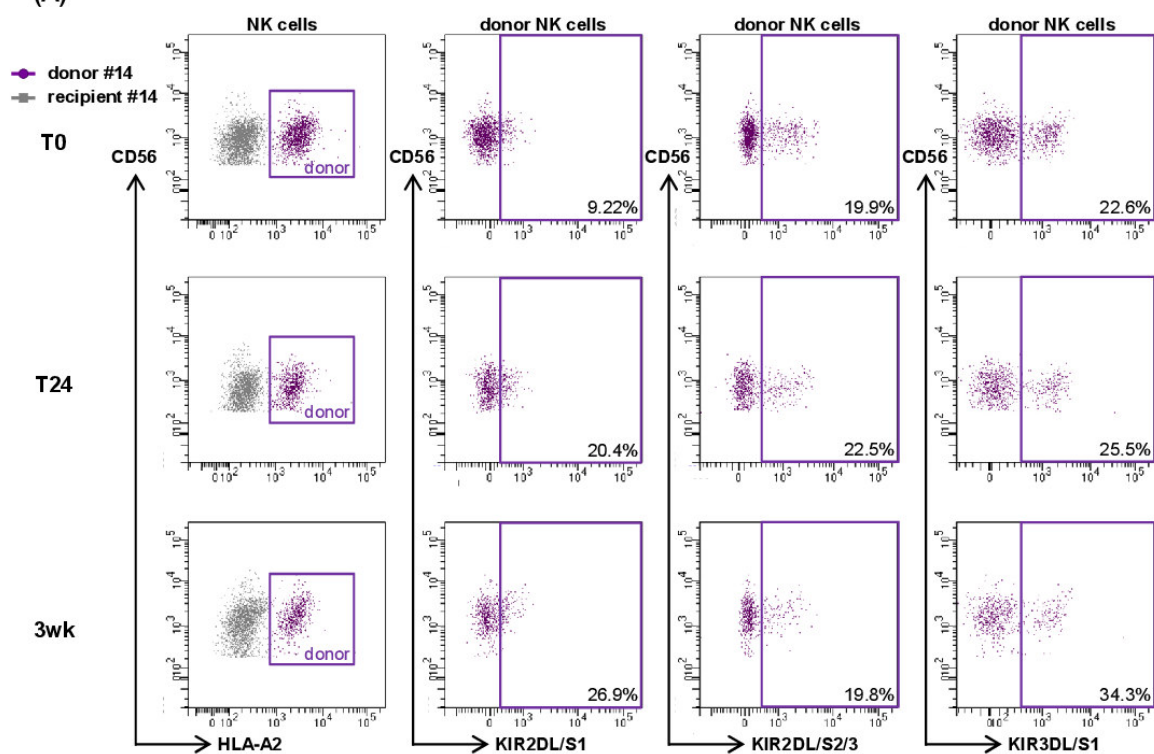


(B)

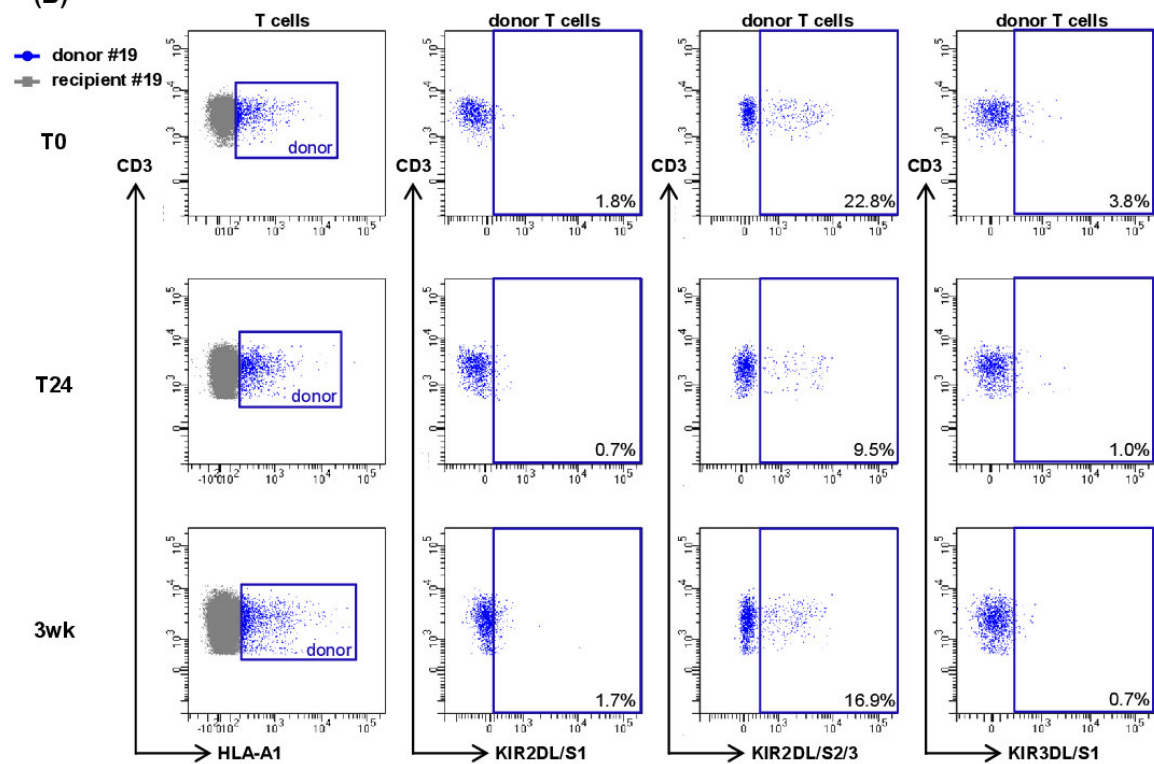


Suppl. Figure S2

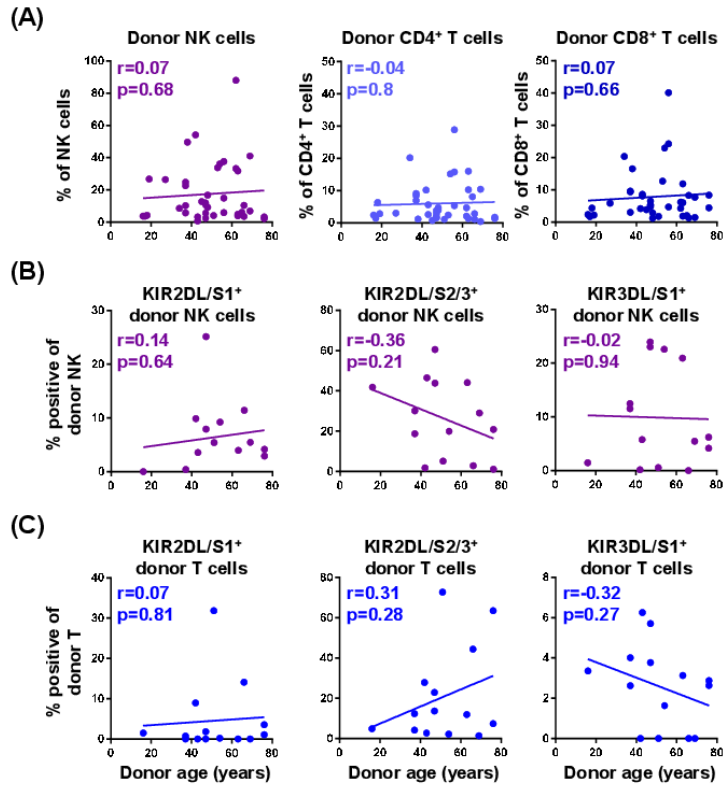
(A)



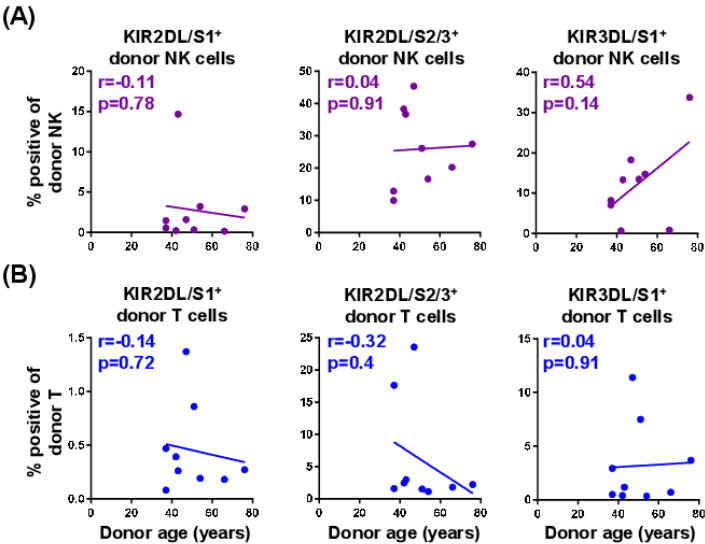
(B)



Suppl. Figure S3



Suppl. Figure S4



7. Discussion

7.1. Passenger leukocytes in LTx

Since the 1990s, it is known that donor-derived passenger leukocytes can be found in the peripheral blood of lung transplant recipients (Richter et al., 1994; Richter et al., 1995), but the understanding of their kinetics, importance and phenotypes is rather poor. Therefore, in this thesis these aspects regarding donor NK and T cells in recipient blood within the first three weeks after LTx with a defined focus on their KIR repertoire and clinical relevance in the transplant setting were examined more precisely. The analyzed period — first three weeks directly after LTx — was also observed by other researchers to detect donor cells after LTx (Snyder et al., 2019).

Surprisingly fundamental changes in the composition of the leukocyte subsets directly after LTx, but also over the course of three weeks, could be discovered. Directly after LTx the proportion of NK cells among all CD45⁺ leukocytes increases, while the proportion of T cells decreases over the same time in relation to prior to LTx, indicating baseline. Predominantly CD4⁺ T cells vanish compared to CD8⁺ T cells causing an inverted CD4⁺/CD8⁺ ratio. NK cells mostly showed a CD56^{dim}CD16^{high} phenotype.

Due to these profound changes in the composition of leukocyte subsets directly after LTx, it is worthy a discussion, if donor cells might be the cause of those findings in the peripheral blood of LTx patients.

Mainly because of organ shortage in LTx and in contrast to for example kidney transplantation, in LTx donors and recipients are not HLA-matched, allowing a discrimination between donor and recipient cells. Directly after LTx primarily donor NK and T cells can be detected in the peripheral blood of lung transplanted patients, whereas levels of donor B cells and donor myeloid cells are rather low. Donor NK cells represent the highest proportion at all observed time points followed by firstly CD8⁺ donor T cells and secondly CD4⁺ donor T cells. Those observations might partly explain the above mentioned changes in the leukocyte subsets. As the proportion of CD8⁺ donor T cells among all CD8⁺ T cells is higher compared to the one

regarding CD4⁺ donor T cells, this might originate the inverse in the CD4⁺/CD8⁺ ratio directly after LTx. The higher frequency of NK cells and lower frequency of T cells in the peripheral blood of lung transplant recipients otherwise, can only insufficiently be explained by the large frequency of donor NK cells leaving the transplanted organ into the peripheral blood of the lung transplant recipient. Therefore, in fact it is imaginable that T cells may emigrate out of the peripheral blood into the transplanted lung, attracted by inflammation caused by immune responses during the transplantation procedure. This scenario has been suggested for NK cells in this review (Fildes et al., 2008). Other locations of attraction might be lymph nodes or the spleen.

In conclusion, movements of immune cells in both directions — out of the transplanted lung into the peripheral blood and *vice versa* — evoke fundamental changes in leukocyte subsets directly after LTx, but more research regarding the relevance of those changes in leukocyte subsets in the transplant setting is needed.

7.2. KIR on donor NK and T cells

NK and T cells play a crucial role in the LTx setting, as their amount, timing and context of activation determines, whether they induce graft tolerance or graft damage (Calabrese et al., 2019; Hadad et al., 2014). In addition, NK and T cells are regulated, among others, by KIR. Thus, high affinity to the ligand and/or the amount of aKIR and iKIR define NK and T cell activity and therefore, might influence LTx outcomes (Greenland et al., 2017; Rajalingam, 2016). Recently a study could show the impact of NK cells associated with KIR ligand mismatch in kidney transplantation (Callemeyn et al., 2021). Hence, KIR are good objectives to focus on in LTx, especially because of the existence of tissue-resident cells in the lung.

To receive more information about the KIR repertoire on donor NK and T cells, the expression of the most relevant KIR (KIR2DL/S1, KIR2DL/S2/3 and KIR3DL/S1) on donor and recipient NK and T cells within the first three weeks after LTx in the peripheral blood of lung transplant recipients was analyzed. Overall, a higher frequencies of donor NK and T cells expressing

KIR2DL/S1, KIR2DL/S2/3 and KIR3DL/S1 compared to recipient NK and T cells could be shown. Moreover, the results revealed a significant increase in the proportion of KIR2DL/S1⁺ donor NK cells over the observed time of three weeks after LTx. In contrast, the proportion of KIR2DL/S1⁺ recipient NK cells remained stable at low baseline levels. Comparing the KIR repertoire on donor and recipient T cells three weeks after LTx shown results demonstrated a significant higher frequency of KIR2DL/S1⁺ and KIR3DL/S1⁺ donor T cells.

As NK and T cells accumulate higher frequencies of KIR on their surface during maturation (Boelen et al., 2018; Colucci et al., 2003) our results regarding the analyzed KIR repertoire on donor NK and T cells probably indicate a more activated and functional phenotype of donor NK and T cells compared to recipient NK and T cells. Furthermore, it might be suggested that donor NK and T cells underlie a stronger regulation by the immune system.

7.3. CD16 and CD25 on donor NK and T cells

Further, we characterized donor NK cells regarding the major NK cell subsets according to CD56 and CD16 in CD56^{dim} and CD56^{bright} NK cells, as well as via CD25. In general, our results underline the above mentioned findings of a more functional and activated phenotype of donor NK cells during the first three weeks after LTx.

In a previous publication, our group showed that down-regulation of CD16 in kidney transplantation leads to cell activation and induction of γ -interferon (Hoffmann et al., 2015). For donor NK cells within the first three weeks after LTx we observed the same effect, as CD16 is down-regulated over the observed time. This impact is even more noticeable on donor NK cells compared to recipient NK cells indicating a stronger activation.

In addition the expression of the activation marker CD25 (Leong et al., 2014) was significant higher on donor NK and T cells three weeks after LTx compared to recipient cells supporting the conclusion of a stronger activated and more functional phenotype of donor NK and T cells. An appropriate explanation for those more activated and more functional donor NK and T cells may be their long-term exposure to recipient cells, as there exists a HLA mismatch and even

more relevant a KIR ligand mismatch between donor and recipient in the LTx setting. According to the study design, this is very likely in the present sample cohort, as more than 90% of all recipients display more than three HLA class I and HLA class II mismatches.

In summary, via a higher KIR and CD25 expression, as well as a down-regulation of CD16 expression donor NK and T cells are characterized to exhibit a more activated and functional phenotype compared to recipient cells in the peripheral blood of lung transplant recipients within the first three weeks after LTx. Doubtless more research needs to be implemented regarding their clinical relevance for example in the field of rejection as well as tolerance induction.

7.4. Perfusion solution and donor NK and T cells

To find out more about the origin and phenotype as well as the chronology of donor NK cell emigration, we examined the perfusion solution, in which the explanted organ is transported to the recipient hospital.

The comparison between NK cells in the perfusion solution and donor NK cells in patients' periphery revealed profound differences. The main proportion of NK cells in perfusion solution are CD56^{dim}CD16^{hi} NK cells, whereas the highest frequency of donor NK cells are CD56^{dim}CD16^{lo} suggesting that the time post LTx represents a crucial variable concerning the migration of distinct donor NK cell subsets out of the transplanted lung. Our results showed that the CD56^{dim}CD16^{hi} NK cell subset leaves the transplanted lung earlier during preservation, while the CD56^{dim}CD16^{lo} donor NK cell subset moves subsequently into the peripheral blood of the lung transplant recipient.

Furthermore, we noticed CD56^{dim}CD16^{lo} NK cells to be the main NK cell subset in the peripheral blood after LTx. As down-regulation of CD16, as already mentioned, is associated with NK cell activation this supports our findings, that NK cells are activated during the transplantation procedure.

Additionally, a certain subset of CD56^{dim} NK cells has been identified as a lung-resident subset in human lungs with an effector phenotype underlining our hypothesis of a more functional phenotype of donor NK cells in the peripheral blood of lung transplant recipients after LTx (Marquardt et al., 2017).

7.5. Clinical impact

Primarily due to early and late onset graft rejection survival rates of lung transplant recipients are rather low, also in comparison to other solid organ transplantations. Recently, a study could show that certain proportions of donor-derived T cells with a tissue-resident phenotype in bronchioalveolar lavage are associated with a smaller presence of PGD (Snyder et al., 2019). As, to our knowledge, the clinical effect of donor-derived passenger leukocytes has not been investigated in detail, the presented data was correlated to CIT and PGD to identify potential targets for immunotherapy in the field of LTx. As the results suggest a transient leukocyte chimerism, the amount of detectable donor NK and T cells decreased over the course of more than four weeks. These findings are also supported by other groups (Snyder et al., 2019) therefore, the shown results were correlated to clinical events early after LTx.

Furthermore, we aimed to identify a potential link between the analyzed KIR repertoire on donor NK and T cells and PGD severity to elucidate short-term outcome, as well as CIT to illustrate effects during organ preservation. Perhaps also due to the small sample size, no correlation between the severity of PGD and the proportions of KIR⁺ donor NK and T cell subsets could be found. In patients, suffering PGD grade II and III higher or lower proportions of KIR⁺ donor NK and T cells could not be discovered indicating no effect of high frequencies of donor NK and T cells on PGD severity.

Contrarily, a positive correlation between high frequencies of KIR⁺ donor NK cells and a longer CIT was observed. Surprisingly this determination could not be made for donor T cells. As IRI occurs during the time when the transplanted lungs are located out of the human body and therefore, during CIT, the proceeded ischemic mechanisms (Hamilton et al., 2017) might

influence donor NK cells or *vice versa*. Inflammation processes occurring during IRI cause migration of especially NK but also T cells. Thus, a longer CIT during transplantation procedure might be harmful and may affect the transient leukocyte chimerism and early outcome after LTx.

Our results are also supported by a previous study demonstrating a crucial role of NK cells on clinical outcomes in early phases after kidney transplantation (Kunert et al., 2007), though this is attenuated to the fact that a transient leukocyte chimerism could not be found in the peripheral blood of kidney transplant recipients (Moroso et al., 2010).

Moreover, CD8⁺ T cells are regulated, among others, by iKIR, which affect survival and function for example in viral infections (Boelen et al., 2018; Ferrini et al., 1994; Phillips et al., 1995). There exists a complex interplay between all receptors on T cells and clearly iKIR are only able to partly down-regulate T cell function, as described in the following mice model (Cambiaggi et al., 1999), and therefore, perform assignments like fine-tuning and adjustments of T cell functions.

For our study group a significantly higher KIR2DL/S1 expression on donor compared to recipient T cells could be shown. As alloreactive CD8⁺ T cells mainly cause acute cellular rejection (ACR) and this is one of the driving factors for CLAD (Koutsokera et al., 2018), it would be interesting to correlate our findings to long-term outcomes.

In addition, it has been shown that NK and T cells share many functional and phenotypical properties (Mingari et al., 1995). Thus, it might be possible to transfer – at least in part – conclusions for donor NK cells onto donor T cells.

Many research groups postulate that KIR expression accumulates with older age indicating enhancement in maturation (Boelen et al., 2018; Colucci et al., 2003). Unexpectedly, in our cohort we could not reveal an effect of donor age on the expression of the analyzed KIR repertoire on donor NK and T cells suggesting a low impact of age on KIR on donor NK and T cells. As donor lungs are very limited in availability, our results might imply a source of donor extension criteria, in which elderly people may be included as donors.

Overall, our results indicate donor NK and T cells to be a strongly activated and functional subset in the lung transplant setting and therefore, might serve as targets for immunomodulation to induce graft tolerance and improvements in lung transplant survival. Still, further investigations regarding regulatory receptors on donor NK and T cells are needed.

7.6. Limitations and outlook of the study

All results were acquired from a single transplant center, at Hannover Medical School. Furthermore, the sample size was relatively small, even though an expanding in patient number is highly probable to confirm our results, as strong significances had been detected. In our study, a clarification of all HLA-C KIR mismatches in detail was not possible because of missing HLA-C genotyping. Additionally, only certain donor-recipient combinations, expressing different HLA class I alleles, could be used to discriminate donor from recipient cells due to the restricted availability of HLA-allele-specific antibodies.

Clinical correlations were limited due to accessibility of only short-term clinical data like CIT and PGD. Ongoing research in our group focusses on the long-term relevance of donor NK and T cells.

8. Summary

LTx remains the ultimate treatment option for patients suffering end-stage lung diseases. Due to multiple factors, for example improvements in the operation techniques and better knowledge about immunosuppression after transplantation, nowadays LTx can be performed more successfully than in the past. Nevertheless, survival rates after LTx are among the poorest of all organ transplantations, mainly due to acute and chronic rejection as well as chronic organ dysfunction.

Since only little is known about the contributions of the immune system in the early phase after LTx, a better understanding in this field is needed.

In this thesis, we investigated the leukocyte subsets, especially NK and T cells, in the peripheral blood of lung transplant recipients within the first three weeks after LTx. Immunophenotyping was performed via flow cytometry.

We revealed fundamental changes in the composition of the leukocyte subsets within the first hours after LTx. While the proportion of NK cells increases directly after LTx, the frequency of T cells decreases during the same time.

Furthermore, we could confirm the, since the 1990s known, donor-derived leukocyte chimerism in the peripheral blood of lung transplant recipients directly after LTx for our patient cohort. Directly after LTx procedure leukocytes emigrate out of the transplanted lung into the peripheral blood of the lung transplant recipient. We elucidated that donor NK and T cells directly after LTx partly evoke the changes within the NK and T cell subsets.

To analyze the phenotype of those donor-derived passenger NK and T cells the expressions of CD56, CD16, CD25 and the most relevant KIR (KIR2DL/S1, KIR2DL/S2/3 and KIR3DL/S1) were determined and compared to their expression on recipient NK and T cells. Summarizing, a down-regulation of the CD16 expression on donor NK cells, an up-regulation of the CD25 expression on donor NK and T cells and a higher expression of the analyzed KIR repertoire on donor NK and T cells could be observed, indicating for a more activated and functional phenotype of donor NK and T cells compared to recipient cells.

To clarify the clinical relevance of our findings, we correlated the observed results to clinical data like CIT and severity of PGD. Whereas no correlation between a higher KIR expression on donor NK and T cells could be detected, a positive correlation between a higher KIR expression on donor NK, but not donor T cells, and longer CIT could be revealed, suggesting IRI, occurring during CIT, to be relevant in this setting.

Overall, our results indicate donor NK and T cells to be a strongly activated and functional subset in the lung transplant setting and therefore, might serve as targets for immunomodulation to induce graft tolerance and improvements in lung transplant survival. Doubtless further investigations regarding regulatory receptors on donor NK and T cells are needed.

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10. Contribution to the paper

As Kim-Alina Bläsing and I, Anna-Maria Hitz, both contributed equally to the publication 'Donor NK and T cells in the periphery of lung transplant recipients contain high frequencies of killer cell immunoglobulin-like receptor-positive subsets', in the following I would like to illustrate my contribution to the publication more detailed.

I implemented the majority of the experiments and analyzed all data for figure 1A, B and E. Furthermore, I performed and analyzed 16 of the 39 HLA-specific FACS experiments, presented in figure 1D. Moreover, I would like to express my gratitude to Kim-Alina Bläsing and Lisa-Marie Horn for performing the rest of the HLA experiments and allowing me to use their results in this publication.

In addition, I executed all experiments regarding figure 2, as well as the assessment of the obtained data.

According to figure 3, I only conducted the experiments and data analysis for figure 3A and B, the rest was done by Kim-Alina Bläsing.

In figure 4 I firstly, analyzed the data, which was already collected by my group, regarding the perfusion solution and secondly, correlated it to data, gained in the previously mentioned experiments.

For figure 5 and 6, I correlated the present results of my experiments to clinical data compiled by the Department of Cardiothoracic, Transplantation and Visceral Surgery (HTTG) at Hannover Medical School.

Moreover, I wrote major parts of the manuscript highlighting my own results and the discussion. Additionally, I participated in the conceptualizing of the manuscript and the revision process with the incorporation of the reviewer's comments.

11. Acknowledgments

Hiermit möchte ich mich ganz herzlich bei allen Personen bedanken, die mich bei der Erstellung meiner Dissertation begleitet und unterstützt haben.

Zunächst möchte ich mich besonders bei Professorin Dr. rer. nat. Christine S. Falk bedanken, die mir dieses spannende Projekt für meine Dissertation zur Verfügung gestellt hat. Herzlichen Dank für die hervorragende Unterstützung, vertrauensvolle Zusammenarbeit und das stets offene Ohr für Anliegen meinerseits. Ihr Enthusiasmus und Passion für die Immunologie hat mich sehr begeistert und inspiriert.

Darüber hinaus möchte ich Dr. Jenny Franziska Kühne mein großes Dankeschön aussprechen. Vielen Dank für das Korrekturlesen meiner Publikation und meiner Dissertation, die konstruktiven Korrekturvorschläge, die viele investierte Zeit und die vertrauensvolle, ausgezeichnete Zusammenarbeit. Dies hat entscheidend zur Fertigstellung meiner Arbeiten beigetragen.

Des Weiteren möchte ich mich bei Kerstin Daemen und Jana Keil für ihre tatkräftige Unterstützung bei experimentellen Fragen und ihre Hilfe im Labor sowie für ihre herzliche Art bedanken. Es war sehr gut zu wissen, dass da immer jemand ist, der einem aus der experimentellen Patsche hilft.

Ein großer Dank gilt auch den anderen Doktoranden der AG Falk. Besonders hervorheben möchte ich Dr. Ramon Bellmàs Sanz, der während der gesamten Zeit im Labor ein wichtiger Ansprechpartner für mich war, und Dr. Franziska Wandrer für das Korrekturlesen meines Manuskriptes, sowie für ihre Expertise. Vielen Dank auch an Jasper Iske und Kim-Alina Bläsing für die lustige Zeit im Labor und die gegenseitige Unterstützung.

Zudem möchte ich der gesamten AG Falk für die herzliche Aufnahme und die schöne Zeit danken. Ich habe mich stets sehr wohl gefühlt.

Ein herzliches Dankeschön geht außerdem an die Klinik für Herz-, Thorax-, Transplantations- und Gefäßchirurgie (HTTG) an der Medizinischen Hochschule Hannover. Ohne deren

Expertise sowie Bereitstellung von Proben und klinischen Daten dieses Projekt nicht möglich gewesen wäre.

Zuletzt möchte ich nun auch meinen Eltern, meiner Schwester und meinen Freunden für ihre emotionale, liebevolle Unterstützung danken. Vielen Dank für das Zuhören, die Geduld, die Ermutigungen, das Verständnis und den Glauben an mich.

13. Declaration according to § 2 sec. 2 no. 7 + 8

PromO

Ich erkläre, dass ich die der Medizinischen Hochschule Hannover zur Promotion eingereichte Dissertation mit dem Titel

Phenotypical and functional characterization of donor NK and T cells in the peripheral blood of lung transplant recipients

im Institut für Transplantationsimmunologie unter Betreuung von Prof. Dr. rer. nat. Christine S. Falk, ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe. Die Gelegenheit zum vorliegenden Promotionsverfahren ist mir nicht kommerziell vermittelt worden. Insbesondere habe ich keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen und Betreuer für die Anfertigung von Dissertationen sucht oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt. Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht. Weiterhin versichere ich, dass ich den beantragten Titel bisher noch nicht erworben habe. Ergebnisse der Dissertation wurden in folgendem Publikationsorgan veröffentlicht: Frontiers in Immunology

Hannover, den _____

(Anna-Maria Hitz)