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# Evaluating the role of niche-derived growth arrest-specific gene 6 (Gas6) in an aggressive mouse model of B-ALL

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# Table of contents

# 1.0 Introduction

## 1.1 Leukemia

It has been more than 170 years from now when leukemia was identified as a distinct clinical entity by John Hughes Bennet and Rudolh Wirchow independently. Bennet identified blood abnormalities distinct from inflammation by staining using acetic acid and microscopic examination. His drawings of round cells with diverse nuclei was published and became the first demonstration of leukemic blood cells. Six weeks after Bennet's finding, Rudolph Wirchow described another case of chronic granulocytic leukemia in his publication "Weißes Blut". Another milestone discovery, which advanced the leukemia research, has been made in 1877 when Paul Ehrlich developed triacid staining, allowing to clearly distinguish morphologically different blood cells (Piller 2001). This pioneering discovery started the era of leukemia research, which evolved drastically over the decades. Today the term 'leukemia' represents a group of life-threatening hematological malignancies, characterized by abnormal proliferation and differentiation of a clonal population of hematopoietic stem or progenitor cells (HSPCs). The classification is based on the clinical course and origin of affected hematopoietic lineages and can be subdivided into four major subtypes: acute lymphoblastic (ALL), acute myeloid (AML), chronic lymphocytic (CLL), and chronic myelogenous leukemia (CML) (Szczepański, van der Velden, and van Dongen 2003). The diagnostic of leukemia is historically based on morphological differences of blood cells, which fundamentally relies on the knowledge of normal hematopoiesis (Zou 2007).

Although the concept of hematopoietic stem cell being an origin of the whole hematopoietic system was introduced already by the Russian pathologist Alexander Maximov in 1909 (Maximov 1909), the first functional identification of HSCs in

multilineage reconstitution, using *in vivo* repopulation assay, was performed only in the early 60s by Till and McCulloch (Till and McCulloch 1963; Till, McCulloch, and Siminovitch 1964). Identification of the small unique self-renewing HSC population as an apex of the hematological hierarchy proceeded along with the understanding, that cancer might mirror the hierarchy and heterogeneity of normal tissue. The evidence of cancer stem cells, with unique self-renewing capacity, was shown in studies of human leukemia proliferation by Clarkson et al. (Clarkson et al. 1967). The authors showed that upon $H^3$ thymidine infusion, the majority of blasts were labeled that originated from the small non-labeled slowly proliferative subset of leukemic stem cells, similar to normal HSCs. Moreover, the researchers noted, that this leukemia stem cell (LSC) fraction reacts to the chemotherapeutical de-bulking of leukemic blasts by entering the cell cycle. The authors hypothesized that this clonal heterogeneity, and in particular the existence of dormant LSCs, will be the major obstacle to treat leukemia, which was proven true and remains a relevant problem up to date. Decades later, using a patient-derived xenograft model with severe combined immunodeficient (SCID) mice, J. Dick published several studies identifying a rare AML initiating subset, responsible for the engraftment and propagation of the disease and demonstrating that AML has a hierarchical organization (Bonnet and Dick 1997). The leukemia origin and evolution is schematically depicted in fig. 1, showing how the leukemic hierarchy and heterogeneity develops similar to normal hematopoiesis and is shaped by clonal evolution. This involves a complex interplay of acquired additional genetic lesions, epigenetic changes, competing with non-transformed hematopoietic counterparts and modifying microenvironmental cross-talk.

Indeed, in the last decade, the role of the bone marrow microenvironment in leukemia pathogenesis has been increasingly appreciated. A tightly regulated cross-

talk between hematopoietic cells and their bone marrow niche counterpart is critical for the maintenance of normal hematopoiesis and such interactions are often hijacked in the context of leukemic transformation (Korn and Méndez-Ferrer 2017). The prominent role of the microenvironment in leukemogenesis has been demonstrated in several studies, showing that genetic alterations in non-hematopoietic bone marrow cells can induce myeloid proliferation or myelodysplasia and acute myeloid leukemia (Raaijmakers et al. 2010). Although these studies have been performed using a murine model, there are rare reports of donor-derived malignancies after bone marrow transplant, making it possible to speculate that such cases may be an example of niche-induced leukemia development in man (Wiseman 2011). The clear evidence of mesenchymal stromal cell reprogramming by human myelodysplastic syndrome (MDS) stem cells has been shown using patient-derived xenograft (PDX) model. This first successful attempt to engraft low-risk MDS was strictly dependent on co-transplantation of HSCs with patient-derived MSCs, demonstrating critical essence of bidirectional cross-talk between niche and MDS-initiating cells for disease maintenance and propagation (Medyouf et al. 2014).

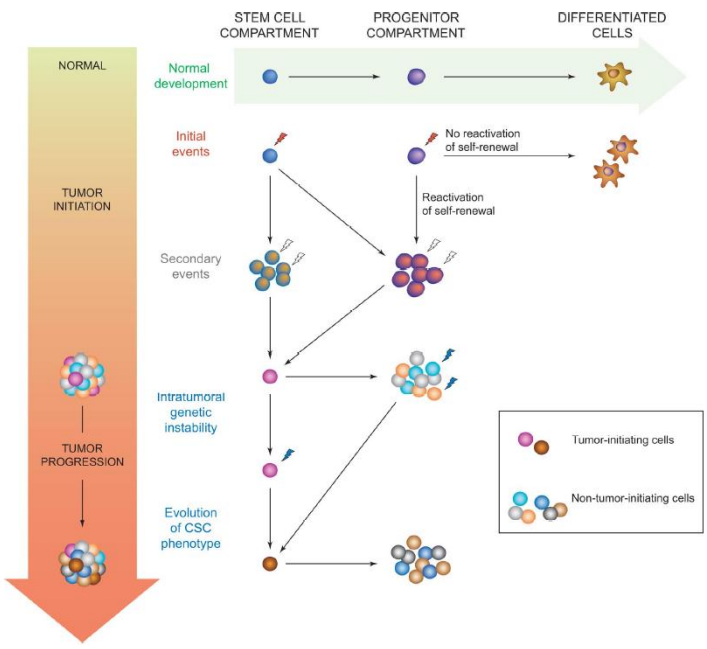


Figure 1. Leukemia origin and evolution.

The primary genetic lesion leading to a (pre-)malignant transformation might occur within the HSC pool or a more differentiated downstream progenitor with consequent expansion of the affected subset. Additionally, secondary or tertiary events might also affect already mutated clones. The tumor progression may be powered by intrinsic genetic instability or progressive additional events in non-tumorigenic progenies, demonstrating the ways of cancer evolution (Dick 2008).

## 1.2 Acute leukemia

### 1.2.1 Clinical phenotype

Acute leukemias are characterized by abnormal proliferation of malignant stem/progenitor cells in bone marrow (BM), blood, and secondary extramedullary sites. As a result, the initial clinical presentation is detected by the presence of anemia, thrombocytopenia, and neutropenia due to severe suppression of normal hematopoiesis by the malignant clone. Once a leukemic clone entered the blood, the major sites of infiltration are spleen, liver, lymph nodes and central nervous system. Depending on the predominant hematopoietic lineage affected, acute leukemia is further subdivided into acute myeloid or lymphoid type (Devine and Larson 1994; Terwilliger and Abdul-Hay 2017).

### 1.2.2 Acute lymphoblastic leukemia.

Acute lymphoblastic leukemia occurs in both children (80%) and adults (20%) with strikingly different prognosis based on age factor. While the outcome in pediatric patients undeniably improved and 5 years overall survival (OS) rate is almost 90%

(Inaba, Greaves, and Mullighan 2013), the treatment of young adults and elderly patients remains a big challenge and OS is the function of age: 15–54 years, OS >50%; 55–64: OS <30%; older than 65 years: OS<20% (Sant et al. 2014), despite the intensification of chemotherapy and use of allogeneic hematopoietic stem cell transplantation (allo-HSCT) depending on patients fitness. At the initial diagnosis patients have to be stratified whether they have B- or T-cell acute leukemia (B-ALL or T-ALL), based on the investigation of blasts morphology and immune phenotype using flow cytometry. The following critical step is the identification of possible chromosomal aberrations with prognostic and therapeutic values as *BCR-ABL1*, *ETV6-RUNX1*, *KMT2A*, etc. An additional molecular investigation may uncover accompanying mutations leading to pathological survival, proliferation advantage or therapy resistance of cancer cells, including *IKZF1* deletions, *CDKN2A/B* locus deletion as well as mutations in *KRAS*, *NRAS* and *FLT3* (Jabbour et al. 2015).

BCR-ABL1 positive B-ALL

B-cell acute leukemia harboring a chromosomal translocation t(9;22)(q34;q11) so-called BCR-ABL1 rearrangements (or Philadelphia positive chromosome) has a historically poor prognosis. BCR-ABL1 positive (BA+) ALLs constitute 5% of pediatric B-ALL and approximately 40% of adult ALLs (Fig. 2).

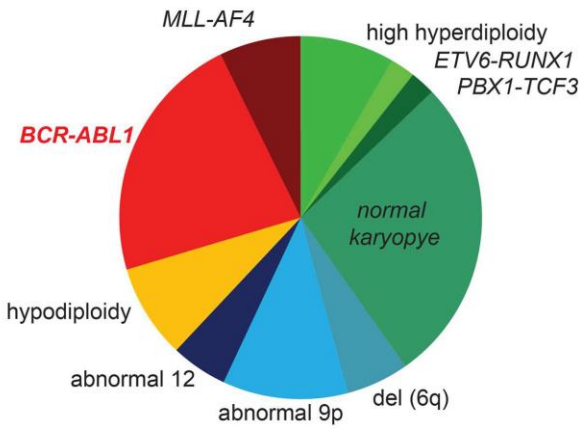


Figure 2. Frequency of BCR-ABL among adult ALL (Bernt and Hunger 2014)

The fusion of the "breakpoint cluster region" (*BCR*) gene on chromosome 22 with the Abelson Murine Leukemia Viral Oncogene Homolog 1 (*ABL*) gene on chromosome 9 leads to a BCR-ABL fusion protein with a constitutively active tyrosine kinase, involving downstream signaling of RAS, PI3K/AKT/mTOR, NF-κB and JAK/STAT, Bcl-$X_L$ and Bcl-2 leading to cell-cycle acceleration and promoting the anti-apoptotic activity of cancer cells (Zou and Calame 1999; Malouf and Ottersbach 2018). One of the typical features of BA+ B-ALL is the accumulation of additional mutational events, affecting lymphoid development such as genetic aberrations in *IKZF1, PAX5, EBF1*, *ETV6,* and *RUNX1*. In particular, complete or partial deletion or mutation of *IKZF1* is detected in more than 70% of BA+ B-ALL patients and is associated with adverse outcome and a higher risk of treatment failure (Mullighan et al. 2009). *IKAROS (IKZF1)* is a zinc finger transcriptional factor, important for the development and commitment of B-cell lineage. Expression of IKAROS in BA+ leukemia acts as a tumor suppressor, thus alteration of IKAROS indicates a less differentiated and more aggressive nature of leukemia. Another group of frequently mutated genes in BA+ B-ALL is related to cell cycle regulation, apoptosis or transcriptional regulation: *CDKN2A/CDKN2B*, *RB1*, *CD200, BTLA/TOX, BTG1* (Mullighan 2012; Roberts et al. 2017; Schjerven et al. 2017; Witkowski et al. 2017). Although treatments, that include targeted BCR-ABL1 tyrosine kinase inhibitors (TKIs) and intensive chemotherapy have revolutionized the therapy, stem cell transplantation post-remission remains the only potential chance of cure for these patients, pointing towards the unmet need for new therapeutic strategies.

### 1.2.3 Acute myeloid leukemia

The most frequent acute leukemia among adults is AML. The cure rate represents around 35-40% among patients of 60 years or younger and only 5-15% for patients

older than 60. Importantly, elderly patients who are not capable to stand intensive chemotherapy, have a devastating median survival of 5-10 months (Döhner, Weisdorf, and Bloomfield 2015). Further, genetic studies revealed an enormous complexity of genomic alterations leading to AML development. It is believed that

| Risk category* | Genetic abnormality |
|---|---|
| Favorable | t(8;21)(q22;q22.1); *RUNX1-RUNX1T1* |
| | inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* |
| | Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD$^{low}$ = allelic ratio < 0.5 |
| | Biallelic mutated *CEBPA* |
| Intermediate | Mutated *NPM1* and *FLT3*-ITD$^{high}$ = allelic ratio > 0.5 |
| | Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD$^{low}$ (without adverse-risk genetic lesions) |
| | t(9;11)(p21.3;q23.3); *MLLT3-KMT2A* |
| | Cytogenetic abnormalities not classified as favorable or adverse |
| Adverse | t(6;9)(p23;q34.1); *DEK-NUP214* |
| | t(v;11q23.3); *KMT2A* rearranged |
| | t(9;22)(q34.1;q11.2); *BCR-ABL1* |
| | inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM(EVI1)* |
| | −5 or del(5q); −7; −17/abn(17p) |
| | Complex karyotype monosomal karyotype |
| | Wild-type *NPM1* and *FLT3*-ITD$^{high†}$ |
| | Mutated *RUNX1* |
| | Mutated *ASXL1* |
| | Mutated *TP53* |

Table 1. The European Leukemia Net stratification of AML by genetics 2017 (Döhner et al. 2017)

clonal evolution is the major driver of disease progression and therapy resistance. Therefore, molecular screening for mutations is part of the diagnostic procedure of AML. The origin of AML, based on a clinical ontogeny, can be defined as *de novo* AML, secondary AML as a transformation of myeloproliferation or MDS, or therapy-related AML (Mossner et al. 2016; Lindsley et al. 2015). Conventional cytogenetic testing gives a stratification of 50% of patients with an abnormal karyotype (favorable outcome: *PML-RARA*, *RUNX1-RUNX1T1*, or *MYH11-CBFB* fusions; adverse outcome: patients with monosomy karyotype) and therefore defines the appropriate therapeutic regime. Yet the remaining 50% of patients with normal karyotype require additional identification of mutations for risk stratification and therapeutic decisions (‘Genomic and Epigenomic Landscapes of Adult De Novo Acute Myeloid Leukemia’ 2013). With the recent advances in next-generation

sequencing, it has been identified that in a cohort of "intermediate" risk patients there are on average three AML driver mutations (DiNardo and Cortes 2016). Taken together, cytogenetic and molecular diagnostics are the cornerstone for risk stratification and choice of the therapeutic option (Table 1).

### 1.2.4 Cancer immunity cycle

The development of the anti-cancer immune response is a complex and dynamic process and usually initiated during the early stages of malignant transformation (Hanahan and Weinberg 2000). It includes a series of orchestrated events which are schematically depicted in fig. 3 with the emphasis on the innate immune response. This cycling process (mainly based on solid tumor models) reinforcing itself and theoretically should amplify and broaden T-cell response by the accumulation of immune-stimulatory signals leading to tumor elimination. However, the immune response is occurring on the interface between the host immune system and the evolving tumor, which creates selective pressure on tumor cells when less immunogenic cells escape the immune control. The result of this interaction is known as the immunoediting process and consists of three phases: elimination, equilibrium and escape (Dunn, Old, and Schreiber 2004; Efremova et al. 2018). This concept stems from the discovery that tumors generated in an immunodeficient host are much more immunogenic than tumors from an immunocompetent host (Shankaran et al. 2001). It is important to state this dual role of immunity in the context of cancer, however, here we consider the immunity cycle irrespectively of its immunoediting phase.

The first stage of the immune response (Fig 3.) originates when innate immune cells being alerted by stromal remodeling and tumor cell death when tumor neoantigens and "danger signals" (damage-associated molecular patterns; DAMPs) are released into the tumor bed. Neoantigens produced during cancerogenesis are captured by

dendritic cells (DCs) for processing while DAMPs are commonly sensed by adenosine, ATP and toll-like receptors (TLRs) widely expressed on innate immune cells, like DCs and macrophages (Mph) (D. S. Chen and Mellman 2013; Demaria et al. 2019). Additionally natural killer (NK) or natural killer T-cells (NKT) cells might recognize the malignant cells via surface-expressed NKG2D ligand (Nakata et al. 2014). Independently of the initial sensation and recognition event, the critical step for the mounting of the immune response is the production of type I interferons (IFNs) (Kang et al. 2019; Fuertes et al. 2011; E. Curran et al. 2016; E. K. Curran, Godfrey, and Kline 2017; Dunn, Old, and Schreiber 2004; Woo et al. 2014). The magnitude of the proinflammatory activation of an innate compartment dictates the success of empowering the adaptive immunity to elicit its effector function. Importantly, activation of TLRs on antigen presenting cells (APCs) stimulates the proinflammatory program via activating *NF-κB* and IFN regulatory factor (*IRF3*, *IRF5* and *IRF7*) signaling pathways (Rakoff-Nahoum and Medzhitov 2009). In particular, it has been shown, that enhanced maturation of DCs, antigen presentation and subsequent cytotoxic activity of T cells is mediated via TLR9 sensing tumor-derived DNA (Kang et al. 2019). Notably, the initial amount of released IFNs acts as a chemoattractant and activator for other innate immune cells like Mph. They, in turn, accelerate IFNs loop via acquiring the pro-inflammatory (M1) phenotype and releasing even more of IFNγ along with interleukin (IL)-12, which is the key cytokine boosting IFNy synthesis by NK cells, what enhances tumor lysis (Fig 3.) (Dunn, Old, and Schreiber 2004). Another critical pathway that boosts IFN production also initiated via sensing of tumor DNA is the cGAS-STING pathway (Deng et al. 2014). The stimulator of interferon gene complex (STING) is an endoplasmatic reticulum transmembrane protein that does not sense DNA directly, but instead captures cyclic-GMP-AMP synthase (cGAS). It has been demonstrated

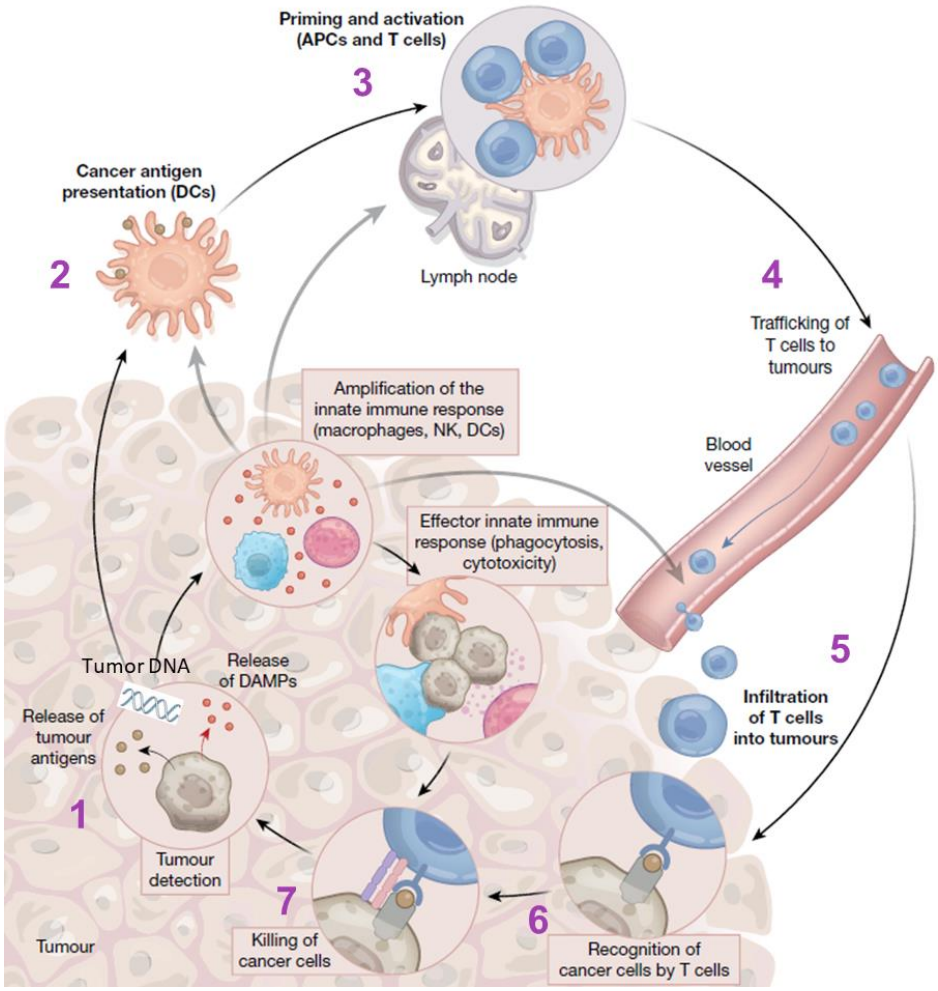


Figure 3. Scheme of the cancer immunity cycle. Adapted from Demaria O. et al., Nature 2019

The process is described in the text. DAMPs represent molecules and mediators that in the healthy state of cells are preserved intracellularly such as High Mobility Group Box 1 (HMGB1), S100 proteins, hyaluronan, heat-shock proteins, adenosine triphosphate (ATP) and calreticulin (Hernandez, Huebener, and Schwabe 2016).

that STING activation is an absolute prerequisite in order to mount spontaneous tumor-specific CD8+ T-cell response in the melanoma model via APC activation (Woo et al. 2014). Therefore, the two next steps of the cancer immunity cycle: maturation of DCs and tumor antigen presentation in complex with major histocompatibility complex (MHC) class I and II (2) together with migration into draining lymph nodes and priming T-cells (3) are sequential events strongly dependent on the magnitude of IFN response. In particular, the tumor-antigen

priming phase (3) of T-cells requires not only T-cell receptor (TCR) engagement with tumor peptide-loaded MHC but co-stimulation via CD28/B7-1/2 axis, while for sustaining the Th1 response CD40/CD40L interaction was found to be critical (Howland et al. 2000; Vonderheide and Glennie 2013). Essentially, the activation of T-cells by immature DCs without B7 co-stimulation renders a tolerogenic response towards the tumor (Quezada et al. 2004). Once T-cells are educated by APCs in the lymph nodes, they extravasate and migrate to the tumor site (phase 4-5) where tumor cells are recognized via cognate antigen on MHC complex (phase 6) and killed by cytotoxic CD8+ T-cells (phase 7), thus starting the next enhanced loop of the immunity cycle.

Essentially, the efficiency of the T-cell effector function is strongly moderated by the balance between co-stimulatory and inhibitory signals namely, immune checkpoints (Fig. 4). Immune checkpoints play a fundamental role in preventing tissue damage during pathogen infection as well as governing the self-tolerance avoiding autoimmunity. In the context of cancer, this immunoregulatory mechanism is hijacked and severely contributes to the tumor immune escape and resistance. Therefore, developing antagonists for blocking inhibitory receptors (PD1, CTLA4, LAG3, TIM3) in order to restore the T-cell effector function is the primary focus of cancer immunotherapy (Pardoll 2012; Havel, Chowell, and Chan 2019; Efremova et al. 2018; Ok and Young 2017)

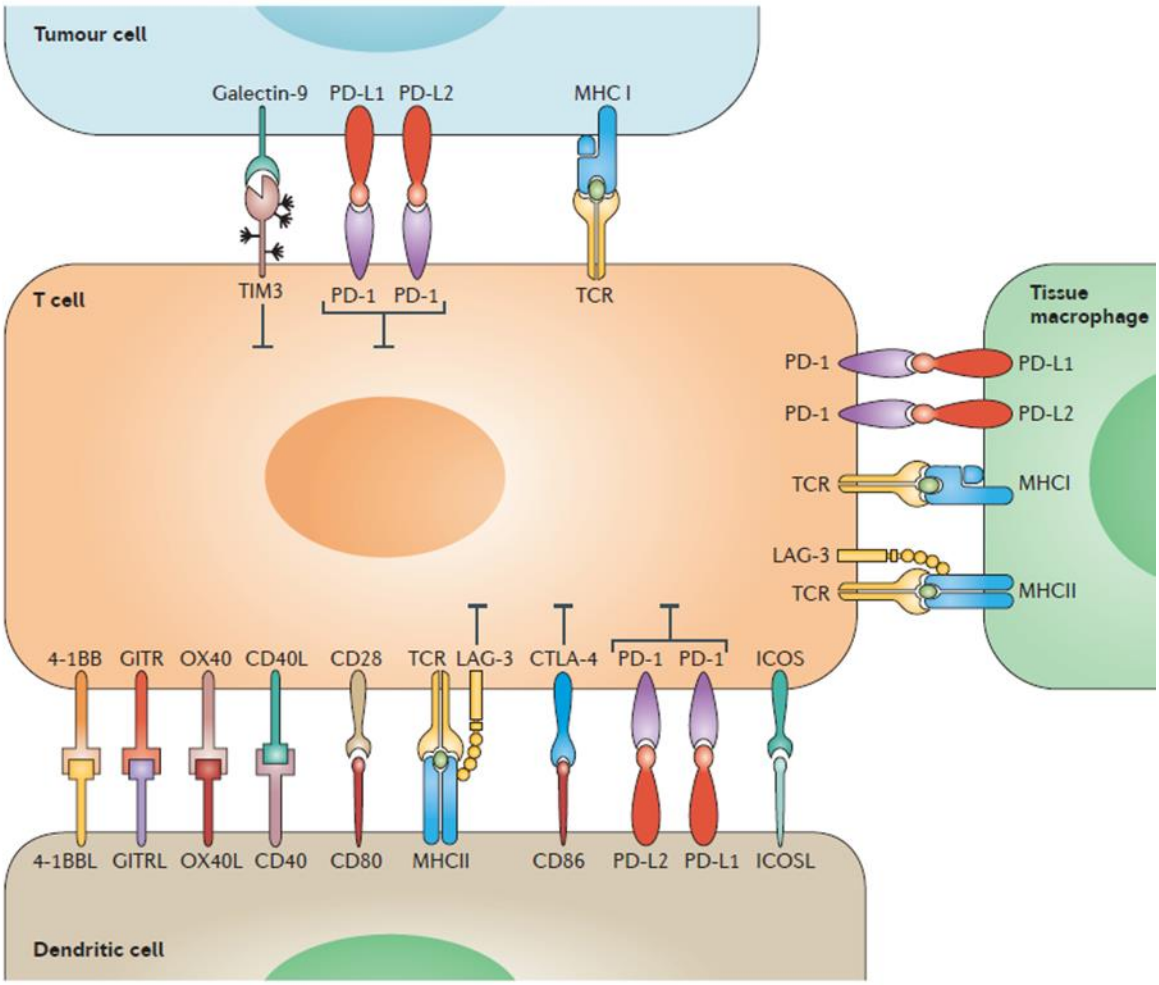


Figure 4. Immune-checkpoint molecules involved in the regulation of the antitumor immune response. Nishino et al., Nature, 2017

Multiple ligand-receptor interactions between antigen-recognizing T-cells and APCs/ tumor cells are depicted. Inhibitory T-cell co-stimulatory receptors, commonly expressed in response to antigen-specific activation: lymphocyte activation gene-3 - LAG3; cytotoxic T-lymphocyte-associated protein 4 - CTLA4; programmed death protein 1- PD1; T cell membrane protein 3 - TIM3. Activation of antigen-specific T-cell response exhibited via following co-stimulation: constitutively expressed CD28; tumor necrosis factor (TNF) super-family receptors: OX40 (CD134), 4-1BB (CD137), glucocorticoid-induced tumor necrosis factor receptor – GITR; inducible T-cell costimulatory receptor - ICOS.

### 1.2.5 Bone Marrow “immune niche”. Implication for leukemia

BM as a primary site of leukemic transformation is an immune-privileged organ. It has been already appreciated the existence and importance not only of a “stromal niche” compartment but also “immune niches” of HSCs (see fig. 3). Although it is not correct to dissect the certain cell type, which does not resemble the complexity

of all interactions *in vivo*, and call it a "niche", it makes it easier to unify certain cellular compartments for the discussion. Despite the primary function of BM being

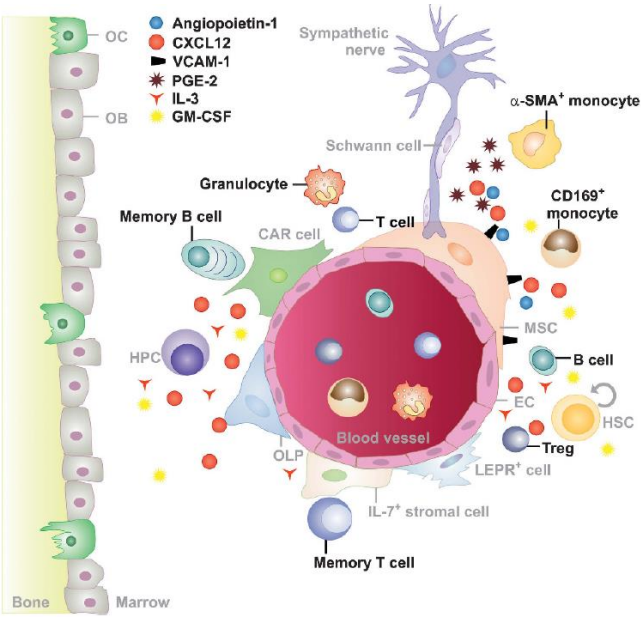


Figure 5. The immune niche of BM microenvironment.

Diverse immune cells (T-cells, B-cells, monocytes, granulocytes, macrophages) regulate the function of HSPCs under homeostasis and stress signals (indicated by interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) expression).

a-SMA - monocytes/macrophages expressing high levels of a-smooth muscle actin; CAR cell, CXCL12-abundant reticular cell; EC - endothelial cell; HPC-hematopoietic progenitor cell; OB - osteoblast; OC - osteoclast; OLP - osteolineage progenitor cell; Treg - CD4+CD25+FOXP3+ regulatory T cell (Riether, Schürch, and Ochsenbein 2015)

a source of the life-long hematopoiesis and maintenance of an unique HSCs pool, it has been hypothesized that it might play the role of a secondary lymphoid organ as well. Thus, the lymphocytes within the BM, have certain organization patterns and distinct functional features, similar to lymphoid follicles. The T-cell pool, which can be sub-divided into CD4+ and CD8+ T cells show a unique composition in the BM, which is inverted in comparison to peripheral blood with a CD4/CD8 ratio of 1/2, where T-regulatory cells (Tregs; CD4+CD25+Foxp3+) constitute one-third of the CD4 population (Zhao et al. 2012). Interestingly, Tregs co-localize with HSCs in the perivascular niche, creating a protective harbor for HSCs from the immune disruption (Fujisaki et al. 2011). Additionally, CD4 cells are essential for BM engraftment in the context of allogeneic transplantation. It has been demonstrated that BM graft, lacking CD4, fails to restore productive myelopoiesis. Furthermore, the maintenance of normal hematopoiesis depends on a specific activated stage of CD4 cells by cognate BM-antigen and production of an indispensable cytokine profile (Monteiro et al. 2005). The BM serves as a reservoir for antigen-specific

CD8 and CD4 memory T-cells, as well as for long-lived antigen-specific B-plasma cells. These cells are residential and persist in the BM long after they disappear from the periphery (Zhao et al. 2012; Chang, Tokoyoda, and Radbruch 2018).

Myeloid cells in the BM represent a complex population, including granulocytes, monocytes, Mph and DCs. They have been implicated in their pivotal role in the pathogenesis of hematological malignancies. Accumulation and expansion of so-called myeloid-derived suppressor cells (MDSCs) of granulocytic or monocytic origin is a well-known feature in solid as well as hematological tumors. MDSCs are represented by myeloid precursor cells with an immature phenotype and exhibit an immunosuppressive cytokine profile by high production of IL-10, transforming growth factor-beta (TGFb), arginase 1 (ARG1) and reactive oxygen species (ROS) (Senovilla et al. 2014). Phenotypically there are no specific cell surface markers to solely identify MDCSs. In mice, these populations may be captured by CD11b and Gr1 (Ly6G) surface expression. In humans, MDSCs are detected as CD14-CD11b+CD33+HLADR- cells and negative for mature lymphocyte markers (CD3-CD19-CD56-). Of note, the only phenotypic characteristic is not essential to dissect MDCSs and distinguish them from normal progenitors. This population develops during the long-lasting pathological process. Therefore, only in combination with functional behavior, it is possible to identify such a subset (e.g. specific cytokine production, suppression of T cell proliferation, etc.) (Yazdani et al. 2015). The accepted phenomenon of MDSCs appearance in cancer has been implicated in its clinical relevance when high MDSC frequencies in patients correlate with poor prognosis and immune response upon anti-cancer therapy (Millrud, Bergenfelz, and Leandersson 2016). Mechanistically, the immunosuppressive profile of myeloid cells in both solid and hematological cancers may be achieved by upregulation of signal transducer and activator of transcription 3 (STAT3) in myeloid cells, leading

to immature/tolerogenic DCs accumulation, skewing of Mph profile towards the anti-inflammatory program (M2) and accumulation of MDCSs (Kortylewski and Moreira 2017; Wang et al. 2004). STAT3 initially recognized as an oncogene, is known to be constitutively activated in 70% of solid and hematological tumors, empowering survival, proliferation and transformation of tumor cells (Boudny and Trbusek 2020; Dysthe and Parihar 2020; Wang et al. 2004; Hossain et al. 2014,). At the same time, in the tumor immune compartment, STAT3 is activated upon immunosuppressive signals (e.g. IL-10, ARG1, IL-6 etc.) thus establishing a tumor-promoting immune milieu (C. Liu et al. 2019; Trovato et al. 2019; Chrisikos et al. 2020). Inhibition of STAT3 in hematopoietic cells and tumor cells in a B16 melanoma and breast cancer model respectively, led to multicomponent anti-tumor activity and significant restraining of the tumor metastasis and growth (Kortylewski et al. 2005; Zerdes et al. 2019). Upon leukemic transformation, the cytokine milieu in the BM changes with increased levels of macrophage colony-stimulating factor (M-CSF), GM-CSF, vascular endothelial growth factor (VEGF), INFy and prostaglandin 2 (PGE2). This further creates an environment, favorable for MDCSs accumulation and expansion. In particular, a high dose of GM-CSF abrogates maturation of DCs and enhances CD11b+Ly6G+ MDSCs proliferation with consequent suppression of T-cell activation and induction of T-regs (Jitschin et al. 2014). It has been previously shown that BM resident CD11c+ DCs cluster with T-cells in order to prime tumor-specific T-cell response via MHCI or II presentation of blood-borne antigens. Therefore, evidence points towards BM being capable of mounting a primary immune response as a secondary lymphoid organ (Feuerer et al. 2003).

Mph are not only essential regulators of immunity but also one of the key components of HSC maintenance in the BM. Previously, it has been shown, that

depletion of CD169+ BM resident Mph leads to egress of HSPCs due to disruption of the crosstalk between Mph and Nestin+ MSCs, responsible for HSCs retention (Chow et al. 2011). The role of Mph in erythropoiesis by engulfment of expelled nuclei during the process of erythrocyte maturation has also been established. Specifically, the critical function of erythroid island Mph appears during stress erythropoiesis, facilitating the immediate response to anemia (Heideveld and van den Akker 2017). Although the critical role of tumor-associated Mph (TAMs) has been widely accepted in the field of solid cancer, the importance and function of these cells in the pathogenesis of acute leukemia remain largely elusive. It is expected, that Mph participate and dramatically change their functional and molecular landscape within leukemia-niche crosstalk. To test this hypothesis, Calletti et al., demonstrated that, Mph support leukemia growth and that lack of Mph significantly extends survival of the recipient in CLL xenograft leukemia model (Galletti et al. 2016). Other studies implicated the role of Mph as tumor promoters *ex vivo*, when direct cell contact of CLL cells with Mph accelerated blast proliferation (Komohara et al. 2015). There are several observations that high Mph infiltrates in lymphomas correlate with worse clinical outcome and shorten overall survival (Steidl et al. 2010; Casulo et al. 2013). Taken together it is appealing to assume that leukemia-associated Mph exhibit a similar tumor-promoting phase of polarization (M2-like phenotype) as found in solid tumors, which severely contributes to the failure of mounting a productive innate immune response - the critical base of generation Antigen (Ag)-specific T-cell response.

### 1.2.6 The immune response against leukemia

In contrast to solid tumors, representing a defined cellular mass in a well-defined “tumor nest”, leukemia is a disseminated malignancy and develops in the BM. Due

to this unique nature of leukemia, it is easy to assume pronounced differences in an immune response in the context of solid tumors and hematological malignancies.

The historical evidence of immune activation against leukemia lays in the phenomenon of graft-versus-leukemia (GVL) response in the context of allo-HSC transplantation, when residual tumor cells after chemotherapy have been eliminated by donor immune cells. However, GVL is usually associated with severe graft-versus-host-disease (GVHD) reaction, a life-threatening complication of allo-HSC transplantation (Weiden et al. 1981).

It is commonly accepted that the immune system can generate a spontaneous Ag-specific T-cell response. This understanding stems from multiple studies using subcutaneously injected solid cancers (Palucka and Coussens 2016). The success of the immune checkpoint blockade therapy is based on such preclinical models, achieving a remarkable treatment advantage by combination of anti-PD1 and anti-CTLA4 in melanoma patients (Chae et al. 2018). Although therapy of hematological malignancies benefits from the application of checkpoint inhibitors, as in Hodgkin Lymphoma patients (Pianko, Goldberg, and Lesokhin 2018), the mechanism of immunological tolerance is dramatically different from solid tumors. Of note, the classic Hodgkin lymphoma (cHL) is exceptional among others, with an extraordinarily positive response to checkpoint inhibitor (CKI) therapy, possibly due to immunogenicity acquired by Epstein-Barr virus (EBV) (E. K. Curran, Godfrey, and Kline 2017). Well in line with these data, it has been shown that virus-associated tumors exhibit increased T-cell infiltration rates, which is linked to a better prognosis in comparison to virus-negative cancers. Therefore, it has been hypothesized that viral infection triggers the immune response along with upregulation of checkpoint receptors (PD1, CTLA4, TIM3) what makes it susceptible for CKI therapy (Cao et al. 2019)

It has been proposed, that localized tumor possesses a certain level of inflammation due to an intratumoral cell death, which results in the release of “danger signals”. This further creates a window for proper activation of APCs (CD8+aDCs) in a type I IFN dependent fashion, which successfully translates in the generation of an Ag-specific cytotoxic T-cell response (Fuertes et al. 2011). The group of Justin Cline demonstrated, that subcutaneously injected OVA-expressing AML was capable to mount an Ag-specific T-cell response and significantly prolonged the survival of recipients in contrast to OVA-AML injected intravenously. Moreover, the administration of anti-CD40 antibody partially rescued the generation of Ag-specific T-cell response, highlighting the role of poorly activated APCs in the context of intravenously injected AML (Zhang et al. 2013). It has also been shown that BM-derived APCs are responsible for cross-presentation of B-cell lymphoma Ags without a “second signal” (co-stimulation through B7 molecules and CD28 on T-cells) leading to the establishment of tolerogenic T-cell response (Sotomayor et al. 2001). The role of the type I IFN response has been established in order to bridge the innate and adaptive immune response since such is not triggered in leukemia. Well in line with this statement the rescue of IFN response by activation of the STING pathway promotes APC activation and T-cell-specific response against AML (E. Curran et al. 2016).

The recognition of cancer cells by the immune system depends on the expression of MHC class I and II molecules, the mechanism being frequently subverted and altering tumor immunosurveillance. By NGS analysis, it has been shown that a large fraction of cHL patients show a loss of the ß-2-microglobulin unit – the key component of MHC class I (Reichel et al. 2015). Rare to solid cancer but typical for B cell lymphoma the expression of MHC II is also frequently downregulated or completely silent (Brown et al. 2016) along with an unique amplification of PDL1

locus on chromosome 9p24.1 (Green et al. 2010; Twa et al. 2015). Interestingly, the loss of MHC expression in acute leukemia is frequently seen in relapse, but not at the primary diagnosis (Masuda et al. 2007; Stölzel et al. 2012).

Another essential difference of hematological malignancies, severely contributing to immune tolerance, is a low mutational burden in contrast to solid tumors (Alexandrov et al. 2013). The occurrence of somatic mutation in cancer cells may potentially lead to the generation of new proteins (neoantigens) with consequent recognition by the immune system (Rooney et al. 2015). Thus, accumulation of such events, so-called “mutational load”, is a surrogate marker of tumor immunogenicity (Austin, Smyth, and Lane 2016). Thus, it has been demonstrated by NGS that the mutational profile in melanoma and lung cancer correlates with the response to CKI therapy (Snyder et al. 2014; Rizvi et al. 2015).

Nevertheless, there are rare pieces of evidence that the immune system can efficiently control or even eradicate AML. A positive correlation has been shown with fast lymphocyte recovery and a low rate of relapse levels (Behl et al. 2006). The most intriguing data of spontaneous AML remission are associated with pathogen infection (Müller et al. 2004), it is tempting to speculate that infection-associated immunity was unleashed against cancer cells.

Taken together, it is clear, that despite all limitations and challenges for immunotherapy in hematological malignancies, there is a window for reactivating the power of the host's immune system to fight leukemia.

### 1.2.7 Immunotherapy of acute leukemia

Currently, immunotherapy is a fast-evolving field and with emerging wide application in ALL. In particular, a significant contribution has been made to advance immunotherapy in relapsed/refractory B-ALL cases (Shang and Zhou

2019). The major clinical advantage was acquired by the use of genetically engineered chimeric-antigen-receptor (CAR) T-cells against Ags expressed on a transformed cancer cell. CAR T-cells represent autologous patient-derived T-cells which were transduced *ex vivo* with gamma retroviral or lentiviral vectors in order to introduce an Ag-specific single-chain fragment variable (scFv) recognition domain into the T cell receptor (TCR) machinery. Such modified CAR T-cells exhibit target-specific killing in an HLA-independent fashion. The second generation of CAR T-cells includes additional co-stimulatory signals through CD28 or 4-1BB currently used in the clinic (Mohty et al. 2019). After *ex vivo* expansion, the cells are re-injected into the patient with subsequent elimination of tumor cells expressing the Ag via perforin-granzyme dependent lysis of a target cell (Benmebarek et al. 2019). The immunotherapy breakthrough happened when in 2017 the FDA approved CD19 CAR T-cell for relapsed and refractory B-ALL in children and young adults (Y. Liu et al. 2017). It has been demonstrated, that CAR T-cell therapy shows a remarkable complete remission rate among adult B-ALL patients up to 93%. In children with B-ALL, treated with CAR T cells, the effect was as well extremely potent in a range of 63-90% of complete remission (Lesch et al. 2019). However, the major obstacle, that frequently occurs upon CAR T-cell therapy is the cytokine-release-syndrome (CRS), a life-threatening complication (Murthy et al. 2019). The prognostic role of minimal residual disease (MRD) in the treatment of adult ALL patients has been established, thus MRD positive patients after chemotherapy are predicted to have a relapse (Gökbuget et al. 2019). For MRD positive patients, the application of bi-specific T-cell-engaging single-chain antibodies (BiTEs) has been approved by the FDA with an efficacy of 81% achieving MRD negativity. This bi-specific antibody recognizes CD3 on the surface of T-cells and CD19, expressed on leukemic blasts, bringing effector and target cells to close proximity and ensuring the tumor-specific killing (Jen et al. 2019).

Notably, the therapy of AML remains extremely challenging and has not evolved dramatically over the last decade. Regarding the treatment, chemotherapy (cytarabine, daunorubicin) is rarely a cure and allo-HSCTs are limited to transplant eligible patients and associated with high risk and life-threatening complications. In contrast, immunotherapy may offer a new therapeutic strategy to harness the patient's own immune system in order to overcome relapse as a major course of morbidity. A promising application is personalized DC vaccination after chemotherapy for patients in clinical remission, which has recently been shown by Rosenblatt et. al. By fusing autologous DCs with AML cells, researchers created hybridoma, subsequently used as a vaccine. This approach resulted in a significant expansion of leukemia-specific T-cells in the circulation for up to 6 months and 4 years survival of 71% (Rosenblatt et al. 2016).

Although the significant upregulation of PD1 by T-cells in a group of AML patients compared to the healthy cohort has been shown by Daver et. al., the implication of CKI in AML and MDS remains limited (Daver et al. 2016). The highest expression of PDL1 on AML cells was found in patients with a poor-risk prognosis, including cases with mutations in the gene of the tumor suppressor protein p53 (Haroun et al. 2017). A phase I clinical trial using the humanized antibody against PD1 showed clinical benefit for 33% of the patients (Berger et al. 2008). Thus, these data provide a rationale for the feasibility of new large clinical trials with CKI in AML/MDS patients. The application of CAR T-cells in AML is particularly challenging due to the rare nature of leukemia-specific Ags, exclusively expressed on cancer cells. Thus, many of the antigens are shared with the normal HSPC compartment or other tissues. There was one phase I clinical trial estimating the potential targeting of Lewis-Y (LeY) Ag by CAR T-cells in AML. The study demonstrated the safety of CAR T-cell therapy, however, only one patient demonstrated molecular remission and others ultimately relapsed (Ritchie et al. 2013). There is a number of preclinical

studies in development and testing of the efficacy of CAR T-cells against CD123 and CD33 for AML treatment (Austin, Smyth, and Lane 2016). With the use of BiTE targeting CD33/CD3 showed its promising therapeutic efficacy by induction of T-cell expansion and antibody-mediated cytotoxicity (Krupka et al. 2014).

### 1.3 TAM receptors and their ligands. Molecular structure

Receptor tyrosine kinases (RTKs) are a superfamily of receptors characterized by similar structural architecture consisting of an extracellular domain with a ligand-binding region, transmembrane domain and cytoplasmatic domain with the tyrosine kinase (TK) region. Generally, the activation of the receptors occurs through the binding of a corresponding ligand with typical dimerization of RTKs, activation of the kinase activity and initiation of certain downstream signaling pathways. RTKs are key players in defining cellular fate and playing a critical role in fundamental processes of growth, differentiation and metabolism (Lemmon and Schlessinger 2010). The TAM receptors family, consisting of TYRO3, AXL and MERTK receptors (TAMRs), belongs to the RTK superfamily. TAMRs were initially identified in 1991 by G. Lemke and Lai and exhibit a highly pleiotropic biological action (discussed in section 1.4) (Lai and Lemke 1991). There are two well-described ligands: growth arrest-specific factor (GAS6) and protein S (PROS1), which bind to the receptors with different affinity. GAS6 is binding to all three receptors with the highest affinity to AXL and lowest to MERTK, while PROS1 was believed to bind only TYRO3 and MERTK (Tsou et al. 2014; Nagata et al. 1996). Although, recently it has been indicated that in glioblastoma PROS1 can also activate AXL (Sadahiro et al. 2018). Both ligands are secreted glycoproteins and share 42% of amino-acid homology. Structurally, the ligands have a Gla amino-terminal domain (rich in glutamic residues), followed by four tandem epidermal growth factor domains (EGF) and a C-terminal sex hormone-binding globulin

(SHBG) domain, required for TAMR binding (Fig. 4) While the function of GAS6 is mainly restricted to TAMRs, PROS1 has a separate function as an anticoagulant and serves as a cofactor of protein C in the coagulation cascade (Burstyn-Cohen 2017). Importantly, a critical event in the activation of TK activity is $Ca^{+}$-dependent sensing of phosphatidylserine (PtdSer) on dying cells by gamma-carboxylated Gla-domains, thus making GAS6 and PROS1 sort of adaptors (Tsou et al. 2014) The receptors consist of extracellular immunoglobulin-like domains followed by tandem fibronectin type III repeats, responsible for receptor dimerization, a single transmembrane domain and a cytoplasmic catalytic domain with 75% amino-acid identity (Fig. 4).

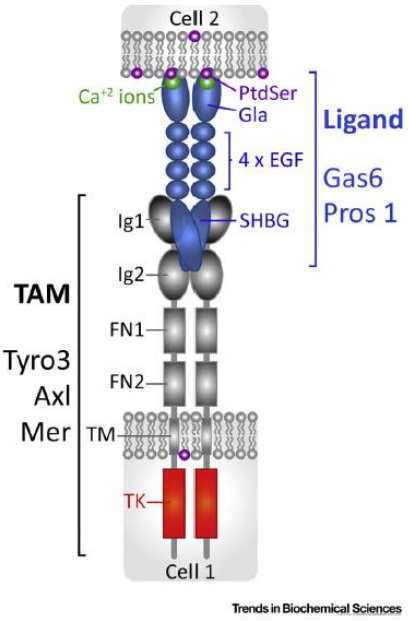


Figure 6. Scheme of TAMR/GAS6&PROS1 organization and interaction. (Lemke 2017)

PtdSer – phosphatidylserine (purple)

Gla amino-terminal domain (rich in glutamic residues)

EGF epidermal growth factors domains

SHBG sex hormone-binding globulin (SHBG)-like domain

Ig1/Ig2 Immunoglobulin-like domain

FN1/FN2 Fibronectin type III repeats

TM transmembrane domain

TK tyrosine kinase

## 1.4 The role of TAMR in homeostasis and immunity

The expression pattern of TAMR is widely distributed through mature mammalian tissues (tissue-resident Mph, DCs, nervous tissue, vessels, muscles, platelets, etc.) and expression of more than one receptor on the same cell type is typical in combination with ligand co-expression (Lemke and Rothlin 2008).

The generation and characterization of “loss-of-function” receptor triple knockout mutant (TKO *Axl*$^{-/-}$*Mer*$^{-/-}$*Tyro*$^{-/-}$) mice shed the light on the biological function of TAMR. Remarkably, generated single/double or even triple knockout animals had no developmental abnormalities, were born with normal Mendelian distribution and were indistinguishable from wild type counterparts up to 2-3 weeks of age. However, later on, the TKO offspring revealed multiple abnormalities across the organs histologically characterized by an accumulation of apoptotic cells. In particular, researchers observed this phenotypic characteristic in the brain (hippocampus, neocortex, cerebellum), liver, blood vessel walls, epithelium of the prostate, and spleen. Additionally, adult TKO animals became blind at the age of 2-3 months due to the apoptotic loss of all photoreceptors. The detailed investigation was dedicated to severe abnormalities in spermatogenesis, where the loss of TAMRs by Sertoli cells (expressing all three TAMR) was detrimental in removing the apoptotic debris during spermatogenesis (Lu et al. 1999, 3). Taken together, this data revealed the essential role of TAMRs in homeostatic support in the processes associated with a high rate of apoptosis. The process of removing apoptotic cells fundamentally relies on the exposure of the “eat-me” signal on the cell surface represented by PtdSer. Normally, the PtdSer asymmetry is strictly regulated and its flipping to the outer membrane layer is controlled by $Ca^{+}$-dependent phospholipid scramblases (Bevers and Williamson 2016). One well-known domain capable of binding PtdSer is the Gla-domain, as mentioned above, an indispensable structural part of both GAS6 and PROS1. It has been shown that this signaling exposure might be exploited by enveloped viruses and TAMR in this scenario serve as a docking site for viral entry (Meertens et al. 2012). A summary of the key physiological processes for immunologically-silent engulfment of apoptotic cells by phagocytes, which require TAMRs is depicted in fig. 7.

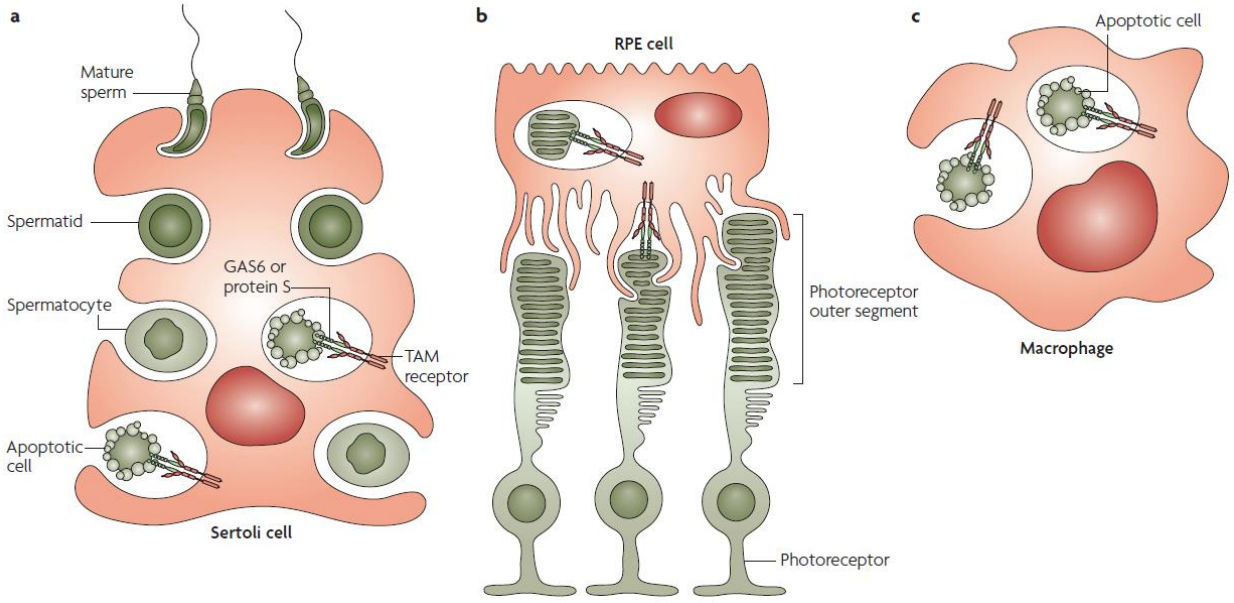


Figure 7. TAM signaling in "homeostatic phagocytosis" (Lemke and Rothlin 2008)

a. Sertoli cells expressing AXL/MER/TYRO3 clearing apoptotic cells generated during spermatogenesis
b. Retinal pigment epithelial RPE cells pruning photoreceptor outer segments
c. Removal of apoptotic cells in lymphoid organs ( e.g. dying erythrocytes in reg pulp of the spleen or erythroid islands in BM (Yoshida et al. 2005))

Additionally, it has been shown that the "murder by phagocytosis" or killing of PdtSer positive alive cells is also TAM dependent (Segawa et al. 2018). Moreover, the phenomenon of pruning of synaptic boutons in the postnatal brain in order to fine-tune the functional neuronal synapses is provided by microglia and requires TAMR functionality (Lemke 2019; 2017). It has been also shown that TYRO, AXL and MERTK have critical importance for the late stage of NK cell development and regulate maturation of functional activating/inhibitory receptors of NK cells (Caraux et al. 2006). Although the role of TAMR during homeostasis is well established, there exists another even more prominent function of TAMR – control of inflammatory feedback mechanisms (Rothlin et al. 2015). The first *in vivo* evidence of increased sensitivity towards inflammatory signal in *MerTK*$^{-/-}$ mice was performed by Camenisch et al. Researchers challenged the *MerTK*$^{-/-}$ animals with

lipopolysaccharide (LPS) and observed increased lethal septic shock susceptibility compared to wild type. Molecularly it was manifested by accelerated production of TNFa *in vivo* and *in vitro* by *MerTK*-deficient Mph (Camenisch et al. 1999). Further characterization of TKO mice revealed abnormal T- and B-cell proliferation, splenomegaly, lymphadenopathy, increased IFN gamma levels and eventually the development of autoimmune syndromes (similar to systemic lupus erythematosus (SLE) and rheumatoid arthritis) (Lu and Lemke 2001; Cohen et al. 2002). Mechanistically, it has been shown that TAMR activation in conjunction with type I IFN receptor induce expression of suppressors of cytokine production SOCS1/SOCS3, providing an inhibitory signal of TLR engagement in DCs (Fig. 8b) (Rothlin et al. 2007). This signaling cascade is believed to be responsible for dampening inflammation in innate immune cells and strikingly differs from conventional Akt pathway, initiated by oligomer TAMR for “homeostatic phagocytosis” (Fig. 8a) (Lemke 2013).

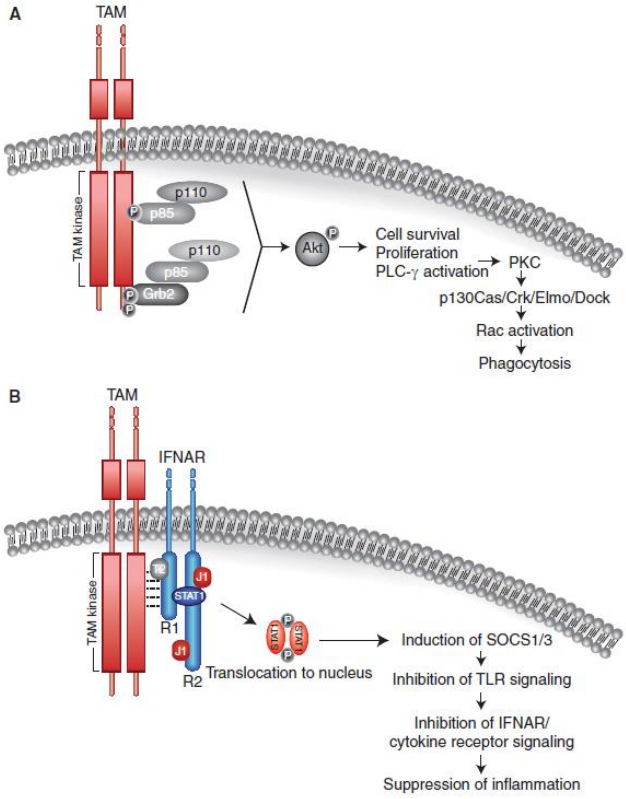


Figure 8. TAMR signaling pathways.

a – AKT pathway. Mobilization of p85/p110 complex leads to phosphorylation and activation of Akt. The pathway required for cell proliferation and modifying cytoskeleton for phagocytosis.

b – TAM receptor complex with type I interferon alpha receptor IFNAR. Activation of TAM-IFNAR by GAS6 leads to rapid phosphorylation of STAT1, followed up translocation pSTAT1 dimer into nucleus and induction of *SOCS1/3* expression (Lemke 2013).

## 1.5. TAMR/GAS6&PROS1 implication in cancer

Interestingly, all TAMR were cloned and identified in multiple cancer cell lines (Linger et al. 2008) and the overexpression of AXL and MERTK induces neoplastic transformation in the NIH3T3 cell line and hematopoietic cells, respectively (O'Bryan et al. 1991; Keating et al. 2006). The abnormal expression of TAMR and their ligands have been reported in a vast majority of cancers including hematological malignancies (A. B. Lee-Sherick et al. 2013; Linger et al. 2013; Brandao et al. 2013; Waizenegger et al. 2015). Thus, around 30-50% of pediatric B-ALL show overexpression of MERTK as well as 70-90% of patients with AML, while AXL expression is predominantly linked to AML (Graham et al. 2014; Neubauer et al. 1994; Linger et al. 2013). The high expression level of GAS6 is associated with a poor prognosis in AML patients and proposed as a novel therapeutic target (Whitman et al. 2014; Loges et al. 2010). The less investigated

PROS1/TYRO3 axis was shown to activate anti-apoptotic signaling, promoting cell survival in head and neck cancer cell lines (Al Kafri and Hafizi 2019). The major signaling axis acting in cancer cells initiated via TAMRs includes MEK/ERK, PI3K/AKT, JAK/STAT, p38, NFκB and FAK/RAC downstream pathways, functionally manifested by promoting cell survival, proliferation, metastasis (Rankin et al. 2014; Zhu, Wei, and Wei 2019) as well as therapy resistance (Giles et al. 2013; Hong et al. 2008). As it is evident from above, the majority of studies were dedicated to cell-intrinsic mechanisms of TAMR and ligands being pro-oncogenic, however, there is an emerging concept of TAMR as modulators of cancer immunity (Paolino and Penninger 2016).

The first evidence of immunomodulation in cancer via TAMR-signaling was shown by Cook et al. when in the syngeneic cancer model of melanoma, breast and colon cancer the tumor growth was delayed when infiltrated with *MerTK*$^{-/-}$ CD11b lymphocytes. The researchers demonstrated that MERTK expression on tumor infiltrated lymphocytes promotes an immunosuppressive phenotype by an increase of IL-10 and GAS6 and correlate with poor infiltration of CD8+ T-cells (Cook et al. 2013). In another study by Paolino et al., it has been demonstrated that NK cells genetically lacking Cbl-b kinase were capable to reject metastatic breast and melanoma tumors. Researchers uncovered the TAMR/Cbl-b axis which constitutes an inhibitory pathway for NK activation via NKD2G receptor (Paolino et al. 2014). Mechanistically the attenuation of NK activity achieved upon GAS6 ligation to AXL/MERTK/TYRO3 phosphorylates Cbl-b subsequently and promotes the degradation of NK activation receptor LAT1 (Chirino et al. 2019). The profound immunosuppressive function of PROS1 was established in regard to tumor-associated macrophages. It has been demonstrated that secretion of PROS1 by tumor cells dictates Mph-M2 polarization fate *ex vivo* and *in vivo*, as well as dampening of inflammation by TLR stimulation (Ubil et al. 2018). However, little is known about

the role of TAMRs in the anti-leukemic immune response. The only study by Lee-Sherick et al. reports that inhibition of MERTK signaling in the syngeneic B-ALL model is associated with a decrease of PDL expression in CD11b positive cells and prolonged survival of *MERTK*$^{-/-}$ recipients. These results demonstrate the descriptive population differences in *MERTK*$^{-/-}$ recipients with B-ALL in comparison to wild type, although the cellular and molecular mechanism of an immune-mediated leukemia clearance remained unclear (A. B. Lee-Sherick et al. 2013; Alisa B. Lee-Sherick et al. 2018b).

## 1.6 Aim of the work

Therapeutic progress in the treatment of acute leukemia drastically improved the survival outcome in pediatric patients, however, the prognosis in older adults remains dismal. Thus, according to the American Cancer Society in 2019 statistical analysis revealed that 5-year overall survival for patients older 20 years remains only 24% for AML and 35% for ALL (American Cancer Society, Cancer Facts &Figures 2019). These data undeniably point towards the unmet clinical need for the development of new therapeutic approaches. The immune suppression is one of the typical hallmarks of leukemia and little is known about the molecular mechanism and cellular mediators underlying leukemic immune evasion. The fundamental difference in the immune response associated with leukemia in contrast to solid cancers was devised by J. Cline and characterized by the failure to bridge innate and adaptive immune response. Therefore, in the frame of the current project, we sought to explore the role of the GAS6/TAMR axis, essentially controlling pro-inflammatory activation of innate immune cells, in the context of ALL using a syngeneic mouse model. Previously the role of GAS6 and TAM receptors was mainly studied in their pro-oncogenic cancer-intrinsic aspect but not as essential players in cancer immunosurveillance. The only study implementing the function of

TAMR on the surface of NK cells, inhibiting NK-dependent cancer cell killing in the melanoma model was demonstrated by Paolino et. al. (Paolino et al. 2014). Thus, the current work provides mechanistic insights into the role of TAMR and their ligands in the pathogenesis of BA+ B-ALL and uncovers the following aspects:

- Cell-intrinsic versus immunomodulatory function of BM-derived GAS6 in the context of BA+ B-ALL using newly generated constitutive *Gas6*$^{-/-}$ hosts (both immunocompetent and immunodeficient);
- Identifying the cellular mediators of GAS6-driven immunosuppression in leukemia;
- Compensatory PROS1 upregulation in *Gas6*-deficient host inhibits ant-leukemic immune response;
- Determining AXL as a potential therapeutic target in order to boost patients own immune system to combat leukemia;
- Revealing that the circulating level of the TAMR ligands in the human system is a function of age and its increase is associated with aging-driven immune dysfunction.

# 2.0 Materials and Methods

## 2.1 Materials

### 2.1.1 Mice

Immune competent C57BL/6N *Gas6* knock out mice ($Gas6^{tm1.1(KOMP)Vlcg}$) were obtained from the Knock Out Mouse Project (KOMP) Repository (https://www.komp.org/index.php). A schematic view of the knockout allele is indicated in fig. 9. Validation of the absence of *Gas6* expression was performed by qPCR and ELISA (Fig. 9 b and c, respectively). A detailed description of the knock out allele is available at http://www.mousephenotype.org/data/alleles/MGI:95660/tm1.1(KOMP)Vlcg.

Immune-deficient NSG $Gas6^{-/-}$ mice were generated by inactivation of *Gas6* gene using CRISPR-Cas9 editing in NSG zygotes as indicated in the scheme (Fig. 9 d) using a workflow recently described by our group (Tirado-González et al. 2018). Briefly, *Gas6* editing was carried out by homology-directed repair using a donor DNA template that introduces a stop codon in exon 2 of the *Gas6* gene, in addition to an ECoR-I site for screening purposes and a silent mutation in the PAM sequence to avoid editing of a productively edited allele. Four independent *Gas6*-deficient mouse lines were generated (697-21, 27, 29 and 31). Knockout successfully resulted in the absence of GAS6 in circulation, verified by ELISA (Fig. 9 e)

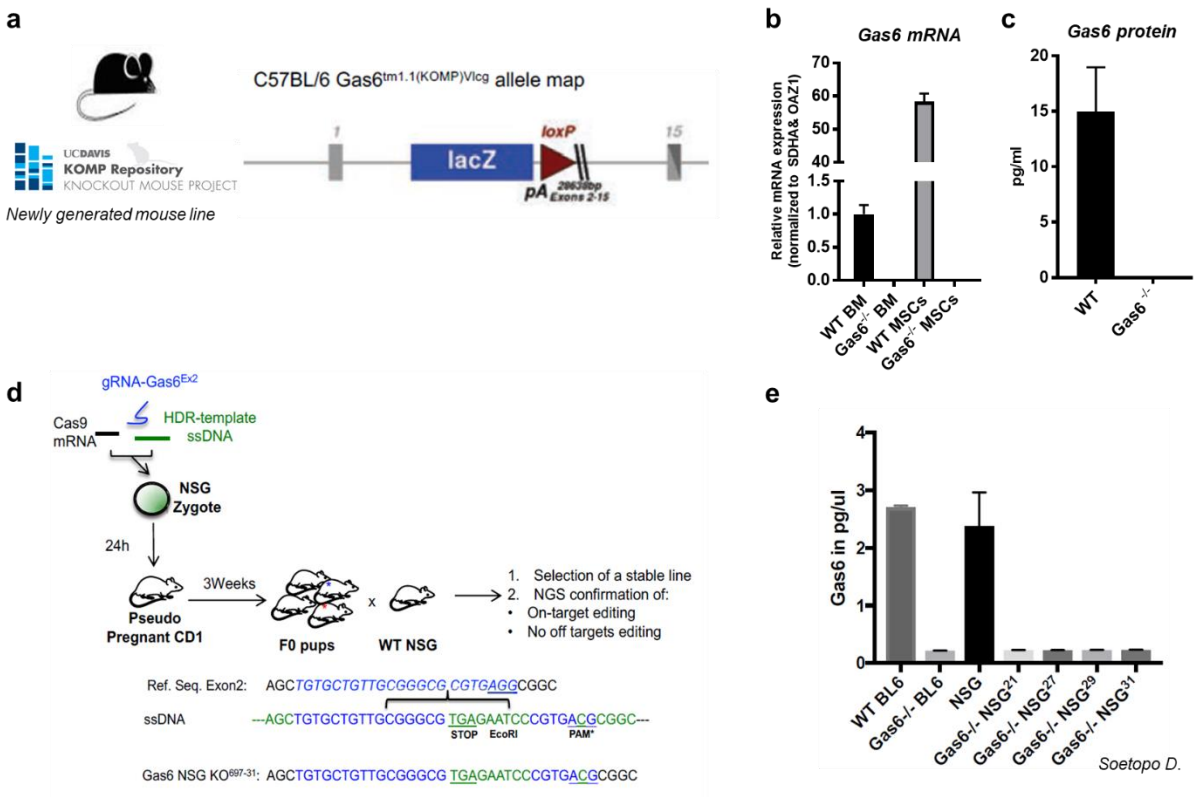


Figure 9. Immunocompetent C57BL/6N *Gas6*$^{-/-}$ and immunodeficient NSG *Gas6*$^{-/-}$ mice generation and validation of GAS6 knockout.

a- Knockout allele map of C57BL/6N Gas6$^{-/-}$; b- *Gas6* mRNA by quantitative qPCR was not detectable in BM and MSCs of C57BL/6N Gas6$^{-/-}$. Data are mean ± s.d after normalization to SDHA& OAZ1; c- Gas6 protein was not detected in BM plasma of C57BL/6N *Gas6*$^{-/-}$; d- Immune-deficient *Gas6*$^{-/-}$ mice (line 927-31) were generated by inactivation of the *Gas6* gene using CRISPR-Cas9 editing in *NOD Prkdc*$^{scid}$ *Il2rg*$^{-/-}$ (NSG) zygotes. gRNA targeting exon 2 of the *Gas6* gene, *Cas9* mRNA (5meC, Psi) (Tebu-Bio#L-6125-100) and a single strand DNA donor template were microinjected in NSG zygotes. The ssDNA template was designed to introduce an in-frame stop codon, an EcoRI restriction site and a mutation in the PAM sequence. Insertion of the stop codon in an early translated exon expectedly halted translation while concomitantly inducing nonsense-mediated mRNA decay (NMD) of the edited transcript. The EcoRI site was used to screen edited mice by RFLP assay. Mutation of the PAM site was used to prevent the secondary editing of a productively edited allele. Genome editing of NSG zygotes using CRISPR/Cas9 was approved by the Regierungspräsidium Karlsruhe under animal protocol number G50/15 (Tirado-Gonzalez et al. 2018); e- GAS6 protein was not detected in PB serum of NSG *Gas6*$^{-/-}$ (lines 697-21, 27, 29 and 31).

Control wild-type mice were purchased from the Jackson laboratories (Jackson laboratories; line: C57BL/6NJ#005304). *Csf1r-CreAxl*$^{f/f}$*Mertk*$^{f/f}$ mice were a kind gift from Dr. Carla Rothlin (Yale University, New York, USA) and described previously (Bosurgi et al. 2017; Schmid et al. 2016). *Socs3*$^{f/f}$ were provided by Dr. Sevenich (Georg-Speyer Haus). Animals were bred and maintained under the

specific pathogen-free facility at the Institute of Tumor Biology and Experimental Therapy. All animal experiments were performed in accordance with regulatory guidelines under TVA number F123/1034.

### 2.1.2 Cell lines

Table 2. List of cell lines

| | |
|---|---|
| MS5, murine stromal cell line | DSMZ-German Collection of Microorganisms and Cell Cultures, Cat# ACC-441; RRID:CVCL_2128 |
| HDMEC, human dermal microvascular endothelial cells | Gift from RepairLab, University Clinic, Mainz, N/A |

### 2.1.3 Primers and plasmids

TaqMan probes, all purchased from Thermo Fisher Scientific.

Table 3. List of TagMan probes

| Target gene | Probe ID |
|---|---|
| mGas6 | Mm00490378_m1 |
| mPros1 | Mm01343426_m1 |
| mAxl | Mm00437221_m1 |
| mMertk | Mm00434920_m1 |
| mTyro3 | Mm00444547_m1 |
| mIL12b | Mm01288989_m1 |
| mSOCS3 | Mm00545913_s1 |
| mTNFa | Mm00443258_m1 |
| mSDHA | Mm01352366_m1 |
| mUbc | Mm02525934_g1 |
| hGas6 | Hs01090305 |
| hPros1 | Hs00165590_m1 |
| hGusb | Hs00939627 |

## Primers for SybrGreen assay, purchased from Sigma Aldrich

Table 4. List of SybrGreen primers

| **Target gene** | **Sequence** |
|---|---|
| mGas6-9 | Fw: TGCAGCTTCGGTACAATGG<br>Rv: CCAGCTCTTCCACGGAGATA |
| mPros1-99 | Fw: ATTCTCGCTCTGGAACGTCT<br>Rv: GCATTTCAGTTGGGATTGCT |
| mSDHA | Fw: AAGTTGAGATTTGCCGATGG<br>Rv: TGGTTCTGCATCGACTTCTG |
| mOAZ1 | Fw: TTTCAGCTACATCCTGTACT<br>Rv: GACCCTGGTCTTGTCGTTAGA |

## Plasmid

| | |
|---|---|
| MSCV-BCR-ABL1-IRES-GFP | Retroviral expression vector that contains an IRES site and allows translation of GFP as a reporter for BCR-ABL $^{p190}$ expression.<br>Provided by Jacques Ghysdael & Filippo Della Marina, Institut Curie, PSL Research University, Orsay, France. |

## 2.1.4 Antibodies, FACS Dye

Table 5. List of FACS antibodies and dyes

| **Name** | **Manufacturer** |
|---|---|
| mCD16/32 FcB | BD Biosciences, #553441 |
| mCD45 BV605 | BD Biosciences, #563053 |
| mCD45 BV786 | BD Biosciences, #564225 |
| mCD3 APC-CY7 | Biolegend, #100222 |
| mCD8a AF700 | BD Biosciences, #564986 |
| mCD8 PECY7 | eBioscience, #25-0081-82 |
| mCD4 PECY7 | eBioscience, #25-0041-82 |
| mB220 BV786 | BD Biosciences, #563894 |

| | |
|---|---|
| mB220 BV711 | BD Biosciences, #563892 |
| mNK1.1 PECF594 | BD Biosciences, #562864 |
| mNK1.1 PE | BD Biosciences, #557391 |
| mLy6G APC-CY7 | BD Biosciences, #560600 |
| mLy6G BV421 | BD Biosciences, #562737 |
| mCD11c AF700 | BD Biosciences, #560583 |
| mB220 APC | BD Biosciences, #553092 |
| mCD11b PECF594 | BD Biosciences, #562317 |
| mCD11b PECY7 | eBioscience, #25-0112-82 |
| mF4/80 PE | BD Biosciences, #565410 |
| mMHC-II BV650 | BD Biosciences, # 563415 |
| mLy6C BV421 | BD Biosciences, #562727 |
| mLy6C PECY7 | BD Biosciences, #560593 |
| mIFNg APC | eBioscience, #17-7311-82 |
| mTNFa BV711 | BD Biosciences, #563944 |
| mGr1 PE-Cy5 | eBiosciences #15-5931-83 |
| mCD8 FITC | BD Bioscience #553031 |
| mCD4 FITC | eBiosciences #11-0041-85 |
| mTer119 APCeF780 | eBioscience #APCeF780 47-5921-82 |
| mCD45.1 PE | eBiosciences #12-0453-81 |
| mCD45.2 BV786 | BDBioscience #563686 |
| mCD71 PB | eBiosciences #48-0711-82 |
| mCD45.1 FITC | BD Bioscience #553775 |
| mTer119 FITC | BD Bioscience #557915 |
| mCD31 BV421 | BD Bioscience #562939 |
| mSca1 APC-Cy7 | BD Bioscience #560654 |
| mCD51 PE | BD Bioscience #551187 |
| mPDGFRa APC | Abcam AF488 #ab196376 |
| mCD44 BV711 | eBioscience #563971 |
| m&h Ki 67AF647 | BD Biosciences #558615 |

| | |
|---|---|
| hCD45 PE | BD Biosciences #555483 |
| hCD14 APC-Cy7 | BD Bioscience #557831 |
| hCD11b PE-Cy7 | BD Bioscience #561785 |
| hHLA-DR BV786 | BDBioscience #564041 |
| hCD90 | BDBioscience BV421 #562556<br>BDBioscience PE #561970 |
| hCD146 | BDBioscience PECy7 #562135 |
| CD105 | BDBioscience FITC #561443 |
| Fixable Viability Dye AF700 | BD Biosciences, #564997 |
| Cell Trace Violet Proliferation Dye | Thermo Fisher Scientific, #C34571 |
| Hoechst | Life technologies #H3570 |
| Propidium Iodide | Sigma, #P4170 |
| hFc block | Miltenyibiotec. #130-059-901 |
| One CompBeads | Thermo Fisher Scientific, #01-1111-41 |

### 2.1.5 Antibodies for *In Vivo* Depletion

Table 6. List of antibodies used *in vivo*

| **Name** | **Manufacturer** |
|---|---|
| Rat anti-mouse CD3 (clone 17A2) | Biolegend, #100202 |
| Rat IgG2b, k Isotype control | Biolegend, #400602 |
| InVivoMab anti-mouse CD8 alpha | Hölzel Diagnostika, #BE0061 (Köln, Germany) |
| InVivoMab rat IgG2b kappa isotype control | Hölzel Diagnostika, #BE0090 (Köln, Germany) |

| | |
|---|---|
| InVivoPure pH7.0 dilution buffer | Hölzel Diagnostika, #IP0070 (Köln, Germany) |
| InVivoMab anti-mouse CD4 | Hölzel Diagnostika, #BE0119 (Köln, Germany) |
| InVivoMab anti-mouse NK1.1 | Hölzel Diagnostika, #BE0036 (Köln, Germany) |
| InVivoMab mouse IgG2a isotype control | Hölzel Diagnostika, #BE0085 (Köln, Germany) |

### 2.1.6 Reagents, chemicals

Table 7. List of reagents

| **Name** | **Manufacturer** |
|---|---|
| PBS, pH 7.4 | Gibco, Thermo Fischer Scientific, #10010031 |
| RPMI 1640 | Gibco, Thermo Fischer Scientific, #11875085 |
| DMEM + 4,5 g/L D-Glucose media | Gibco, Life Technologies, #21969-035 |
| MEM | Sigma, #M2279 |
| StemSpan SFEM | STEMCELL Technologies, #09600 |
| Fetal calf serum | Gibco, Thermo Fischer Scientific, #10270106 |
| L-Glutamine | Gibco, Thermo Fischer Scientific, #25030-024 |
| Hepes | Sigma-Aldrich, #H0887 |
| Penicillin-streptomycin | Gibco, Thermo Fischer Scientific, #15140-122 |
| Trypsin-EDTA (0,25%) | Gibco, Thermo Fischer Scientific, #25200056 |
| EBM™-2 Basal Medium | Lonza, #CC-3156 |
| EGM™-2 SingleQuots™ Supplements | Lonza, #CC-4176 |
| StemMACS MSCs expansion Media, human | Miltenyi Biotech, #130-091-680 |

| | |
|---|---|
| Ficoll-Pague ™ PLUS. Density 1.077+/- 0.001g/ml | GE Healthcare #17-1440-03 |
| Antibiotic - Antimycotic (100x) | Gibco, Thermo Fischer Scientific, #15240062 |
| DMSO (dimethyl sulfoxide) | Sigma, #D8418 |
| Acetic Acid | Sigma, #A6283 |
| Clodronate /PBS Liposomes | ClodronateLiposomes (Amsterdam, Netherlands) |
| LPS | Sigma, #L4391 |
| Golgi Plug | BD biosciences, #555029 |
| Bemcemtinib (BGB324) | ApexBio, cas Nr-1037624-75-1 |
| ß-Mercaptoethanol | Sigma, #M3148 |
| RBC Lysis Buffer, 10x | BD biosciences, #555899 |
| Isopropanol | Roth |
| Ethanol, 99% | Roth |
| 10x PBS sterile | Gibco, Thermo Fisher Scientific #70011044 |
| Human recombinant IL2 | Peprotech, #200-02 |
| Mouse recombinant M-CSF | Thermo Fisher Scientific, #14-8983-80 |
| Mouse recombinant INFg | Peprotech, #315-05 |
| Human recombinant bFGF | Peprotech #100-18B |
| Cas9 mRNA NLS – 5-methyl-C, pseudo-U | Tebu-Bio, # 040L-6125-100 |

### 2.1.7 Commercially available Kits

Table 8. List of Kits

| **Name** | **Manufacturer** |
|---|---|
| RNeasy mini kit | Qiagen, #74106 |

| | |
|---|---|
| Arcturus PicoPure™ RNA Isolation Kit | Applied Biosystems, #KIT0204 |
| cDNA High Capacity Reverse Transcriptase Kit | Thermo Fisher Scientific, #4368814 |
| VILO™ cDNA Synthesis Kit | Thermo Fischer Scientific, #11754050 |
| SYBR Green Master Mix | Thermo Fischer Scientific, #4368702 |
| TaqMan™ Gene Expression Master Mix | Thermo Fisher Scientific, #4369016 |
| Cytofix/Cytoperm plus Kit | BD Biosciences, #555028 |
| Mouse CD45 MicroBeads | MACS Milteny Biotec, #130-052-301 |
| Human CD14 Microbeads | MACS Milteny Biotec, #130-050-201 |
| Mouse PROS1 ELISA Kit | Aviva Systems Biology, #OKEH01403 |
| Mouse GAS6 DuoSet ELISA | R&D, #DY986 |
| DuoSet ELISA Ancillary Reagent Kit | R&D, #DY008 |
| Luminex Human Magnetic Assay (1-Plex) Gas6 | R&D, #LXSAHM-01 |
| Luminex Human Magnetic Assay (1-Plex) Pros1 | R&D, #LXSAHM-01 |

| | |
|---|---|
| Luminex Human Magnetic Assay (14-Plex) | R&D, #LXSAHM-14 |

### 2.1.8 Equipment & Consumables

*Table 9. List of equipment and consumables*

| **Name** | **Manufacturer** |
|---|---|
| Viia7 Real-Time PCR system | Thermo Fisher Scientific |
| FACSAria™ Fusion | BD Biosciences, Heidelberg, Germany |
| BD LSRFortessa™ | BD Biosciences |
| Bio-Plex 200 | BIO-RAD |
| Irradiation device | Eckert & Ziegler, Model BIOBEAM2000 |
| Thermocycler for cDNA synthesis | Biometra, Thermocycler model T3 |
| Centrifuge | Thermo Scientific. Heraeus Megafuge40R |
| Sterile Hood | Thermo Scientific, Model Safe 2020 |
| CO2 incubator | Thermo Scientific, Heracell Vios 160i |
| Water Bath | GFL |
| ELISA plate reader | TECAN |
| Microscope Light | Nikon ECLIPSE TS100 |
| NanoDrop 2000 | Thermo Fisher Scientific |
| Vortex | Bender & Hobein AG |
| Shaker | Eppendorf, Thermomixer comfort |
| Table centrifuge | VWR, Mini Star |
| Vi-CELL XR Cell Viability Analyzer | Beckman Coulter |
| Falcon tubes (15, 50 ml) | Greiner |
| FACS tubes | SARSTEDT |

| | |
|---|---|
| Cell culture flasks (T25, T75, T175 ) | SARSTEDT |
| Well plates | Greiner |
| Cell strainer 40µm, 70µm | Greiner |
| Cryovials | Greiner |
| Graduated pipette | Greiner, Sardtstedt |
| LS column, LD columns | Milteny Biotech |
| Pipette filter tips | Fisherbrand |
| Pipette tips | Starlab |
| Syringes for i.v. /i.p injections | Beckton Dickinson |
| Needles for bleeding | BD Microlance #302200 |
| Teflon bags, 30ml | PermaLife, Origen |
| UpCell™ 24 well plates | Thermo Fischer Scientific |
| Microvette® 500 Z-Gel | SARSTEDT |
| Microvette® 200 K3E | SARSTEDT |

### 2.1.9 Software

BD FACS Diva

Viia7 Real-Time PCR analysis software

FlowJo V10

GraphPad Prism 7

Zotero

Microsoft Office

## 2.2 Methods

### 2.2.1 Leukemia transplantation experiments

All leukemia transplantation experiments were performed in non-irradiated secondary recipients, to maintain the integrity of the microenvironment. The number of cells injected is indicated in each figure legend. Transplantations were used for the generation of primary BA+ B-ALL as described in section 3.1. The MLL-ENL leukemia model has been described previously (Horton et al. 2009).

### 2.2.2 Normal bone marrow transplantation into lethally irradiated hosts

The recipient mice (C57BL/6N *Gas6*$^{-/-}$ and C57BL/6N 8-12 weeks old) were subjected to 2 rounds of irradiation with 4.5 Gy (morning and evening, 9 Gy in total). On the next day, irradiated recipients received $3x10^6$ of rescue donor whole BM cells (CD45.1/CD45.2 mixture of 2 donor animals) injected into tail vein in 200 µl of PBS. The donor chimerism was estimated by FACS in the peripheral blood monthly.

### 2.2.3 Analysis of murine peripheral blood, bone marrow and spleen

Blood was collected by bleeding from the *vena facialis* using an EDTA containing microvette and subjected to red blood cell lysis using with RBC lysis buffer (BD biosciences) prior to FACS antibody staining. Mice were sacrificed by cervical dislocation when they developed clinical signs of disease or at specified time points after transplantation. For the isolation of cells from the BM, the isolated two femurs, two tibiae and two hips were crushed using a mortar and pestle. To obtain the cells from the mouse spleen, the spleen was homogenized by smashing the organ through a 70 µm cell strainer with 10 ml PBS into a 50 ml Falcon tube. The cells were resuspended in PBS/RPMI 2%FBS and filtered through a 70 µm cell strainer. The cells from the spleen and BM were washed with 10 ml PBS (400 g, 5 min, room temperature (RT)), resuspended, counted and used in downstream FACS analysis. In some cases, in order to improve the quality of FACS staining, the cells from the

spleen and BM were lysed for 1 min with RBC lysis buffer (BD biosciences), washed and filtered before FACS staining.

### 2.2.4 Flow cytometry

Cells were prepared as single-cell suspension and blocked with CD16/32 Fc Block and then subjected to multicolor panel staining. Surface staining was performed for 45 min on ice in the dark. Antibodies and secondary reagents were titrated to determine optimal concentrations. After surface staining was completed in some cases cells were subjected to intracellular staining (Ki67, TNFa, INFy). For that purpose, cells were fixed and permeabilized using Cytofix/Cytoperm Kit (BD Biosciences) according to manufacturers instruction and stained overnight in the dark at +4°C. In the context of cytokine staining, prior fixation cells were kept in PBS 2% FBS with Golgi Plug (1:1000) (BD Biosciences). CompBeads (BD Biosciences) were used for single-color compensation to create multi-color compensation matrices. For gating, fluorescence minus one (FMO) controls were used. The instrument calibration was controlled daily using Cytometer Setup and Tracking beads (BD Biosciences). In the case of cell sorting, cells were sorted either into 50 µl Arcturus lysis buffer (up to 10,000 cells) or in PBS 2% FBS, 50 mM EDTA (> 10,000 cells) and further processed in downstream RNA isolation. Post-sort purity was >95% and determined by re-analysis of sorted cells.

### 2.2.5 Depletion of CD3, CD4, CD8, Nk1.1 cells *in vivo* using monoclonal antibodies

All antibodies were rigorously titrated in order to optimize the minimal dosage for the efficient and specific clearance of cells of interest. The efficiency and administration mode were estimated in peripheral blood, spleen and BM by FACS analysis using a staining covering all mature cell subsets (CD45/CD3/CD4/CD8/B220/NK1.1/CD11b/Ly6G). The chosen concentration and

administration regime of antibodies used in the experiments is indicated in each figure legend. All antibodies were used along with appropriate isotype controls recommended by the manufacturer and injected i.p. (100 µl left + 100 µl right side) in antibody diluent recommended by the manufacturer.

### 2.2.6 Administration of liposome suspension

Clodronate liposomes were purchased from Liposoma Research (Amsterdam, Netherlands) and injected intravenously (i.v.) at a dose of 250 µl/mouse as indicated in figure legends. PBS loaded liposomes were used as control.

The dosage and administration mode were chosen in the test experiment, the efficiency of macrophage depletion was verified by FACS in the spleen and BM using following cell surface staining: CD45/B220/Ly6C/Ly6G/CD11b/F4/80/CD11c/MHCII/CD8.

### 2.2.7 RNA isolation and real-time PCR

RNA was isolated using the PicoPure™ RNA Isolation Kit or RNeasy Mini Kit according to the manufacturer's instructions and converted into cDNA using the SuperScript® VILO™ cDNA Synthesis Kit or cDNA High Capacity Reverse Transcriptase Kit, respectively. All samples of cDNA were diluted 1:4 before usage. For some cases real-time PCR for the assessment of *Gas6* and *Pros1* expression was carried out using SYBR Green Master Mix with primers from Sigma-Aldrich. For all other real-time PCR experiments, TaqMan based real-time PCR assays using the TaqMan™ Gene Expression Master Mix and TaqMan probes, all purchased from Thermo Fisher Scientific were used.

### 2.2.8 Generation of bone marrow-derived macrophages and polarization experiments

BM cells obtained from 2 femurs were cultured for 6 days in 30 ml size Teflon bags in DMEM + 4,5 g/L D-Glucose media complemented with 10% fetal calf serum, 1% L-glutamine, 1% HEPES 1 M, 1% penicillin-streptomycin and 10 ng/ml mouse M-CSF (M0). The media was exchanged every 2 days. For polarization experiments, cells were seeded in 24 well plates with 10 ng/ml mouse M-CSF for M0 or 10 ng/ml mouse INFy and 10 ng/ml LPS for M1. Polarization was done for 24 h in the presence or absence of 0.5 μM of Bemcentinib.

### 2.2.9 ELISA and Luminex assay

Serum from mouse peripheral blood was isolated using 788 Microvette® 500 Z-Gel, containing a serum gel with clotting activator as per the manufacturer's instructions. Samples were then analyzed using the Mouse Gas6 DuoSet ELISA (R&D Systems) and Mouse Pros1 ELISA Kit (Aviva Systems Biology) according to the manufacturer's instructions.

Plasma samples from human peripheral blood were collected in collaboration with Prof. H. Bönig (DRK-Blutspendedienst) and processed with informed consent in compliance with the institutional review board. Human plasma samples were thawed in a water bath at 37°C, gently vortexed to dissolve precipitates, spun down for 15 min at 12000 rpm and immediately placed on ice prior to dilution according to manufacturer instruction. For the assessment of the plasma level for the following proteins using Luminex magnetic assays (R&D) samples were diluted 1:50 (GAS6), 1:4000 (PROS1), 1:2 (TNFa, IL17a, IL-13, IL7, IL6, IL4, IL2, IL1b, FGF1, CXCL10, CCL2). The analysis was performed using Bio-Plex 200 (BIO-RAD). Results were plotted using GraphPad Prism 7 software.

### 2.2.10 Human samples and ethical compliance

All human BM or peripheral blood samples from patients were processed in compliance with the institutional review board at the Faculty of Medicine of the Technical University of Munich (ethics vote number 538/16) and the university hospital Carl Gustav Carus (ethics vote number EK49022018).

### 2.2.11 Human co-culture experiments with donor-derived monocytes

Peripheral blood samples from healthy donors and patient BM were diluted with PBS 1:1 and 35 ml was gently layered on 15 ml Ficoll-Pague (p= 1.0077, GE Healthcare) in 50 ml Falcon. Samples were centrifuged at RT for 30 min at 2300 rpm without brake. The mononuclear cells were collected, washed with PBS 2% FBS, resuspended and counted. Healthy donor mononuclear cells were used to obtain mature cells by positive selection using human CD14 microbeads (Milteny Biotech) according to manufacturer's instruction. The enrichment purity was estimated by FACS, the CD45+CD11b+CD14+ fraction represented >95%. Before starting the co-culture, MDS/AML mononuclear cells were labeled with 1 µM CFSE in 1 ml of PBS at 37°C for 15 min. The staining was stopped by adding 10 ml of 10% FBS in PBS and cells were washed twice. The efficiency of the labeling was estimated by FACS, showing that the dye was incorporated in >99% of cells. CD14 monocytes and CFSE labeled AML/MDS cells were mixed in a ratio of 2:1 in 1 ml of serum-free StemSpan media and co-cultured for 24 h in 24-well plate in a $CO_2$ incubator at 37°C. After incubation, the cells were collected, washed and stained with viability dye (FixVia Dye AF700) and antibody mix (CD45/CD11b/CD14). Monocytes were re-purified using FACSAria™ Fusion. At least 10,000 $CD14^+$ cells were sorted (purity >95%), pelleted down and used in the downstream RNA isolation using Arcturus PicoPure Kit (Applied Biosystems) for qPCR analysis.

### 2.2.12 Culture conditions of human MSCs and HDMEC

To generate primary human BM-derived MSCs 3-5x$10^6$ whole BM cells obtained from healthy donors were resuspended in 5 ml of StemMACS media for MSC expansion (supplemented with 1% Antibiotic - Antimycotic, 1% L-glutamine) and seeded into T25 flasks in the cell culture incubator. The next day non-attached cells were removed and media was replaced every second day. Healthy donor MSCs (HY-GSH17) were expanded (p2) and immortalized by Ewelina Członka as previously described (Matsumura et al. 2004). Briefly, human MSCs were infected with lentiviral vectors expressing simian virus 40 (SV40) T antigen and human telomerase reverse transcriptase protein (hTERT). Immortalized human MSCs were passaged at least 20 times while maintaining functional, morphological and phenotypic (CD45-CD105+CD90+CD146+) characteristics similar to its primary progeny (data are not shown). Importantly, the oncogenic potential of immortalized MSCs was carefully excluded *in vivo.* Immortalized MSCs were pre-seeded in 24 well plates in the amount of 10,000 cells per well and on the next day were co-cultured with MDS/AML mononuclear cells.

Human dermal endothelial microvascular cells (HDMEC) were isolated and expanded in REPAIR-Lab, Universitätsmedizin Mainz and were a kind gift from Thomas Böse. Cells were cultured in endothelial expansion media EGM2 (Lonza), which was replaced every second day. HDMEC were used in co-culture experiments at passage six. HDMEC were pre-seeded in 24 well plates in the amount of 30,000 cells per well and on the next day were co-cultured with MDS/AML mononuclear cells.

### 2.2.13 Generation of BM-derived murine MSCs

To generate primary murine BM-derived MSCs $5x10^6$ whole BM cells obtained from BL6 wild type animals were resuspended in 5 ml of MEM (Sigma) (supplemented with 10% FBS, 1% P/S, 1% l-Glutamin) and placed into T25 flasks in the cell culture incubator. The next day the non-attached cells were removed and media was replaced every second day. Cells were passaged three times until MSCs fraction reached >85%, verified by FACS staining (CD45/CD140a/CD51/Sca1).

### 2.2.14 Quantification and statistical analysis

All statistical analyses were carried out using version 7 of the GraphPad Prism software. Kaplan-Meier survival curves with two-sided log-rank Mantel-cox analysis was used to evaluate the difference in survival *in vivo*. Comparison of leukemic burdens and target expression levels were carried out using two-sided Student's t-tests.

# 3.0 Results

## 3.1 Experimental model of BCR-ABL+ B-ALL

As an experimental model of acute lymphoblastic leukemia, Ph+ B-ALL was chosen, due to its adverse outcome associated with the high risk of recurrence and acute need for new therapeutic strategies (Roberts et al. 2017). To faithfully mimic the patient situation *BCR-ABL1* (BA) oncogene was combined with the loss *CDKN2A (Arf$^{-/-}$)* with or without *IKZF1* (*Ikaros$^{L/+}$*) haploinsufficiency, resembling the genetic characteristics typical for relapses in the clinic (Roberts and Mullighan 2015). Thus, BM cells from *Arf$^{-/-}$* or *Arf$^{-/-}$Ikaros$^{L/+}$* mice were infected *ex vivo* using a retroviral construct containing the human BA$^{p190}$ fusion protein with a GFP reporter (MSCV-BCR-ABL1-IRES-GFP). After 24h the cells were intravenously transplanted into lethally irradiated C57BL/6 host recipients. All recipients succumbed to B-ALL characterized by expansion of GFP$^{+}$B220$^{dim}$ expression levels as demonstrated in a representative FACS-plot (Fig. 10a). All generated leukemias were subsequently transplanted into non-conditioned C57BL/6 hosts (to preserve microenvironmental integrity) as little as $10^3$ cells and led to the death of recipients within 35 days (Fig. 10b). Moreover, at the stage of full-blown leukemia animals manifested with limb paralysis, indicating CNS involvement a typical feature of BA$^{+}$ B-ALL in the clinic (Sanchez et al. 2017). Leukemic blasts harvested from terminally sick animals were initially co-cultured with MS5 for 2-4 days enabling them to grow further as stroma-independent culture (Fig. 10c).

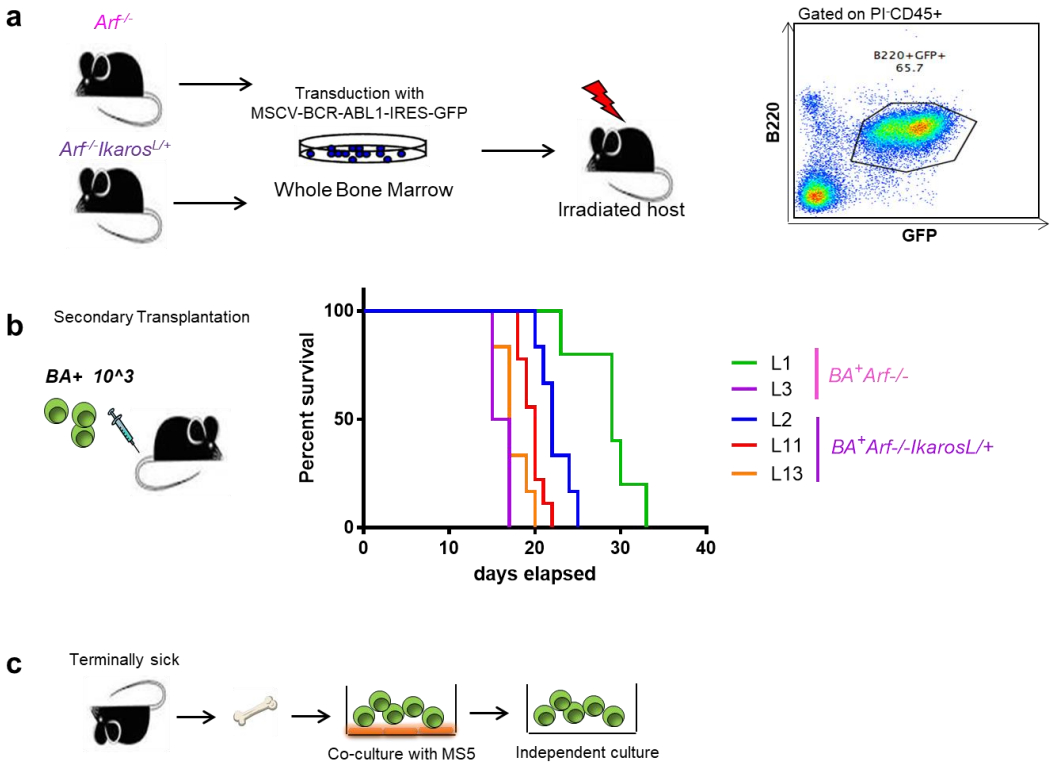


Figure 10. Experimental mouse BCR-ABL1 positive B-ALL model.

a- Retroviral transduction/transplantation model for the generation of BCR-ABL1 positive B-ALL (in collaboration with Filippo Della Marina & Jacques Ghysdael); b- Survival analysis of non-irradiated mice transplanted with a 1,000 $GFP^+BA^+$ cells from independent primary leukemia lines generated in (a); c- Generation of stroma-independent primary leukemia cell lines.

## 3.2 TAMR and their ligands expression in generated $BA^+$ leukemias

It has been reported, that normal B-cell progenitors and mature cells lack expression of TAMR (Huey et al. 2016) while AXL is mainly associated with AML. In turn, *MERTK*, *PROS1* and *GAS6* are aberrantly expressed in a subset of ALL (Brandão et al. 2011) in particular, *MerTK* expression was linked to t(1;19) translocation (Krause et al. 2015) and to T-cell ALL (Brandao et al. 2013). Therefore, the level of expression of TAMR and their ligands *Gas6* and *Pros1* was analyzed in generated leukemias to estimate the potential contribution to cancer cell survival via TAMR-dependent signaling. In the generated BA+ leukemia model, no expression of *Axl* and *Mer* mRNA was observed as indicated in fig. 11a, while all leukemias expressed

a significant level of *Tyro3*. The expression of *TYRO3* has been previously reported in B-CLL, however, the functional consequences of its upregulation are not known (Sinha et al. 2015). Concerning the TAMR ligands, *Gas6* expression was barely detected in leukemic blasts but rather restricted to other cellular compartments of non-leukemic BM (Fig. 11b). In regards to *Pros1* level, a comparable amount of mRNA was detected in BA+ blasts and wild type BM, pointing towards the possibility of beneficial survival signaling in cancer cells via TYRO3/PROS1 as well as TYRO3/GAS6 axis.

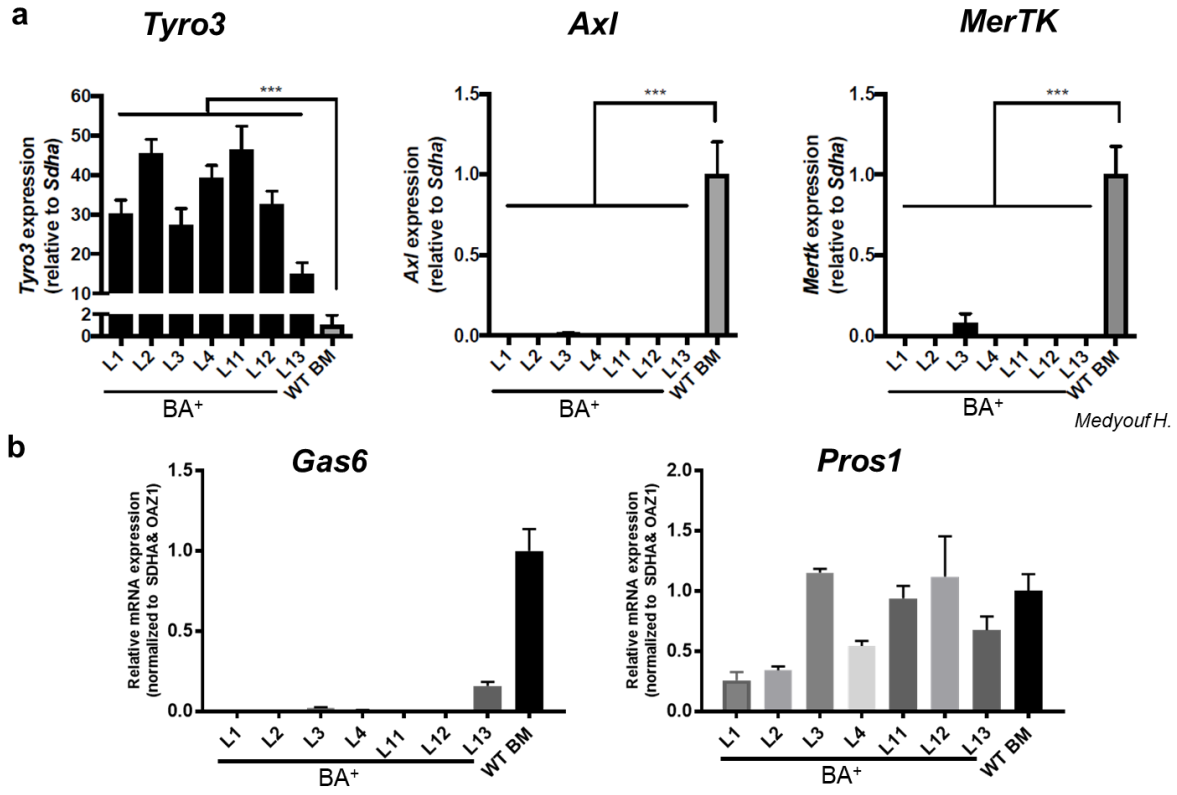


Figure 11. The expression level of TAMR and their ligands in generated leukemia.

Quantitative PCR (qPCR) estimation of *Tyro3, Axl, MerTK* (a) and *Pros1* and *Gas6* (b) in generated leukemias in comparison to wild type whole bone marrow (WT BM). Data are mean ± s.d after normalization to *Sdha* (a) or *Sdha+Oaz1* (b). Data were compared using unpaired two-tailed Student's t-test. ***, p<0,001.

### 3.3 *Gas6*-deficient host environment hampers BCR-ABL+ leukemia propagation and results in 50% long-term survival

Taking into account the role of the GAS6/TAMR axis in dampening the immune response and their emerging role in cancer immunity (Lemke 2013), it was hypothesized that the absence of GAS6 may affect anti-leukemic immune response. To explore this possibility immunocompetent C57BL/6N *Gas6*$^{-/-}$ hosts were challenged with two independent leukemias L1$^{BA+\ Arf-/-}$ and L2$^{BA+\ Arf-/-IkarosL/+}$ without prior conditioning to preserve BM environment integrity. As expected, all control cohorts of wild type recipients ultimately succumbed to full-blown disease within 40 days in case of L1$^{BA+Arf-/-}$ and 25 days in case of a more aggressive clone due to additional Ikaros haploinsufficiency L2$^{BA+\ Arf-/-IkarosL/+}$ (Fig. 12a). In striking contrast to the control group, 50% of *Gas6*$^{-/-}$ recipients were protected from leukemia, importantly, the effect was not limited to a particular leukemic clone (Fig. 12a). To estimate the kinetic of leukemia propagation at the early time point BM aspiration was performed on day 17 after the leukemia challenge (Fig. 12b). By flow cytometry, GFP positive cells in the BM of both cohorts were readily detected. The astonishing observation in *Gas6*$^{-/-}$ cohort depicted in fig. 12c, showing that despite the presence of an expanded leukemia clone in the BM at the early time point some

mice were able to demonstrate superior disease-free survival and the absence of BA+ cells in the BM on the day of sacrifice by FACS analysis (d225).

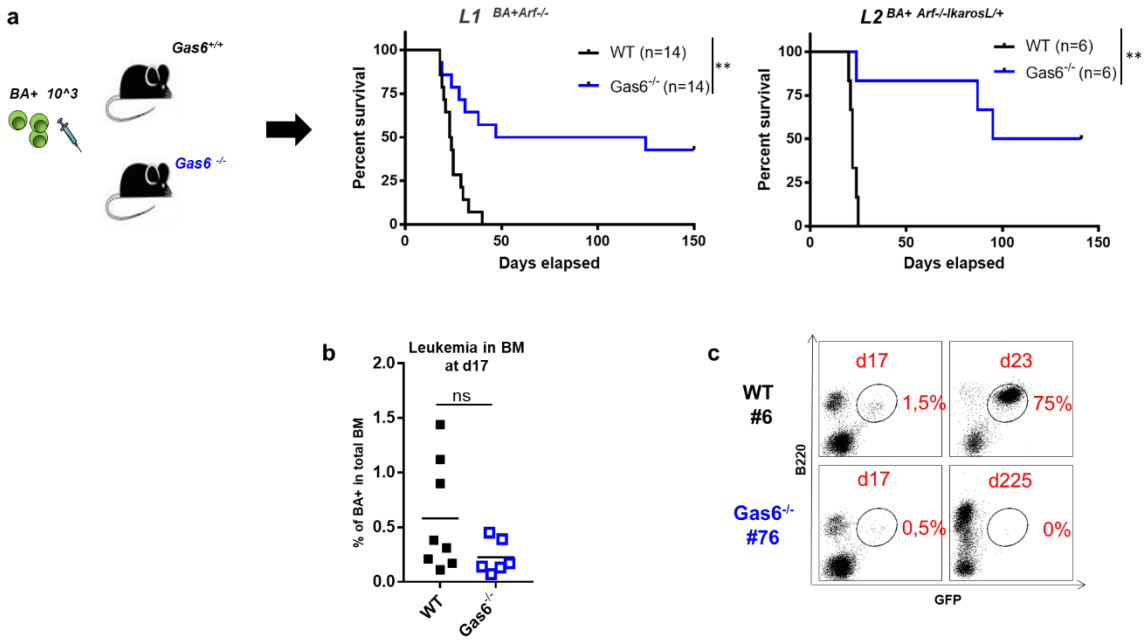


Figure 12. *Gas6*-deficiency rescues 50% of recipients in the syngeneic BCR-ABL1 positive B-ALL model.

a- Transplantation of BA+ cells into non-conditioned recipients with or without Gas6 deficiency. Survival curve represented for L1 (2 independent experiments pooled) and L2; b- Frequency of BA+ cells in WBM analyzed by FACS after BM aspiration. Data were compared using unpaired two-tailed Student's t-test; c- Leukemia clearance example in Gas6-deficient recipients, FACS plots represented at the early time point versus final analysis in contrast to wild type, succumbing to leukemia after 23 days. Survivals were analyzed using the Log-rank test. **, $p<0,01$.

## 3.4 *Gas6*-deficient environment has no effect on the long-term multilineage reconstitution of HSCs

To verify that the observed phenotype in section 3.3 is not provided by any indirect environmental GAS6-driven insufficiency to support leukemic cell maintenance, the effect of GAS6-deficiency on the multilineage reconstitution ability of HSCs was tested in transplant settings. Therefore, C57BL/6N *Gas6*$^{-/-}$ versus C57BL/6N animals were used and subjected to lethal irradiation with subsequent transplantation of rescue WBM with CD45.1/CD45.2 phenotype, in order to track donor chimerism

by FACS (Fig. 13a). Recipient peripheral blood (PB) was evaluated for donor cell chimerism (CD45.1/CD45.2) at 4, 8 and 16 weeks after the transplant by flow cytometry (Fig. 13a). As depicted in fig. 13b, the donor reconstitution of T-, B- and myeloid cells in the control cohort was indistinguishably high in *Gas6*$^{-/-}$ compared to wild type recipients, suggesting that the absence of GAS6 is dispensable for successful HSPCs engraftment. Thus, the phenomenon of the protective effect against B-cell leukemia cannot be explained by the failure of the maintenance of pro-B-cells due to GAS6 deficiency.

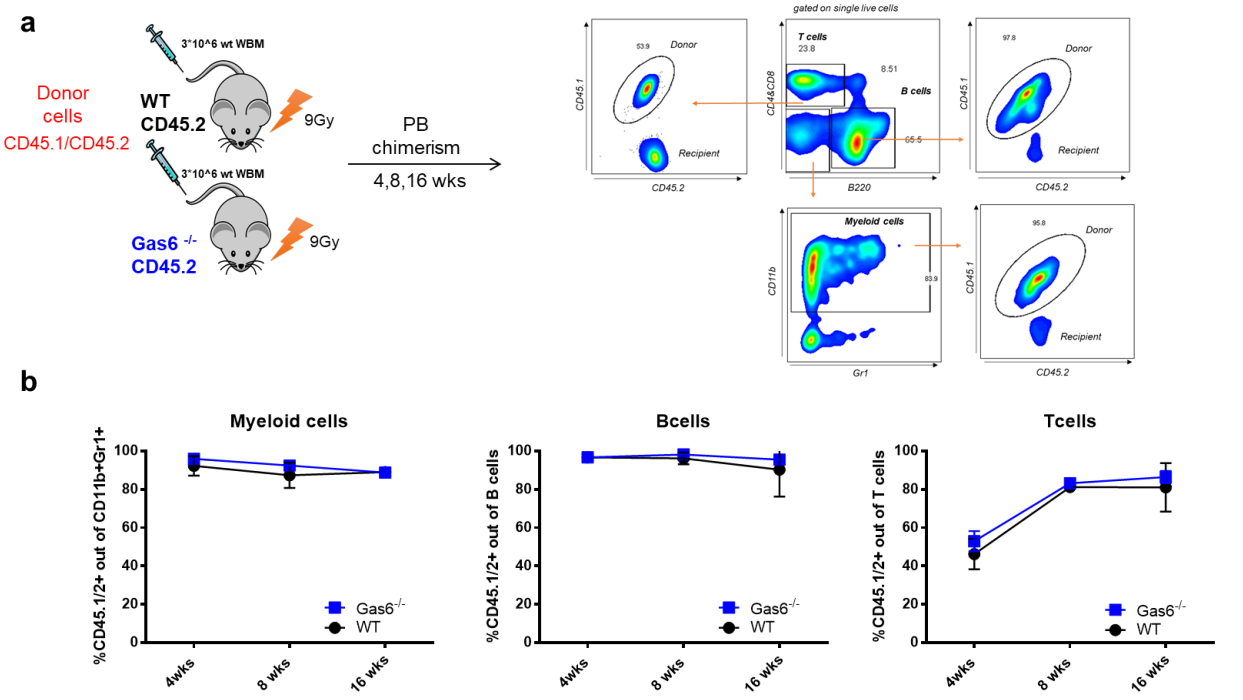


Figure 13. *Gas6*-deficient environment has no defect in multilineage reconstitution in transplant settings.

a- Host recipients (CD45.2) were irradiated with 9 Gy and intravenously transplanted with rescue 3x10$^6$ WBM cells (CD45.1/CD45.2). The donor chimerism in peripheral blood was estimated by demonstrated staining and gating scheme; b- Donor cell chimerism in B-, T- and myeloid lineage depicted for C57BL/6N (black, n=5) C57BL/6N *Gas6*$^{-/-}$ recipients (blue, n=5). Data are mean ± s.d.

## 3.5 GAS6 deficiency does not prolong survival in NSG mice

After exclusion of engraftment defects causing leukemia clearance, we moved back to the initial hypothesis of GAS6 being a modulator of the immune response. To test

this theory and to differentiate between leukemia cell-intrinsic versus immune-modulatory GAS6 effect, we took advantage of an immunodeficient NSG *Gas6*$^{-/-}$ line previously generated in our laboratory. Thus, NSG versus NSG *Gas6*$^{-/-}$ mice were challenged with 1,000 of BA+ cells (Fig. 14a). On the final analysis it was observed an equal leukemia burden across all organs-PB, BM and Sp (Fig. 14b). Moreover, it was also evaluated whether the cell cycle of leukemic blasts changes depending on GAS6 presence in the environment (Fig. 14c) and any significant differences it was detected. These data strongly demonstrate that GAS6-deficiency in the host environment promotes leukemia clearance in strictly immune-dependent fashion and despite TYRO3 being expressed on leukemic blasts (Fig. 11), the direct effect of the TYRO3-GAS6-axis is not essential for leukemia maintenance.

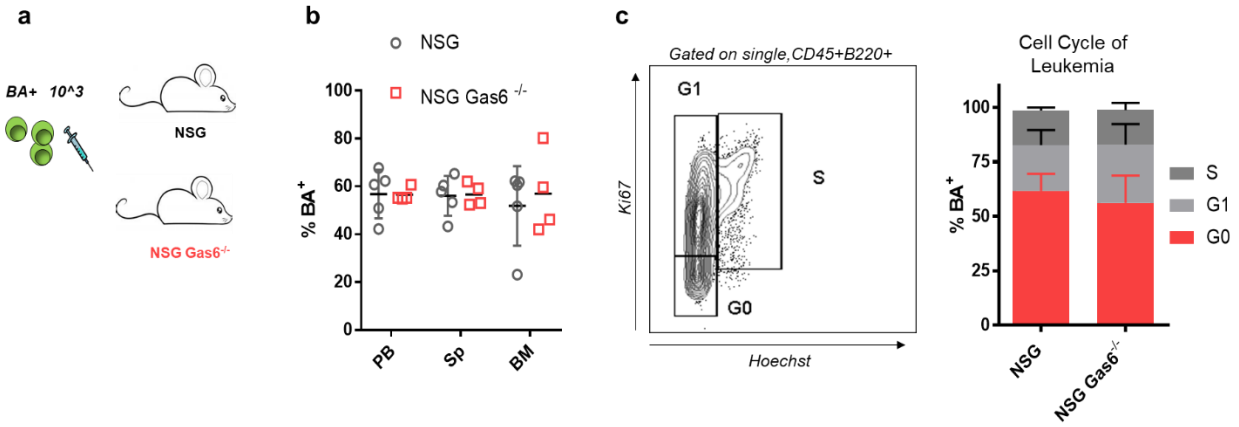


Figure 14. Leukemia clearance mediated by *Gas6*-deficiency is an immune-dependent process.

a- Scheme of the experiment; b- graph representing the frequency of GFP+ (BA+) cells in the peripheral blood (PB), spleen (Sp) and bone marrow (BM) in NSG (grey) and NSG *Gas6*$^{-/-}$ (coral) by FACS analysis, mean ±sd; c- FACS contour plot shows the gating scheme for the cell cycle analysis of BM isolated leukemic cells intracellularly stained with Ki67/Hoechst, graph represents cell cycle composition (S – synthesis phase, G1 – growth phase, G0 – non-proliferative phases) in two cohorts of NSG (n=5) and NSG *Gas6*$^{-/-}$ (n=4), mean±sd.

## 3.6 Adoptive transfer of splenocytes from *Gas6*$^{-/-}$ survivors prolongs the survival of the immunodeficient host

After establishing the role of GAS6 being an immune suppressor in the context of the anti-leukemic immune response, it was decided to test whether long-term survivors from *Gas6*$^{-/-}$ cohort mounted any kind of immune response which can be transferred. Hence, the adoptive transfer of splenocytes from *Gas6*$^{-/-}$ survivors after leukemia (L2$^{BA+Arf-/-IkarosL/+}$) challenge (Fig 15a) was initiated. To enable engraftment of adaptive immune cells it was chosen to use B- and T-cell deficient *Rag*$^{-/-}$ recipients. Donors of splenocytes for control cohorts served age-matched C57BL/6N and C57BL/6N *Gas6*$^{-/-}$ animals not experienced BA+ leukemia, so-called "naïve". Notably, the reconstitution of T-cells in all three *Rag*$^{-/-}$ cohorts was in the normal homeostatic range as compared to an age-matched cohort of C57BL/6N mice (Fig. 15b). Survival analysis revealed that recipients injected with naïve splenocytes died within 35 days (Fig. 15c) in contrast to mice reconstituted with leukemia "primed" splenocytes which demonstrated by the two-fold increase of survival (50%). These data have indicated that GAS6 deficiency indirectly activates the host immune system enabling to mount a productive immune response against BA+ leukemia, which can be at least partially transferred to an immune-compromised recipient.

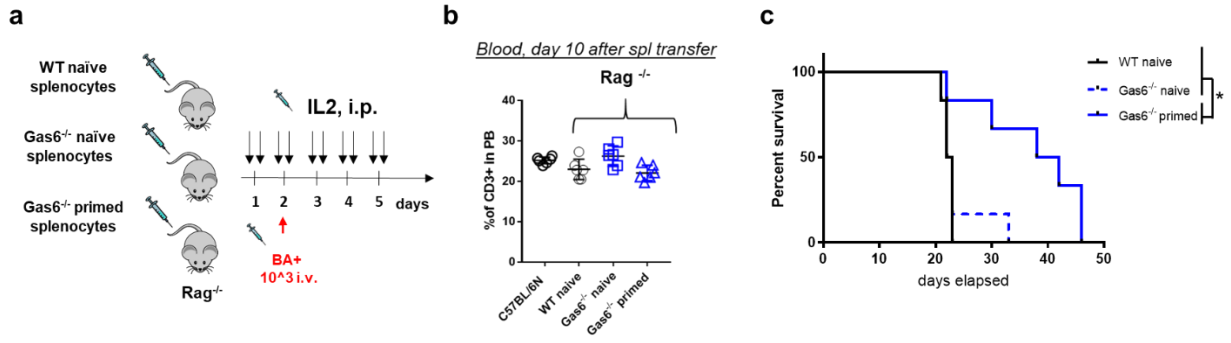


Figure 15. Adoptive transfer of splenocytes of *Gas6*$^{-/-}$ long-term survivors prolongs the survival of *Rag*$^{-/-}$ recipients upon BA+ challenge.

a- Workflow for adoptive splenocyte transfer: three cohorts of *Rag*$^{-/-}$ recipients were i.v. transplanted with $3x10^6$ non-experienced unpurified (naïve) splenocytes obtained from wild type and *Gas6*$^{-/-}$ donors or splenocytes from the sacrificed long-term *Gas6*$^{-/-}$ survivors (survival curve depicted in fig 12a, L2$^{BA+ Arf-/-IkarosL/+}$). To support the homeostatic reconstitution of transferred T-cells, mice received human recombinant IL-2 i.p., twice per day for 5 days. All mice received L2$^{BA+ Arf-/-IkarosL/+}$ i.v. at the dose of $10^3$ at day 2 after

splenocyte transfer; b- Frequency of recovered T-cells in PB of *Rag*$^{-/-}$ mice after the transfer was determined at day 10 by flow cytometry. Data are presented as mean ±sd; c- Survival curve depicted, n=6 in each cohort. Survivals were analyzed using the Log-rank test. *, p<0,05.

### 3.7 Leukemia clearance in *Gas6*-deficient hosts is T-cell dependent

To gain further mechanistic insights into downstream cellular mediators of GAS6 action, we employed a loss of function approach by depletion of T-cells. The data from the previous section (3.6) are pointing towards T-cells being effectors in leukemia eradication. Therefore, to dissect the T-cell role in GAS6-mediated immune response CD3 positive cells were eliminated in *Gas6*$^{-/-}$ mice by CD3-antibody (aCD3) administration and subsequently injection of mice with BA+ leukemia (Fig. 16a). Thus, two cohorts of *Gas6*$^{-/-}$ mice were treated with either isotype control or aCD3 antibody prior to the injection of BA+ cells at day 7. The depletion of T-cells was monitored every week and remained less than 3% (Fig. 16a). The survival analysis revealed that 50% of *Gas6*$^{-/-}$ mice treated with isotype control remained alive as it was demonstrated in previous experiments (Fig. 12a) while in the absence of T-cells the survival advantage was completely lost (Fig. 16b). These data demonstrate that T-cells are essential players in GAS6-mediated immune response against BA+ B-ALL.

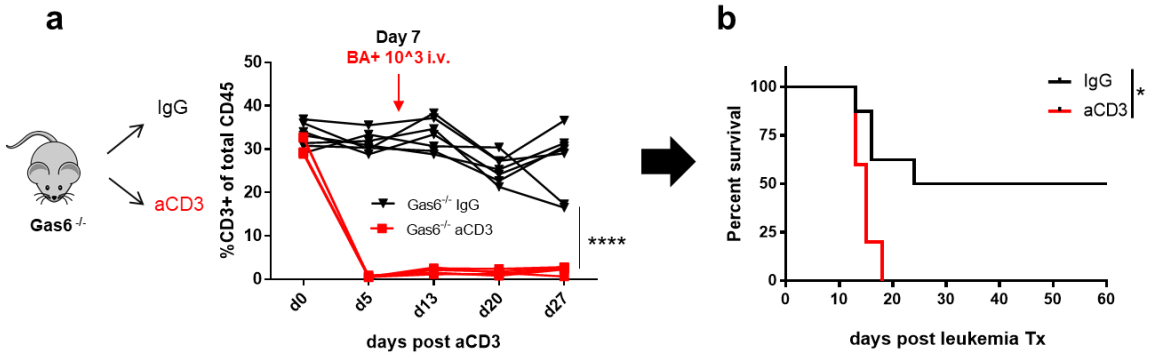


Figure 16. Gas6-deficient environment is no longer protective against BA+ leukemia in the absence of T-cells.

a- Scheme of aCD3 experiment: 8-old weeks C57BL/6N *Gas6*$^{-/-}$ animals were treated either with 100 µg of Rat IgG2b, κ isotype control or rat anti-mouse CD3 (clone 17A2) antibodies three times i.p. once per week. The graph demonstrates the total frequency of T-cells in PB of all individual mice determined by flow cytometry every 6-7 days. Data were compared using unpaired two-tailed Student's t-test. *** p<0,001. Mice were challenged with L2$^{BA+\ Arf-/-IkarosL/+}$ leukemia i.v. in the dose of $10^3$ cells at day 7 post antibody treatment; b- Survival analysis depicted, isotype control cohort in black, aCD3 cohort in red (n = 5 in each cohort). Survivals were analyzed using the Log-rank test. *, p<0,05.

## 3.8 Monocytes/macrophages in *Gas6*$^{-/-}$ environment have a skewed M1/M2 ratio at steady-state

In order to decipher the consequences of GAS6 deficiency and define the direct downstream target of GAS6, the expression of TAMR among murine immune cells was analyzed. Thus, as depicted in the heat map (Fig. 17) generated from the publicly available data set *Axl* and *MerTK* are highly expressed in the macrophage/monocyte subsets and DCs in the spleen. Notably, the lymphoid populations of T- and B-cells remained largely negative for *Axl* and *MerTK*, which made us hypothesize that the T-cell involvement in the GAS6-deficient phenotype (section 3.6 and 3.7) is rather mediated via an enhanced innate immune response. Therefore, next it was estimated whether GAS6 deficiency shapes differently activation and inflammatory status of

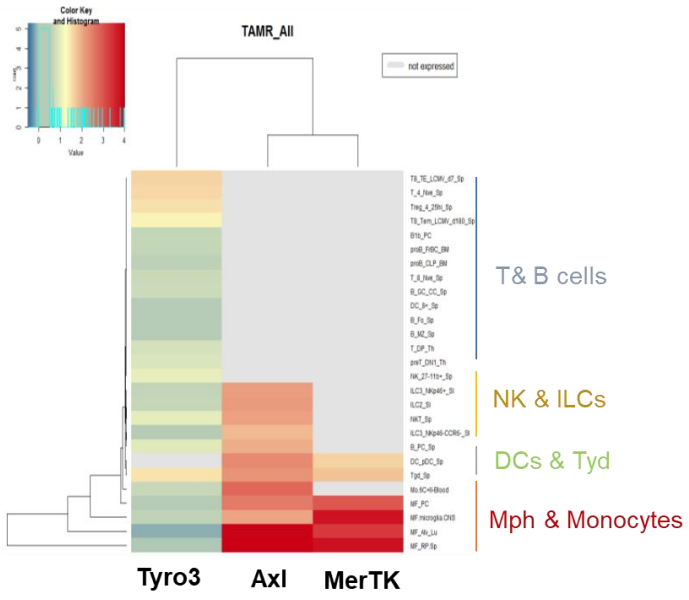


Figure 17. Expression of TAMR in mouse immune cells.

Heat map analysis of publicly available data from the immunological genome project (http://www.immgen.org/databrowser/) depicting the expression levels of *Tyro3*, *Axl* and *MerTK* in different murine immune cells. The highest expression of *Axl* and *MerTK* displayed in red pulp macrophages (MF_RP-Sp), lung alveolar macrophages (MF_Alv_Lu) and microglia (MF.microglia.CNS). Both receptors highly expressed in plasmacytoid dendritic cells in the spleen (DC_pDC_Sp).

*(Heat map by Birgitta E. Michels)*

Mph and monocytes. In order to do so, the M1/M2 phenotype of myeloid cells was analyzed in the blood of wild type versus *Gas6*$^{-/-}$ animals by flow cytometry according to the gating strategy published by De Henau et al. (De Henau et al. 2016) (Fig. 18a). The data revealed that indeed, even at the steady-state the M1/M2 ratio

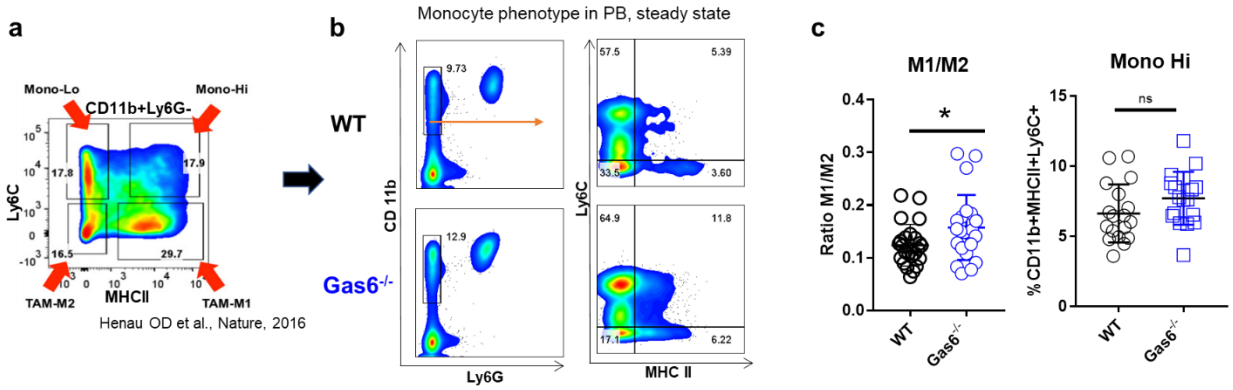


Figure 18. M1/M2 phenotypic analysis of monocyte/macrophages in *Gas6*$^{-/-}$ versus wild type PB at the steady-state.

a- Representative flow cytometry analysis of Ly6C, MHC class II expression in CD11b+Ly6G− cells (TAM-tumor associated macrophages M2- or M1-like) in the study by De Henau et al. (De Henau et al. 2016). b- Own data: flow cytometry analysis of PB in C57BL/6N versus C57BL/6N Gas6$^{-/-}$ at steady state according to the gating in (a); c- Quantification of flow cytometry analysis. Data represent the analysis of 18-24 mice per group, mean±sd, M1/M2 p=0,03, MonoHi p=0.08.

was significantly higher in *Gas6*$^{-/-}$ mice (Fig 18b and c). Taken together, the data from section 3.6-7 and the current analysis, it is tempting to speculate that GAS6 deficiency triggers potent inflammatory priming of monocyte/macrophages, which in turn translates to the potent adaptive immune response against leukemia.

## 3.9 The loss of survival advantage in newly generated C57BL/6N *Gas6*$^{-/-}$ mouse line mediated by compensatory upregulation of *Pros1*

Despite robust data, which were observed in 4 independent experiments (Fig. 12a, 16b) in regards to superior survival of *Gas6*$^{-/-}$ recipients upon challenge with an aggressive B-ALL model, one year after initial experiments it was detected that all *Gas6*$^{-/-}$ succumbed to disease equally to the wild type cohort (Fig. 19a). However, the leukemic burden at the end-point of sacrifice was still significantly lower across

all organs analyzed in the *Gas6*-deficient host as demonstrated by my colleague Tirado-Gonzalez I. (Fig. 19b).

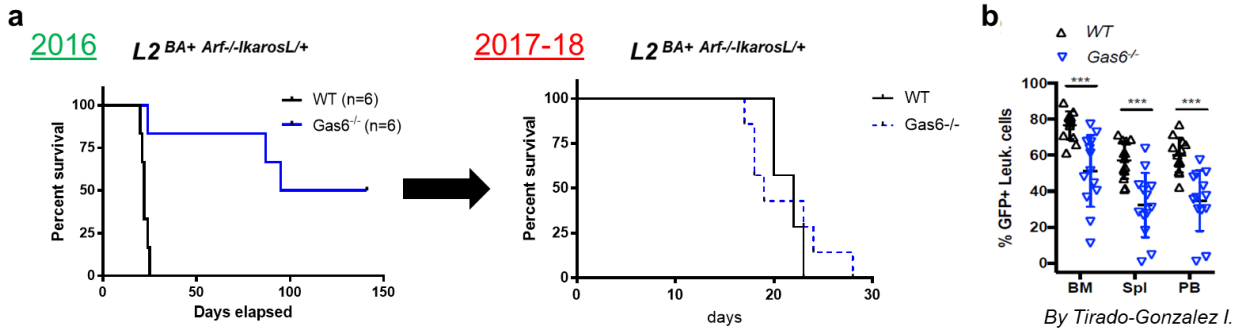


Figure 19. Loss of survival advantage in *Gas6*$^{-/-}$ 1 year after initial experiments.

a- Survival curve from fig 4a, experiments performed soon after import of a newly generated C57BL/6N *Gas6*$^{-/}$ mouse line and survival curve of a representative technical repeat of the same experimental setup at the end of 2017, n=6 in each cohort. b- Quantification of GFP+ leukemic cells by flow cytometry analysis in WT (black) or *Gas6*$^{-/-}$ (blue) recipients, n=11, mean±sd. Data were compared using unpaired two-tailed Student's t-test. *** p<0,001.

Thus, having established the prominent effect of GAS6-deficiency in previous experiments it was hypothesized that the loss of phenotype must underlie the biological changes occurred in the new knockout line over time. Therefore, it was decided to determine the level of GAS6 agonist PROS1 in the MSCs generated in 2016 versus 2018 from *Gas6*$^{-/-}$ BM. *Ex vivo* generated MSCs were subjected to phenotypic analysis by flow cytometry at passage three and 85% were CD45-CD140a+CD51+Sca1+ (Fig 20a) (Rostovskaya and Anastassiadis 2012). Next, the level of mRNA expression of *Pros1* in MSCs obtained from *Gas6*$^{-/-}$ offspring in 2016 and 2018 was compared. As demonstrated in fig. 20b the level of *Pros1* was already 2-fold higher in 2016 compared to wild type MSCs. Strikingly, over 2 years the *Pros1* mRNA level doubled in *Gas6*-deficient host, suggesting that *Gas6* deficiency was overcompensated by the upregulation of agonist *Pros1*. We speculated that a dramatic increase of PROS1 in the system abrogated benefits of an immune response against leukemia in *Gas6*$^{-/-}$ mice. In line with this hypothesis, in

2018 a study has been published demonstrating the immunosuppressive role of tumor-secreted PROS1 by inhibiting M1 macrophage polarization (Ubil et al. 2018).

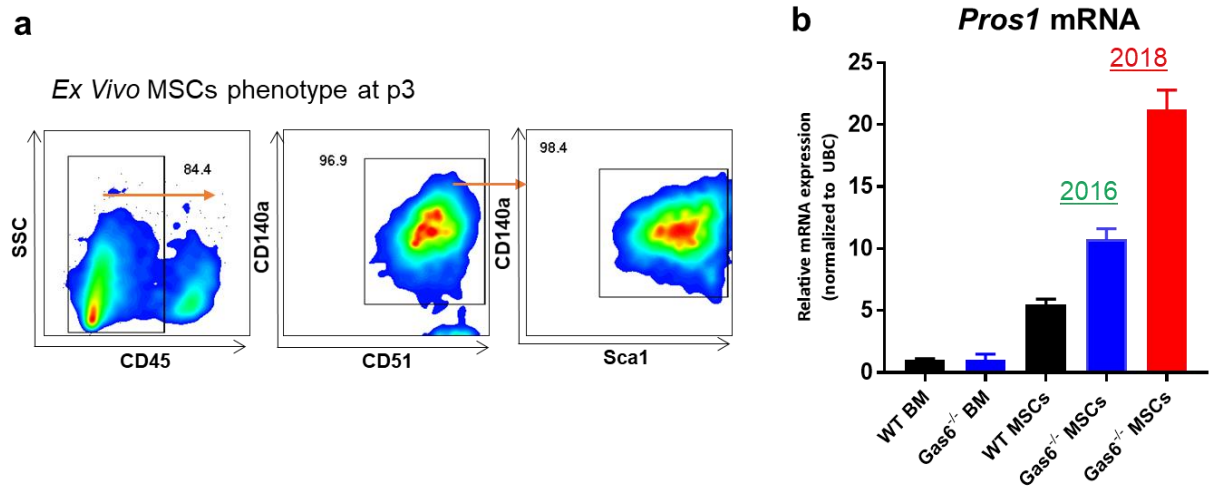


Figure 20. *Pros1* upregulation in *Gas6*-deficient host.

a- An example of phenotypic characteristics of *ex vivo Gas6*$^{-/-}$ - derived MSCs at passage 3. b- *Pros1* mRNA by quantitative qPCR in the BM-derived MSCs of C57BL/6N Gas6$^{-/-}$ in 2016 and 2018, compared to Pros1 expression in wild type BM and MSCs. Data are mean ± s.d after normalization to *Ubiquitin*.

## 3.10 BA+ leukemia induces a change in the expression of *Pros1* and *Gas6* in the bone marrow microenvironment

Having identified the compensatory PROS1 upregulation over time in the absence of GAS6 we sought to characterize whether the expression of these proteins was differentially regulated in response to the leukemic challenge. Therefore, a cohort of wild type mice was injected with either BA+ leukemia or PBS. Animals were sacrificed at day 13 post-transplant and MSCs from BM were FACS sorted for downstream qPCR along with the collection of the BM plasma for ELISA (Fig. 21a,b). Surprisingly, the gene expression and protein level of GAS6 were downregulated (Fig. 21 c,d) or not changed (Fig. 21d) in response to both BA+ leukemic clones.

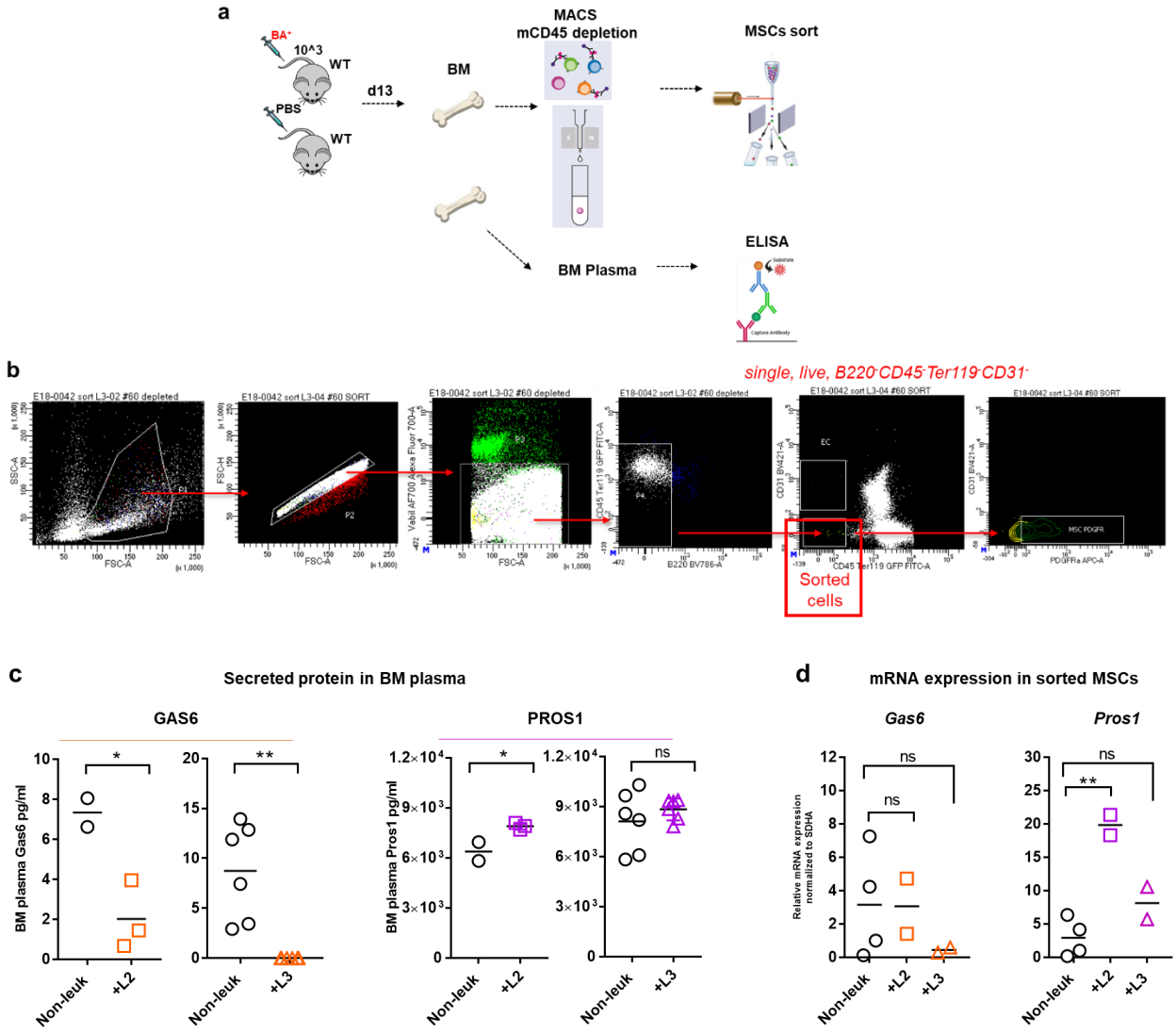


Figure 21. Leukemia-induced changes of *Gas6* and *Pros1* expression in the bone marrow.

a- Scheme of the experimental setup. Wild type mice were i.v. injected either with 1,000 cells of $L2^{BA+\ Arf-/-IkarosL/+}$, $L3^{BA+\ Arf-/-}$ or 200 µl of PBS. The final analysis was performed on day 13 post-leukemia. BM plasma was collected and snap-frozen for ELISA, while BM cells were subjected to depletion of mouse CD45+ hematopoietic cells using MACS separation column; b- Representative gating scheme for MSCs sort. CD45 depleted fraction of BM was used for sorting of MSCs subset CD45-B220-Ter119-CD31-, as indicated on the gating scheme around 50% of sorted cells expressed CD140a (PDGFRa); c- Concentration of GAS6 (pg/ml) and PROS1 (pg/ml) in the BM plasma in PBS injected wild type cohort versus $L2^{BA+\ Arf-/-IkarosL/+}$ or $L3^{BA+\ Arf-/-}$. Data are mean; d- *Pros1 and Gas6* mRNA by qPCR in the sorted MSCs of C57BL/6N injected with PBS or BA+ leukemia, Data are mean after normalization to *Sdha*. Data were compared using unpaired two-tailed Student's t-test. *, $p < 0,05$; **, $p<0,01$.

However, the PROS1 analysis exposed the leukemia-specific pattern of response, opposite to GAS6. Thus, mRNA and protein level of PROS1 was upregulated in the case of an aggressive $L2^{BA+\ Arf-/-IkarosL/+}$ clone, while upon less aggressive $L3^{\ BA+\ Arf-/-}$ challenge there were no apparent variations in comparison to the PBS-injected cohort (Fig. 21c,d). Taken together, PROS1 is a putative essential player in establishing of a leukemia-induced immunosuppressive milieu in the BM.

### 3.11 Leukemia clone-specific upregulation of PROS1 abrogates the early anti-cancer immune response in *Gas6*-deficient hosts

Having identified the critical contribution of PROS1 in the dysregulation of a productive immune response against leukemia: from one side, overexpression of PROS1 leads to the loss of survival advantage in *Gas6*-deficient mice and from the other side, an instructive increase of PROS1 by leukemia was detected in BM. Therefore, in order to prove the detrimental immunosuppressive effect of PROS1 the $L2^{BA+Arf-/-IkarosL/+}$ clone as verified PROS1 inducer and $L3^{BA+Arf-/-}$ as not increasing PROS1 expression control clone were used. To pinpoint the early immune changes in response to leukemia, the experiment was terminated at day 7 post-transplant (Fig. 22a). Phenotypic analysis of the monocyte/macrophage population revealed a significantly increased M1/M2 ratio in the BM of *Gas6*$^{-/-}$ mice compared to wild type recipients challenged with $L3^{BA+Arf-/-}$ (Fig. 22 b,c). Notably, after transplantation of $L3^{BA+Arf-/-}$ cells the frequency of $MHCII^{hi}$ Ly6C+CD11b+ monocytes was significantly higher in BM as well as in PB, highlighting the activation of antigen presentation by MHCII (Fig. 22 b,c), which was not observed at the steady-state analysis (Fig 18c). Such differences in the activation status of innate cells translated into an upregulated production of TNFa by T-cells in the spleen of *Gas6*-deficient hosts along with an increased frequency of INFy in the non- lymphoid CD45

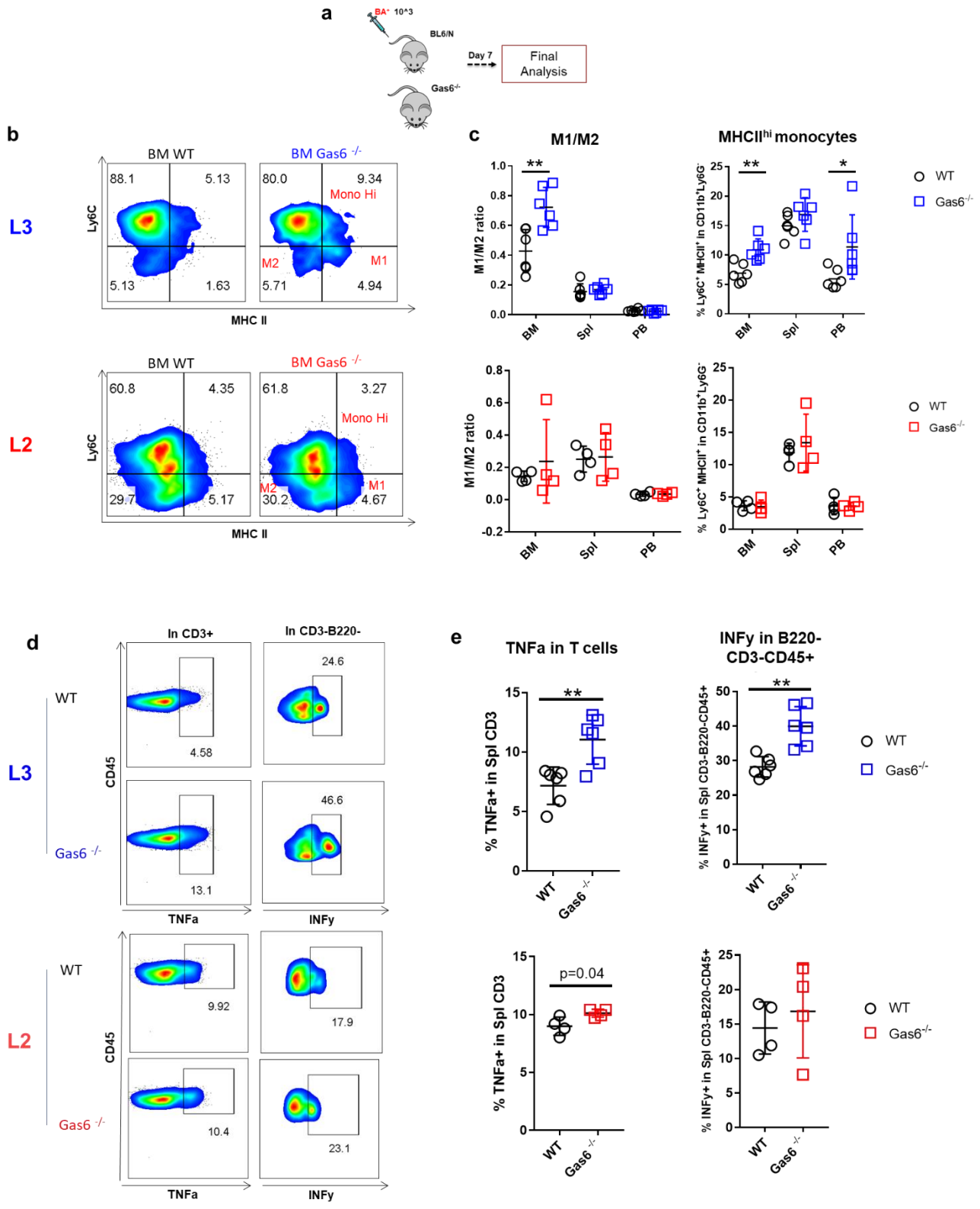


Figure 22. Leukemia- induced PROS1 upregulation effectively blocks the early immune response in *Gas6*$^{-/-}$ host.

a- Scheme of the experimental setup. C57BL/6N or C57BL/6N *Gas6*$^{-/-}$ mice were i.v. injected with 1,000 cells of L2$^{BA+Arf-/-IkarosL/+}$ or L3$^{BA+Arf-/-}$. The final analysis was performed on day 7 post-leukemia; b- Representative FACS plots of M1/M2 phenotypic analysis of monocyte/macrophages population (CD45+CD11b+Ly6G-) in BM of wild type and *Gas6*$^{-/-}$ challenged with L2$^{BA+Arf-/-IkarosL/+}$ and L3$^{BA+Arf-/-}$; c- Quantification of M1/M2 ratio and frequency of MonoHi population injected with L3$^{BA+Arf-/-}$ (C57BL/6N n=6; C57BL/6N *Gas6*$^{-/-}$ n=6) and with L2$^{BA+Arf-/-IkarosL/+}$ (C57BL/6N n=4; C57BL/6N *Gas6*$^{-/-}$ n=4); d- Representative FACS plots of intracellular cytokine staining TNFa within CD45+CD3+ subset and INFy within CD45+CD3-B220- subset in splenocytes of wild type and *Gas6*$^{-/-}$ mice challenged with L2$^{BA+Arf-/-IkarosL/+}$ or L3$^{BA+Arf-/-}$, staining was performed without additional *ex-vivo* stimulation; e- Quantification of results obtained in (d). Data are mean ± s.d. Data were compared using unpaired two-tailed Student's t-test. *, p < 0,05; **, p<0,01.

splenocyte fraction in comparison to control (Fig. 22 d,e). However, all these aforementioned signatures of the early immune response against L3$^{BA+\ Arf-/-}$ clone were essentially abrogated in the case of a more aggressive L2$^{BA+\ Arf-/-IkarosL/+}$ clone, potentially due to the induced PROS1 upregulation by MSCs in the BM. Taken together, these data suggest that PROS1, but not GAS6 can be instructively upregulated in response to aggressive BA-driven leukemia with the genetic makeup of relapse phenotype *(Arf*$^{-/-}$*Ikaros*$^{L/+}$*)*, thus co-opting with leukemia to corrupt cancer immune sensation.

## 3.12 GAS6 and PROS1 effect mediated through AXL and/or MERTK in *Csf1r*-expressing mononuclear cells

To further dissect the benefits of the absence of GAS6 and PROS1 for the anti-leukemic immune response mice with floxed alleles of *Axl* and/or *MerTK* plus Cre recombinase under the control of the *Csf1r* promoter were used. The chosen model allowed to exclude the contribution of both ligands and simultaneously target the cell populations of mononuclear phagocytes carrying the highest expression of AXL and MERTK – Mph, monocytes and DCs (Fig. 17, http://www.immgen.org/databrowser/). Of note, constitutive *Pros1* knockout mice are embryonically lethal due to the heavy coagulation defects (Burstyn-Cohen, Heeb, and Lemke 2009). Two weeks after injection with BA+ leukemia control mice

(*Csf1r-Cre*$^{-}$*Axl*$^{f/f}$) demonstrated full-blown disease burden in BM, Spl and PB, while *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ showed no sign of leukemia as shown by flow cytometry (Fig. 23a). Importantly, the potential *Csf1r-Cre*$^{+}$ toxicity was carefully excluded – both *Csf1r-Cre*$^{-}$ and *Csf1r-Cre*$^{+}$*Axl*$^{f/+}$ mice had indistinguishable leukemic burden at the final analysis (Fig. 23b). Interestingly, it was shown by my colleague Tirado-González I. that either use of *Csf1r-Cre*$^{+}$ mice with floxed alleles of *Axl* and/or *MerTK* results in an unprecedented long-term survival rate of 60% compared to control (data are not shown).

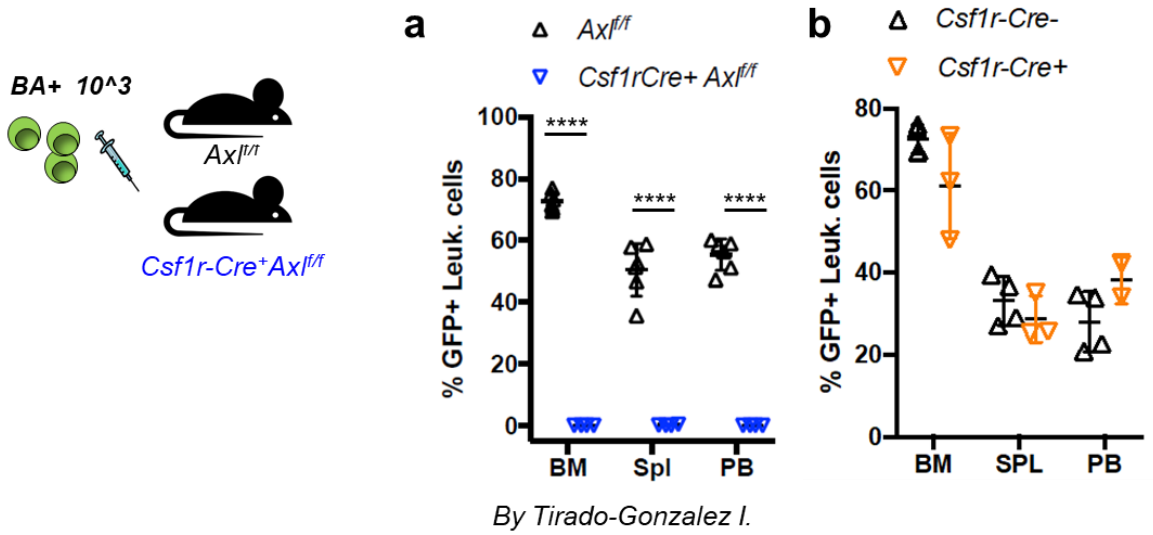


Figure 23. *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ are protected from BA+ leukemia.

a- Scheme of the experiment and leukemic burden in the bone marrow (BM), spleen (Spl) and peripheral blood (PB) in *Csf1r-Cre*$^{-}$*Axl*$^{f/f}$ (n=6) and *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ (n=4) analysis by flow cytometry at day 13 post-transplant, data are mean ± s.d.; b- Leukemic burden in the bone marrow (BM), spleen (Spl) and peripheral blood (PB) in *Csf1r-Cre*$^{-}$*Axl*$^{f/f}$ (n=4) and *Csf1r+Cre*$^{+}$*Axl*$^{f/+}$ (n=3), data are mean ± s.d. Data were compared using unpaired two-tailed Student's t-test ****, p<0,0001.

## 3.13 *Csf1r*-expressing macrophages are essential to confer protection against BA+ leukemia via AXL/MERTK

The "black and white" phenotype of *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ (and/or *Mer* $^{f/f}$) in response to BA+ challenge makes it the feasible model to explore the necessary cellular mediators of GAS6/PROS1-mediated immune response. Therefore, to test whether

TAMR-deficient Mph are required for leukemia resistance, Mph were depleted via clodronate liposome injection prior to leukemia challenge (Fig. 24a). The treatment drastically depleted F4/80+ mature Mph in the system as depicted in the representative FACS plot (Fig 24a) and completely abrogated protection against leukemia in *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ hosts (Fig. 24b,c). This data strongly points towards Mph being indispensable downstream cellular mediators of GAS6 and PROS1. Taken together, inactivation of TAMR in *Csf1r*-expressing Mph unleashes an efficient innate immune response against leukemia, in line with the data from section 3.12.

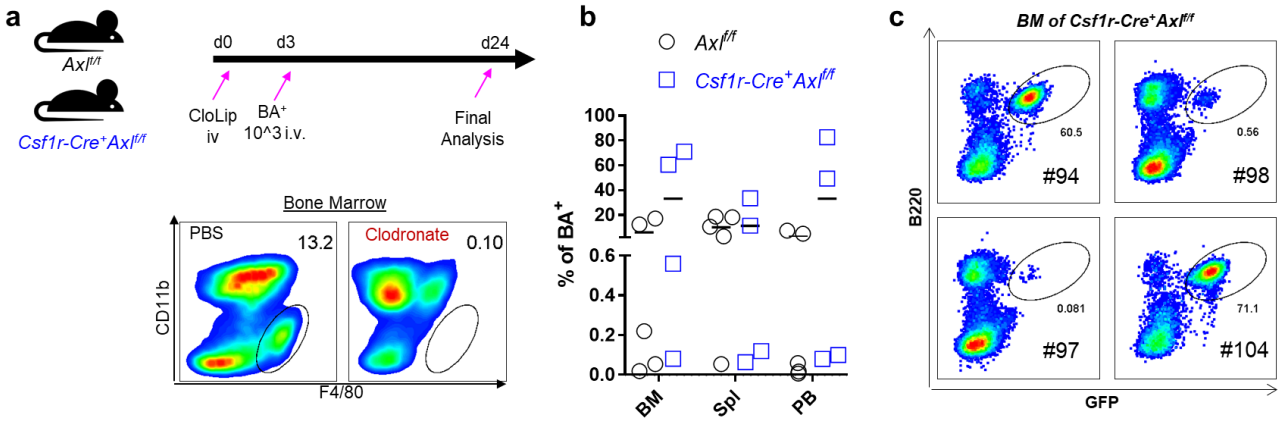


Figure 24. Macrophages are indispensable cellular downstream mediators of the anti-leukemic immune response in *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ model.

a- Scheme depicting the experiment setup. Control *Axl*$^{f/f}$ (n=5) mice and *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ (n=4) received one shot of clodronate liposomes (250 µl, i.v.) three days prior to 1,000 BA+ injections. Mice were analyzed at day 21 post-leukemia when the first mouse was terminally ill. b- Leukemic burden was estimated in bone marrow (BM), spleen (Spl) and peripheral blood (PB) by flow cytometry, data as mean. c- Representative FACS plots demonstrating the variable frequency of GFP+ leukemia cells in BM of *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ mice.

## 3.14 Pharmacological inhibition of Axl in wild type bone marrow-derived macrophages enhances M1-polarization *ex vivo*

According to genetic data, inhibition of TAMR signaling in Mph leads to an enhanced M1 polarization. In order to test whether this effect can be mimicked in wild type cells, bemcentinib (known as R428 or BGB324 (Holland et al. 2010)) a

selective AXL inhibitor was tested. Thus, wild type BM-derived Mph (BMDM) were generated and their response to LPS and INFg was evaluated in the presence of vehicle or bemcentinib (Fig 25a). LPS and INFg are two conventional stimulators commonly used to induce canonical M1-polarization (Orecchioni et al. 2019). Treatment with bemcentinib significantly increased the expression of both key M1-cytokines *TNFa* and *IL-12*, in contrast, the expression of *Socs3*, the well-known downstream target of AXL (and MERTK) that dampens the production of inflammatory cytokines, was consistently decreased (Fig 25b). These data suggest, that selective AXL targeting using orally available small molecular compound bemcentinib, might be a promising therapeutic approach in order to unleash the efficient priming of macrophages in response to leukemia.

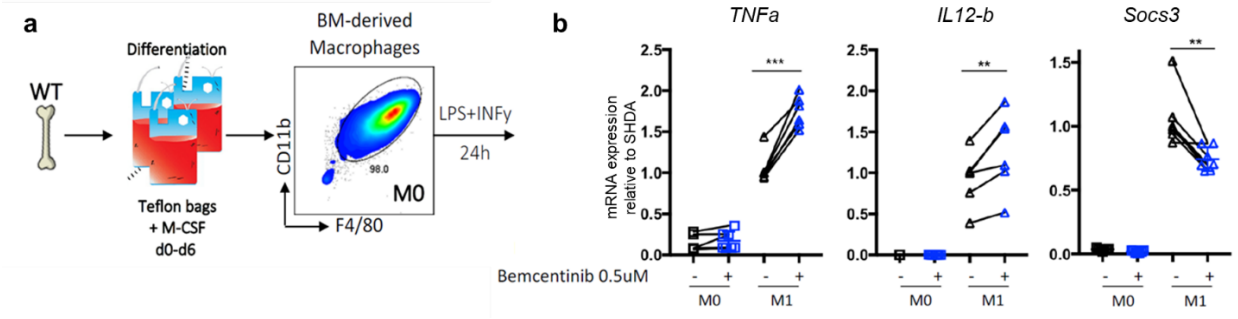


Figure 25. Pharmacological blockade of AXL promotes enhanced inflammatory priming in wild type BMDMs.

a- Scheme of the experiment setup. The whole BM was obtained from the femurs of C57BL/6N (n=5 independent donors) and cultured in teflon bags in the presence of M-CSF (10 ng/ml). Representative FACS plot demonstrates that 98% of cells constitute of mature Mph at day 6, indicated by $CD11b^{hi}F4/80^{hi}$ expression. The polarisation lasted for 24 h: M0 -stimulated with M-CSF (10 ng/ml) M1- stimulated with LPS (10 ng/ml) and INFg (10 ng/ml), both conditions were treated with vehicle or bemcentinib (0.5 µM) b- qPCR estimation of *TNFa*, *IL-12b* and *Socs3* in BMDMs. Data are mean after normalization to *Sdha*. Data were compared using paired two-tailed Student's t-test. **, p<0,01; ***, p<0,001.

## 3.15. AXL/SOCS3 axis alters the inflammatory priming of macrophages in the context of BA+ leukemia

*Ex vivo* data from the previous section suggest that mechanistically, the blockade of AXL on Mph induced enhanced M1-priming with the consequent downregulation of *Socs3* (Fig 25b). If the production of inflammatory cytokines by Mph is essential for leukemia resistance the phenotype we have seen in *Csf1r-Cre*$^{+}$*Axl* $^{f/f}$ mice should be recapitulated if *Axl* floxed alleles will be replaced with *Socs3*. Indeed, in line with the downregulation of *Socs3* in BMDMs treated with bemcentinib, *Csf1r-Cre*$^{+}$*Socs3*$^{f/f}$ mice were protected from BA+ leukemia as indicated in fig. 26a in comparison to the control cohort. Thus, downstream of TAMR signaling engaged in Mph in the syngeneic model of BA+ B-ALL blocks the inflammation via interaction with type I interferon receptor IFNaR and downstream activation of *Socs1/Socs3*, as has been previously shown in the context of DCs by Rothlin et al. (Rothlin et al. 2007) (Fig 26b).

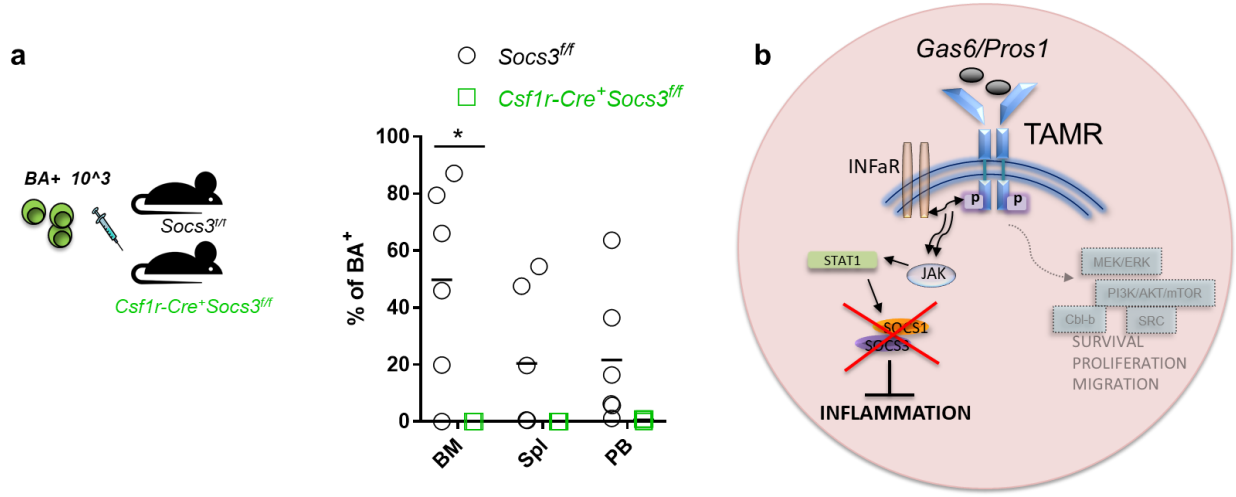


Figure 26. *Csf1r-Cre*$^{+}$*Socs3*$^{f/f}$ mice are protected from BA+ leukemia.

a- Scheme of the experiment. *Csf1r-Cre*$^{+}$*Socs3*$^{f/f}$ (n=4) and Socs3$^{f/f}$ control mice (n=5) were injected with 1,000 BA+ cells. Mice were analyzed 21 days post-leukemia, when the first mouse was terminally sick. Leukemic burden was estimated by flow cytometry in bone marrow (BM), spleen (Spl) and peripheral blood. Data presented as mean. b- Own drawings depicting downstream signaling of TAM receptors in macrophages, which putatively underlying the phenotype observed in (a). Data were compared using paired two-tailed Student's t-test. *, p<0,05;

## 3.16 NK cells are essential to combat ALL and AML in *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ model

In order to estimate the contribution of other cellular effectors, reported to be important in anti-leukemic immunity, such as NK (Mattias Carlsten and Järås 2019) and T-cells (Teague and Kline 2013), we used the loss of function approach by depletion of these cell compartments using monoclonal antibodies *in vivo*. Two different leukemia entities BA+ B-ALL and MLL-ENL-driven AML were used in order to set up the baseline of an immune response in the syngeneic mouse models. To test whether leukemia accelerates expansion in wild type recipients in the absence of CD4-, CD8- and NK- cells, mice were challenged with leukemia at day 4 after the subset of interest was efficiently cleared out. The leukemic burden was analyzed early on day 10 for ALL and day 29 for AML (Fig. 27a,b). The experiment was terminated at the early time point due to the fact of aggressive nature of tested leukemia types leading to the ultimate death of wild type hosts, otherwise, at the stage of full-blown leukemia, the differences of a subtle immune response might be simply saturated. Additionally, the early time point was successfully used to detect immune changes in myeloid and T-cells in *Gas6*-deficient recipients earlier, as have been shown in section 3.12. The depletion of CD4, CD8 and NK subsets were extremely efficient in both ALL and AML cases (Fig. 27b and e, respectively). The final analysis of the leukemic burden revealed that in both cases of AML and ALL

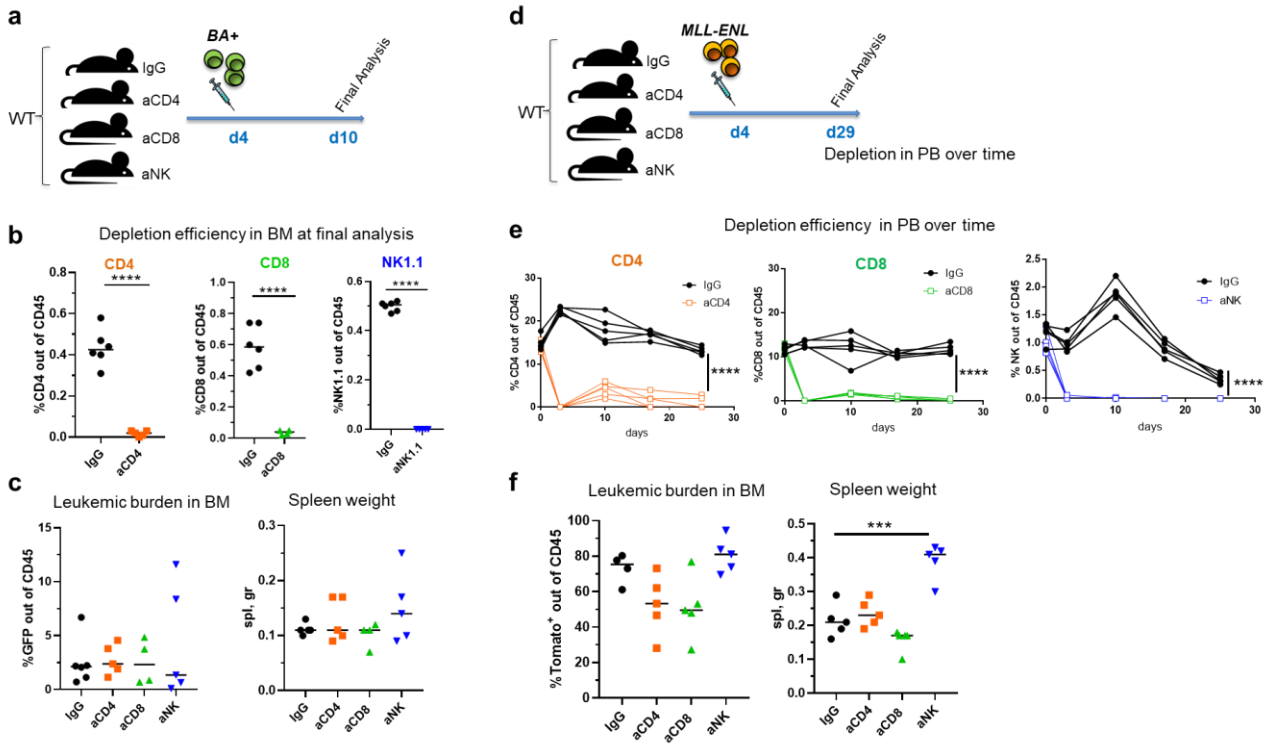


Figure 27. Contribution of CD8 and NK cell in control of B-ALL and AML in wild type recipients.

a- Scheme of the experiment. Four cohorts of C57BL/6N mice were pre-treated (i.p.) with 50 µg of IgG (n=5), 100 µg of aCD4 (n=5), 50 µg of aCD8 (n=5) and 50 µg of aNK1.1 (n=5) prior to injection of B-ALL (BA+) or (d) AML (MLL-ENL). The experiment was terminated at day 10 (a) or 29 (d); b- Efficiency of depletion CD4-, CD8- and NK-cells determined at the final analysis in BM by flow cytometry in the case of B-ALL, for AML (e) depletion was monitored over time; c- Quantification of B-ALL burden in bone marrow (BM) was evaluated by FACS and spleen weight was measured, data for AML depicted in (f). Data are mean. Data were compared using unpaired two-tailed Student's t-test. ***, p<0,001; ****, p<0,0001.

the absence of either CD4 or CD8 cells did not accelerate the leukemia burden (Fig. 27c,f). In contrast, NK-cell depletion leads to a significantly higher leukemic burden for AML. Next, it was tested how the depletion of CD8 cells may affect the survival in *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ mouse model challenged with BA+ leukemia (Fig 28a). As depicted in the survival curve in fig. 28c, depletion of CD8 T-cells did not accelerate disease in the control cohort. Intriguingly, in anti-CD8 treated *Csf1r-Cre*$^{+}$ *Axl*$^{f/f}$ group less than 20% of recipients survived versus 50% in the IgG-treated cohort. These data are indicative that CD8 T-cells might be involved in leukemia eradication in *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ mice. However, this aspect certainly requires further

investigation using genetically encoded depletion of T cells (e.g. *Rag1*$^{-/-}$ crossed to *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ ).

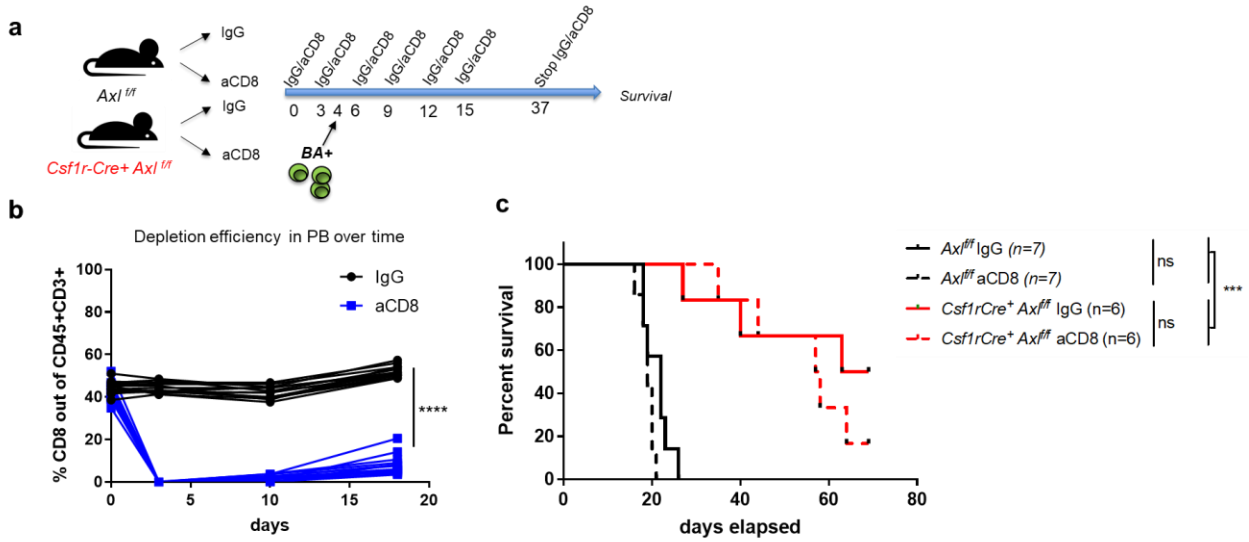


Figure 28. Contribution of CD8-cells in B-ALL leukemia protection in the *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ model.

a- Scheme of the experimental setup. Control *Axl*$^{f/f}$ (n=14) mice and *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ (n=12) received two shots of IgG or aCD8 (50 µg, i.p.) prior to injection of 1,000 BA+ cells. Injection of isotype control and depleting antibodies were maintained every 3 days and stopped at day 37. Mice were left for survival analysis; b- The depletion efficiency of CD8-cells monitored in peripheral blood (PB) by FACS analysis. Data were compared using unpaired two-tailed Student's t-test. ****, p<0,0001; c- Representative survival curve depicted for IgG-treated *Axl*$^{f/f}$ (n=7, in solid black), aCD8-treated *Axl*$^{f/f}$ (n=7, in dashed black), IgG-treated *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ (n=6, in solid red) and aCD8-treated *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ (n=6, in dashed red). Survivals were analyzed using the Log-rank test.***, p<0,001

To evaluate the contribution of NK cells in our genetic model (*Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ or *Axl*$^{f/f}$*Mer*$^{f/f}$) NK were depleted using the NK1.1 antibody and terminating the experiment when the first mouse was terminally ill (Fig 29 a,c). Interestingly, in both leukemia models B-ALL and AML, the lack of NK cells led to a complete failure of disease control (Fig 29 b,d) in comparison to IgG recipients.

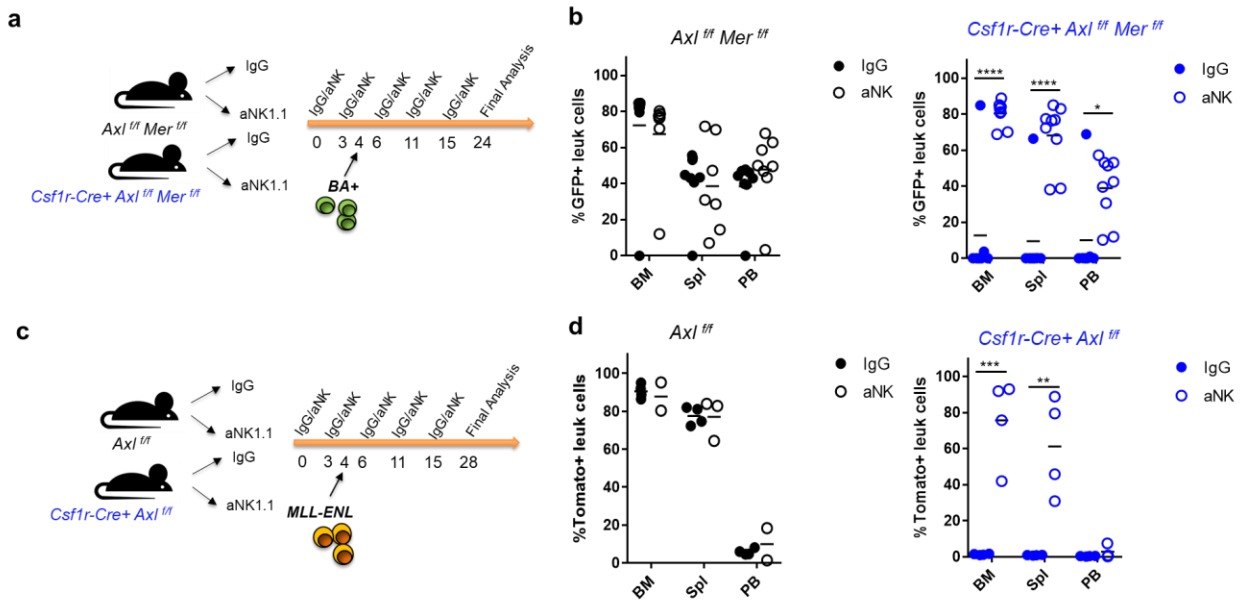


Figure 29. NK-cells are essential to confer protection against B-ALL and AML in *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ model.

a- Scheme of the experimental setup. Control *Axl*$^{f/f}$*Mer*$^{f/f}$ (n=16) mice and *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$*Mer*$^{f/f}$ (n=16) received two shots of IgG or aNK1.1 (50 µg, i.p.) prior to injections of 1,000 BA+ cells. Injection of isotype control and depleting antibodies were maintained every 5 days after 2$^{nd}$ shot of IgG and aNK1.1. Animals were analyzed when the first mouse demonstrated full-blown B-ALL; b- Leukemic burden in (a) was estimated in bone marrow (BM), spleen (Spl) and peripheral blood (PB) by FACS analysis; c- Scheme of the experimental setup. Control *Axl*$^{f/f}$ (n=7) mice and *Csf1r-Cre*$^{+}$ *Axl*$^{f/f}$ (n=8) received two shots of IgG or aNK1.1 (50 µg, i.p.) prior to 10^5 MLL-ENL leukemia injections. The administration mode of IgG/aNK1.1 as in (a). d- Leukemic burden in (c) was estimated in bone marrow (BM), spleen (Spl) and peripheral blood (PB) by FACS analysis; data as mean. Data were compared using unpaired two-tailed Student's t-test. *, p < 0,05; **, p<0,01; ***, p<0,001; ****, p<0,0001.

## 3.17 Macrophages and NK-cells upregulate TAMR in response to leukemia *in vivo*

Having established the critical role of TAMR acting as suppressors of anti-leukemic potential in Mph and NK-cells, we sought to identify which of the receptors will be the best druggable target. Therefore, the expression kinetic of TAMR in NK and Mph upon B-ALL challenge was evaluated in wild type mice. The analysis revealed that the expression of *Axl* and *MerTK* was significantly upregulated in the leukemia-associated Mph, which could potentiate a non-inflammatory, tissue-repair pro-tumor phenotype (Fig. 30a). However, in NK cells only *Axl* expression was found to be significanly increased in comparison to leukemia-free mice (Fig. 30a). As expected,

the expression of *Tyro3* was not modulated upon the leukemic challenge in both Mph and NK-cells (Fig. 30a,b). At the time of analyses, leukemia challenged mice had a low disease burden (0.1-0.7%), possibly suggesting that induction of *Axl* (and *MerTK* in Mph) expression is an early event post-leukemia emergence.

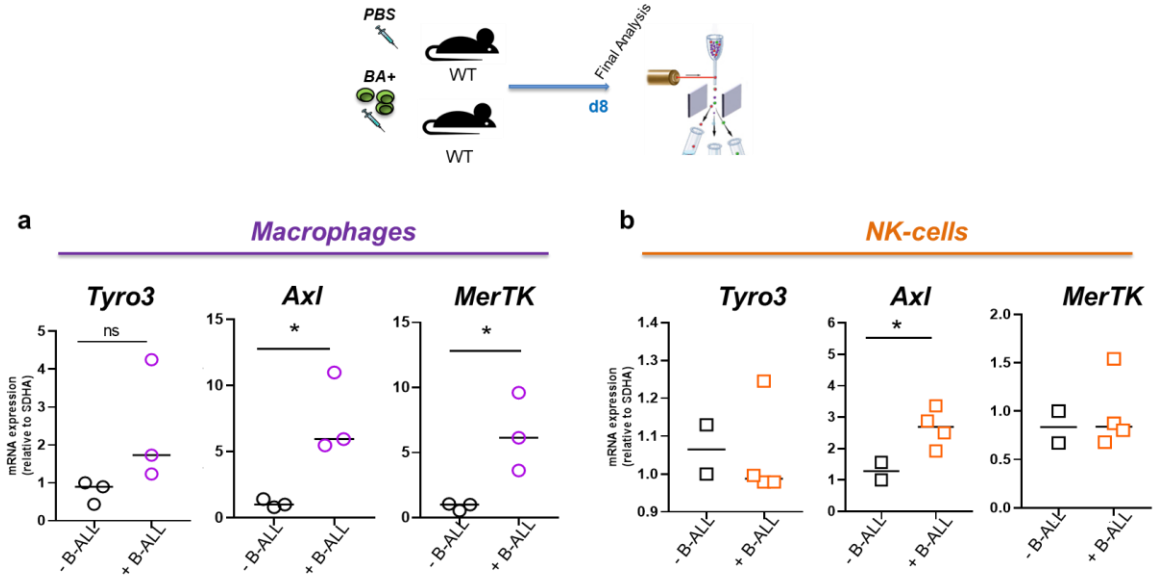


Figure 30. Leukemia induces the upregulation of *Axl* in macrophages and NK-cells.

a- Macrophages were FACS purified (CD45+CD11c-CD11b$^{dim}$F4/80+) from the spleen of naïve (n = 3) and BA+ B-ALL challenged (n = 3) mice and subsequently analyzed by real-time PCR to evaluate *Tyro3*, *Axl* and *MerTK* expression. b- NK-cells were FACS purified (CD45+CD11b-B220-CD3-NK1.1+) from the spleen of naïve (n = 2) and BA+ B-ALL challenged (n = 3) mice and subsequently analyzed by real-time PCR to evaluate *Tyro3*, *Axl* and *MerTK* expression Data are mean after normalization to *Sdha*. Each dot represents an independent mouse. Data were compared using unpaired two-tailed Student's t-test. *, p < 0,05.

## 3.18 Human CD14+ monocytes upregulate *GAS6* in response to HLA-mismatched co-culture with B-ALL, MDS and healthy bone marrow

In the BM microenvironment, there are multiple sources of TAMR ligands expression: myeloid cells, megakaryocytes, endothelial cells, and MSCs (Lemke and Rothlin 2008). Having established the prominent role of TAMR signaling in the syngeneic mouse models it was hypothesized that in ALL patients GAS6/PROS1 expression might be modulated by leukemia cells. First, the autologous *ex vivo*

system was formed where healthy donor-derived monocytes were co-cultured either with the primary BA+ B-ALL/high-risk, MDS/AML (or xenografted) samples or alone (Fig 31a). It was shown that *GAS6* expression is significantly induced in CD14+ peripheral blood monocytes after co-culture with both B-ALL and MDS/AML cells (Fig 31b). In contrast to *GAS6*, the *PROS1* level remained unchanged after co-culture with BA+ B-ALL, therefore it was decided to primarily focus on *GAS6*. Due to the allogeneic settings of the *ex vivo* system, it was tested whether *GAS6* upregulation is instructively induced by leukemia cells or a rather general immunosuppressive mechanism in response to HLA-mismatch. Therefore, an additional control condition was created – co-culture of CD14+ cells with healthy BM. As indicated in fig. 31c it was detected that in both co-cultures with healthy BM and B-ALL samples the level of *GAS6* increases at least 2-fold in comparison to control condition. *GAS6* was likely found upregulated due to HLA-mismatched allogeneic settings, rather than leukemia instructive immunosuppression.

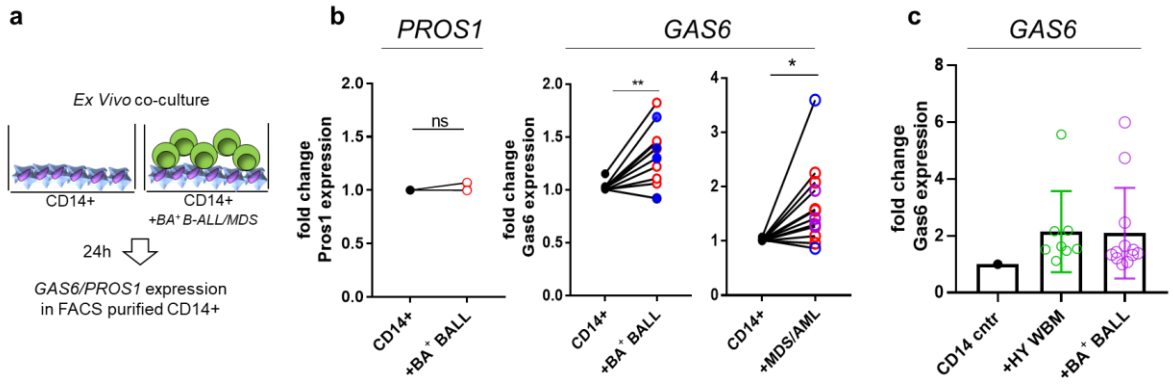


Figure 31. Expression of *GAS6* and *PROS1* by healthy monocytes after 24h exposure to patient-derived B-ALL/AML/MDS cells.

a- Scheme of the experimental patient-derived ex-vivo system. MACS-purified from healthy donor peripheral blood CD14+ positive monocytes (purity > 95%) were co-cultured alone for 24 h in serum-free StemSpan media or with $2x10^5$ of healthy donor whole BM (c), patient-derived BA+ B-ALL or MDS/AML cells; b- qPCR showing *PROS1* expression in CD14+ peripheral blood monocytes (n=4 independent healthy donors, mean age 39.25 ± 3.038) cultured with BA+ B-ALL patient-derived cells (n=1), *GAS6* expression in CD14+ monocytes (n=11 independent healthy donors, mean age 46.67 ± 3.941 with BA+ B-ALL; n=12 independent healthy donors, mean age 43.75 ± 4.72, with MDS/AML) cultured with BA+ B-ALL patient-

derived cells (n=2 red&blue) and MDS/AML patient-derived cells (MDS n=1 in red, AML n=2 in blue&purple); c- qPCR showing *GAS6* expression in CD14+ monocytes (n=8 independent healthy donors, mean age 47.25 ± 6.957 with HY WBM, n=12 independent healthy donors, mean age 43.75 ± 4.72 with BA+ B-ALL) cultured with healthy whole bone marrow (HY WBM n=2) and BA+ B-ALL patient-derived cells (n=2). Each data point represents a mean value obtained from at least 2 technical replicates, after normalization to reference gene *GUSB*. Data were compared using the paired two-tailed Student's t-test. *, $p < 0,05$; **, $p<0,01$;

## 3.19 *GAS6* and *PROS1* expression by human MSCs and ECs are not modulated by exposure to primary patient B-ALL cells *ex vivo*

To shed the light into molecular changes regarding PROS1 and GAS6 expression by a non-hematopoietic compartment of the BM environment occurring upon leukemic transformation in patients, an allogeneic *ex vivo* system was used based on the co-culture of human primary BA+ leukemic blasts (as well as high-risk MDS) with healthy donor-derived MSCs and microvascular endothelial cells, HDMEC

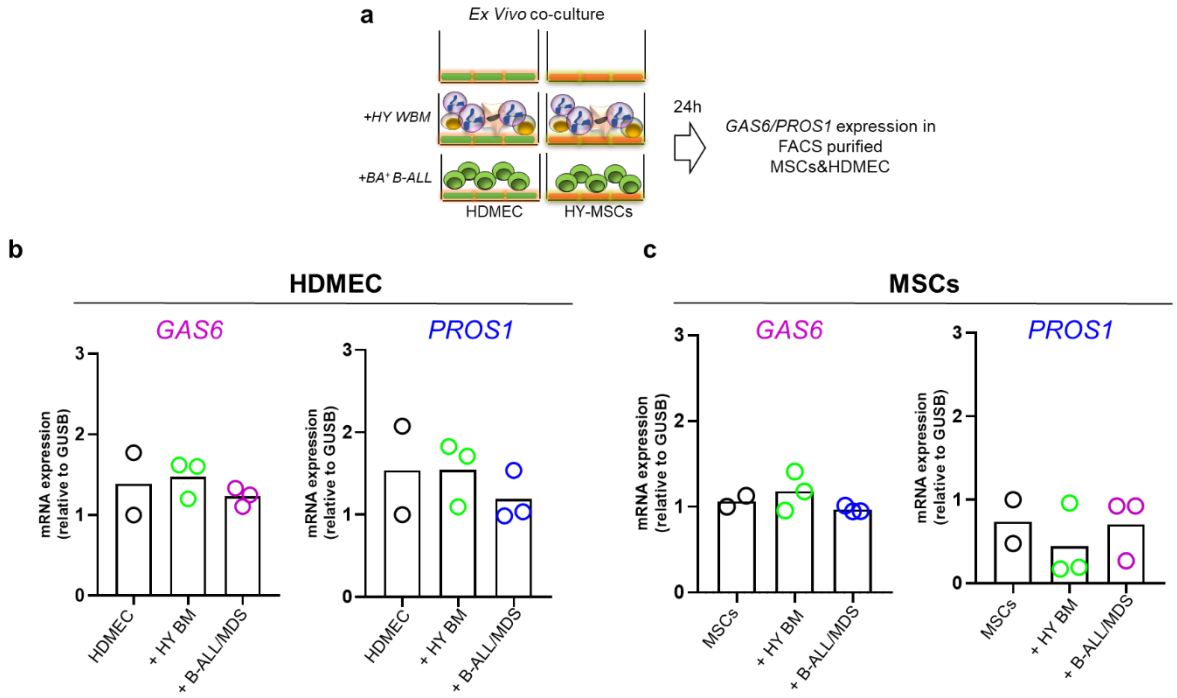


Figure 32. Expression of *GAS6* and *PROS1* by hMSCs and HDMEC after 24 h exposure to patient-derived B-ALL/MDS cells.

a- Scheme of the experimental patient-derived ex-vivo system. Immortalized healthy donor-derived MSCs and HDMEC were co-cultured for 24 h in serum-free StemSpan media with $2x10^5$ of healthy donor whole BM, patient-derived BA+ B-ALL and high-risk MDS cells. HDMEC and MSCs were FACS-repurified and used in downstream qPCR to determine *GAS6* and *PROS1* expression; b- qPCR to evaluate *GAS6* and *PROS1* expression in HDMEC and MSCs (c). Results summarize two independent experiments. Each data point represents a mean value obtained from at least 2 technical replicates, after normalization to reference gene *GUSB.*

(Fig. 32a). To verify whether changes in expression of *GAS6* and *PROS1* are specifically induced by cancer cells healthy whole BM cells was used as a control. The gene expression analysis revealed that *GAS6* and *PROS1* levels largely remain unchanged in both ECs and MSCs upon exposure to leukemia (Fig 32b).

## 3.20 Shifts in the circulating level of GAS6 and PROS1 associated with aging-related immunosuppression in mice and humans

The overall survival of patients with hematological malignancies ultimately declines in the elderly (Roberts et al. 2017). It has been commonly accepted the profound aging-linked establishment of a systemic pro-inflammatory environment due to the progressive dysfunction of the immune system (López-Otín et al. 2013). Hence, to investigate whether GAS6 and PROS1 secretion is regulated by the function of age, the circulating level of TAMR ligands was compared in the murine and human system depending on age. Interestingly, the peripheral blood level of GAS6 in old versus young mice found to be significantly increased in contrast to human with significantly downregulated GAS6 levels in the “old” cohort (Fig. 33a, b). However, in the cohort of healthy donors aged over 60 years, the concentration of PROS1 appeared to be significantly increased (Fig. 33b). Along with PROS1 upregulation, a significant increase of CCL2, Eotaxin and CCL3 inflammatory cytokines in older human donors was detected.

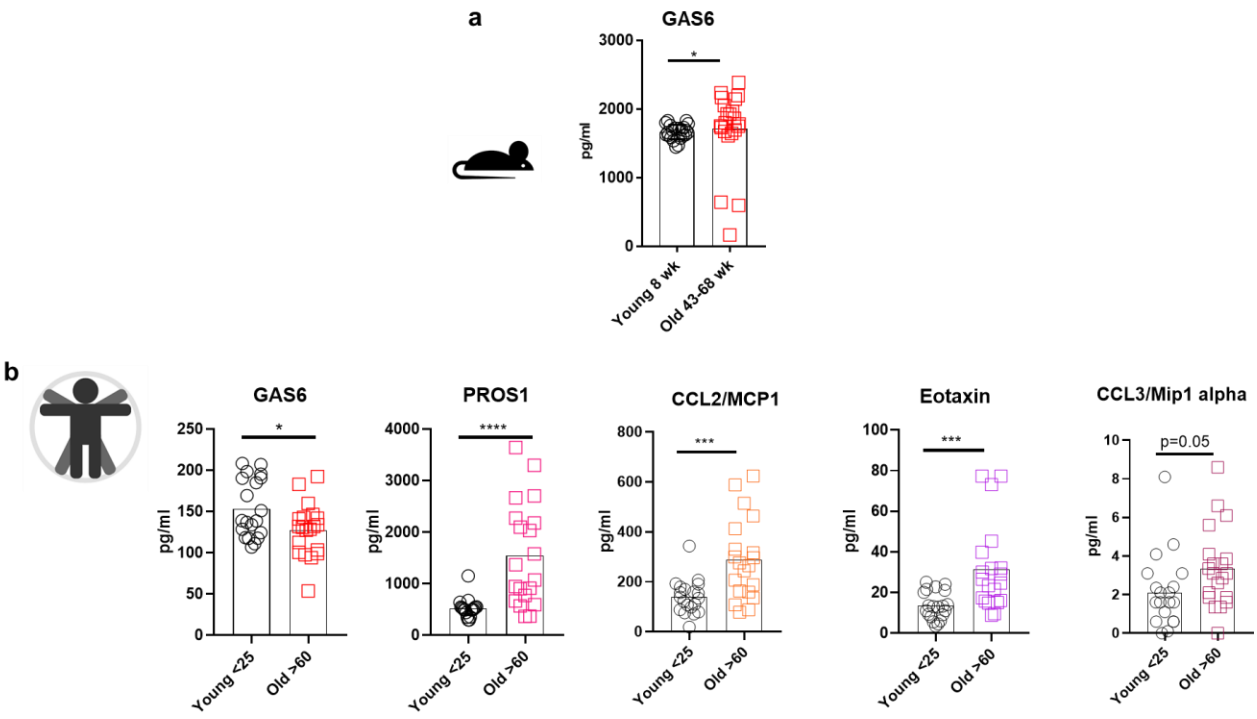


Figure 33. Aging-associated changes of circulating cytokines in mice and humans.

a- The protein level of Gas6 determined by ELISA in peripheral blood serum of young 8 wk (n=22) versus old, 43-48 wk old (n=22) C57BL/6N mice; b- The concentration of GAS6, PROS1, CCL2/MCP1, eotaxin, CCL3/Mip1alpha in peripheral blood serum of young (n=20) versus old (n=20) healthy donors were determined using Luminex multiplex bead assay. Data were compared using the paired two-tailed Student's t-test. *, $p < 0,05$; ***, $p<0,001$; ****, $p<0,0001$

# 4.0 Discussion

## 4.1 GAS6-deficiency in the leukemia environment hampers the progression of BCR-ABL+ B-ALL in an immune dependent fashion *in vivo*

The rise in survival rate among patients diagnosed with acute leukemia has been achieved through increased use of allo-HSCT (Kersey 2010) and implementation of small molecular inhibitors, e.g. against BCR-ABL1 in B-ALL (Roberts and Mullighan 2015; Cortes et al. 2012; Druker et al. 2001) and FLT3-internal tandem duplication (FLT3-ITD) in AML (DiNardo and Cortes 2016; Daver et al. 2016; 2019) however OS remains dismal in particular in older patients. The progress in allo-HSCT constitutes of multiple factors: improved conditioning regime, increased donor-availability, innovative transplant technologies in combination with the precise risk-stratification of the patients. Regardless of all improvements, the high rate of disease relapse is the major reason for transplant failure (Loke et al. 2020). The disease-free long-term survival after allo-HSCT stems from the phenomenon of graft-versus-leukemia (GVL) when the residual leukemia cells are eliminated by donor-derived immune cells. Therefore, the fact of disease recurrence is strongly associated with the ability of tumor cells to escape the immunosurveillance and the establishment of an immunosuppressive environment (Witkowski et al. 2017). This points to the unmet necessity for new immunotherapeutic strategies implemented in standard clinical practice as well as delineating the mechanism of leukemia-induced immunosuppression. Following this assertion, it has been shown that the administration of Sorafenib for FLT3-ITD AML patients subjected to allo-HSCT empowers GVL reaction in mice and humans via enhanced IL-15 production by leukemia cells (Mathew et al. 2018). Another branch of actively evolving therapeutic strategies in acute leukemia being FDA approved or in clinical trials is represented by CAR-T cells or BiTE antibody along with CKI (Topp et al. 2011; O'Leary et al.

2019; Gore et al. 2018; Austin, Smyth, and Lane 2016). All of these revolutionary approaches facing various pitfalls of the treatment resistance in part associated with the unknown mechanisms of cancer immune-suppression. Therefore, the current study has been dedicated to explore the role of the GAS6&/TAMR axis, primarily known to be responsible for tissue repair and resolving inflammation (Rothlin et al. 2015), in the context of acute leukemia.

The present project is focused on one of the most aggressive types of acute leukemias with the historically poor outcome – BA+ B-ALL. In particular, the genetic landscape of generated mouse leukemia represented a relapse phenotype in patients (Roberts and Mullighan 2015), namely BCR-ABL$^{p190}$ in combination with the loss of *CDKN2A (Arf$^{-/-}$)* with or without *IKZF1 (Ikaros$^{L/+}$)* haploinsufficiency. Next, we identified that generated leukemias express a substantial level of TYRO3 in comparison to healthy BM. Previously, it has been shown the existence of aberrant expression of TAMR and their ligands across hematological malignancies and this signaling axis was mainly associated with fueling the proliferation and survival potential of cancer cells (Brandão et al. 2011; Brandao et al. 2013; Krause et al. 2015; Loges et al. 2010; Sinha et al. 2015; Waizenegger et al. 2015). In contrast to previous studies, it was aimed to identify the differential mode of action of the GAS6/TAMR axis in modulation leukemia immunity and whether this axis could be exploited by the leukemic blast to augment the immunosuppression. Hence, for this purpose newly generated constitutively *Gas6*-deficient immunocompetent mice were used versus severely immunodeficient (NSG background) animals and transplanted in both cohorts as little as 1,000 cells of BA+ leukemia cells. Importantly, all of the transplant experiments were performed without prior conditioning in order to preserve microenvironment integrity. This comprehensive *in vivo* approach allowed us to confirm that an unprecedented 50% survival of *Gas6*-

deficient hosts requires a functional immune system, while this survival advantage was abrogated in the context of immunodeficient animals (Fig. 12, 14). Additionally, transplant experiments performed in NSG versus NSG *Gas6*$^{-/-}$ showed a lack of the differences in leukemia burden as well as in the proliferation status of cancer cells, allowing us to putatively rule out a direct effect of the GAS6/TYRO3 axis for leukemia maintenance (Fig. 14). Notably, we carefully controlled that the survival advantage in leukemia transplant experiments was not due to an inappropriate BM environment created by GAS6 insufficiency for BA+ cell propagation. Thus, we demonstrated that *Gas6*-deficiency was not altering the reconstitution of normal HSPCs and all mature hematopoietic lineages, including B-cell recovery (Fig. 13). In our attempt to identify whether adaptive immunity acts as a downstream mediator of GAS6-mediated immune suppression, we performed adaptive splenocyte transfer and antibody-mediated CD3+ cell depletion. Hence, we showed that splenocyte transfer from leukemia long-term survivors into *Rag*$^{-/-}$ recipients significantly prolongs survival after secondary BA+ challenge in comparison to *Rag*$^{-/-}$ cohort reconstituted with naïve splenocytes (Fig. 15). Additionally, these data were complemented by the loss of survival advantage of C57BL/6N *Gas6*$^{-/-}$ treated with anti-CD3 antibody in comparison to isotype treated C57BL/6N *Gas6*$^{-/-}$ cohort (Fig. 16) before BA+ challenge. Taken together this data are highlighting an essential role of T-cells in GAS6-mediated immune suppression, although it is unlikely that this effect is mediated via a direct influence of GAS6 on T-cells, as they are largely negative for all three TAMRs. Therefore, we investigated the monocyte-macrophage population, as they are known to highly express AXL and MERTK across the murine hematopoietic hierarchy (Fig. 17). The phenotypical analysis of monocyte-macrophages revealed that *Gas6*-deficient mice harbor skewed towards the M1-polarisation phenotype of myeloid cells in comparison to the age-matched control cohort (Fig. 18). Thus, the current data were the first hint to suspect that Mph may

be a first-line downstream target of an immunosuppressive GAS6 effect, which in turn renders insufficient priming of T-cells in wild-type situation in B-ALL context. It has been indicated that GAS6 is an important factor in creating an immunosuppressive environment in solid tumor models (Cook et al. 2013; Aguilera et al. 2016) in contrast, in hematological malignancies, there are very limited data available (Lee-Sherick et al. 2013). In line with our data, there is only one study regarding BA+ B-ALL where researchers demonstrate that in constitutive *MerTK* knockout animals the propagation of leukemia is significantly delayed in an immune-dependent fashion (Alisa B. Lee-Sherick et al. 2018a). Regarding to the translational aspect, it is interesting that recently it has been shown that high *GAS6*-mRNA level among AML patients undergoing allo-HSCT has been associated with worse OS (Yang et al. 2019). In light of our data, it is tempting to speculate, that a high GAS6 level may severely affect GVL reaction by altering anti-leukemic immunity.

## 4.2 Upregulation of PROS1 by bone marrow stromal cells in response to *Gas6* deficiency impedes anti-leukemic immune response *in vivo*

Using a newly generated immunocompetent *Gas6*$^{-/-}$ mouse line we faced the loss of the survival advantage upon leukemia challenge after one year, while the leukemia burden in all organs analyzed appeared to stay significantly lower in comparison to wild type controls (Fig. 19). Therefore, it was speculated that this transformation is underlying molecular changes in the murine system that compensate the absence of GAS6 during steady-state and potentially enhance leukemia-induced immunosuppression. In fact, it has been identified a two-fold increase of GAS6 agonist, PROS1, produced by BM-derived MSCs. Strikingly, this difference exaggerated over two years of homozygous breeding C57BL/6N *Gas6*$^{-/-}$ and *Pros1* level became 4-fold higher than its physiological level in wild type mice (Fig. 20).

Thus, having established this previously not recognized interdependency of GAS6 and PROS1 in the murine system, we sought to determine how their expression changes *in vivo* in wild type situation with the presence of B-ALL in non-hematopoietic BM environment. Interestingly, the mRNA and protein level of GAS6 was found to be decreased after the injection of two individual leukemias in comparison to PBS-injected control mice. Conversely, the PROS1 level was upregulated in case of the more aggressive $L2^{BA+Arf-/-IkarosL/+}$ leukemia and rather remained unchanged in the case of $L3^{BA+Arf-/-}$ (Fig. 21). To further confirm the functional consequences of the PROS1 increase for anti-leukemic immune response, we analyzed the early immune changes in wild type and *Gas6*-deficient mice challenged with $L2^{BA+Arf-/-IkarosL/+}$ and $L3^{BA+Arf-/-}$. In agreement with the expression changes of *Pros1* upon leukemia challenge, inflammatory activation of BM Mph and T-cells was essentially blocked in *Gas6*-deficient mice when transplanted with L2 (established Pros1-inducer). In contrast, using L3-clone, the frequency of M1-polarised Mph and $MHCII^{hi}$ subset were found significantly higher in $Gas6^{-/-}$ recipients compared to wild type controls accompanied by significantly increased levels of inflammatory cytokines TNFa and IFNy in the spleen (Fig. 22). Thus, these data identify for the first time a differential mode of production of two immunosuppressive ligands GAS6 and PROS1 by BM MSCs in response to BA+ leukemia *in vivo*. Importantly, that current finding is indicative that two ligands can be responsible for the execution of different biological programs, which yet remained unknown. The fact of PROS1 upregulation induced by leukemia cells implying that PROS1 is playing an essential role in co-opting with leukemia by subverting the immune response into immunological tolerance. In agreement with this finding, it has been previously shown in the context of the melanoma model that PROS1 secreted by cancer cells was promoting tumor growth and inhibiting M1 polarization of tumor-associated Mph (Ubil et al. 2018). Interestingly, that in our

model PROS1 induction was linked to B-ALL clone with *Ikaros*$^{L/+}$ haploinsufficiency, arising the awareness that depending on the genetic makeup leukemia may hijack a particular signaling axis to escape the immune response. The current study would certainly benefit from experiments where the deficiency of both ligands will be combined. However, mouse models with lineage-specific deletion (e.g. *LepR-Cre*, *VE-Cadherin-Cre, Mx1-Cre*) of both ligands are required while constitutive *Pros1* knockout is embryonic lethal due to severe coagulation defects (Burstyn-Cohen, Heeb, and Lemke 2009). The more feasible alternative was chosen to follow – targeting of TAMRs on putative effector cells thus, omitting the contribution of both ligands simultaneously (discussed in the following section).

## 4.3 AXL-deficient macrophages and NK cells are downstream cellular mediators of GAS6 and PROS1 mediated immunosuppression in leukemia

It was identified that the BM-derived ligands GAS6 and PROS1 significantly alter anti-leukemic immunity in the context of murine BA+ B-ALL. Moreover, phenotypical analysis of monocyte-macrophage subsets in *Gas6*$^{-/-}$ mice (Fig. 18, 22) together with TAMR being highly expressed in those (Fig. 17) led us to choose *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ (and/or *Mer* $^{f/f}$) mouse line as a model to deplete AXL and MERTK receptors in mononuclear phagocytes, putatively responsible for the anti-leukemic response. Initial experiments using *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ (and/or *Mer* $^{f/f}$) mice challenged with B-ALL showed that the *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ mice were fully protected while the Cre-negative cohort suffered from full-blown leukemia (Fig. 23). Remarkably, this advantage observed in the *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ cohort translated in >60% survival difference (Fig. 28). Of note, it was carefully excluded potential Cre-toxicity effects in the observed phenotype (Fig. 23). We sought to identify the differential role of selective depletion of AXL or MERTK in *Csf1R*-expressing cells. Impressively, the leukemia protection was equally prominent irrespectively of the deleted receptor

(data are not shown). Therefore, mice were used in the experiment according to offspring availability. It was speculated, that this receptor dispensible phenotype might be due to the described phenomenon of AXL to heterodimerize with other RTKs (Scaltriti, Elkabets, and Baselga 2016) possibly blocking MERTK in the current scenario.

Being aware that *Csf1R-Cre*$^{+}$ targets a heterogeneous cell population we preconditioned mice with clodronate liposomes prior to B-ALL challenge in order to deplete mature macrophages in BM and spleen. This approach revealed that *Axl* (or *Mer*)-deficient *Csf1R*-expressing Mph are critical subsets to confer leukemia protection (Fig. 24). Essentially, the contribution of TAMR-deficient DCs was excluded in our laboratory when B-ALL was injected into *CD11c-Cre*$^{+}$*Axl*$^{f/f}$ mice versus *CD11c-Cre*$^{-}$ *(*by Tirado-González I. et al, the paper in preparation*)*. Thus, it has been concluded that *Axl*-deficient DCs are not sufficient to combat B-ALL (data are not shown). In the recent decade, the role of leukemia-associated macrophages (LAMs) has been increasingly recognized in their anti-inflammatory polarization program favorable to leukemia progression (Li et al. 2020). Here we identified that Mph in the context of extremely aggressive B-ALL may act as a powerful innate immune checkpoint via ablation of AXL (MERTK) signaling. Current *in vivo* data using genetic means are supported by selective AXL inhibition *ex vivo* in wild type Mph, where Bemcentinib treatment gradually enhances the production of the key M1 cytokines (*TNFa*, *IL-12*) and simultaneously inhibits the expression of *Socs3* (Fig. 25). Last but not least we were able to demonstrate *in vivo,* that *Socs3* deleted in *Csf1R*-expressing Mph mimics AXL ablation, delineating the mechanistic importance of inflammatory cytokine production induced in Mph (Fig. 26).

In the frame of the current project, it was aimed to continuously identify all cellular components involved in anti-leukemic immunity using the *Csf1R-Cre*$^{+}$ model.

Interestingly, another subset of innate immune cells was determined, namely *Axl*-deficient NK cells, being of critical importance to grant anti-leukemic protection (Fig. 29). Our data indicate that *Csf1r-Cre*$^{+}$ does not specifically deplete *Axl* in mononuclear phagocytes but also in NK cells, despite the relatively low level of expression of these receptors at steady-state (depicted in the heat map, fig. 17). The fact of non-specific *Csf1r-Cre*$^{+}$ targeting was reported by McCubbrey et. al, when researchers demonstrated by reporter evaluation that Cre-recombinase under Csf1r-promoter targets almost all leukocyte subsets, including NK cells (McCubbrey et al. 2017). Despite this non-specific targeting using the *Csf1r-Cre*$^{+}$ model, current data interpreted relying on the fact of selected subsets expressing *Axl* and *MerTK* (indicated in the heat map, fig. 17) in combination with the loss of function approaches (chlodronate depletion, antibody depletion). NK cells are known to be an explicit first-line defense in the context of hematological malignancies (Fig. 27) (Mattias Carlsten and Järås 2019). Hence, along with other immune cells, NK cells are falling into the immunosuppressive trap induced by leukemia and found to be functionally defective in patients with AML (M. Carlsten et al. 2010; Fauriat et al. 2007). Therefore, our data proposing a new avenue in order to revert the anti-cancer potential of endogenous NK cells in patients with B-ALL and AML via targeting AXL. In agreement with these data, it has been previously shown that altering TAMR-signaling in NK cells enhances its anti-metastatic activity in melanoma model (Paolino et al. 2014).

Taken together it have been identified that AXL/MERTK ablation in Mph and NK cells empowers the innate immune system to mount a productive response against leukemia. Remarkably, the uncovered mechanism was not restricted to B-ALL as we observed very similar results in the context of a murine AML model (Fig. 27). The efficient priming of the innate compartment in the context of leukemia is the

major challenge to overcome the tolerogenic T-cell response, frequently observed in leukemia (Zhang et al. 2013; Fuertes et al. 2011; Teague and Kline 2013; E. K. Curran, Godfrey, and Kline 2017). Well in line with these statements, our data demonstrate that in wild type recipients the absence of T-cells does not accelerate B-ALL and AML course (Fig. 27). However, when the innate compartment, namely Mph and NK cells, has been activated via the absence of GAS6 in the environment, we were able to show that the T-cell pool became essential for leukemia eradication (when GAS6 deficiency was not yet overcompensated by PROS1 upregulation) (Fig. 15 and 16). Interestingly, using anti-CD8 treatment we haven't seen the same outcome using the *Csf1r-Cre*$^{+}$ *Axl*$^{f/f}$ model. We speculate, that these discrepancies might be linked to the resistance towards the anti-CD8 treatment reservoir of cytotoxic T-cells. In fact, prior to leukemia challenge FACS analysis of PB showed a non-detectable level of CD8+ T cells, however soon after the leukemia challenge, CD8-cells re-appeared in the circulation and in 2 weeks reached up to 20% in some recipients despite continuous administration of anti-CD8 (Fig. 28). Therefore, these data do not argue for T-cells being dispensable due to a technical caveat of the experiment. Moreover, when the analogous experiment was performed using a genetic approach, 90% of the *Csf1r-Cre*$^{+}$*Axl* $^{f/f}$ *Rag1*$^{-/-}$ recipients succumbed to leukemia in comparison to only 20% of T-cell proficient *Csf1r-Cre*$^{+}$*Axl* $^{f/f}$ mice (data are not shown, by Tirado-González I. et al, the paper in preparation). Hence, these pre-clinical data are uncovering the new prospect to efficiently bridge innate and adaptive immune responses in the context of leukemia via M1-reprogramming of Mph. Mechanistically, we propose AXL as the most specific therapeutic target based on the uniform leukemia-induced upregulation of AXL in Mph and NK cells (Fig. 30). The treatment using orally available selective AXL inhibitor Bemcentinib was evaluated in the AML/MDS clinical trial and was well tolerated by the patients and exhibited anti-leukemic activity (Loges et al. 2016). Our data emphasize that

Bemcentinib treatment may serve also as a promising strategy to unleash endogeneous patients innate immune response. Essentially, the continuous administration of Bemcentinib in combination with the standard of care Nilotinib has cured 100% of wild type recipients injected with BA+ B-ALL (data are not shown, by Tirado-González I. et al, the paper in preparation). We also hypothesize that AXL inhibition represents an encouraging therapeutic approach for post-HSCT patients to boost the GVL in order to eliminate residual tumor cells and significantly prolong OS.

## 4.4 Modulation of the expression level of TAM ligands in different compartments of BM environment by patient-derived leukemia cells

In order to evaluate the promising data which were obtained using the murine pre-clinical model, a patient-derived *ex vivo* system was established. It is essential to understand whether certain compartments of the human BM environment change the expression of PROS1 and GAS6 once engaged into the direct contact with leukemia cells. There are multiple production sites of TAM ligands in the BM: hematopoietic compartment (myeloid cells, Mk, activated T-cells) and stromal compartment (ECs, MSCs) (Lemke and Rothlin 2008), therefore both types have been tested. The analysis revealed that the expression level of both ligands was not modulated in ECs and MSCs in co-culture with primary B-ALL/MDS samples in comparison to healthy BM control (Fig. 32). Due to the limited lifespan of the primary patient samples *in vitro*, the mRNA expression of *GAS6* and *PROS1* was estimated only after a short-time (24h) in co-culture. Taking into account the technical limitation to study microenvironmental changes in human it is challenging to rule out the contribution of one or another ligand to modulate the magnitude of anti-leukemic immune response among ALL patients. Interestingly, it has been demonstrated that primary human osteoblasts increase GAS6 secretion in response to E2A/PBX1-

positive ALL cells, although cancer cells highly expressed MERTK (Shiozawa, Pedersen, and Taichman 2010). It was hypothesized, that multiple factors influence the kinetic of GAS6 and PROS1 in the BM niche upon leukemic transformation: type of leukemia, the expression level of TAMR on cancer cells and the stage of disease progression. It is of critical importance to identify the prognostic implication of TAM ligands in different types of leukemia, as it has already been shown that high GAS6 levels are associated with poor OS among post-allo-HSCT patients (Yang et al. 2019).

To test whether myeloid compartment responds by TAM ligands upregulation we used allogeneic co-culture of patient B-ALL/MDS samples with healthy donor-derived monocytes. Here we identified, that the PROS1 expression level in healthy monocytes was not modulated in the presence of B-ALL and MDS cells (Fig. 31). In contrast, *GAS6* was significantly induced in response to different leukemia entities, like BA+ B-ALL and MDS/AML cells. Interestingly, healthy whole BM has induced *GAS6* levels comparable to leukemia cells in donor-derived monocytes, arguing that *GAS6*-upregulation was not the result of an instructive signal emanating from cancer cells. From a different perspective, we have to consider that healthy whole BM represents a too heterogeneous cell population to serve as an “ideal” control condition. In fact, patient-derived leukemia samples constituted from almost 90% of blasts, therefore the improved control conditions for B-ALL and AML/MDS would be purified healthy pro-B-cells and HSCs, respectively. The *GAS6* upregulation in this experiment most likely manifests the feedback immunosuppressive mechanism in response to monocyte activation due to HLA-mismatching allogeneic settings. Although it is interesting, yet unknown, whether the same tendency will be present using HLA-matching setup (high resolution typing at the HLA-A, -B, -C, -DRB1, and -DQB1 loci 9/10 or 10/10 match) according to

criteria applied for donor selection for allo-HSCT (Nakata et al. 2014). It is well-established that HLA-disparity has been associated with poor OS in allo-HSCT settings (Ayuk et al. 2018). Therefore, we speculate that GAS6 upregulation might have contributed to the cases with early leukemia relapse, which certainly requires further investigation.

### 4.5 Upregulation of circulating PROS1 among elderly individuals is associated with the aging-related immune malfunction

It is well recognized that aging represents the paradoxical contradiction between immune suppression and inflammation accompanied by an increased rate of cancer incidence among elderly individuals (Gupta et al. 2006). In particular, in the aging society, the rate of "elderly" hematological diseases as MDS increases exponentially. Importantly, patients with MDS are frequently characterized by profound dysfunction of the immune system (Winter et al. 2020). Although the detrimental effect of aging-related changes in adaptive immunity associated with thymic involution is known (Weng 2006; Thomas, Wang, and Su 2020), the details characterizing alterations in the innate compartment are less investigated. TAMRs and their ligands are indispensable regulators of innate immunity, therefore it is increasingly clear that TAM-signalling playing a critical role in the age-associated dysfunction of phagocytosis and immune tolerance. Hence, it has been reported that aging causes the functional impairment of basic Mph functions: decreases phagocytic ability, antigen presentation, skewed towards M2 polarization program together with altered signal transduction from TLRs (Linehan and Fitzgerald 2015; Solana, Pawelec, and Tarazona 2006). Taken together, we sought to compare the level of TAM ligands in the systemic circulation in the "young" versus "elderly" healthy volunteers as surrogate markers of age-driven modification in TAM-signaling. The analysis revealed the contrast relationship between the circulating

level of GAS6 and PROS1: while GAS6 significantly decreased in the donors >60 y.o., the PROS1 level found to be significantly upregulated in comparison to young <25y.o. control cohort (Fig. 33b). Interestingly, that in mouse system GAS6 kinetic turned out to be opposite to humans, we identified significantly higher GAS6 level in plasma in aged wild type mice in comparison to the young cohort (Fig. 33a). That notion awakens the awareness of the functional heterogeneity of the analogous signaling molecules that may lead to different biological outcomes across different species. Notwithstanding, along with PROS1 upregulation in elderly cohort chemokines responsible for the infiltration of monocyte and macrophages (MCP1, MIP1 alpha) found to be increased (Fig. 33b). In agreement with our finding, it has been demonstrated that an increased level of MIP1 in circulation is linked to advanced biological age, frailty in humans and senescence phenotype of MSCs (Bettcher et al. 2019; Yousefzadeh et al. 2018; Jin et al. 2015). Remarkably, MIP1 alpha was implicated as a crucial player in the leukemogenesis and known to inhibit normal HSC maintenance while creating a BM environment favorable to cancer progression (Baba and Mukaida 2014; Baba et al. 2013). The concentration of eotaxin in plasma was also significantly higher in the elderly cohort (Fig 33b). Initially identified as eosinophil chemoattractant, eotaxin recently has been connected to neurogenerative processes and it's increase positively correlated with the rising age of blood donors (Hoefer et al. 2017; Teixeira et al. 2018). Taken together, our analysis of aging-associated changes in circulation are well confirmed by previously published data. Moreover, here we have additionally explored the age-related kinetic of immunoregulatory proteins GAS6 and PROS1, being a surrogate readout of the involved TAMR signaling in the immune system dysfunction. The valuable data we have obtained require further investigation in order to shed the light whether GAS6 and PROS1 concentration may serve as predictive or diagnostic

means in various immune- or cancer-related pathological processes, in particular among elderly individuals.

## 4.6 Concluding remarks

The current work was dedicated to shed light on universal mechanisms of leukemia immune escape, being acknowledged as one of the major treatment obstacles (Witkowski et al. 2019). Here it was shown, using an aggressive preclinical BA+ B-ALL model that TAMR and their ligand are the novel immunosuppressive axis, being hijacked in the leukemic environment. In the frame of this project, it was established that blocking GAS6/AXL-signaling primarily on Mph and NK-cells rewires the host immunity and confers unprecedented leukemia protection. These data being an exceptional translational value and propose AXL as the novel therapeutic target in particular, due to the receptor upregulation in response to leukemia *in vivo*. Therefore, the application of orally available AXL inhibitor bemcentinib in combination with standard of care treatment creates a safe and specific therapeutic window to boost host immunity. These preclinical data were complemented by the indication that in the human system dysregulated levels of GAS6 and PROS1 associated with aging-related immune system dysfunction in the cohort of individuals being at the most risk once diagnosed with leukemia.

# 5.0 Summary

## 5.1 Abstract

The overall survival for patients with acute lymphoblastic leukemia (ALL) often is the function of age, in particular in 2019 analysis revealed that 5-year overall survival for patients older than 20 years remains below 35% (American Cancer Society, Cancer Facts &Figures 2019). Importantly, one of the major issues in ALL therapy is the ability of tumor cells to escape the treatment via the establishment of an immunosuppressive environment. The tumor microenvironment has gained tremendous importance in the past decade. This is largely based on the reasoning that, in order to devise better therapeutic strategies for patients, we need to gain better understanding into how malignant cells transform their microenvironment to promote growth, escape immune control and gain therapeutic resistance.

TAM receptors (TAMRs) are engaged in innate immune cells as a feed-back mechanism to terminate the immune response and promote the return to homeostasis (Rothlin et al. 2007). In the context of cancers, aberrant TAMR signaling was mainly explored concerning its pro-oncogenic function (Paolino and Penninger 2016). There are only limited data available suggesting the modulation of cancer immune response via TAMR signaling in highly immunogenic solid tumor models (Paolino et al. 2014; Ubil et al. 2018). So far, however, little is known about their potential indirect immune-modulatory function in hematological malignancies. Taking into account the pronounced importance of TAMR signaling in immune cells combined with the leukemic immune tolerance, the current study focused on the function of TAMR and their ligands in anti-leukemic immunity.

This work uncovers the mechanism of dampening anti-leukemic immune response via TAMR signaling on macrophages using the syngeneic BCR-ABL1 B-ALL

mouse model. Using genetic depletion of GAS6 in the host environment or ablation of AXL and/or MERTK receptors in macrophages the bone marrow microenvironment could be rewired in order to achieve an efficient anti-leukemic immune response. In particular, the GAS6/AXL blockade triggers an effective NK- and T- cell-dependent anti-leukemic response that results in prolonged survival. This finding specifically tackles the obstacle of inefficient bridging between innate and adaptive immune response typical for hematological malignancies in contrast to solid tumors (E. K. Curran, Godfrey, and Kline 2017).

Besides establishing the vital function of TAMR signaling in anti-leukemic immunity using murine models, the analysis of human blood plasma revealed that age-related immune dysregulation was manifested by significant GAS6 decrease and PROS1 upregulation among elderly donors (>60 y.o.) compared to controls (<25 y.o.). These data are indicative that TAMR signaling likely favors the age-dependent immune system decline, which in turn is associated with a poor survival rate of elderly patients diagnosed with leukemia.

In conclusion, using a preclinical ALL model here it was identified *in vivo*, that *Axl* significantly increases upon B-ALL challenge in Mph and NK cells. Therefore, AXL targeting, using the orally bioavailable selective inhibitor Bemcentinib, could serve as a powerful approach to revert early immunosuppression created by leukemia.

Taken together these data propose the AXL receptor as a novel immune checkpoint and attractive candidate for the development of a new therapeutic approach via unleashing the patient’s own immune system to combat leukemic cells.

## 5.2 Zusammenfassung

Die Lebenserwartung von Patienten mit akuter lymphoblastische Leukämie (ALL) hängt häufig vom Alter ab. Insbesondere ergab eine Studie aus dem Jahr 2019 , dass das 5-Jahres-Gesamtüberleben für Patienten über 20 Jahre unter 35% liegt (American Cancer Society, Cancer Facts & Figures 2019). Dabei ist eines der Hauptprobleme bei der ALL-Therapie die Fähigkeit maligner Zellen, der Behandlung durch die Schaffung einer immunsuppressiven Umgebung zu entkommen. Daher hat die Erforschung der Tumor-Mikroumgebung in den letzten zehn Jahren enorm an Bedeutung gewonnen. Um gezielte Therapiestrategien für Patienten zu entwickeln, ist ein besseres Verständnis, wie maligne Zellen ihre Umgebung transformieren und so ihr Wachstum fördern, der Immunregulation entkommen und therapeutische Resistenzen entwickeln, eine Grundvoraussetzung.

TAM-Rezeptoren (TAMRs) sind bei der angeborenen Immunantwort als Rückkopplungsmechanismus beteiligt, um diese zu beenden und die Rückkehr zur Homöostase zu fördern (Rothlin et al. 2007). Im Zusammenhang mit Krebserkrankungen wurde die aberrante TAMR-Signalübertragung hauptsächlich im Hinblick auf dessen pro-onkogene Funktion untersucht. Insbesondere wurde der Zusammenhang der Expression von TAMRs und ihrer Liganden mit erhöhter Tumorproliferation, dem Überleben, der Therapieresistenz, der Metastasierung und der Tumorinvasion identifiziert (Paolino und Penninger 2016). Es liegen allerdings nur begrenzt Daten vor, die auf eine Modulation der Immunantwort über die TAMR-Signalübertragung in hoch immunogenen soliden Tumormodellen hinweisen (Paolino et al. 2014; Ubil et al. 2018). Auch ist bisher wenig über ihre mögliche indirekte immunmodulierende Funktion bei hämatologischen Neoplasienbekannt. Unter Berücksichtigung der entscheidenden Bedeutung der TAMR-Signalübertragung in Immunzellen in Kombination mit der leukämischen

Immuntoleranz konzentriert sich diese Arbeit auf die Funktion von TAMRs und ihren Liganden bei der anti-leukämischen Immunität. Zur Untersuchung der leukämischen Immunantwort wurde das aggressive murine B-ALL-Modell verwendet, das einen in der Klinik beobachteten aggressiven Rezidivphänotyp genetisch rekapituliert, indem es neben dem BCR-ABL1- (BA) Fusionstranskript sich durch den Verlust von *CDKN2A* (*Arf*$^{-/-}$) und heterozygoten Knockout von *IKZF1* (*Ikaros*$^{L/+}$) auszeichnet (Mullighan et al. 2009). Zunächst wurde eine mögliche direkte bzw. immunmodulierende Wirkung von GAS6, einem TAMR-Liganden, untersucht. Dazuwurden B-ALL-Zellen sowohl in immunkompetente als auch immundefiziente *Gas6*$^{-/-}$ Rezipienten transplantiert. Dieser Ansatz ergab eine immunabhängige Wirkung, da in Abwesenheit von GAS6 das Überleben immunkompetenter, nicht aber immundefizienter Empfänger, deutlich verlängert war. Darüber hinaus gab es trotz der Expression vom TAMR TYRO3 auf der Oberfläche von Leukämiezellen in Abwesenheit von GAS6 bei immundefizienten NSG-Mäusen keine Veränderung der Tumorzellproliferation, so dass eine direkte Wirkung ausgeschlossen werden konnte. Ferner wurde gezeigt, dass das Fehlen von GAS6 im immunkompetenten Wirt zu einer Verschiebung myeloider Zellen in Richtung des M1-Phänotyps führt, was zu einer T-Zell-abhängigen Immunantwort gegen die Leukämie führt. Interessanterweise kam es beim konstitutiven *Gas6*-Knockout über die Zeit zu einer Kompensation der Abwesenheit von GAS6 und führte zu einer Zunahme der Genexpression des alternativen TAMR-Liganden *Pros1* um das Vierfache. In diesem Zusammenhang wurde auch gezeigt, dass die *Pros1*-Hochregulation in Verbindung mit der aggressiveren genetischen B-ALL-Variante (*BA*$^{+}$*Arf*$^{-/-}$*Ikaros*$^{L/+}$) instruktiv induziert wurde, wodurch der *Gas6*$^{-/-}$-Effekt vollständig kompensiert wurde. Zusammengenommen zeigen diese Daten, dass beide TAMR-Liganden, GAS6 und PROS1, im Kontext des präklinischen BA+ B-ALL-Modells eine tiefgreifende immunsuppressive Rolle spielen.

Um weitere zelluläre Effektoren zu identifizieren, die GAS6 und PROS1 nachgeschaltet sind, wurde die *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ Mauslinie, die sich durch die Abwesenheit des TAMR AXL auf CSF1R-exprimierenden Zellen auszeichnet, als Rezipient für BA+ B-ALL-Zellen verwendet. Dieses Mausmodell wurde insbesondere aufgrund des Targetings von mononukleären Phagozyten ausgewählt, wobei die Population das höchste Expressionsniveau von TAMRs in hämatopoetischen Zellen aufweist. Die Endpunkt- und Überlebensanalyse, die nach der B-ALL-Transplantation in *Csf1r-Cre*$^{+}$*Axl*$^{f/f}$ -Tiere gegenüber der *Cre*-negativen Kontrollkohorte durchgeführt wurde, hat einen beispiellosen Leukämieschutz der *Csf1r-Cre*$^{+}$ *Axl*$^{f/f}$ -Gruppe bestätigt. Die entscheidende Rolle von Makrophagen für diesen Mechanismus wurde durch Depletion dieser Zellen bestätigt. Die Behandlung von *Csf1r-Cre*$^{+}$ *Axl*$^{f/f}$ -Mäusen mit Clodronat vor Transplantation der B-ALL-Zellen führte zum kompletten Verlust des Leukämieschutzes  Damit wurden Makrophagen als essentielles Kompartiment zur Erhöhung der Immunität gegen B-ALL identifiziert. Darüber hinaus konnte die antileukämische Aktivität von Makrophagen *ex vivo* mittels Blockade von AXL durch Bemcentinib-Behandlung (selektiver AXL-Inhibitor) von M1-polarisiertem Wildtyp-Makrophagen rekapituliert werden. Dies führte zu einer signifikant erhöhten Produktion der wichtigsten pro-entzündlichen Zytokine *TNFa* und *IL-12*, während *Socs3*, welches über TAMR induziert wird, deutlich gehemmt wurde. Bemerkenswerterweise bestätigt die genetische *in-vivo*-Depletion von *Socs3* in *Csf1R*-exprimierenden Makrophagen die *Axl*-Ablation. Zusammenfassend zeigen diese Daten, dass die Immunantwort von Makrophagen durch die Blockade von AXL zumindest teilweise über eine verstärkte entzündliche Zytokinproduktion erreicht wird.

Neben der entscheidenden Rolle von Makrophagen konnte in dieser Arbeit weiterhin gezeigt werden, dass natürliche Killerzellen (NK-Zellen) für die anti-leukämische

Immunität von entscheidender Bedeutung sind und ihre Aktivität über die AXL-Signalübertragung weitgehend unterdrückt wird. Obwohl die *Axl*-Expression in NK-Zellen im Normalzustand relativ gering ist, steigt sie *in vivo* nach Transplantation von B-ALL-Zellen wie auch bei Makrophagen signifikant an. Daher könnte eine spezifische Hemmung von AXL als wirksamer Ansatz dienen, um die durch die Leukämie verursachte frühe Immunsuppression umzukehren. Dieser Mechanismus konnte hier sowohl für das präklinische B-ALL- als auch für das Modell der akuten myeloischen Leukämie (AML) nachgewiesen werden.

Es konnte damit gezeigt werden, dass die immunsuppressive Signalübertragung über TAMRs durch die Leukämie verstärkt wird, was in der Wildtyp-Situation zu einer frühen Inhibierung der proinflammatorischen angeborenen Immunantwort durch Makrophagen und NK-Zellen führt. Durch die Blockade der TAMR-Signalkaskade konnte hier die Inhibierung der Immunantwort durch die Leukämie aufgehoben werden und eine T-Zell-abhängige Immunantwort zur Bekämpfung der Leukämie induziert werden. Damit wurde die ineffiziente Induktion der adaptiven Immunantwort durch das angeborene Immunsystem überbrückt, was im Gegensatz zu soliden Tumoren für hämatologische Neoplasien typisch ist(E. K. Curran, Godfrey und Kline 2017).

Diese Arbeit hat die entscheidende Funktion der TAMR-Signalübertragung als Suppressor der anti-leukämischen Immunität in präklinischen ALL-Modellen gezeigt. Um diese Resultate auf das humane System zu übertragen, wurde ein vollständig humanisiertes *ex vivo*-System verwendet. Hierfür wurde untersucht, wie sich das Expressionsniveau von GAS6 und PROS1 bei direktem Kontakt mit humanen primären Leukämiezellen in der humanen Knochenmarkumgebung (hämatopoetisches und stromales Kompartiment) verändert. Die zeitlich begrenzte Co-Kultur von humanen endothelialen Zellen und mesenchymalen Stammzellen mit

von Patienten stammenden B-ALL-Zellen oder Proben von Patienten mit myelodysplatischen Syndrom (MDS) hat im Vergleich zur Co-Kultur mit gesunden Knochenmarkzellen keine signifikanten Veränderungen des Expressionslevels von *GAS6* und *PROS1* induziert. Interessanterweise wurde *GAS6*, jedoch nicht *PROS1*, in isolierten CD14+ Monozyten nach Co-Kultur sowohl mit Leukämiezellen als auch gesunden Knochenmarkzellen hochreguliert. Das deutet darauf hin, dass die *GAS6*-Induktion aufgrund einer unzureichenden HLA-Übereinstimmung auftrat. Daher erfordert dieser Aspekt weitere Untersuchungen unter Berücksichtigung der begrenzten Lebensdauer der primären humanen Leukämieproben *ex vivo*.

Darüber hinaus wurde hier das Niveau von GAS6 und PROS1 im Blutserum von gesunden Spendern im Alter von unter 25 Jahren und über 60 Jahren analysiert. Diese Untersuchung ergab, dass sich eine altersbedingte Immunschwäche in der älteren Kohorte im Vergleich zur jungen Gruppe in signifikant verringerten GAS6-Spiegeln und erhöhten Werten für PROS1, CCL2/MCP1a, Eotaxin und CCL3/Mip1a-Spiegeln manifestiert. Diese Daten deuten darauf hin, dass die TAMR-Signalübertragung wahrscheinlich den altersabhängigen Rückgang des Immunsystems begünstigt, was wiederum mit einer schlechteren Überlebensrate bei älteren Leukämie-Patienten verbunden ist.

Zusammenfassend identifiziert diese Arbeit den Mechanismus der Inhibierung der anti-leukämischen Immunantwort über die TAMR-Signalkaskade in Makrophagen unter Verwendung des syngenen BCR-ABL1 B-ALL-Mausmodells. Durch genetische Depletion von GAS6 in der Wirtsumgebung oder Ablation von AXL- und/oder MERTK-Rezeptoren in Makrophagen wirdeine effiziente anti-leukämische Immunantwort induziert. Insbesondere löst die GAS6/AXL-Blockade eine wirksame NK- und T-Zell-abhängige Anti-Leukämie-Reaktion aus, die zu einem verlängerten Überleben führt.

Zusammengenommen schlagen diese Daten den AXL-Rezeptor als neuartigen Immun-Checkpoint und attraktiven Kandidaten für die Entwicklung eines neuen therapeutischen Ansatzes vor, indem das eigene Immunsystem des Patienten gegen die Leukämie gerichtet wird.

# 6.0 Addendum

## 6.1 List of figures

## 6.2 List of tables



## 6.3 List of abbreviations

| | |
|---|---|
| AKT | Protein kinase B |
| ALL | Acute lymphoblastic leukemia |
| Allo-HSCT | Allogeneic hematopoietic stem cell transplantation |
| AML | Acute myeloid leukemia |
| APCs | Antigen-presenting cells |
| ARG1 | Arginase 1 |
| BA+ | BCR-ABL+ |
| B-ALL | B-cell acute lymphoblastic leukemia |
| BCR | B-cell receptor |
| bFGF | basic fibroblast growth factor |
| BiTEs | bi-specific T-cell-engaging single-chain antibodies |
| BM | Bone marrow |
| BMDM | Bone marrow-derived macrophages |
| CAR | chimeric-antigen-receptor |
| Cbl-b | Casitas B-lineage lymphoma proto-oncogene-b |
| CCL2/MCP1 | C-C Motif chemokine ligand 2/monocyte chemoattractant protein-1 |
| CCL3/Mip1alpha | C-C Motif Chemokine ligand 3/macrophage inflammatory protein 1-alpha |
| CDKN2A/B | cyclin-dependent kinase 2A/B |

| | |
|---|---|
| cDNA | Complementary DNA |
| cHL | Classic Hodgkin Lymphoma |
| CKI | Checkpoint inhibitor |
| Csf1R | Colony Stimulating Factor 1 Receptor |
| CTLA4 | cytotoxic T-lymphocyte-associated protein 4 |
| DCs | Dendritic cells |
| DNA | deoxyribonucleic acid |
| EC | Endothelial cells |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| FACS | Fluorescence-activated cell sorting ( |
| FBS | Fetal bovine serum |
| FDA | Food and Drug Administration |
| FLT3 | FMS-like tyrosine kinase 3 |
| FLT3-ITD | FLT3-internal tandem duplication |
| g | Gramm |
| Gas6 | Growth-arrest specific factor 6 |
| GFP | Green fluorescent protein |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| gRNA | Guide RNA |
| GVHD | Graft-versus-host-disease |
| GVL | Gravt-versus-leukemia |
| Gy | Grey |
| h | hours |
| HDMEC | Human dermal microvascular endothelial cells |
| HSCs | Hematopoietic stem cells |
| HSPCs | Hematopoietic stem and progenitor cells |
| i.p. | intraperitoneal |
| i.v. | intraveneously |
| IgG | Immunoglobulin G |
| IKFZ1 | IKAROS family zinc finger 1 |
| IL | Interleukin |
| INFy | Interferon gamma |
| JAK | Janus kinase |
| l | liter |
| LPS | Lypopolisacharide |
| LSCs | Leukemia stem cells |
| m | Mili- |
| m | meter |
| M | mol |
| M-CSF | Macrophage colony-stimulating factor |
| MDCSs | Myeloid-derived suppressor cells |
| MDS | Myelodysplastic syndrome |
| MHC | Major histocompatibility complex |
| min | minitues |
| Mk | Megakaryocytes |

| | |
|---|---|
| MLL-ENL | Mixed-lineage leukemia–eleven nineteen fusion protein |
| Mph | Macrophages |
| MRD | Minimal residual disease |
| mRNA | Messenger RNA |
| MSCs | Mesenchymal stromal cells |
| NF-κB | Nuclear factor-κB |
| NGS | Next generation sequencing |
| NK | Natural killer |
| NKG2D | Natural killer group 2D receptor |
| NSG | non-obese diabetic/severe combined immunodeficiency/gamma chain depleted (NOD.Cg-Prkdc$^{scid}$ IL2rg$^{tm1Wjl/}$SzJ) |
| OS | Overall survival |
| OVA | Ovalbumin |
| p | pico |
| PB | Peripheral blood |
| PBS | Phosphate buffered saline |
| PD1 | Programmed cell death protein 1 |
| PDGFRa | Platelet-derived growth factor receptor alpha |
| PDX | Patient-derived xenograft |
| PI3K | Phosphoinositol-3-kinase |
| Pros1 | Protein S |
| PtdSer | phosphoditilserine |
| qPCR | quantitative polymerase chain reaction |
| Rag | Recombination activating gene 1 |
| rpm | Revolutions per minute |
| RT | Romm temperature |
| RTKs | Receptor tyrosine kinases |
| SCID | Severe combined immunodeficient |
| SD | Standart deviation |
| SOCS | suppressors of cytokine synthesis |
| Spl | Spleen |
| STAT | Signal transducers and activators of transcription |
| STING | Stimulator of interferon genes pathway |
| TAMRs | Tyro Axl Mer receptors |
| TCR | T-cell receptor |
| TGFb | Transforming growth factor beta |
| TK | Tyrosine kines |
| TKI | Tirosine kinase inhibitor |
| TKO | Triple knockout |
| TLR | Toll-like receptor |
| Treg | Regulatory T-cells |
| TVA | Tierversuchsantrag |
| WT | Wild type |
| µ | Micro ($10^{-6}$) |

# 7.0 References


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# 9.0 Declaration of authorship

I, Aleksandra Nevmerzhitskaya, hereby declare that this submitted thesis has been composed entirely by myself and is based on my own work. All direct and indirect sources of information used are cited in the text and acknowledged as references in the bibliography. I confirm that I have respected the principles of good scientific practice and have not made use of the services of any commercial agency in respect of my doctorate. I herewith declare that I have not previously participated in any doctoral examination procedure in a mathematics or natural science discipline.

_____ _____

City, Date Signature

# 10.0 Curriculum Vitae

## ALEKSANDRA NEVMERZHITSKAYA

**EDUCATION**

**Dr. rer. nat.** **01/2016 – 10/2020**

**Faculty of Biochemistry**

**Johann Wolfgang Goethe-Universität/**

**Georg-Speyer-Haus, Frankfurt**

Dissertation title: Evaluating the role of niche-derived growth arrest-specific gene 6 (Gas6) in an aggressive mouse model of B-ALL

Supervisors: Prof. Dr. F. Greten, Prof. Dr. R. Marschalek

**Diplom of Biologist** **09/2006 – 07/2011**

**Specialization Immunology**

International Sakharov Environmental University, Minsk

Thesis title: Factors of non-specific resistance in children with acid-dependent disorders of the gastrointestinal tract

Supervisor: Dr. T. Suhan

State exam with distinction

**PROFESSIONAL & RESEARCH EXPERIENCE**

**Ph.D. student** **01/2016 – 10/2019**

**Georg-Speyer-Haus, FFM**

**Project:** Evaluated the role of niche-derived growth arrest-specific gene 6 (Gas6) in an aggressive mouse model of B-ALL

**TECHNICAL SKILLS**

Full independence in:

- Flow cytometry
- 2- and 3D cell culture
- ELISA & Luminex
- qPCR, PCR
- Light & fluorescent microscopy
- *In Vivo* animal techniques

**LANGUAGES**

- English (Advanced)
- Russian (Native)
- German (Basic)

**COMPUTING SKILLS**

- GraphPad Prism
- FlowJo
- Excel, Power Point, Word

**CERTIFICATION**

- Advanced BD FACSAria Operator Course
- FELASA (module rodents+surgeries)

**Key achievements:**

- Designed and executed 100+ *In Vivo & Ex Vivo* experiments and identified the novel mechanism of dampening anti-leukemic immune response via modulating GAS6/TAMR signaling in macrophages in preclinical B-cell leukemia model
- Processed and FACS analyzed 40+ primary human PB & BM samples within MDS/AML clinical trial
- Analyzed 200+ scientific papers on B-cell leukemia, innate immune checkpoints, anti-leukemic immune response
- Shared-first authorship in *Leukemia*
- Presented grant proposal on PIs behalf SFB, 2016; selected speaker at 9$^{th}$ UCT Science Day, Frankfurt am Main, 2019; monthly presented results and publications at internal and external institute meetings

**Scientist** **09/2014 – 12/2015**

**MTZ, TU Dresden**

**Project:** Studied the mechanism of acute and long-term immune rejection of xenotransplant in NOD-Scid-IL2r$^{null}$ Kit$^{W41/W41}$ humanized mice

**Key achievements:**

- Established and performed the porcine islet intraportal transplantation assay into humanized NSG Kit$^{W41/W41}$ mice and investigated by FACS & immunohistology assay the mechanism of early xenograft immune rejection
- Attended the 18$^{th}$ International Summer School on Immunology "Immune system: Genes, Receptors and Regulations" Rabac, Croatia, 2015
- Authorship in *Stem Cell Reports*
- Reported the project on behalf of two PIs in trans-regional project meeting TRR127, Mariensee, 2015; monthly presented results and publications in internal and external group meetings

**Guest scientist** **09/2013 – 08/2014**

**Universitätsmedizin Mainz**

**Project:** Investigated the markers to detect latent placental insufficiency

**Key achievements:**

- Isolated, cultured and functionally characterized microvascular endothelial cells from the human placenta
- Authorship in *Placenta*
- Presented results and publications in internal group meetings

**Junior scientist** **08/2011 – 08/2013**

**Belarusian Research Center for Pediatric Oncology, Hematology and Immunology, Minsk**

**Project:** Diagnostic of children primary immunodeficiency

**Key achievements:**

- Analyzed 50+ BM & PB samples using flow cytometry from patients after allo-HSCT and with primary immunodeficiency
- Authorship in 3+ international conferences, regular reports

## PUBLICATIONS

- **Tirado-Gonzalez I*, Czlonka E*, Nevmerzhitskaya A*,** Soetopo D, Bergonzani E, Mahmoud A, Contreras A, Jeremias I, Platzbecker U, Bourquin JP, Kloz U, Van der Hoeven F, Medyouf H. *CRISPR/Cas9-edited NSG mice as PDX models of human leukemia to address the role of niche-derived SPARC* //Leukemia, 2018

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