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Sex-Dependent Impact of Cytokines on Cytotoxic Lymphocyte Function during Aging

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Chantal Hof

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Dekan: Univ.-Prof. Dr. med. Michael D. Menger
Berichterstatter: Prof. Dr. Markus Hoth
Prof. Dr. Sandra Iden

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Summary

Cytotoxic T lymphocytes and natural killer cells are effector immune cells which are affiliated with the adaptive and innate immune system, respectively. Their primary tasks are the identification and active elimination of their target cells, including infected or degenerated cells, through different killing pathways. One of these killing pathways is driven by the secretion of effector molecules perforins and granzymes that are stored in lytic granules within the immune cells, inducing cell death. Both cytotoxic T lymphocytes and natural killer cell activity can be induced by supplementation with the cytokine interleukin-2, which then creates a positive feedback loop to further enhance activation. Interleukin-2 signaling begins at the interleukin-2 receptor, consisting of the three subunits CD25, CD122, and CD132, which then activate different downstream pathways, controlling differentiation, proliferation, and survival of the immune cells. Two other fundamental properties influencing the immune response of effector immune cells are age and sex/gender, which impact the abundance and functionality of effector cells, resulting in different susceptibilities to diseases. However, little is known about the sex- and age- differences in interleukin-2 regulation in both mouse and human; therefore, this study aims to investigate the differences in activation, exhaustion, effector molecule expression and secretion in cytotoxic T lymphocytes and natural killer cells of mice and human across age and sex.

Cytotoxic T lymphocytes and natural killer cells were isolated from mouse and human donors of varies ages and both sexes, stimulated accordingly with interleukin-2, and analyzed regarding cytotoxicity using a real time killing assay, expression of surface markers and effector molecules using flow cytometry. Lastly, the secretion of effector molecules and cytokines was quantified using the LegendPlex™ system.

Analysis of murine cytotoxic T lymphocytes from both sexes revealed that while there was no difference in interleukin-2 response in the adult mice, the elderly mice showed a strong sex-specific interleukin-2-mediated decline cytotoxicity in the male mice that was absent in the females. In general, adult mice cytotoxic T lymphocytes were more susceptible to fluctuations in interleukin-2 concentration compared to their elderly counterparts. Only the neutralization of autocrine interleukin-2 inhibited cytotoxicity in all tested cohorts except elderly female mice. These differences are due to enhanced activation as well as expression and secretion of effector molecules but are independent of viability, subtype distribution, and proliferation.

In murine spleen natural killer cells, no general interleukin-2-dependence was observed. However, in all but one cohort, cytotoxicity peaked at 500U IL-2, suggesting that this is an ideal interleukin-2 concentration for natural killer cell stimulation. Both the subtype distribution and the expression of activating natural killer cell receptors showed tendencies depending on the interleukin-2 concentration, with an increasing abundance in mature natural killer cells with

decreasing interleukin-2 concentrations. In contrast to cytotoxic T lymphocytes, a sex-specific differences were not found in the elderly but rather in adult mice, where the females exhibited significantly higher cytotoxicity compared to their male counterparts.
Murine blood natural killer cells, though more abundant percentagewise, showed almost no cytotoxic activity. This was generally contradicting the higher abundance of mature natural killer cells but in accordance with the higher expression of inhibitory receptors. Notably, elderly female mice's blood natural killer cells exhibited lower cytotoxicity due to lower expression of activating receptors.
In natural killer cells healthy human donors, interleukin-2 significantly influenced almost all analyzed parameters, such as cytotoxicity, the expression of activating receptors and exhaustion markers, as well as the expression and secretion of effector molecules, which significantly increased under interleukin-2 supplementation. However, only few sex-specific differences were observed as cytotoxicity tended to be higher in women after interleukin-2 treatment most likely due to a higher expression of activating receptors.

This study functionally characterized cytotoxic T lymphocytes and natural killer cells in the context of interleukin-2 dependence across age and sex. It reveals species-, tissue-, age- and sex-specific differences in interleukin-2 response, indicating diverging regulatory mechanisms of the immune system in mouse and human.

Zusammenfassung

Zytotoxische T-Lymphozyten und natürliche Killerzellen sind Effektorzellen des Immunsystems, welche jeweils zum erworbenen und angeborenen Immunsystem gehören. Ihre primären Aufgaben liegen in der Identifikation und aktive Eliminierung ihrer Zielzellen, welche infizierte aber auch entartete Zellen beinhalten, durch verschiedene zytotoxische Signalwege. Einer dieser Signalwege nutzt die Sekretion von Effektormolekülen wie Perforin und Granzymen, welche in den Zellen in Form von lytischen Granula gelagert werden, um den Tod der Zielzelle zu induzieren. Sowohl T-Zellen als auch natürliche Killerzellen können durch die Gabe des Zytokins Interleukin-2 aktiviert werden, welches eine positiven Rückkopplungsschleife auslösen kann, um die Aktivierung zu verstärken. Der IL-2 Signalweg beginnt bei dem Interleukin-2 Rezeptor, bestehend aus den drei Untereinheiten CD25, CD122 und CD132, über den anschließend verschiedene nachgeschaltete Signalwege aktiviert werden, welche unter anderem die Differenzierung, Proliferation und das Überleben der Immunzellen kontrollieren. Zwei weitere fundamentale Eigenschaften, welche die Immunantwort beeinflussen sind das Alter und das Geschlecht (englisch *sex* und *gender*), die unter anderem Einfluss auf die Verteilung und Funktionalität dieser Effektorzellen haben, wodurch es zu verschiedenen Prädispositionen in Bezug auf Erkrankungen kommt. Jedoch ist nur sehr wenig zu der Interleukin-2 Regulation dieser Alters- und Geschlechtsbedingter Unterschiede bekannt, weder in der Maus noch im Menschen. Daher soll im Rahmen dieser Arbeit die Aktivierung, Erschöpfung, sowie die Expression und Sekretion von Effektormolekülen in humanen und murinen T-Zellen und natürliche Killerzellen in Abhängigkeit vom Alter und Geschlecht untersucht werden.
Die T-Zellen und natürliche Killerzellen wurden dafür zunächst aus der Maus oder menschlichen Spendern unterschiedlichen Alters und Geschlechts isoliert, mit Interleukin-2 stimuliert und anschließend in Bezug auf Zytotoxizität mittels Echtzeit Zytotoxizitätsassays, sowie der Expression von Oberflächenmarkern und Effektormolekülen mittels Durchflusszytometrie analysiert. Zuletzt wurde die Sekretion der Effektormoleküle und anderer Zytokine mit Hilfe des LegendPlex™ Systems quantifiziert.

Die Analyse muriner T-Zellen aus beiden Geschlechtern zeigte, dass es keine geschlechtsbedingten Unterschiede bei den adulten Mäusen gab, allerdings gab es in den älteren männlichen Mäusen einen starken Interleukin-2-abhängigen Abfall in der Zytotoxizität, welcher bei den weiblichen Mäusen nicht feststellbar war. Grundsätzlich waren die T-Zellen aus den adulten Mäusen bedeutend anfälliger für Schwankungen in der Interleukin-2 Konzentration im Vergleich zu den älteren. Jedoch, lediglich die Neutralisation von autokrinem Interleukin-2 erreichte eine vollständige Inhibition der Zytotoxizität in allen getesteten Kohorten außer den gealterten Weibchen. Dieser Unterschied ist auf eine erhöhte Aktivierung, sowie

Expression und Sekretion von Effektormolekülen zurückzuführen, ist allerdings unabhängig von Viabilität, Subtypenverteilung und Proliferation.
In murinen natürliche Killerzellen konnte hingegen keine generelle Interleukin-2-Abhängigkeit beobachtet werden. Jedoch zeigte sich in allen Kohorten außer einer, dass die Zytotoxizität bei einer Interleukin-2 Konzentration von 500U am höchsten ist, was darauf hindeutet, dass es sich hierbei um eine ideale Interleukin-2 Konzentration zur Stimulation von natürliche Killerzellen handeln könnte. Sowohl die Subtypenverteilung als auch die Expression der aktivierenden natürliche Killerzell-Rezeptoren zeigte Interleukin-2-abbhängige Tendenzen mit steigendem Vorkommen reifer natürliche Killerzellen mit fallenden Interleukin-2 Konzentrationen. Im Gegensatz zu T-Zellen konnte kein geschlechtsspezifischer Unterschied in den älteren Mäusen gefunden werden, sondern in den adulten Mäusen, bei denen erneut die Zellen aus den Weibchen eine erhöhte Zytotoxizität zeigten.
Murine natürliche Killerzellen aus dem Blut, obwohl sie einen höheren prozentualen Anteil haben, zeigten fast keine zytotoxische Aktivität. Dies steht in Widerspruch zu dem erhöhten Vorkommen reifer natürliche Killerzellen, entspricht allerdings der erhöhten Expression inhibitorischer natürliche Killerzell-Rezeptoren.
In natürliche Killerzellen aus gesunden menschlichen Probanden hatte die Gabe von Interleukin-2 einen signifikanten Einfluss auf alle untersuchten Parameter, unter anderem auf die Zytotoxizität, die Expression aktivierender Rezeptoren und Erschöpfungsmarker, sowie der Expression und Sekretion von Effektormolekülen, welche alle unter Interleukin-2 Gabe zunahmen. Allerdings zeigten sich hier fast keine geschlechts-spezifischen Veränderung, wobei eine leichte Tendenz zur erhöhten Zytotoxizität nach Interleukin-2 Gabe in Frauen zu finden war, vermutlich aufgrund der erhöhten Expression aktivierender natürliche Killerzell-Rezeptoren.

In dieser Studie wurden zytotoxische T-Lymphozyten und natürliche Killerzellen auf ihre Interleukin-2 Abhängigkeit im Kontext des Alterns und des Geschlechts funktionell charakterisiert. Dabei konnten Spezies-, Gewebe-, Alters- und Geschlechtsspezifische Unterschiede in der Interleukin-2 Antwort festgestellt werden, welche auf Divergenzen in den zugrundeliegenden regulatorischen Mechanismen des Immunsystems von Maus und Mensch hindeuten.

1 Introduction

1.1 The Immune System

The immune systems in both mice and humans consists of the innate and the adaptive immune responses, although the composition differs between the two species (Bjornson-Hooper et al., 2022; Mestas & Hughes, 2004). Their primary purpose is to prevent and eliminate infected or degenerated cells in the body through a cell- and humoral-mediated response (Marshall et al., 2018). The innate immune system, crucial for the first line of defense with key players including natural killer (NK) cells, macrophages, mast cells, and granulocytes (Diamond & Kanneganti, 2022; Iwasaki & Medzhitov, 2010; Lacy & Stow, 2011). Due to its unspecific response, the innate immune system can control viral loads in the early phases of infection until the adaptive immune system, comprising T and B cells that are specific for their target cells, is activated (Sego et al., 2020). For such specificity, the so-called priming, antigen-presenting cells (APCs) like dendritic cells (DCs) that are components of the innate immune system are necessary as they present antigens on the major histocompatibility complex (MHC) class I or II (Inaba et al., 1983; Steinman & Witmer, 1978). Therefore, APCs serve as a bridge between the innate and the adaptive immune systems. Following antigen presentation, T and B cells can recognize this specific antigen and eliminate all cells presenting this antigen (Engleman et al., 1981; Norbury et al., 1997; Platt et al., 2010; Sallusto et al., 1999). Notably, after the adaptive immune system's response, some immune cells remain in the body, enabling a faster secondary immune response. This forms the fundament of the immunological memory or trained immunity (Netea et al., 2020).

1.1.1 Cytotoxic Lymphocytes

Cytotoxic T lymphocytes (CTLs) or $CD8^+$ T cells are part of the adaptive immune system and are responsible for cytotoxic elimination of infected cells and degenerated cells (Raskov et al., 2021). Besides CD8 expression, human CTLs are further distinguished by CD45RO/RA and CCR7/CD62L expression, categorizing them into various memory and effector subtypes that show differences in cytotoxic capacity (Knörck et al., 2022; Maecker et al., 2012; Sallusto et al., 1999). In mice, these subtypes are classified based on CD44 and CD62L expression: naïve $CD44^{hi}CD62L^{lo}$; central memory (CM) $CD44^{hi}CD62L^{hi}$; and effector memory (EM) $CD44^{lo}CD62L^{hi}$ (Nakajima et al., 2021). However, differences in cytotoxicity among different CTL subtypes in mice have not been observed (Zöphel et al., 2022).

NK cells, functioning similarly to CTLs but acting in an antigen-independent manner, are linked with the innate immune system (Friedmann et al., 2022). In mice, NK cells are identified by NK1.1 (CD161) and NKp46 (CD335) expression, although NK1.1 is not expressed in all strains

due to genetic variations (Carlyle et al., 1999, 2006; Koo & Peppard, 1984; Miao et al., 2021; Walzer et al., 2007). Another possible marker for NK cell identification is DX5 (CD49b) (Arase et al., 2001). Murine NK cell subtypes, defined by CD27 and CD11b expression, indicate different maturation stages: $CD11b^{lo}CD27^{lo}$; $CD11b^{lo}CD27^{hi}$; $CD11b^{hi}CD27^{hi}$; and $CD11b^{hi}CD27^{lo}$ (Chiossone et al., 2009). Unlike in mice, human NK cells are primarily identified by CD56 expression in the absence of CD3 (Gunesch et al., 2020; Robertson & Ritz, 1990). Two major subtypes are differentiated by the expression levels of CD56 and CD16 with $CD56^{hi}CD16^{-}$ NK cells being cytokine producers and $CD56^{dim}CD16^{+}$ NK cells known for their cytotoxic abilities (Cooper et al., 2001; Ziegler et al., 2017). In human blood, $CD56^{dim}CD16^{+}$ NK cells make up about at least 90% of total NK cells, with $CD56^{hi}CD16^{-}$ comprising up to 10% (Dogra et al., 2020; Pascal et al., 2004). NK cells represent between 5% and 20%, occasionally up to 30%, of blood leukocytes (Bisset et al., 2004; Freud et al., 2017; Merkt et al., 2021).

An imbalance in effector lymphocyte numbers or malfunction can lead to various conditions, ranging from a higher susceptibility to infections and tumors with increased severity to autoimmune diseases in both mice and humans (Bagot et al., 2004; Begolka et al., 2001; Imai et al., 2000; Ishigami et al., 2000; Jira et al., 1988; S. Kim et al., 2000; S.-J. Lin et al., 2003; Pender et al., 2012; Sullivan et al., 1994). Therefore, both CTLs and NK cells are prime targets for immunotherapy approaches, including CAR-T and CAR-NK therapies (Chu et al., 2022; Du et al., 2021; Farhood et al., 2019; Miliotou & Papadopoulou, 2018; Page et al., 2024).

1.2 Activation of Cytotoxic Lymphocytes

1.2.1 Cytotoxic T Lymphocytes

Activation of CTLs involves a complex interplay of signals and cellular interactions, crucial for their role in the adaptive immune response. CTLs mediate MHC-I-dependent cytotoxicity in an antigen-dependent manner, requiring prior “priming” to recognize specific target cells (Pavelic et al., 2009). This priming is performed by antigen-presenting cells, such as dendritic cells, which process and present specific antigen on their MHC-I molecule. This presentation is crucial for the initial engagement of CTLs (Blum et al., 2013; Joffre et al., 2012). CTLs bind to the presented antigen with the T cell receptor (TCR) that consists of four different CD3 molecules (γ, δ, ε, ζ). The required variety in TCRs is accomplished through random V-D-J segment combinations in the α and β chain which allows each TCR to uniquely recognize specific antigens (Menon et al., 2023; Snir et al., 2023). Activation occurs when the TCR and its co-receptor CD8 bind to the antigen-MHC-I complex, leading to the phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytoplasmic tail of CD3 by the CD8-associated lymphocyte-specific protein tyrosine kinase (Lck) (Love & Hayes, 2010). This

phosphorylation event recruits and activates zeta-chain-associated protein kinase 70 (ZAP-70), further propagating the activation signal. Through different pathways including calcineurin–nuclear factor of activated T cell (NFAT), nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB), mitogen-activated protein kinases (MAPK)-extracellular signal-regulated kinases (ERK), or mammalian target of rapamycin (mTOR) distinct T cell functions are regulated. These pathways control the expression of critical effector molecules and IL-2, modulating T cell proliferation, differentiation, and response to antigens (Blanchett et al., 2021; Macian, 2005).

Additionally, CTL activation is enhanced by costimulatory signals through CD28, which colocalizes with the TCR at the immunological synapse, to improve activation (Sanchez-Lockhart et al., 2008). CD28 signaling is induced after ligation with CD80/CD86 and activates mainly the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mTOR but also the NFAT or NF-κB pathways, similar to TCR signaling (Bour-Jordan et al., 2011). Notably, CD28 together with CD3 stabilizes mRNA of key effector molecules like IL-2, tumor necrosis factor α (TNF-α), and interferon-γ (IFN-γ) (Lindstein et al., 1989).

1.2.2 Natural Killer Cells

Unlike CTLs, NK cells identify their target cells through an antigen-independent mechanism as they express a variety of activating and inhibitory receptors (Quatrini et al., 2021). NK cell activation can be categorized into three different modes, which are determined by the balance of receptor engagement signals (Myers & Miller, 2021). A state of tolerance is induced when inhibitory signals predominate, preventing NK cell activation. One major inhibitory signal is the ligation of MHC molecules with their respective receptors, for example members of the Ly49-family in mouse, the KIR-family in humans, and NKG2A in both species (Raulet & Vance, 2006). Activation through the ‘missing self’ occurs when the negative signal is absent, and the activating signals outweigh the inhibitory ones (Thielens et al., 2012). Loss or downregulation of MHC molecules appears on many tumor cells like breast, colorectal, and prostate cancer as well as melanoma and hepatocellular carcinoma which allows then to evade the CTL-mediated immune response (Dhatchinamoorthy et al., 2021). However, not all tumor cells decrease MHC expression. Therefore, NK cells can also be activated through the ‘induced self’ mechanism, where the presence of activating signal, independent of MHC ligation, dominates (Long & Rajagopalan, 2002). This activation can occur when tumor or infected cells upregulate ligands for activating receptors, such as viral hemagglutinins or Rae-1, which bind activating receptors NKp46 and NKG2D, respectively (Chisholm & Reyburn, 2006; Long & Rajagopalan, 2002; Mandelboim et al., 2001).

1.3 Interleukin-2 and Its Role in the Immune System

Since its discovery in the 1970s, IL-2 has been extensively researched due to its immunomodulatory properties (Morgan et al., 1976). Although primarily produced by activated $CD4^+$ as the main source, IL-2 can also be secreted by CTLs, B cells, and dendritic cells under specific conditions, highlighting its broad involvement in immune cell activation (Granucci et al., 2003; Kahan et al., 2022; Kindler et al., 1995; Lagoo et al., 1990; Redeker et al., 2015; Sojka et al., 2004; Toumi et al., 2022; Zelante et al., 2012; Y. Zhang, Maksimovic, et al., 2018). IL-2 plays a crucial role, among other cytokines, in enhancing the activation of both T cells and NK cells (Henney et al., 1981; C. Lehmann et al., 2001; Morgan et al., 1976; Ross & Cantrell, 2018; Trinchieri et al., 1984).

1.3.1 Interleukin-2 Receptor

The IL-2 receptor (IL-2R) consists of up to three subunits: CD25 (α chain), CD122 (β chain), and CD132 (common γ chain), which combine in distinct combination to form IL-2Rs with different IL-2 affinities (Figure 1) (Hernandez et al., 2022; X. Wang et al., 2005). CD25 alone or has the lowest affinity for IL-2 followed by a combination of CD122 and CD132. The combination of all three subunits shows the highest affinity for IL-2, and their expression increases upon T and NK cell activation in a time-dependent manner (Beltra et al., 2016a; S.-H. Lee et al., 2012). It's noted that CD132 levels may decrease following CD3/CD28/IL-2 stimulation within the initial 48 hours (Cao et al., 2014).

The intermediate affinity receptor, which includes CD122, also binds IL-15 due to shared receptor components, underscoring a unique cross-functionality that extends beyond IL-2 signaling (Bamford et al., 1994). For optimal IL-15 signaling, the high-affinity IL-15 receptor also requires a cytokine-specific α chain (CD215) which can utilize both *cis*- and *trans*-presentation as opposed to IL-2 and its primary *cis*-presentation (Burkett et al., 2003, 2004; Dubois et al., 2002; Lodolce et al., 2001; Schluns et al., 2004; Wuest et al., 2011; Zanoni et al., 2013).

1.3.2 Interleukin-2 Signaling

Upon ligation with the intermediate- or the high-affinity IL-2R, Janus family kinase 1 (JAK1) and JAK3 bound by CD122 and CD132, phosphorylate the signal transducer and activator of transcription 5 (STAT5) and STAT3 (Beyer et al., 2011; Kolios et al., 2021). Following phosphorylation, STAT5A and STAT5B dimerize and translocate to the nucleus where it binds to the gamma interferon–activated sequence (GAS) DNA sequences and induce transcription

of its target genes like forkhead-box P3 (FoxP3) (relevant for regulatory T cells), CD25, and the negative IL-2 regulator suppressor of cytokine signaling 3 (SOCS3) (Kanai et al., 2014; P. Li et al., 2017; Liao et al., 2008; J.-X. Lin et al., 2012).

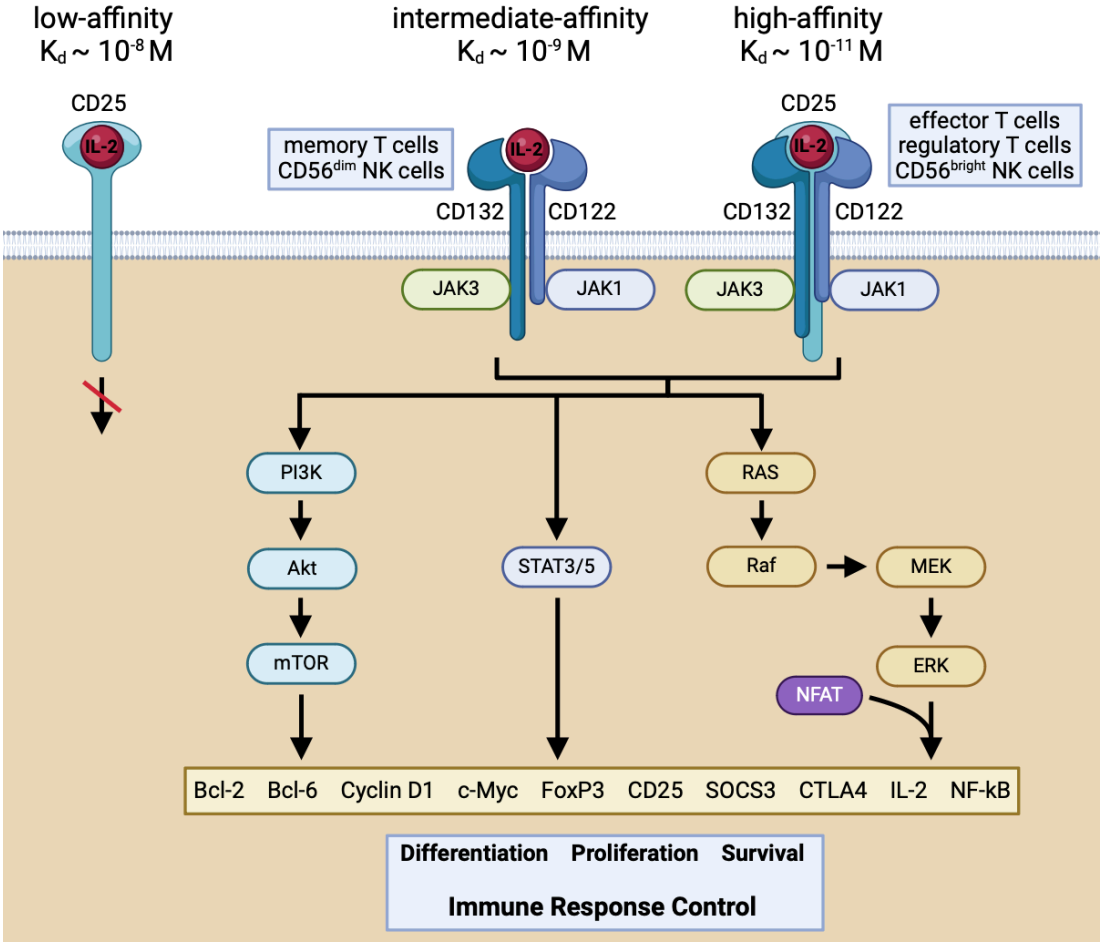


Figure 1: Interleukin-2 receptor and signaling pathways. IL-2 can bind to CD25 alone, but this ligation does not induce intracellular signal transduction. Ligation with the intermediate-affinity receptor (CD122 and CD132) or the high-affinity receptor (CD25, CD122, and CD132) induces signal transduction through JAK1 and JAK3. After that, three possible pathways can be activated with STAT5/STAT3 being the most prominent. Alternatively, the PI3K/Akt/mTOR or the MAPK pathway is used. All pathways regulate different target genes relevant in cell differentiation, proliferation, and survival. Modified after Kolios et al. (2021) and Yang & Lundqvist (2020). Created with BioRender.com.

IL-2 signaling pathway operates mainly through the JAK/STAT pathway (Ross et al., 2016). However, two others are also well researched and can compensate for disruptions in JAK/STAT-mediated signaling (Kolios et al., 2021; Yang & Lundqvist, 2020). Similar to the TCR, IL-2R engagement activates the PI3K/Akt/mTOR pathway signaling, which is pivotal for promoting cell survival and proliferation, in addition to regulating cytokine responses (Castedo et al., 2002; Mori et al., 2014; Nandagopal et al., 2014; Sinclair et al., 2008). Simultaneously, the MAPK-ERK pathway, activated by IL-2R signaling, facilitates cellular proliferation and differentiation (K. Jiang et al., 2000; Sun et al., 2015; T. K. Yu et al., 2000). Common target genes for these pathways are proto-oncogenes B-cell lymphoma 2 (Bcl-2) (Miyazaki et al., 1995), Bcl-6 (W. Yi et al., 2017), and c-Myc (Almajali et al., 2022; Swords et al., 2015), the cell cycle regulator cyclin D1 (F. Wang et al., 2023), and NF-κB (Bai et al., 2009). Moreover, the

synergistic action of NFAT and IL-2 itself establishes a positive feedback loop that amplifies IL-2-mediated lymphocyte activation, highlighting the cytokine's self-regulatory capacity (Busse et al., 2010; L. Zhang & Nabel, 1994). The presence of CD25 alone constitutes the low-affinity IL-2 receptor, which lacks the capacity to induce IL-2 signaling independently, underscoring the necessity of the higher-affinity receptor complexes for functional response (Abbas, 2020).

Besides IL-2, a variety of other cytokines, including IL-4, IL-22, IL-17A, IL-6, TNF-α, and IFN-γ among others, are secreted by activated cytotoxic lymphocytes (Huber et al., 2013; S. Paul & Lal, 2017; Res et al., 2010; Schwaiger et al., 2003; Sellau et al., 2016; W. Wu et al., 2015; Yoshimoto, 2018). These cytokines play a role in regulation of the activation, proliferation, and differentiation while and some influence the effector function of the immune cells.

1.4 Cytotoxicity Pathways

Because CTLs and NK cells serve a similar purpose in active elimination of target cells, they deploy two primary cytotoxic pathways upon stimulation/activation: receptor-mediated and granule-mediated cytotoxicity (Backes et al., 2018; Ramírez-Labrada et al., 2022).

Receptor-mediated cytotoxicity involves the interaction between death receptors on the target cell and their ligands on the effector cell. The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor and the Fas receptor are key components of this pathway (Trauth et al., 1989; Wiley et al., 1995). The binding of TRAIL and Fas ligand (FasL) to their respective receptors activates the receptor's death domain (DD), which recruits the Fas-associated death domain (FADD)/caspase-8 complex, initiating caspase-8 signaling and inducing apoptosis of the target cell (Mandal et al., 2020; Ramírez-Labrada et al., 2022; Tuomela et al., 2022). However, resistance to receptor-induced apoptosis is observed in target cells lacking these death receptors, such as the K-562 cell line (Daniels et al., 2005; Keane et al., 1996; Mühlenbeck et al., 2000; Munker et al., 1997; Wilson et al., 1998).

Granule-mediated cytotoxicity, in contrast, operates independently of target cell receptors. This pathway include effector molecules such as perforin, granzymes, and granulysin (in humans), which are delivered from the effector cell to the target cell via lytic released into the immunological synapse (Pardo et al., 2009; Prager & Watzl, 2019). Several models have been proposed regarding the role of perforin in the internalization of lytic molecules (Chávez-Galán et al., 2009; Voskoboinik et al., 2006). The most commonly used model is that perforin multimerizes in the plasma membrane of the target cell and thereby forming pores into the membrane, a process in which granulysin can also participate (Kaspar et al., 2001). Subsequently, granzymes activate caspase-3, comparable to the receptor-mediated cytotoxicity resulting in apoptosis (Saini et al., 2011). However, excessive or high perforin concentrations in the immunological synapse can lead to rapid necrotic death of target cell,

distinguished as primary necrosis or secondary necrosis following apoptotic processes (Backes et al., 2018; Keefe et al., 2005; Sachet et al., 2017).
Strikingly, while perforin alone can induce target cell death, granzymes require the presence of perforin to be effective. Moreover, CTLs and NK cells can flexibly switch between receptor- and granule-mediated cytotoxicity pathways, highlighting the adaptability of these effector cells in response to different targets (Backes et al., 2018; Prager et al., 2019).

1.5 Impact of Age on the Immune System

Aging is a physiological process occurring in every individual and represents an important biological factor. During aging, the immune response generally weakens, a phenomenon known as immunosenescence, resulting in higher susceptibility to neurological conditions (Dumurgier & Tzourio, 2020; Kesidou et al., 2023), cancer (Berben et al., 2021; López-Otín et al., 2023), infections (Bajaj et al., 2021; Y. Wu et al., 2021), and autoimmune diseases (Zhao et al., 2022; Zheng et al., 2023).
One of the most pronounced changes with age is the involution of the thymus, leading to a decreasing thymic output of immune cells (Hirokawa et al., 1994; Palmer et al., 2018; Tosi et al., 1982). Consequently, there is a shift in immune cell distribution, with an increase in NK cell and a decrease in T cell abundance in human blood (Brauning et al., 2022; Keenan & Allan, 2019). In mice, the abundance of NK and T cell decreases depending on the organ analyzed (Krishnarajah et al., 2022; Menees et al., 2021a; Mogilenko et al., 2022). Specifically, both human and murine CTL pools exhibit a strong decrease in the naïve phenotype, coupled with an accumulation of memory and terminally differentiated effector T cells, thereby limiting the individual's ability to respond to new immunological challenges (Fagnoni et al., 2000; Lazuardi et al., 2005; Zhou & McElhaney, 2011; Zöphel et al., 2022). Beside changes in the composition of the immune system, alterations in the functionality of specific immune cell subsets have also been observed. Aged humans display decreased numbers of perforin$^+$/granzyme B$^+$ CTLs and increased CD57 expression on CTLs and NK cells, which results in the exclusion of the costimulatory CD28 molecule's expression (Alpert et al., 2019; J. Merino et al., 1998; Quan et al., 2023; Zhou & McElhaney, 2011). In mice, IL-2 production, proliferation, and CTL cytotoxicity decreases (Effros & Walford, 1983; Smithey et al., 2011), not because of diminished expression of effector molecules but rather due to decreased cell counts (Zöphel et al., 2022). Murine NK cells also show decreased cytotoxicity with age, potentially due to decreased activation, effector molecule expression, and impaired maturation, along with reduced expression of activating receptors (Beli et al., 2014; W.-N. Jin et al., 2021; Nair et al., 2015; Shehata et al., 2015).

Aging in both species is associated with cellular exhaustion, as evidence by the increased expression of several exhaustion markers, such as programmed cell death 1 (PD-1), TIM-3, T cell immunoreceptor with Ig and ITIM domains (TIGIT), or cytotoxic T-lymphocyte associated protein 4 (CTLA4) leading to impaired immune cell functionality (Alvarez et al., 2019; K. Lee et al., 2016; Parks et al., 2023; Soto-Heredero et al., 2023). However, the lack of clear definition and distinct marker for exhaustion complicates its identification.

Another term often associated with aging is 'inflamm-aging', which was established back in 2000 (Franceschi et al., 2000), and describes a chronic, low-level inflammatory state affecting nearly all levels of the immune system (X. Li et al., 2023). Various factors, including oxidative stress (Martínez de Toda et al., 2021), alterations in the microbiome (Biagi et al., 2010), and inflammatory cell death (e.g., necrosis) (Franceschi et al., 2017; X. Li et al., 2023), have been proposed as drivers of this chronic inflammation. The mechanisms regulating inflamm-aging remain poorly understood, although recent study suggest dietary restriction can mitigate age-induced inflammation by altering chromatin accessibility and downregulating the transcription of innate immune receptors that mediate inflammation (Rasa et al., 2022).

1.6 Impact of Sex on the Immune System

Another fundamental but often neglected biological parameter in biomedical research is the sex of the study object. Since the early 1990s, the National Institute of Health (NIH) and the Food and Drug Administration (FDA) have made effort to include women in clinical trials. Nevertheless, the first trial evaluating the effects of aspirin on preventing of cardiovascular disease in women was not published until 2005 (Mazure & Jones, 2015; Ridker et al., 2005), long after the first study on men appeared in 1989 (Steering Committee of the Physicians' Health Study Research Group, 1989). Most recently, the coronavirus disease 2019 (COVID-19) pandemic highlighted the impact of sex on disease severity and outcomes, with men more likely to develop severe COVID-19 infections, require intensive care, and experience higher mortality rates compared to women(J.-M. Jin et al., 2020; Meng et al., 2020; Salje et al., 2020). Similarly, several studies identified sex biases in various diseases as summarized in Table 1. Despite this, many immunology studies as of 2009 did not evaluate or specify sex-specific results or the sex of laboratory animals used (Beery & Zucker, 2011), possibly due to skewed funding practices (Barlek et al., 2022).

Notably, women tend to have a higher incidence in autoimmune diseases, while men have a higher incidence of cancer (Table 1). The disparity is believed to be found in the genetic, molecular, and cellular differences in the immune response of men and women. A well-documented sex-specific differences involves the enhanced expression of the toll-like

receptors 7 (TLR7) and 8 (TLR8), crucial for pathogen defense and inflammation, encoded on the X chromosome (Bender et al., 2020; Schurz et al., 2019). Although one of the two X chromosomes in females is typically inactivated, certain genes, such as TLR7 gene, can bypass this inactivation (Bhattacharya et al., 2024; Souyris et al., 2018; Youness et al., 2021). This mechanism is implicated in the pathogenesis of systemic lupus erythematosus (SLE), an autoimmune disease with a notable female bias (Berghöfer et al., 2006; Infante et al., 2022; Preble et al., 1982).

On the cellular level, women display a lower abundance of CTLs but exhibit enhanced cytotoxicity, characterized by higher expression of effector molecules and activation in response to the stimuli (Abdullah et al., 2012; Hewagama et al., 2009; Uppal et al., 2003). Conversely, men and male mice consistently have a higher abundance of NK cells (Abdullah et al., 2012; Cheng et al., 2023; Z. Huang et al., 2021).

Table 1: Short overview of human diseases with a published sex bias in incidence and prevalence. Varied sex-biases are due to age-related changes in incidence.

Type	Disease	Sex Bias	Reference
Autoimmune and Inflammation (reviewed in Xing et al., 2022)	Ankylosing spondylitis	Male	(Haroon et al., 2014; Xing et al., 2022)
	Graves' disease	Female	(Donner et al., 2023)
	Hashimoto thyroiditis	Female	(Calcaterra et al., 2020)
	Rheumatoid arthritis	Female	(Calcaterra et al., 2020) (Kvien et al., 2006)
	Systemic lupus erythematosus (SLE)	Female	(Linos et al., 1980) (Xing et al., 2022)
	Crohn's disease	Male Varies	(Danchenko et al., 2006) (Shah et al., 2019) (Shah et al., 2018)
Neurological	Alzheimer's disease	Female	(Lopez-Lee et al., 2024)
	Parkinson's disease	Male Varies	(Elbaz et al., 2002) (Taylor et al., 2007)
Cancer (reviewed in Sung et al., 2021 and Lopes-Ramos et al., 2020)	Colorectal	Male	(Abancens et al., 2020)
	Respiratory System	Male	(Siegel et al., 2022)
	Leukemia	Male	(Stabellini et al., 2022)
	Bladder	Male	(Doshi et al., 2023)
	Breast	Female	(Siegel et al., 2022)
Infection	Urinary tract infections (UTI)	Female	(Deltourbe et al., 2022)
	Influenza	Varies	(Eshima et al., 2011)
	Tuberculosis	Male	(Min et al., 2023)
Cardiovascular	Stroke	Varies	(Reeves et al., 2008)
	Pulmonary embolism	Varies	(Jarman et al., 2021)

Additionally, sex hormones such estrogens, progesterone, and testosterone are known to modulate immune function at various levels (Ben-Batalla et al., 2020; Mauvais-Jarvis et al., 2020; Nolasco-Pérez et al., 2023; Ramírez-de-Arellano et al., 2021; Trumble et al., 2016; T. Wang et al., 2021), and while sex and age are distinct biological parameters, their interplay is crucial, particularly in the context of sex-specific alterations. In both sexes, aging is accompanied by a loss of sex hormones, however, this loss is gradual in men, who lose testosterone, but more abrupt in women, who experience a rapid loss of estrogen during menopause (Lamberts et al., 1997). Consequently, immunological changes, particularly after

menopause in women, are observed, with a decline in numbers of CTLs in the women's endometrium but an increase in their cytotoxicity, as evidenced by higher expression of granzymes A and B (Rodriguez-Garcia et al., 2020; Shen et al., 2022). Additionally, CTLs cytotoxicity in women varies within the menstrual cycle, diminishing during the secretory or postovulatory phase from day 14 to day 28 (Tsolova et al., 2022; White et al., 1997). Therefore, age plays a significant role, especially for women, who experience more fundamental changes in the hormone levels than men which might render them as an unfavorable study object.

However, only a few changes in IL-2 signaling are known with women, unlike men, showing a sex-specific upregulation of STAT5B post-vaccination. Similarly, in rats, females exhibit a more pronounced expression of STAT3 upon viral challenge, both of which are critical for IL-2 signaling (Figure 1) (Hannah et al., 2008; Klein et al., 2010). Moreover, female participants and rodents exhibited increased IL-2 plasma levels and secretion following infection or stimulation (Davila & Kelley, 1988; Klingström et al., 2008; Kosyreva et al., 2020), indicating that IL-2 signaling is enhanced in females on multiple levels. Additionally, IL-2-mediated proliferation of type 2 innate lymphoid cells (ILC2) was found to be enhanced in adult female mice compared to their male counterparts and a transcriptional role of testosterone has been implicated (Cephus et al., 2017; C. Wang et al., 2020). However, further research in necessary to investigate the interplay between sex hormones and IL-2 signaling.

Regarding the definition of sex and gender, which one of these parameters is analyzed in human studies is disputable and difficult to determine. In the human system, sex and gender are oftentimes connected and directly dependent on each other. For simplicity purposes, this study only uses the terms of ‚female' and ‚male', indicating the effect of sex though the effect of gender cannot be excluded.

1.7 Aim of the Study

The objective of this study is to deepen our understanding of the distinct molecular and cellular mechanisms that define the cytotoxic activity of CTLs and NK cells, focusing on the differential effects attributed to sex and age across both mouse and human subjects. Despite existing evidence underscoring the significant roles sex and age play in modulating immune responses, detailed insights into how these factors specifically influence the functionality of CTLs and NK cells are lacking. This research aims to analyze these lymphocytes isolated from individuals of varying sexes and ages, thereby shedding light on sex-related variances within age groups and unveiling the dynamics of immune change throughout the aging process—a phenomenon where notable shifts in immune functionality have been preliminarily observed (Angenendt et al., 2020; Verschoor et al., 2023; Zöphel et al., 2022).

Historically, immunological studies have often overlooked the necessity of including both sexes or have failed to specify the sex of animal models used (Beery & Zucker, 2011), potentially obscuring vital sex-specific insights. Moreover, research investigating the underlying cause for sex-specific differences is mostly restricted to alterations in sex hormone abundance. In addressing these oversights, this study will not only focus on identifying differences in cell distribution as influenced by sex and age but will also explore functional changes, particularly in cell activation, exhaustion, and the production and secretion of effector molecules. Through a combination of cellular and molecular assays, this study aims to provide new insights into the intricate interplay between sex, age, and immune function, determining their significance in modulating immune responses and influencing susceptibility for various diseases.

2 Materials and Methods

2.1 Materials

2.1.1 Antibodies

Table 2: List of used flow cytometry and cell culture antibodies with additional information regarding reactivity, fluorochrome, clone, manufacturer, and ordering number.

Specificity	Reactivity	Fluorochrome	Clone	Manufacturer	Ordering No.
CD3ε	mouse	PerCP	145-2C11	BioLegend	100326
CD3	human	PerCP	SK7	BioLegend	344814
CD4	mouse	Pacific Blue™	RM4-5	BioLegend	100531
CD4	human	Brilliant Violet 421™	SK3	BioLegend	344632
CD8	mouse	FITC	53-6.7	BioLegend	100706
CD8	mouse	APC/Cyanine7	53-6.7	BioLegend	100713
CD8	mouse	PerCP	53-6.7	BioLegend	100731
CD8	mouse	Pacific Blue™	53-6.7	BioLegend	100725
CD8	mouse	APC	53-6.7	BioLegend	100711
CD8	human	FITC	SK1	BioLegend	344704
CD11b	mouse/ human	PE	M1/70	BioLegend	101208
CD11b	mouse/ human	PerCP	M1/70	BioLegend	101230
CD14	human	PE/Cyanine7	M5E2	BioLegend	301814
CD16	human	PE	B73.1	BioLegend	360704
CD19	human	Brilliant Violet 421™	HIB19	BioLegend	302234
CD25	mouse	Vio® Bright FITC	REA568	Miltenyi Biotec	130-120-172
CD27	mouse/ rat/human	APC	LG.3A10	BioLegend	124212
CD27	mouse/ rat/human	APC/Cyanine7	LG.3A10	BioLegend	124226
CD44	mouse/ human	PE	IM7	BioLegend	103024
CD44	mouse/ human	FITC	IM7	BioLegend	103005

CD45	human	Brilliant Violet 510™	HI30	BioLegend	304036
CD56	human	APC	HCD56	BioLegend	318310
CD62L	mouse	APC	MEL-14	BioLegend	104412
CD62L	mouse	Pacific Blue™	MEL-14	BioLegend	104424
CD69	mouse	PE-Vio® 770	REA937	Miltenyi Biotec	130-115-577
CD94 (NKG2A)	mouse	PE/Cyanine7	16A11	BioLegend	142809
CD94 (NKG2A)	human	PE/Cyanine7	S19004C	BioLegend	375114
CD122	mouse	APC	TM-ß1	BioLegend	123214
CD161 (NK1.1)	mouse	Pacific Blue™	PK136	BioLegend	108722
CD161 (NK1.1)	human	Pacific Blue™	HP-3G10	BioLegend	339925
CD152 (CTLA4)	mouse	Brilliant Violet 421™	UC10-4B9	BioLegend	106312
CD279 (PD-1)	mouse	PerCP Vio® 700	REA802	Miltenyi Biotec	130-111-957
CD279 (PD-1)	human	Brilliant Violet 421™	NAT105	BioLegend	367421
CD314 (NKG2D)	mouse	APC	CX5	BioLegend	130212
CD314 (NKG2D)	human	FITC	1D11	BioLegend	320819
CD335 (NKp46)	mouse	Brilliant Violet 510™	29A1.4	BioLegend	137623
CD335 (NKp46)	human	Brilliant Violet 510™	9E2	BioLegend	331923
Granulysin	human	PE/Cyanine7	DH2	BioLegend	348011
Granzyme B	mouse/ human	FITC	QA16A2	BioLegend	372206
IL-2	mouse		JES6-1A12	BioLegend	503706
KLRG1	mouse/ human	PE	2F1/KLRG1	BioLegend	138407

KLRG1	human	APC/Cyanine7	SA231A2	BioLegend	367723
Ly49A	mouse	FITC	YE1/48.10.6	BioLegend	116805
Perforin	mouse	Pacific Blue™	S16009B	BioLegend	154407
Perforin	human	Pacific Blue™	B-D48	BioLegend	353305
Perforin	human	Brilliant Violet 510™	dG9	BioLegend	308119
TIGIT	human	PE-Vio® 770	REA1004	Miltenyi Biotec	130-116-937
TIM-3	human	Vio® Bright FITC	F38-2E2	Miltenyi Biotec	130-126-004

2.1.2 Reagents

Table 3: Table of used reagents with information regarding manufacturer and ordering number.

Reagent	Manufacturer	Ordering No.
Acridine Orange / Propidium Iodide Stain	BioCat	F23001-LG
Ammonium chloride	Sigma	A9434
β-Mercaptoethanol	Sigma	M3148
BD FACS™ Clean Solution	BD Biosciences	340345
BD FACSFlow™ Sheath Fluid	BD Biosciences	342003
BSA Fraction V	PAN_Biotech	P06-1391500
Calcein-AM	Invitrogen	C3100MP
DMSO	Sigma Aldrich	15677730
EDTA	Sigma	E9884
FCS	Fisher Scientific	10270-106
HEPES	Sigma	H7523
Histopaque®-1083	Merck Sigma Aldrich	10831-100ML
IL-2	Miltenyi Biotec	130-097-748
IL-15	Miltenyi Biotec	130-095-760
Isopropanol	Fisher Scientific	327270010
Lymphocyte Separation Medium	PromoCell	C-44010
Sodium acide	Sigma Aldrich	S8032
Paraformaldehyde	Polyscience	00380-250
Penicillin/streptomycin	Merck Sigma Aldrich	P4333-100ml
Poly-L-ornithin	Cell Systems	5172-5MG
Potassium bicarbonate	Sigma	237205

ProLong™ Glass Antifade Mountant	Invitrogen™	P36984
Saponin	Sigma Aldrich	S7900-25G
TritonX-100	Fisher Scientific	10287923
TRIzol™ Reagent	Fisher Scientific	15596018

2.1.3 Media and Buffer

Table 4: Table of used media and buffers with information regarding manufacturer and ordering number.

Media	Manufacturer	Ordering No.
AIM-V	Fisher Scientific	12055-083
DPBS (1x)	Fisher Scientific	14190-094
HBSS	SIGMA	H6648-500mL
RPMI-1640	Fisher Scientific	21875-034

2.1.4 Solutions

Table 5: Table of used solutions with information regarding its components and composition.

Solution Name	Component	Concentration
Isolation Buffer	DPBS	
	BSA	0.5%
	EDTA	2 mM
Erythrocyte Lysis Buffer	$KHCO_3$	9.99 mM
	NH_4Cl	155 mM
	EDTA	130 µM
	pH 7.3	
Lysis Control Solution	AIMV	
	Triton X-100	10 %
FACS Buffer	DPBS	
	BSA	0.5 %
FCS FACS Buffer	DPBS	
	BSA	0.5 %
	FCS	5 %
Intracellular Staining Buffer	DPBS	
	BSA	0.5 %
	FCS	5 %
	Saponin	0.1 %
	NaN3	0.007 %

	pH 7.4	
HEPES Buffer	dH2O	
	HEPES	10 mM
	NaCl	150 mM
	pH 7.4	
AIM-V (complete)	AIMV	
	FCS	10 %
	β-Mercaptoethanol	50 µM
RPMI (complete)	RPMI-1640	
	FCS	10 %
	P/S	1 %

2.1.5 Kits

Table 6: Table of used kits with information regarding manufacturer and ordering number.

Kit	Manufacturer	Ordering No.
Dynabeads™ Untouched™ Mouse CD8 Cells Kit	Fisher Scientific	11417D
Dynabeads™ Mouse T-Activator CD3/CD28 for T-Cell Expansion and Activation	Fisher Scientific	11453D
LEGENDplex™ Human CD8/NK Panel (13-plex) w/ VbP	BioLegend	741065
LEGENDplex™ MU Th Cytokine Panel Mix and Match	BioLegend	740368 740065 740059 740061 740054 740738 740055 740057 740066 740446 741045 741046
NK Cell Isolation Kit, human	Miltenyi Biotec	130-092-657
NK Cell Isolation Kit, mouse	Miltenyi Biotec	130-115-818

2.1.6 Equipment and Software

Table 7: Table of used equipment with information regarding software and manufacturer.

Equipment	Description/Software	Manufacturer
Cell Counter	Z2	Beckman Coulter
	LUNA-FL™ Dual Fluorescence Cell Counter	Logos Biosystems
Centrifuge	Universal 32R	Hettich
	Centrifuge 5810R	Eppendorf
	Centrifuge 5418	Eppendorf
	Centrifuge 5415R	Eppendorf
Cell Isolation	autoMACS® Pro Separator	Miltenyi Biotec
Flow Cytometer	FACSVerse™	BD Biosciences
	FACSuite™ Software	BD Biosciences
	MACSQuant® Analyzer 16	Miltenyi Biotec
	MACSQuantify™ Software	Miltenyi Biotec
	FlowJo™ Software V10.8.1	BD Biosciences
Incubator	CO2 Inkubator Heracell 150i	Thermo Scientific
	CO2 Inkubator Heracell 150	Heraeus
Magnet	DynaMag™ - 15	Invitrogen
	DynaMag™ - 50	Invitrogen
	Magnetic Beads Separator	Chang Biosciences
	QuadroMACS™ Separators	Miltenyi Biotec
	OctoMACS™ Separators	Miltenyi Biotec
Microscope	LSM880	Zeiss
Photometer	NanoDrop™ OneC	Fisher Scientific
Plate Reader	CLARIOstar Plus	BMG Labtech

	Reader Control MARS data analysis	BMG Labtech BMG Labtech
Steriel Benches	HERAsafe	Thermo Fisher Scientific
Vortexer	Vortex Genie 2	Scientific Industries
Other Software	Excel 2019 Word 2019 PowerPoint 2019 LEGENDPlex™ V2023-02-15 Prism V10.1.2	Microsoft Microsoft Microsoft Qognti, Inc. GraphPad

2.1.7 Consumables

Table 8: Table of used consumables with information regarding manufacturer and ordering number.

Consumable	Manufacturer	Ordering No.
96 well black clear tissue culture treated plate flat bottom with lid	VWR	734-2480
96-Well Microplatte, PP, V-Boden	Greiner Bio One	651201
B Braun™ Injekt Solo Cone Syringes	Fisher Scientific	4606027V
Corning™ Cell Strainers (40 µm)	VWR	734-2760
Corning™ Cell Strainers (70 µm)	VWR	734-2761
LeucoSep tubes	Greiner Bio One	227290
LUNA™ Cell Counting Slides	Logos Biosystems	L12001
MACS SmartStrainers, 30 µm	Miltenyi Biotec	130-098-458
Sample cup for Coulter	Sarstedt	73.650.500
S-Monovette® EDTA K3E, 9 ml	Sarstedt	02.1066.001

2.1.8 Tumor Cell Lines

Table 9: Table of used tumor cell lines with information regarding provider and origin.

Cell Line	Provided by	Origin
P815	ATCC TIB-64 / DSMZ ACC1	*Mus musculus*; mastocytoma
YAC-1	Leslie Elsner, Institut für Zelluläre & Molekulare Immunologie; Göttingen University	*Mus musculus*; lymphoma

K-562	DSMZ ACC 10	*Homo sapiens*; chronic myeloid leukemia

2.1.9 Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) are mononuclear cells, such as T and NK cells, found in peripheral blood. They can be separated from other components of the blood like plasma, erythrocytes, or granulocytes via density centrifugation. In this work, human blood was either received either from the blood donation service at the local university clinic in cooperation with Prof. Dr. Eichler or from a local general practitioner (Dr. Juliane Wendorf). The identity of all donors was anonymized, but they were considered healthy; the only information provided was the sex and the age cohort.

From the blood donation we received the remains after thrombocyte donation in leukocyte reduction system (LRS) chambers. The work was approved by the ethics committee of the Ärztekammer des Saarlandes (83/15; Prof. Dr. Rettig-Stürmer). From the general practitioner office, we received 27 mL of whole blood in EDTA tubes. The work was approved by the ethics committee of the Ärztekammer des Saarlandes (62/23; Prof. Dr. Grundmann). The samples were used to isolate and stimulate primary human NK cells.

2.2 Methods

2.2.1 Cell Culture of Tumor Cell Lines

Tumor cell lines P815, YAC-1, and K-562 were all cultured and maintained under sterile conditions in RPMI-1640 media complemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (P/S) (see Table 5). All cells were passaged every two to three days und subsequently replaced every three to four months. All cells were cultured at 37°C and 5% CO_2. The cell culture of tumor cell lines was kindly performed by the technician team including Sandra Janku, Cora Hoxha, Gertrud Schwär, and Kathrin Förderer.

2.2.2 Murine System

C57BL6/J (wildtype) mice were purchased from Charles River Laboratories and were kept in-house under specific-pathogen-free (SPF) conditions. Adult mice are defined as mice between 14 and 26 weeks of age and elderly mice were older than 70 weeks. Both sexes were analyzed individually to identify sex-specific differences in immunity.
All animal experiments were approved by local authorities and conducted in strict compliance with national and European guidelines for the welfare of laboratory animals.

2.2.2.1 Isolation of Murine Splenocytes

Mice were sacrificed via cervical dislocation after CO_2 anesthesia. Spleens were removed and strained through a 40 µm or 70 µm cell strainer into isolation buffer (see Table 5). After centrifugation at 240g for 8 min or 300g for 10 min, erythrocytes were removed by incubation with 2 mL erythrocyte lysis buffer for 2 min (see Table 5). Lysis was stopped by adding 48 mL isolation buffer. Living cell count was determined using acridine-orange/PI-staining and the LUNA-FL™ Dual Fluorescence Cell Counter. Isolations not carried out by me were conducted by Sandra Janku.

2.2.2.2 Isolation of Mononuclear Cells from Mouse Blood

Murine mononuclear cells (MNCs) were isolated using a density centrifugation. To separate the cells from the remaining blood components, Histopaque-1083™ was used. Separation media such as Histopaque-1083™ or Lymphocyte Separation Medium 1077 allow separation of cells based on density. High-density cells like erythrocytes pass through, while lymphocytes, having a lower density settle beneath the plasma, following the principles of density gradient centrifugation (McNamara, 2019). Histopaque-1083™, with a density of 1.083 g/mL, is optimized for use with rodent blood as stated by the manufacturer.

For blood collection, mice were sacrificed via cervical dislocation after CO_2 anesthesia. Immediately after sacrifice, the thorax and abdomen were opened and perfusion medium HBSS/4 mM EDTA was injected directly into the left heart chamber using a 21G butterfly needle. Blood was collected from the vena cava either using a 21G butterfly or a transfer pipette.
The blood was then carefully layered over an equal volume of Histopaque®-1083 in a 15 mL tube and centrifuged for at 400g for 30 min without acceleration or brake, according to the manufacturers protocol. The interface containing the leukocytes was transferred into a new reaction tube and washed with isolation buffer. After centrifugation at 250 g for 10 min, erythrocytes were lysed as described before for one minute only. Following another washing step, cells were resuspended in isolation buffer and counted, using the previously used method.

2.2.2.3 Isolation of Murine CTL

Murine CTLs from wildtype mice were isolated using Dynabeads™ Untouched™ Mouse CD8 Cells Kit (Invitrogen™) according to manufacturer protocol. Briefly, splenocytes were resuspended in isolation buffer, FCS, and the antibody cocktail. After a 20 min incubation, cells were washed once with isolation buffer and mixed with magnetic beads. The cell suspension was then incubated for another 15 min and placed within the magnet. The supernatant was transferred into a new tube and the beads were washed three times. The tube containing the cell suspension was again placed in the magnet and the supernatant was transferred into a new tube. Cells were then centrifuged, resuspended in a small volume of AIM-V/10% FCS, and counted using the Z2 cell counter. Isolations were performed with the help of Sandra Janku.

2.2.2.4 Stimulation of Murine CTLs

CTLs from wildtype mice were resuspended in AIM-V10% FCS/50 µM ß-mercaptoethanol with a final cell density between 0.8 - $1*10^6$ cells per mL. Dynabeads Mouse T-Activator CD3/CD28 for T-Cell Expansion and Activation were added in a cell-to-bead ratio of 5:4.
To assess the influence of IL-2 on CTL stimulation, 100U, 10U, or 0U of recombinant human IL-2 was added for 3 days. To neutralize autocrine IL-2, 10 µg anti-mouse IL-2 was added. The effect of the intermediate-affinity IL-2/IL-15 receptor was tested by adding 10 µg anti-mouse IL-2 and 100 ng recombinant human IL-15. Cells were maintained at 37°C and 5% CO_2.

2.2.2.5 Isolation Murine NK Cell

To isolate NK cells from wildtype mice splenocytes and MNCs the NK cell isolation kit, mouse (Miltenyi Biotec) was used according to manufacturer's protocol. Like CTLs, splenocytes were first incubated with the antibody cocktail, washed once with isolation buffer, and then incubated with the MicroBeads. LS or MS columns were used for magnetic separation in the QuadroMACS™ Separator or OctoMACS™ Separator of NK cells from splenocytes and MNCs, respectively. After magnetic separation, columns were washed once with isolation buffer to further enhance the yield. Cells were counted using acridine-orange/PI-staining and the LUNA-FL™ Dual Fluorescence Cell Counter. Isolations not carried out by me were conducted by Sandra Janku.

2.2.2.6 Stimulation of Murine NK Cells

Isolated NK cells were cultured in RPMI-1640 supplemented with 10% FCS, 1% penicillin/streptomycin, and 50 µM ß-mercaptoethanol. Different concentrations of IL-2 were tested, including 1000U, 500U, and 100U of recombinant human IL2. NK cells isolated from MNCs were consistently stimulated with only 100U IL-2. The cells were seeded at a density of $1*10^6$ cells per mL and cultured overnight at 37°C and 5% CO_2.

2.2.3 Human System

For human blood donors, samples were analyzed from healthy individuals aged 18 to 74 years. The donors were divided into 10-year cohorts (18-29, 30-39, 40-49, 50-60, 60+). Both genders were analyzed individually to identify gender-specific differences in immunity.

2.2.3.1 Isolation of Peripheral Blood Mononuclear Cells

In this study, blood samples were provided either in LRS chambers or in EDTA-coated blood collection tubes. To access the blood, the LRS chambers were cut open at both sides. Following the collection of all blood, the LRS chambers were rinsed with HBSS to enhance the yield. Blood from the EDTA-coated blood collection tubes required initial centrifugation for 10 min at 500g with reduced braking to separate the plasma. After the plasma removal, blood from the three EDTA coated blood collection tubes was first pooled into a new reaction tube, followed by rinsing with HBSS to improve the yield.

For isolation, blood was layered over Lymphocyte Separation Medium with a density of 1077 g/mL. After centrifugation for 30 min at 450g without using acceleration or braking, the remaining plasma was discarded, and the leukocyte layer was transferred into a new reaction

tube. Leukocytes were then washed with HBSS to remove excessive separation medium and centrifuged at 250g for 15 min. The supernatant was discarded, and the cells were incubated with erythrocyte lysis buffer for one minute. Lysis was stopped by adding HBSS. To remove thrombocytes, leukocytes underwent centrifuged for 10 min at 130g, a step repeated once for the blood obtained from the LRS chambers. Finally, cells were then resuspended in DPBS/0.5% BSA and strained through a 40 µm cell strainer. Living cell count was determined using acridine-orange/PI-staining with the LUNA-FL™ Dual Fluorescence Cell Counter. Samples from the LRS chambers were kindly isolated by our technicians Carmen Hässig and Kathleen Seelert.

2.2.3.2 Isolation of Human NK Cells from PBMC

To isolate NK cells from human PBMCs, the NK cell isolation kit from Miltenyi Biotec was used according to manufacturer's protocol. Briefly, PBMCs were centrifuged at 300g for 10 min at 4°C and then resuspended in the appropriate volume of DPBS/0.5% BSA. If cell numbers were sufficient, antibody labeling, and separation processes were automated using the autoMACS® Pro Separator. For smaller cell numbers, the cells were first incubated with first the antibody mix, followed by incubation with magnetic beads. The separation was conducted using LS columns in the QuadroMACS™ Separator. Cells counting was performed using the Z2 cell counter. Isolations not carried out by me were conducted by Kathrin Förderer, Gertrud Schäfer, Carmen Hässig or Sandra Janku.

2.2.3.3 Stimulation of Human NK Cells

Isolated NK cells were resuspended in RPMI-1640 supplemented with 10% FCS, 1% penicillin/streptomycin with a cell density of 2-2.5*10^6 cells per mL. Cells were stimulated either without IL-2 or with 50U recombinant human IL-2 overnight at 37°C and 5% CO_2.

2.2.4 General Methods

2.2.4.1 Real Time Killing Assay

To determine cytotoxic efficiency of isolated killer cells, the real time killing assay was employed, as previously described (Zöphel et al., 2022). The calcein-based killing assay, developed in our laboratory allows the quantification of cytotoxicity in a time-dependent manner (Kummerow et al., 2014).

Therefore, tumor target cells were stained for 15 min with 500nM calcein-AM in AIM-V/10 mM HEPES. In the murine system, P815 and YAC-1 were utilized for CTLs and NK cells, respectively, while K-562 were used for human NK cells. Following washing, the cells were

diluted in AIM-V10 mM HEPES to a density of $1.25*10^5$ cells per mL. Subsequently, $2.5*10^4$ cells were seeded into a black 96- or 384-well plate with clear bottom and incubated for at least 20 min in the CLARIOstar Plus plate reader to allow cells to settle at the bottom. Unstained effector cells were then gently added to the wells. For murine CTLs the effector-to-target cell ratio was 10:1, and for murine and human NK cells 5:1. The loss of fluorescence was measured over 4 hours, with measurements taken every 10 min at 37°C and 5% CO_2.

2.2.4.2 Flow Cytometry

For the general and detailed identification and characterization of effector cells, flow cytometry, allowing for the simultaneous quantification of multiple markers using fluorochrome-coupled antibodies. These fluorochromes are then excited by different laser wavelengths. In this study, two different flow cytometers were used. The first was a FACSVerse™, equipped with three lasers at 405 nm (detectors: 450/40, 525/50), 488 nm (detectors: 530/30, 575/26, 670/14, 695/40, 780/60), and 640 nm (detectors: 660/20, 780/60), operated using FACSuite™ software. The second was a MACSQuant® 16 Analyzer, also featuring lasers at 405 nm (detectors: 450/50, 525/50, 579/34, 615/20, 667/30), 488 nm (detectors: 525/50, 579/34, 615/20, 667/30, 725/40, 785/62), and 640 nm (detectors: 667/30, 725/40, 785/62), with MACSQuantify™ as the associated software. Compensation for multi-color panels was performed and managed through the specific software provided with each machine. Gates were set using fluorescence minus one (FMOs) and unstained control cells. Stainings were performed with the help of Sandra Janku.

2.2.4.2.1 Cell Surface Flow Cytometry Staining

For cell surface staining, 0.1 - $1*10^6$ cells were centrifuged at 400g for 4 min and resuspended in 50-100 µL FACS buffer (refer to Table 5). Antibodies, as specified in Table 10 to Table 18, were added and incubated for 15 min in the dark at room temperature (RT). To wash the cells, 1 mL FACS buffer was added, and the cell suspension was centrifuged again. Finally, cells were resuspended in 200 µL FACS buffer and kept at 4°C until measurement, which was performed on the same day. Measurements were conducted using either the FACSVerse™ or the MACSQuant® Analyzer 16, as detailed in the table descriptions. All analyses were performed using FlowJo™ software.

Table 10: CD8 panel. Table of used antibodies to identify different T cell subtype populations within murine splenocytes and isolated CTLs. Measurement was performed at the FACSVerse™.

Antibody	Fluorochrome	Amount

CD3	PerCP	2.5 µL
CD4	Pacific Blue™	1 µL
CD8	FITC	2 µL
CD44	PE	3 µL
CD62L	APC	2.5 µL

Table 11: Activation panel. List of used antibodies to determine activation status of isolated murine CTLs. Measurement was performed at the MACSQuant® Analyzer 16.

Antibody	Fluorochrome	Amount
CD8	Pacific Blue™	2 µL
CD44	PE	3 µL
CD62L	APC	2.5 µL
CD25	Vio® Bright FITC	2 µL
CD69	PE-Vio® 770	2 µL

Table 12: CD122 panel. List of used antibodies to determine CD122 expression on isolated murine CTLs. Measurement was performed at the FACSVerse™.

Antibody	Fluorochrome	Amount
CD44	FITC	2 µL
CD62L	Pacific Blue™	2.5 µL
CD122	APC	1.5 µL

Table 13: Exhaustion panel (mouse). List of used antibodies to determine exhaustion status of isolated murine CTLs. Measurement was performed at the MACSQuant® Analyzer 16.

Antibody	Fluorochrome	Amount
CD8	APC/Cyanine7	5 µL
CD44	PE	3 µL
CD62L	APC	2.5 µL
PD-1	PerCP-Vio® 700	2 µL
CTLA4	Brilliant Violet 421™	5 µL

Table 14: NK panel. List of used antibodies to identify different NK cell subtype populations within murine splenocytes and isolated NK cells. Measurement was performed at the FACSVerse™.

Antibody	Fluorochrome	Amount
CD3	PerCP	2 µL
NKp46	Brilliant Violet 510™	3 µL
NK1.1	Pacific Blue™	3 µL

CD11b	PE	1.5 µL
CD27	APC	1.5 µL

Table 15: NK Receptor panel (mouse). List of used antibodies to determine NK receptor expression on isolated murine NK cells. Measurement was performed at the FACSVerse™.

Antibody	Fluorochrome	Amount
NKp46	Brilliant Violet 510™	3 µL
NK1.1	Pacific Blue™	3 µL
CD11b	PerCP	3 µL
CD27	APC/Cyanine7	3 µL
KLRG1	PE	3 µL
NKG2D	APC	3 µL
NKG2A	PE/Cyanine7	3 µL
Ly49A	FITC	3 µL

Table 16: PBMC panel. List of used antibodies to identify different leukocyte subsets such as T cells, B cells and NK cells within isolated human PBMCs. Measurement was performed at the FACSVerse™.

Antibody	Fluorochrome	Amount
CD3	PerCP	0.5 µL
CD4	Brilliant Violet 421™	2 µL
CD8	FITC	2 µL
CD14	PE/Cyanine7	2 µL
CD19	Brilliant Violet 421™	2 µL
CD45	Brilliant Violet 510™	2 µL
CD56	APC	2 µL
CD16	PE	2 µL

Table 17: NK Receptor panel (human). List of used antibodies to determine NK receptor expression on isolated human NK cells. Measurement was performed at the FACSVerse™.

Antibody	Fluorochrome	Amount
CD3	PerCP	0.5 µL
CD16	PE	2 µL
CD56	APC	2 µL
NKG2A	PE/Cyanine7	2 µL
NKG2D	FITC	2 µL
KLRG1	APC/Cyanine7	2 µL
NKp46	Brilliant Violet 510™	2 µL

CD161	Pacific Blue™	2 µL

Table 18: Exhaustion panel (human). List of used antibodies to determine exhaustion status of isolated human NK cells. Measurement was performed at the MACSQuant® Analyzer 16.

Antibody	Fluorochrome	Amount
CD3	PerCP	0.5 µL
CD16	PE	2 µL
CD56	APC	2 µL
PD-1	Brilliant Violet 421™	5 µL
TIM-3	Vio® Bright FITC	2 µL
TIGIT	PE-Vio® 770	2 µL

2.2.4.2.2 Intracellular Flow Cytometry Staining

To quantify the abundance of effector molecules within the effector cells, they were stained for granzyme B, perforin and granulysin. In mouse CTLs, only granzyme B and perforin were stained, in human NK cells all the previously mentioned were stained with two different antibodies for perforin (clones BD48 and dG9). Before intracellular staining, a cell surface staining was performed as described in 2.2.4.2.1. Following the final wash, cells were resuspended in 4% paraformaldehyde (PFA) and incubated in the dark at RT for 15 min. Subsequently, cells were then centrifuged and washed once with FCS FACS buffer (see Table 5). Then, cells were resuspended in 50 µL intracellular staining buffer and incubated for 10 min in the dark at RT (see Table 5). After this incubation period, antibodies as specified in Table 19 and Table 20, along with 50 µL intracellular staining buffer were added. After 30 min in the dark, 1 mL FCS FACS buffer was added to wash the cells. Following centrifugation, cells were resuspended in 200 µL of FACS buffer and stored at 4°C until measurement, which was performed on the same day using the MACSQuant® Analyzer 16. All analyses were carried out using FlowJo™ software.

Table 19: Intracellular staining panel (mouse). List of used antibodies to quantify effector molecule abundance of isolated murine CTLs.

Antibody	Fluorochrome	Amount
CD44	PE	3 µL
CD62L	APC	2.5 µL
Granzyme B	FITC	5 µL
Perforin	Pacific Blue™	2 µL

Table 20: Intracellular staining panel (human). List of used antibodies to quantify effector molecule abundance of isolated human NK cells.

Antibody	Fluorochrome	Amount
CD16	PE	2 µL
CD56	APC	2 µL
Granulysin	PE/Cyanine7	5 µL
Granzyme B	FITC	5 µL
Perforin (BD48)	Pacific Blue™	5 µL
Perforin (dG9)	Brilliant Violet 510™	5 µL

2.2.4.2.3 Proliferation Assay

Proliferation of isolated murine CTLs was assessed using carboxyfluorescein succinimidyl ester (CFSE) staining. For this purpose, $1*10^6$ cells were stained for 15 min at RT in the dark in 1 mL of a 5 µM CFSE solution in PBS. The 5 mM CFSE stock solution was prepared by dissolving of lyophilized CFSE in 18 µL DMSO. After incubation, at least twice the volume of DPBS was added to wash the cells. The cells were then centrifuged and resuspended in culture medium as detailed in section 2.2.2.4. Proliferation was evaluated after 48 hours. In addition, cells were stained with 3 µL PerCP-coupled anti-mouse anti-CD8 antibody, following the procedure described in section 2.2.4.2.1.

2.2.4.2.4 Cytokine Secretion

During culture and killing, effector cells can secrete various effector molecules and cytokines. A cost-effective and rapid method for quantifying these molecules is through BioLegend's LEGENDplex™. This bead-based assay allows for the simultaneous quantification for up to 14 analytes using flow cytometry, distinguishing analytes by the size and APC-fluorescence intensity of the beads. In this study, murine CTLs cell culture supernatant and human NK cell killing assay supernatants were analyzed. For murine CTLs, IL-2, IL-4, IL-6, IL-10, IL-17A, IL-22, IFN-γ, and TNF-α were quantified. For human NK cells, the assay tested for IL- 2, IL-4, IL-6, IL-10, IL-17A, IFN-γ, TNF-α, soluble Fas, soluble FasL, granzyme A, granzyme B, perforin, and granulysin.

Supernatants were collected from either cell culture or killing assay and centrifuged for 10 min at 14000g to remove cells and debris, then stored at -80°C until measurement. To quantify the analytes afterwards, a standard curve was prepared by reconstitution of the lyophilizted standard, which was stored at -80°C until use. A standard dilution series, with a dilution factor of 1:4, was prepared before the assay and treated similarly to the samples. For the sample preparation, supernatants were thawed, and 12.5 µL were mixed with an equal amount of

Assay Buffer and mixed biotin-coupled beads directly in the 96-well plate. The plate was then shaken overnight in the dark at 4°C. The following day, samples were washed twice in 1x Wash Buffer with centrifugations performed at 400g for 5 min. Then, 12.5 µL of Detection Antibodies was added and incubated for 1 hour in the dark while shaking. Subsequently, 12.5 µL of streptavidin-PE was directly added and incubated for an additional 30 min. After washing as previously described, samples were resuspended in 150 µL 1x Wash Buffer. Measurements were taken using the FACSVerse™ on the same day and analyzed with BioLegend's cloud based LEGENDPlex™ software.

2.2.4.3 Airyscan laser scanning microscopy (LSM)

Intracellular staining of isolated murine CTLs for microscopy was performed on day three of stimulation. Therefore, 3 - 4*10^6 cells were first washed, followed by centrifugation at 900 rpm for 5 min and subsequently stained with APC-conjugated anti-CD8 in PBS on ice. Following incubation for 15 min, cells were washed twice in PBS and centrifuged at 1200 rpm for 5 min at 4°C. The cells were then resuspended in 184 µL PBS and 92 µL of the cell suspension was added onto a 0.01% Poly-L-ornithin-coated coverslip each which was situated in a 4-well-plate. Following incubation for 5 min on ice, 500 µL 4% PFA solution was added and incubated for 20 min to fix the cells. Subsequently, the PFA was removed, and cells were washed thrice with 500 µL PBS. To permeabilize the cells, 500 µL 0.1% TritonX-100 in PBS was added and incubated for another 20 min in the dark. The triton solution was then removed, and the reaction was stopped by adding 500 µL 0.1% TritonX-100 in PBS/5% BSA and incubated for 20 min in the dark. In the meantime, the antibodies were prepared in TritonX-100 in PBS/5% BSA and placed onto parafilm. Following incubation, the coverslips were removed from the well plate and placed heads down onto the antibody mixture and incubated for 45 min in the dark. During this incubation, 500 µL PBS was transferred into a well of the 4-well plate and after incubation the coverslips were placed into these cells heads up. Cells were then washed thrice with 500 µL PBS. Finally, cells were mounted using ProLong™ Glass Antifade Mountant for at least overnight according to manufacturers' protocol.

High resolution Airyscan images were taken using the Zeiss LSM880 system. APC, FITC and Pacific Blue™ coupled antibodies were excited using a multi-line argon 488nm, HeNe 633nm and diode 405nm laser lines with 405 and 488/561/633 multi-beamsplitters, respectively. Similarly, BP570-620+ LP654 (APC) and BP420-480 + BP 495-550 (FITC, Pacific Blue 410) filters were used to collect the emission light and captured using the Airyscan detector. A pixel size of 35-42nm (at least 1.8x zoom) in the 16-bit mode through a Zeiss Plan-Apochromat 63x/1.4 Oil DIC objective with the pinhole set to at least 1.25 AU (corresponding to the FITC

channel). The detector gain was kept between 800 and 900V. Moreover, minimal clipping of intensity values within half of the detector range was achieved by adjustment of the laser power to allow sufficient headroom for image reconstruction (usually <1% relative power). These settings were set according to the 100U IL-2 condition of female CTLs and kept constant for all following samples. The scans were performed with a pixel dwell time of ~1µs and 2x mean averaging and Z-stacks with 0.18µm intervals throughout the cell were taken for all channels in a sequential manner. Raw Airyscan images were aligned from the individual detector elements, summed and Wiener filtered with the suggested settings in the Zeiss Zen Blue software before export. Representative images were imported into Fiji, background-subtraction was performed together with a reduction in dimensionality by maximum intensity projection. Lastly, images were further arranged using the Quickfigure. plug-in for displaying purposes. Microscopy measurements and subsequent analysis were kindly performed by Lukas Jarzembowski.

2.2.4.4 Statistical Analysis

All statistical analyses were performed using GraphPad Prism V10.1.2. Depending on the system, Student's t-test, two-way or three-way ANOVA were used. The calculated tests and sample size are detailed in the respective figure legends. All data shown are mean ± SEM if not stated otherwise. Significance levels were defined as followed: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

3 Results

3.1 Interleukin-2 Dependence of Murine CTLs across Age and Sex

Splenic CTLs are reliant on various cytokines during stimulation. Therefore, the influence of IL-2 and its family member IL-15 during activation and their effects on effector functions were investigated.

3.1.1 Cytotoxicity of Murine Primary CTLs under varying Cytokine Conditions

Interleukin-2 acts as a vital signal during T cell activation and has substantial impact on effector function (Williams et al., 2006). In a previous studies, we discovered that CTLs from elderly mice demonstrate enhanced killing kinetics due to increased effector molecule expression (Zöphel et al., 2022, 2023). However, the influence of IL-2 and IL-15 on CTLs effector functions across sexes and age has not been fully elucidated. Therefore, cytotoxicity of CTLs from adult and elderly mice of both sexes were analyzed after *in vitro* stimulation for three days (Figure 2).

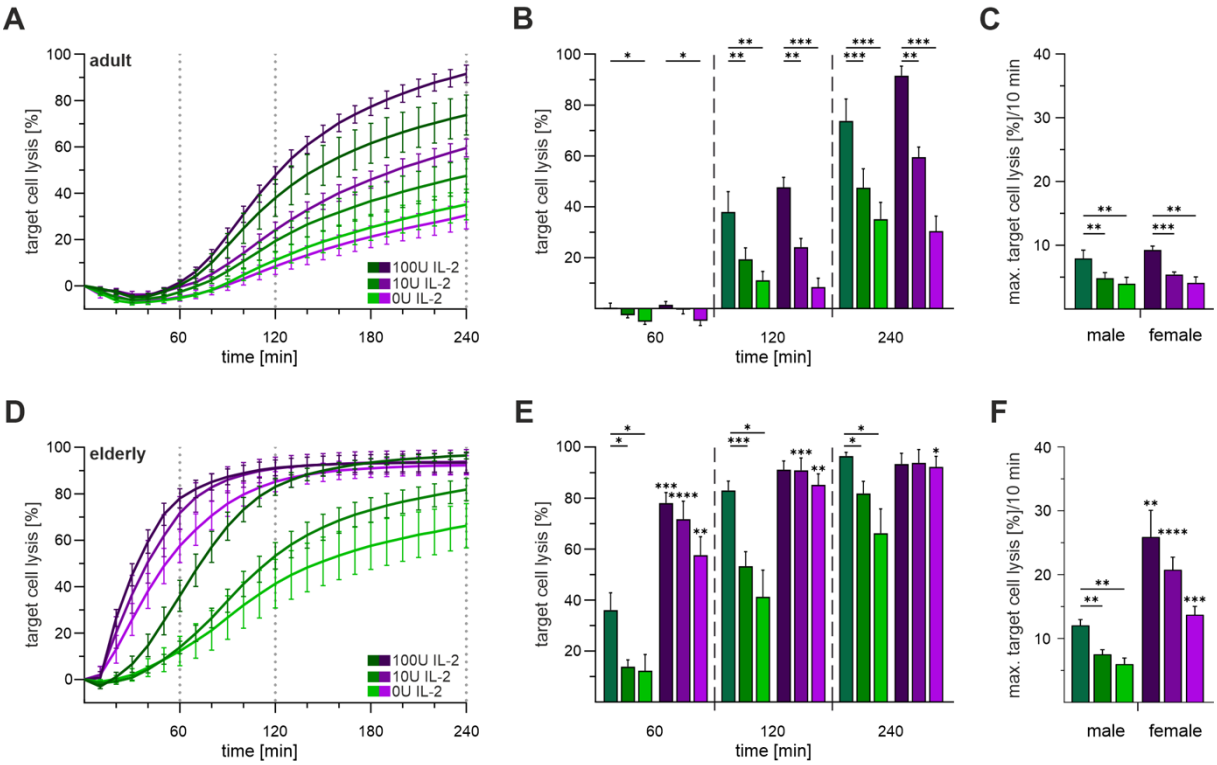


Figure 2: Cytotoxicity of elderly female mice CTLs remains unaltered by loss of external IL-2 during stimulation. Real-time killing assay from adult (**A-C**) and elderly (**D-F**) male (green) and female (purple) mice CTLs on day three of stimulation. CTLs were stimulated with varying IL2 concentrations (100U, 10U, or 0U) as depicted in shades of green and purple. P815 were utilized in a 10:1 effector-to-target cell ratio. Figures **A** and **D** depict killing kinetics over the time course of four hours. Figures **B** and **E** depict mean target cell lysis at 60 min, 120 min, and 240 min. figures **C** and **F** depict maximal target cell lysis per 10 min or killing rate. Significance depicted above bars indicate differences between sexes. Significance between conditions was calculated using paired ordinary one-way ANOVA and differences between sexes using unpaired Student's t-test. Data presented as mean ± SEM. Significance levels are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. *n = 5-6 per group.*

In adult mice CTLs (Figure 2A-C), cytotoxicity was gradually but significantly reduced with decreasing IL-2 concentrations in both sexes. End point lysis at 240 min decreased from 73% to 35% and 91% to 30% in male and female mice CTLs, respectively. Similarly, maximal target cell lysis decreased from 8% to 4% per 10 min and 9% to 4% per 10 min. Hence, adult mice CTLs displayed no discernible sex-specific differences in response to IL-2 loss.
Consistent with our previous findings, elderly mice CTLs exhibited steeper killing kinetics (Zöphel et al., 2022). Strikingly, CTLs from elderly female mice retained their cytotoxic potential regardless of IL-2 concentrations (Figure 2D-F). End point lysis at 240 min in these cells remained stable between 92% and 93%, whereas in elderly male mice, it fell from 96% (100U) to 66% (0U) indicating a gradual reduction in cytotoxicity with age in male. Thus, the differences in end point lysis between male and female CTLs became significant only at 0U IL-2, where mean target cell lysis at 60 min was notably higher in females compared to males. By 120 min, the mean target cell lysis for male mice CTLs stimulated with 100U IL-2 matched that of females and remained similar for the rest of the assay. Maximal target cell lysis decreased from 12% to 6% per 10 min in males and 26% to 14% per 10 min in females, highlighting the robustness of elderly female mice CTLs cytotoxic response to IL-2 depletion.

Despite the absence of supplemented IL-2, CTLs maintain the capability to execute cytotoxic functions. To investigate whether this could be attributed to autocrine IL-2 secretion, a neutralizing IL-2 antibody was utilized. With the exception of CTLs from elderly female mice, treatment with anti-IL-2 led to complete termination of cytotoxic activity (Figure 3). The addition of IL-15 aimed to assess the relevance of the intermediate-affinity IL-2/IL-15 receptor. While elderly female mice CTLs continued to exhibit effector function under anti-IL-2 treatment, their cytotoxicity dropped significantly compared to cells receiving IL-15 supplementation. End point lysis decreased from 94% to 45%, cytotoxicity more than halving the cytotoxicity (Figure 3E). Additionally, maximal target cell lysis saw an even stronger decline from 16% to 5% per 10 min (Figure 3F). This reduction in cytotoxicity was markedly pronounced across other cohorts.

In summary, CTLs from adult male and female mice display a profound reliance on IL-2, yet without noticeable sex-specific differences. Among elderly mice, only male CTLs reduced IL-2 dependency, with female CTLs showing complete independence from external IL-2, possibly due to possible autocrine IL-2 secretion. Nevertheless, the mechanism by which elderly female mice CTLs retain cytotoxic function in the absence of both external and autocrine IL-2, upon anti-IL-2 treatment, remains unclear. Interestingly, the cytotoxicity of cells stimulated with IL-15/anti-IL-2 and 100U IL-2 was comparable, suggesting the activation through the intermediate-affinity receptor alone is adequate for driving CTLs effector function.

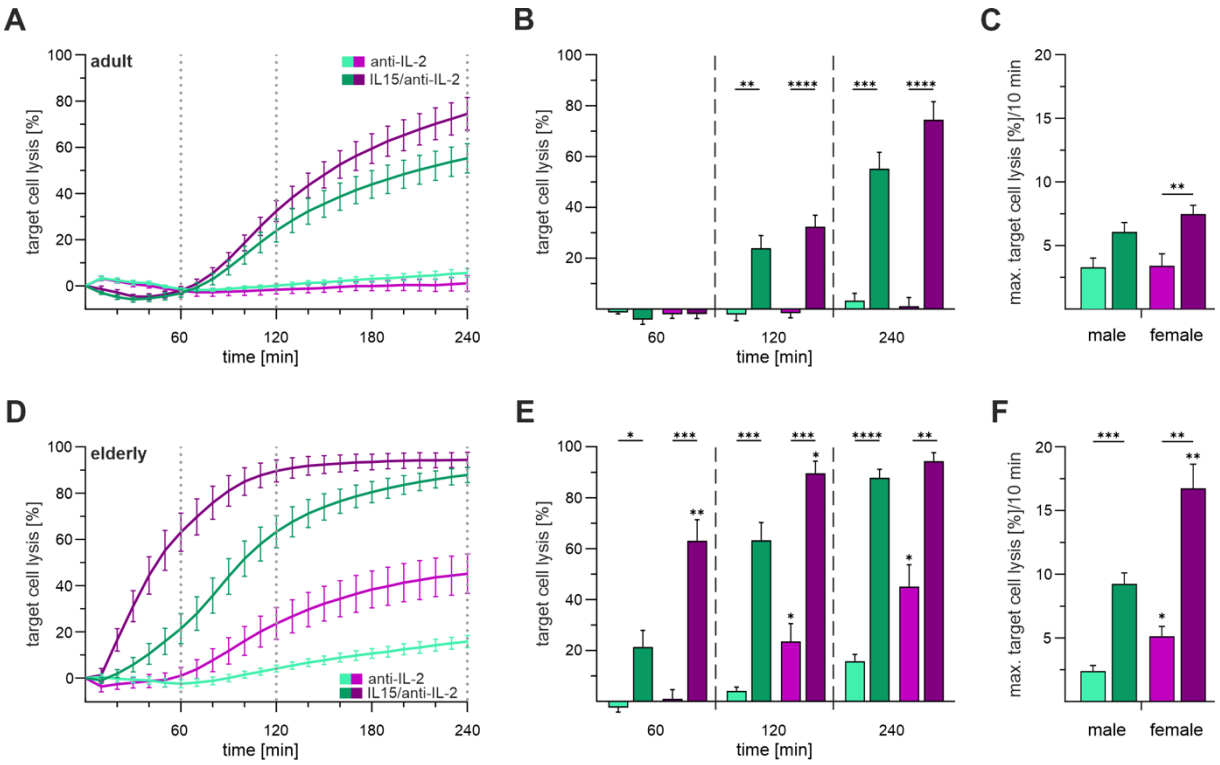


Figure 3: Ablation of autocrine IL-2 severely diminishes cytotoxicity across age and sex with IL-15 supplementation being able to reverse complete loss of IL-2. Real-time killing assay from adult (**A-C**) and elderly (**D-F**) male (green) and female (purple) mice CTLs on day three of stimulation. CTLs were stimulated with neutralizing IL-2 antibody with or without 100 ng IL-15 as depicted in shades of green and purple. P815 were utilized in a 10:1 effector-to-target cell ratio. Figures **A** and **D** depict killing kinetics over the time course of four hours. Figures **B** and **E** depict mean target cell lysis at 60 min, 120 min, and 240 min. Figures **C** and **F** depict maximal target cell lysis per 10 min or killing rate. Significance depicted above bars indicate differences between sexes. Significance between conditions was calculated using paired ordinary one-way ANOVA and differences between sexes using unpaired Student's t-test. Data presented as mean ± SEM. Significance levels are indicated as * $p < 0.05$, ** $p< 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. *n = 5-6 per group.*

3.1.2 IL-2-dependent Modulations of Viability, Proliferation, and Subtype Distribution

Besides its impact on effector function, the role of IL-2 in T cell differentiation, proliferation and viability has been extensively studied (Kaartinen et al., 2017; Rollings et al., 2018). However, little is known about the impact of age and sex on these parameters and were therefore analyzed in this regard.

Viability was assessed under all stimulation conditions across both age and sex (Figure 4A). Adult mice, CTLs stimulated with 100U IL-2 or IL-15/anti-IL-2 displayed the highest viability rates, exceeding 97%. Viability decreased to a minimum of 80% and 75% in male and female mice CTLs, with diminishing IL-2 concentrations, indicating a gradual decrease with no discernible sex difference. Elderly male mice also exhibited a gradual reduction in viability from 95% (100U IL-2) to 75% (anti-IL-2). In contrast, elderly female mice CTLs also showed a notable drop in viability to 78% when cells were treated with anti-IL-2, though their viability with 100U IL-2 (90%) and IL-15/anti-IL-2 (92%) treatments was significantly lower compared to males, suggesting a sex-specific response.

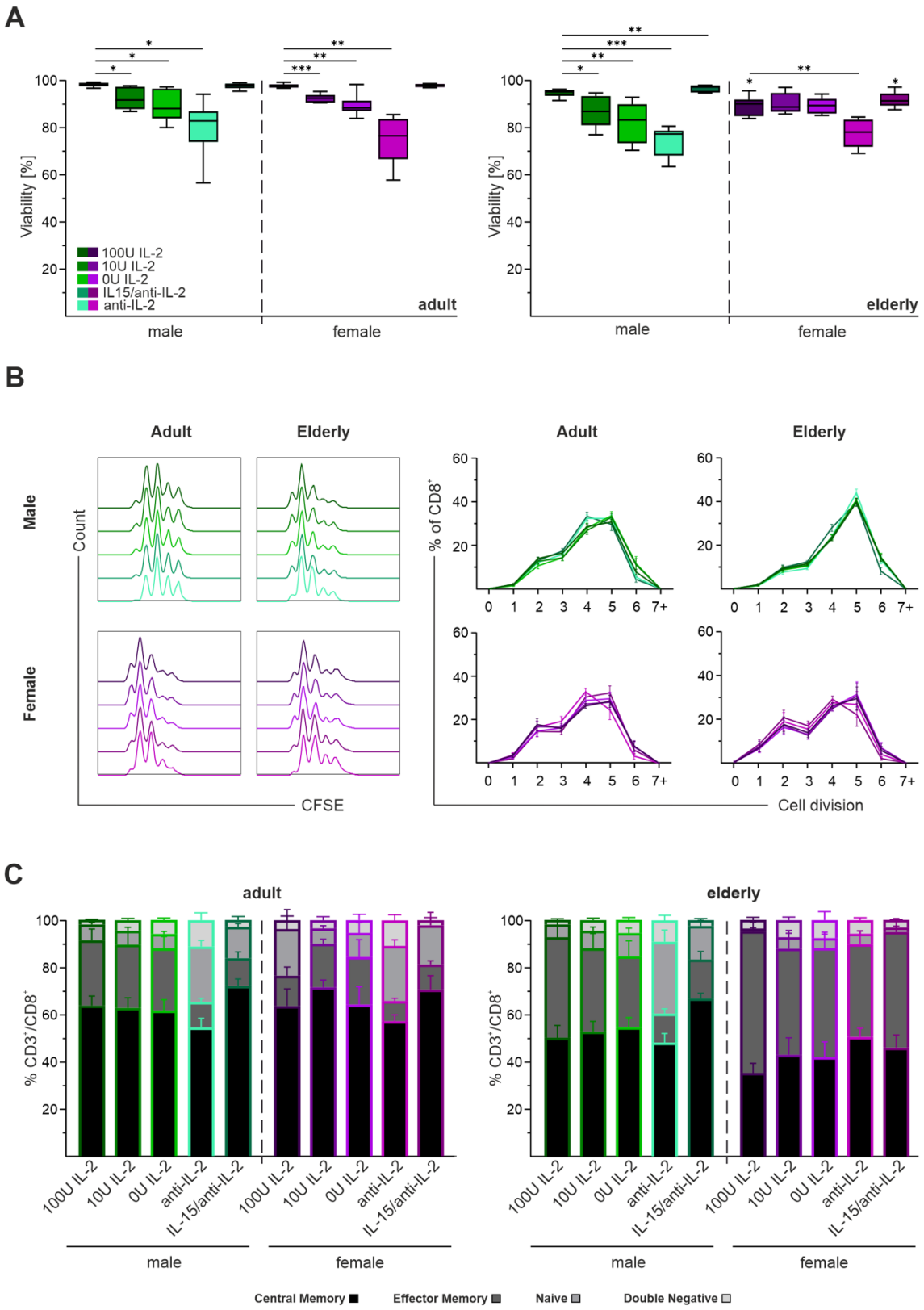


Figure 4: Interleukin-2 influences viability but not proliferation or subtype distribution. Viability (**A**) and flow cytometry stainings investigating proliferation (**B**) on day two of stimulation and CTL subtype distribution (**C**) of adult (left) and elderly (right) CTLs on day three of stimulation. All stimulation conditions were analyzed in both sexes with male mice CTLs depicted in shades of green and female mice in shades of purple. Significance depicted above bars indicate differences between sexes. Significance between conditions was calculated using paired ordinary one-way ANOVA and differences between sexes using unpaired Student's t-test. Tables of p-values of proliferation and subtype distribution are shown in supplementary data. Data presented as mean ± SEM. Significance levels are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. *n = 4-7 per group.*

To avoid the impact of cell viability on target cell lysis, the killing assays previously described were performed using living cell count numbers. This approach ensures that the killing assays accurately reflect the cytotoxic potential of equivalent numbers of viable cells, thereby circumventing any potential confounding effects of differential cell viability.

Proliferation data contrasts with several studies (Ghaffari et al., 2021; Han et al., 2016; Manjunath et al., 2001), showing almost no changes in proliferation with decreasing IL-2 concentrations (Figure 4 B, Supplementary table 21). Only treatments with anti-IL-2 and IL-15/anti-IL-2 led to a significant decrease of adult mice CTLs in divisions five and six, indicating an impaired proliferation. Similar relations can also be found in elderly mice CTLs, with an additional pronounced sex-specific increase in proliferation among male CTLs, as evident by increased percentage of cells in division five and six.

Subtype distribution was explored through the cell surface expression of CD44 and CD62L of $CD3^+CD8^+$ CTLs (Figure 4C) (Zöphel et al., 2022). Neither adult nor elderly mice CTLs showed significant IL-2- or IL-15-dependent differences in subtype distribution could be found (Supplementary table 22). Notably, female mice CTLs treated with anti-IL-2 or IL-15/anti-IL-2 displayed a significantly higher proportion of effector memory cells and lower count of naïve CTLs compared to males, suggesting a heightened activation state in female mice CTLs, though the underlying causes remain to be clarified.

3.1.3 Interleukin-2 is Crucial for Activation of CTLs

To elucidate the reasoning behind the differences observed in the previous sections, flow cytometry analyses were performed to determine the activation status of CTLs. CD69 is widely recognized as an early activation marker, while the onset of CD25 expression marks later stages of activation (Grégoire et al., 2019; Pieren et al., 2019). Additionally, CD122 is known to be constitutively expressed on activated T cells (Beltra et al., 2016). Hence, the presence of activation markers CD69, CD25 and CD122 was quantified across all experimental conditions and mouse cohorts.

In adult mice, a significant reduction in CD69 expression was observed, dropping from approximately 68.7% in conditions with 100U IL-2 and IL-15/anti-IL-2 to a low of 23% under decreasing IL-2 concentrations in both sexes (Figure 5A). Surprisingly, this decrease was not gradual but occurred abruptly. Elderly male mice CTLs mirrored this trend, exhibiting an instant reduction in CD69 expression (Figure 5B). While elderly female mice CTLs also showed a decrease in $CD69^+$ cells, it was not statistically significant compared to cells stimulated with 100U IL-2 (75% vs 59%). Moreover, under reduced IL-2 concentrations, elderly female mice

CTLs had a significantly higher amount of CD69$^+$ cells than their male counterparts. Overall, stimulation of CTLs with IL-15/anti-IL-2 resulted in the highest proportion of CD69$^+$ cells.

In contrast to CD69, CD25 expression showed a gradual decline across all mouse cohorts (Figure 5C/D). CD25 expression decreased from 99% to around 25% in all mice cohorts except elderly female mice. There, the quantity of CD25$^+$ cells only decreased to a minimum of 60.5% when treated with anti-IL-2. Elderly female mice CTLs consistently displayed a higher expression of CD25 under decreasing IL-2 concentrations.

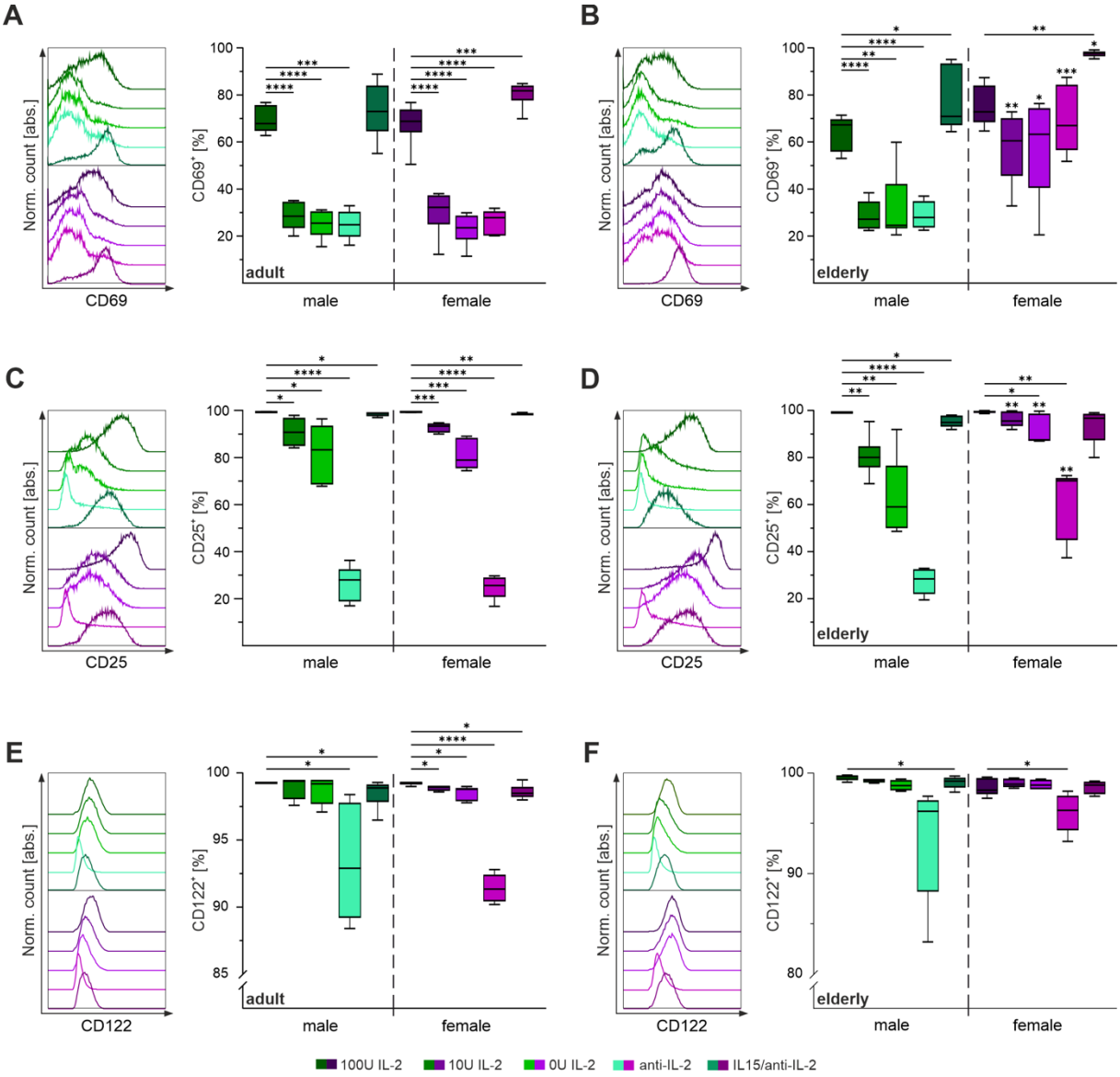


Figure 5: Elderly female mice exhibit higher expression of activation markers CD69 and CD25 but not CD122 under decreasing IL-2 concentrations. Flow cytometry analysis of CD69, CD25, and CD122 in adult (**A**/**C**/**E**) and elderly (**B**/**D**/**F**) mice, respectively. All stimulation conditions were analyzed in both sexes with male mice CTLs depicted in shades of green and female mice in shades of purple. Analysis was performed on day three of stimulation. Representative fluorescence profiles are shown in the left panels and corresponding statistical analysis in the right panels. Significance depicted above bars indicate differences between sexes. Significance between conditions was calculated using paired ordinary one-way ANOVA and differences between sexes using unpaired Student's t-test. Data presented as mean ± SEM. Significance levels are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. *n = 5-6 per group.*

CD122 expression remained consistently high across all conditions with at least 91.4% of CTLs being $CD122^+$. Still, in adult mice CD122 expression gradually decreased from around 99% to 93.4% and 91.4% for males and females, respectively (Figure 5E). Strikingly, adult female mice CTLs displayed a significant but still comparable decrease in CD122 expression to male mice CTLs. Similarly, CD122 expression in elderly male and female mice dropped to 93.4% and 96.1%, respectively. in elderly male and female mice. Although, despite $CD122^+$ CTLs levels were generally high, median fluorescence intensity (MFI) of CD122 expression showed a strong IL-2 dependency (Supplementary Figure 24). With declining IL-2 concentrations, CD122 MFI gradually decreased gradually in all cohorts except for elderly female mice CTLs, where it remained stable. Only CTLs treated with anti-IL-2 exhibited a significant decrease in CD122 MFI.

In summary, these findings indicate that CTLs from elderly female mice, stimulated under decreasing IL-2 concentrations, exhibit a higher activation status due to elevated levels of $CD69^+$ and $CD25^+$ cells, along with higher CD122 MFI. This increase in activation markers and therefore enhanced activation in elderly female mice CTLs could contribute to the improved effector function. The elevated expression of CD69 and CD25 in these CTLs implies that they may either be in an early phase of activation not reached by male mice CTLs or capable to maintain CD69 expression upon advancing later activation stages, as indicated by CD25 expression.

3.1.4 Expression of CTLA-4 and PD-1 Indicate the Exhaustion Stage of CTLs

Exhaustion is a physiological characterized by a gradual loss of immune cell function (Alahdal & Elkord, 2022; Gao et al., 2022; J. S. Yi et al., 2010). Identification of exhausted cells is still challenging since various markers are altered with exhaustion (Y. Wu et al., 2023). However, the dynamics between different exhaustion markers like PD-1 and CTLA4, especially under sub-optimal stimulation conditions remain ambiguous. To address this, the study evaluated the expression of PD-1 and CTLA4 across all experimental conditions and cohorts to shed light on how cellular exhaustion influences effector function.

CTLA4, known to be expressed on regulatory and activated T cells, competes with CD28 for its ligand is CD80/CD86 (Chan et al., 2014). Unlike CD28, which activates T cells, CTLA4 transmits an inhibitory signal, modulating the T cell response (M.-L. Alegre et al., 2001). Its expression typically peaks at around 48 hours post-activation before gradually declining (Deppisch et al., 2016; Shabrish et al., 2024).
Flow cytometry analysis showed that CTLA4 expression decreased from a maximum of 21.9% to a minimum of 3.1% with reduced IL-2 concentrations in both adult and elderly mice (Figure 5A/B). Additionally, supplementing IL-15, even after the neutralization of autocrine IL-2, did not

lead to an increase in CTLA4 expression. This suggests that IL-15 influence on T cell activation may operate independently of the inhibitory pathways associated with CTLA4 Furthermore, elderly female mice CTLs did not exhibit such a pronounced decrease in CTLA4 expression, only decreasing from 23.2% to 9.5%. Strikingly, elderly female mice CTLs treated with anti-IL-2 displayed significantly higher CTLA4 expression compared to their males (9.5% vs 3.1%), a finding that seems at odds with cytotoxicity data.

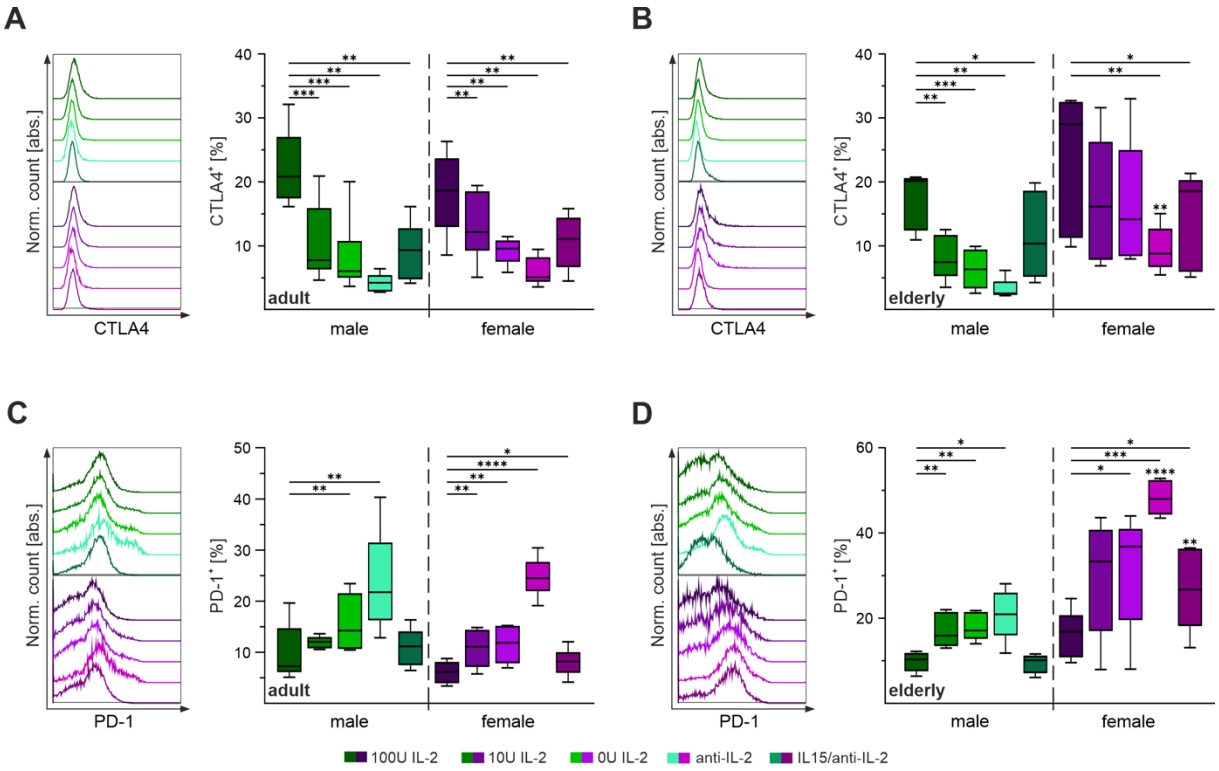


Figure 6: Cellular exhaustion is driven by PD-1 and CTLA4 expression and is IL-2-dependent. Flow cytometry analysis of CTLA4 and PD-1 in adult (**A**/**C**) and elderly (**B**/**D**) mice, respectively. All stimulation conditions were analyzed in both sexes with male mice CTLs depicted in shades of green and female mice in shades of purple. Analysis was performed on day three of stimulation. Representative fluorescence profiles are shown in the left panels and corresponding statistical analysis in the right panels. Significance depicted above bars indicate differences between sexes. Significance between conditions was calculated using paired ordinary one-way ANOVA and differences between sexes using unpaired Student's t-test. Data presented as mean ± SEM. Significance levels are indicated as * $p < 0.05$, ** $p< 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. *n = 5-6 per group.*

PD-1, another member of the CD28 family with its specific ligands PD-L1 and -L2 (Butte et al., 2007; Keir et al., 2008), shows a significant expression during stimulation, peaking after 48 hours and is predominately expressed on effector T cells (Ahn et al., 2018; Chemnitz et al., 2004; Day et al., 2018). Contrary to CTLA4, PD-1 expression significantly increased with decreasing IL-2 concentrations across all cohorts (Figure 5B/C). Adult mice CTLs saw an increase in PD-1 expression from 9.7% and 6% to 23.4% and 24.7% for male and female mice, respectively (Figure 5C). Elderly male mice also displayed an increase from 9.8% to 21% of PD-1$^+$ cells (Figure 5D), with this increase being even more pronounced in elderly female mice CTLs (16% to 48.3%). Interestingly, elderly female mice CTLs, regardless of IL-2 or IL-15 supplementation, exhibited significantly higher PD-1 expression than their male counterparts, challenging the cytotoxicity data.

Taken together, elderly female mice CTLs displayed more markers of exhaustion, however, are still able to maintain their cytotoxic effector function. These findings underline the complex interplay between CTLA4 and PD-1 expression, which are highly IL-2-dependent but may reflect changes in CTL activation status rather than being directly caused by IL-2 itself.

3.1.5 Expression of Perforin and Granzyme B is Sex- and IL-2-Dependent

Effector molecules such as perforin and granzyme B are the key players in granule-mediated cytotoxicity of murine CTLs (Zöphel et al., 2022). High levels of perforin are able to induce necrosis of the target cell, whereas granzyme B primarily triggers apoptosis (Backes et al., 2018). The expression of both perforin and granzyme is directly influenced by IL-2 signaling strength (Janas et al., 2005; J. Zhang et al., 1999). Additionally, expression of perforin is more pronounced in effector T cells compared to naïve T cells (Hamann et al., 1997; Sallusto et al., 1999; Willinger et al., 2005). Therefore, analyzing the expression of these molecules offers insights into the cytotoxic potential of CTLs.

For adult mice CTLs, perforin expression, as quantified by intracellular flow cytometry staining, was consistent across all tested conditions and did not vary by sex (Figure 7A), with the percentage of perforin$^+$ cells ranging between 1.79% and 4.7%. However, in elderly mice, female mice CTLs exhibited significantly higher expression under all conditions (Figure 7B). Notably, expression levels in female mice CTLs stimulated with 100U IL-2 were fourfold higher (15.6% to 62.9%), compared to their male counterparts. Without external IL-2, perforin expression in elderly female mice CTLs was increased threefold (10.3% to 31.1%); and upon neutralization of autocrine IL-2, it was 2.5-times higher compared males (2.9% to 7.6%). Interestingly, IL-15 supplementation did not fully restore perforin expression to levels seen in 100U IL-2 stimulation.

Granzyme B expression was comparable in adult male and female mice CTLs (Figure 7C), and exhibited significant IL-2 dependence, with expression levels decreasing from 84.8% and 77.6% to 18.5% and 18% in male and female mice CTLs, respectively. In elderly mice, this IL-2 dependance was solely observed in male mice CTLs (Figure 7D), where granzyme B expression decreased gradually from 90.3% to 16.6% with diminishing IL-2 concentration. IL-15 supplementation could not counteract the loss of autocrine IL-2. In contrast, elderly female mice maintained stable granzyme B expression between 91.7% and 98.3%. Only neutralization of autocrine IL-2 resulted in a significant decrease to 43.8% of granzyme B$^+$ cells, still significantly higher than in elderly male mice. Confocal laser scanning microscopy further validated these findings, showing that elderly female mice CTLs are less dependent on IL-2 for effector molecule expression (Figure 7E).

In summary, the enhanced expression of perforin and granzyme B in elderly female mice CTLs enables them to eliminate target cells more efficiently compared to male mice CTLs. Whether these sex-specific differences are attributed to. Variations in subtype distribution, particularly the portion of naïve T cells, warrants further investigations. These findings reflect the cytotoxic capabilities of both adult and elderly mice CTLs under varying IL-2 conditions.

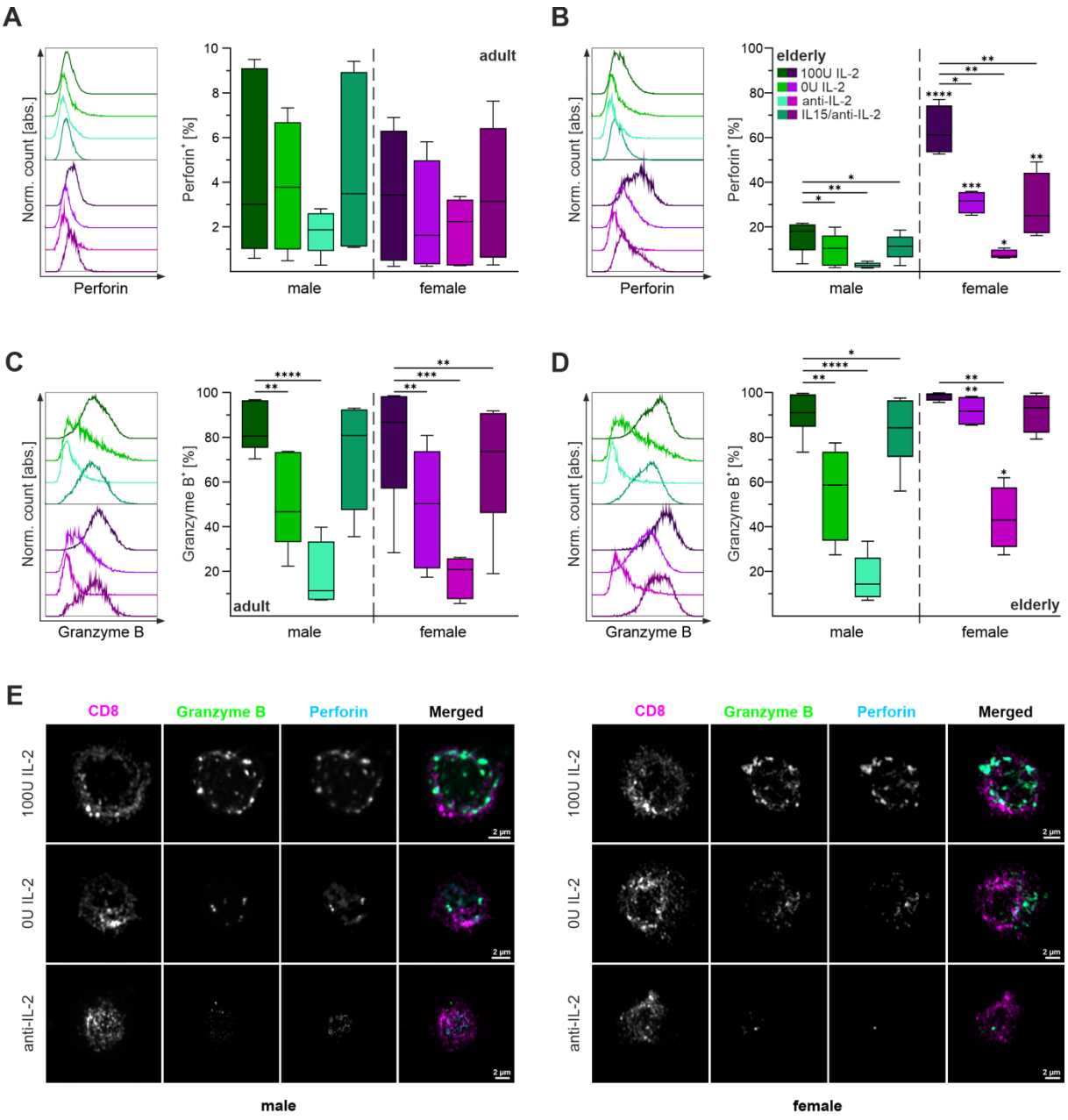


Figure 7: Elderly female mice express significantly more perforin and granzyme B making them efficient effector cells. Intracellular flow cytometry analysis of perforin and granzyme B in adult (**A**/**C**) and elderly (**B**/**D**) mice, respectively. All stimulation conditions, except 10U IL-2, were analyzed in both sexes with male mice CTLs depicted in shades of green and female mice in shades of purple. Figure **E** shows confocal laser microscopy analysis of perforin and granzyme B expression in $CD8^+$ CTLs of male (left) and female (right) elderly mice. Analysis was performed on day three of stimulation. Representative fluorescence profiles are shown in the left panels and corresponding statistical analysis in the right panels. Significance depicted above bars indicate differences between sexes. Significance between conditions was calculated using paired ordinary one-way ANOVA and differences between sexes using unpaired Student's t-test. Data presented as mean ± SEM. Significance levels are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. *n = 4-6 per group.*

3.1.6 Cytokine Secretion Profile of Murine CTLs

Secretion of various cytokines influence stimulation and activation of CTLs *in vivo*, with T helper cells (Th cells) and macrophages being the primary producers and secreters (J.-M. Zhang & An, 2007). CTLs secrete cytokines like IFN-γ, TNF-α, IL-2, IL-6, IL-4, or IL-12 upon stimulation and play a significant role in modulating both their own cytotoxic capabilities and the activity of other immune cells (Koh et al., 2023; M. S. Paul & Ohashi, 2020; M. Wang et al., 2021). Therefore, the cytokine secretion patterns of adult female mice and elderly mice CTLs of both sexes were analyzed in relation to IL-2 stimulation.

Interferon-γ (IFN-γ), known as a pro-inflammatory cytokine that plays an important role in chemotaxis, T cell motility, and cytotoxicity (Bhat et al., 2017). However, evidence hints that IFN-γ can also act as an anti-inflammatory cytokine (Allen et al., 1991; Mühl & Pfeilschifter, 2003; Nicoletti et al., 2000), and its secretion is enhanced by IL-2 in a dose-dependent manner (Bihl et al., 2010; Herr et al., 2014; Rollings et al., 2018). In adult mice, a clear dose-dependent relationship was observed, with IFN-γ secretion decreasing significantly from 13.75 pg/mL to 2 pg/mL (Figure 8A). IL-15 supplementation failed to compensate for the loss of IL-2. Conversely, elderly mice CTLs maintain stable IFN-γ levels (12.5 pg/mL and 14.4 pg/mL) across all conditions without noticeable sex-differences.

Tumor necrosis factor-α (TNF-α), another pro-inflammatory cytokine, is primarily secreted by activated T cells, NK cells, and macrophages (Agbanoma et al., 2012; Carswell et al., 1975; Rodrigues et al., 2021; R. Wang et al., 2012). It is a major regulator of immune response and inflammation (Bradley, 2008). Like IFN-γ, TNF-α production is stimulated by IL-2 (Nedwin et al., 1985; Strieter et al., 1989); but can decrease in exhausted T cells despite IL-2 treatment (Y. Liu et al., 2021). In adult female mice CTLs, TNF-α secretion slightly declined with reduced IL-2 from 0.14 pg/mL to 0.09 pg/mL (Figure 8B). Additionally, IL-15 supplementation could compensate for the loss of autocrine IL-2 and increased from 0.17 pg/mL to 0.21 pg/mL in elderly male mice CTLs. Notably, elderly female mice CTLs secreted significantly more TNF-α than males with secretion levels ranging from 0.31 pg/mL and 0.36 pg/mL. The only exception was observed in cells treated with anti-IL-2, which only secreted only 0.2 pg/mL TNF-α ($p=0.0524$ vs 100U IL-2).

Interleukin-22 (IL-22), is a cytokine initially discovered in activated T cells, but is also secreted by NK cells and natural killer T cells (NKT cells) (Cella et al., 2009; Dumoutier et al., 2000; Paget et al., 2012). It plays an important role in tissue repair and inflammation (Kumar et al., 2013; Pavlidis et al., 2022), with epithelial and stromal cells being the primary targets (Dudakov et al., 2015). However, influence of IL-2 on IL-22 secretion by CTLs is not well understood. In elderly mice CTLs, IL-22 secretion notably limited (Figure 8C), and the secretion levels varied

significantly, ranging from 0.0015 pg/mL and 0.45 pg/mL. Although the decrease in IL-22 secretion with diminishing IL-2 concentrations was not statistically significant, it tended to decrease in both sexes, and supplementation with IL-15 could not compensate for the loss of autocrine IL-2.

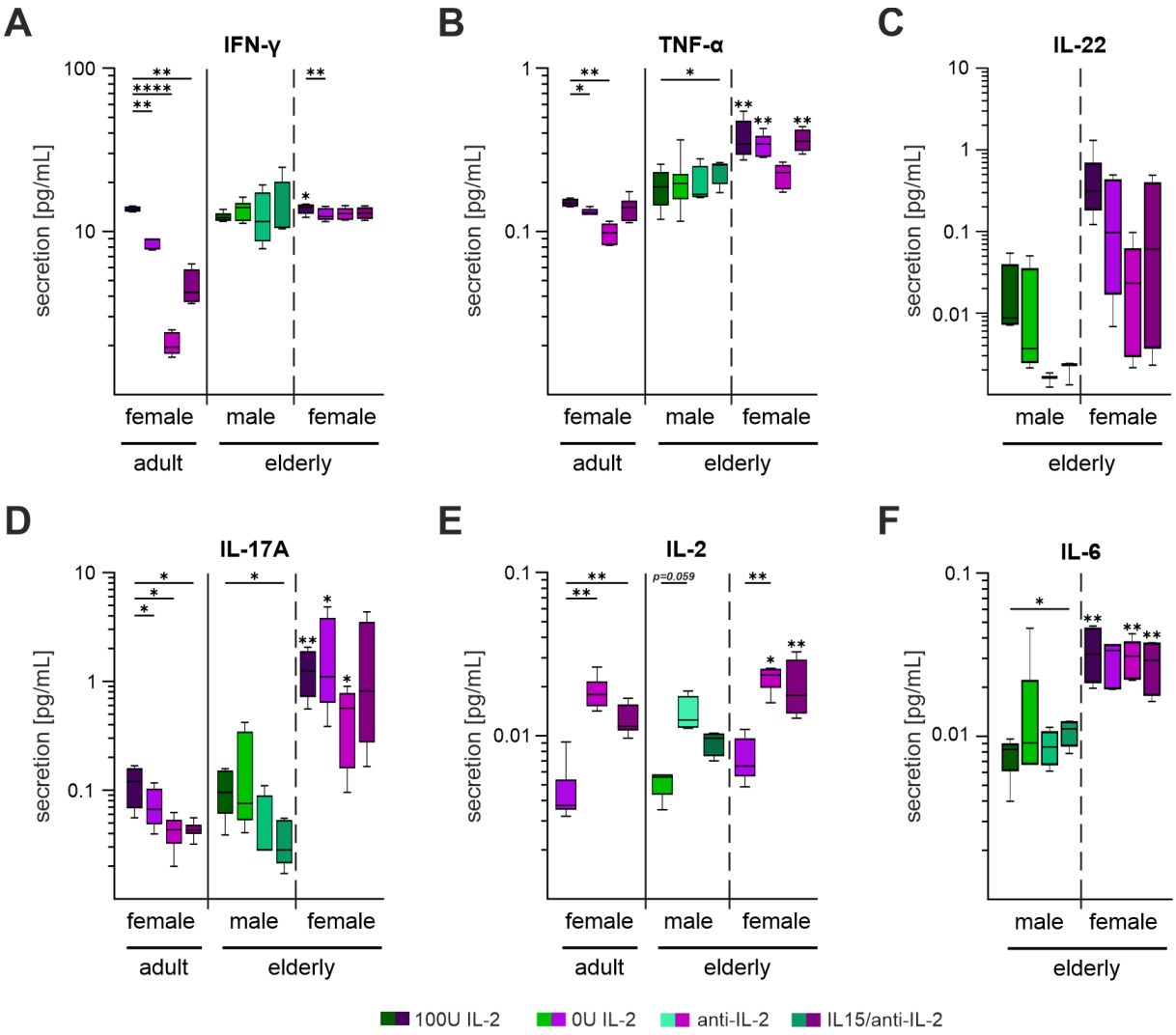


Figure 8: Elderly female mice CTLs secrete higher amounts of pro-inflammatory cytokines TNF-a, IL-2 and IL-17A. LegendPlex™ analysis of CTL cell culture supernatant after three days of stimulation. Secretion of cytokines IFN-g (**A**), TNF-a (**B**), IL-22 (**C**), IL-17A (**D**), IL-2 (**E**), and IL-6 (**F**) in adult female (left) and elderly male and female (right) mice. Analysis of IL-22 and IL-6 secretion was only possible for elderly mice. Various stimulation conditions were analyzed in both sexes with male mice CTLs depicted in shades of green and female mice in shades of purple. Significance depicted above bars indicate differences between sexes. Significance between conditions was calculated using paired ordinary one-way ANOVA and differences between sexes using unpaired Student's t-test. Data presented as mean ± SEM. Significance levels are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. *n = 3-7 per group.*

Interleukin-17A (IL-17A), another pro-inflammatory cytokine, is secreted by various T cell subsets such as Th17 cells, and mast cells, particularly in the context of disease (Hueber et al., 2010; S.-J. Liu et al., 2007; X. Liu et al., 2014; Lockhart et al., 2006). Research indicates that IL-2 may negatively impact IL-17A secretion, likely by inhibiting the generation of Th17 cells *in vitro* (Laurence et al., 2007; Y. Murakami et al., 2020). Across all mouse cohorts tested, a decrease in IL-17A secretion was observed with diminishing IL-2 concentration, and IL-15

supplementation did not counteract this trend (Figure 8D). Specifically, in adult female and elderly male mice CTLs, IL-17A secretion gradually declined from 0.12 pg/mL and 0.1 pg/mL to 0.04 pg/mL and 0.05 pg/mL, respectively. Notably, elderly female mice CTLs demonstrated significantly higher IL-17A secretion, which decreased from 1.3 pg/mL to 0.5 pg/mL as IL-17A secretion is negatively regulated by testosterone (Nolasco-Pérez et al., 2023).

Interleukin-2 production of CTLs is significantly lower compared to $CD4^+$ T cells, underscoring the distinctive regulatory mechanisms within different T cell subsets (Westerhof et al., 2019; Y. Zhang, Maksimovic, et al., 2018). Interestingly, autocrine IL-2 secretion peaked when cells were treated with anti-IL-2, suggesting a compensatory mechanism for impaired IL-2 signaling (Figure 8E). Both adult female mice and elderly male mice CTLs displayed similar IL-2 secretion levels, ranging from 0.005 pg/mL to 0.02 pg/mL and 0.005 pg/mL to 0.014 pg/mL, respectively. Notably, elderly female mice CTLs secreted significantly more IL-2 when treated with anti-IL-2, regardless of IL-15 presence or absence, compared to their male counterparts, with secretion levels ranging from 0.02 pg/mL to 0.023 pg/mL.

Interleukin-6 (IL-6), a pleiotropic cytokine, plays a crucial role in preventing activation induced cell death (AICD) in T cells, thus ensuing their survival and functionality (Atreya et al., 2000; Ayroldi et al., 1998; M. Murakami et al., 2019; Rochman et al., 2005). Produced by monocytes and T cells, among others cell types (L. Kang et al., 2020; Trinschek et al., 2013), IL-6 secretion patterns in CTLs from elderly male and female mice CTLs appears to be largely IL-2-independent but with a strong bias towards female mice (Figure 8F). Specifically, elderly male mice secreted IL-6 in the range of 0.008 pg/mL to 0.015 pg/mL, whereas elderly female mice demonstrated higher secretion levels, ranging from 0.028 pg/mL to 0.033 pg/mL.

In summary, elderly female mice CTLs create a more pronounced pro-inflammatory environment through the enhanced secretion of TNF-α, IL-17a, IL-2, and IL-6, largely independent of IL-2 supplementation. This finding, aligning with cytotoxicity observations, emphasizes the unique cytokine secretion profile in elderly mice, particularly females. The direct impact of these cytokines on CTLs functionality, however, required further investigation, as cytokine receptor expression analysis was not within this study's scope.

3.2 Interleukin-2 Dependence of Murine NK cells across Age and Sex

In addition to CTLs, NK cells play a crucial role in viral defense, tumor elimination, and also tissue damage (Cavalcante-Silva & Koh, 2023; S. Paul & Lal, 2017; Smyth et al., 2001; Zuo & Zhao, 2021). In contrast to CTLs, NK cells do not require antigen-specific stimulation but rather signaling through a variety of activating and inhibitory receptors (Abel et al., 2018; S. Kim et

al., 2005). NK cells serve a similar purpose in the innate immune system as CTLs in the adaptive immune system. Consequently, this study focused on analyzing the IL-2-dependence of murine NK cells isolated from spleen and blood, aiming to uncover potential adaptive strategies employed by the immune system to maintain homeostasis and effective mechanism across ages and sexes.

3.2.1 Cytotoxicity of Splenic NK cells under varying Cytokine Conditions

High concentrations of interleukin-2 are essential for the activation of splenic NK cells (T.-J. Kim et al., 2014), enhancing their viability and cell purity in a dose-dependent manner after isolation (G. Wang et al., 2017). IL-2 stimulated lymphokine-activated-killer (LAK) cells significantly reduced pulmonary and hepatic metastases in murine cancer models, demonstrating the cytokine's role in NK cell-mediated cytotoxicity (Lafreniere & Rosenberg, 1985; Shiloni et al., 1986). Similarly, IL-15 has been found to mediate cytotoxicity by induction of effector molecule expression (Fehniger et al., 2007). However, little is known about *in vitro* target cell lysis itself, especially regarding the influence of age and sex. Therefore, this study evaluated the cytotoxicity of primary splenic NK cells from both adult and elderly mice in both sexes (Figure 9).

Contrary to expectations, NK cells from both adult and elderly mice showed no significant IL-2-dependence. In adult males, end point lysis gradually decreased with reduced IL-2 concentrations from 49.3% to 28.5% (Figure 9A/B). Interestingly, adult females demonstrated a higher resistance to decreasing IL-2 concentrations, with end point lysis minimally affected, decreasing only from 73.8% to 68.3% and peaking at 81.9% with 500U IL-2. The trend for maximal target cell lysis per 10 min in male mice mirrored the gradual decrease from 8.2% to 5% (Figure 9C). However, in adult females, maximal target cell lysis initially increased with 500U IL-2 from 8% to 10.3% per 10 min before dropping to 7.4% per 10 min with 100U IL-2.
Elderly mice, male and female, exhibited similar IL-2-dependent cytotoxic patterns. Both sexes reached their cytotoxic peak with 500U IL-2 stimulation, after which both end point lysis and maximal target cell lysis per 10 min declined. Specifically, end point lysis increased from 56.5% to 74.9% in males and from 60.9% to 74.6% in females before dropping to 55.2% and 63.7%, respectively (Figure 9E). Similarly, maximal target cell lysis per 10 min for elderly male mice rose from 7.5% to 8.5% and then fell to 7% while in female mice, it went from 7.3% to 8.8% before decreasing to 6.2% (Figure 9F).

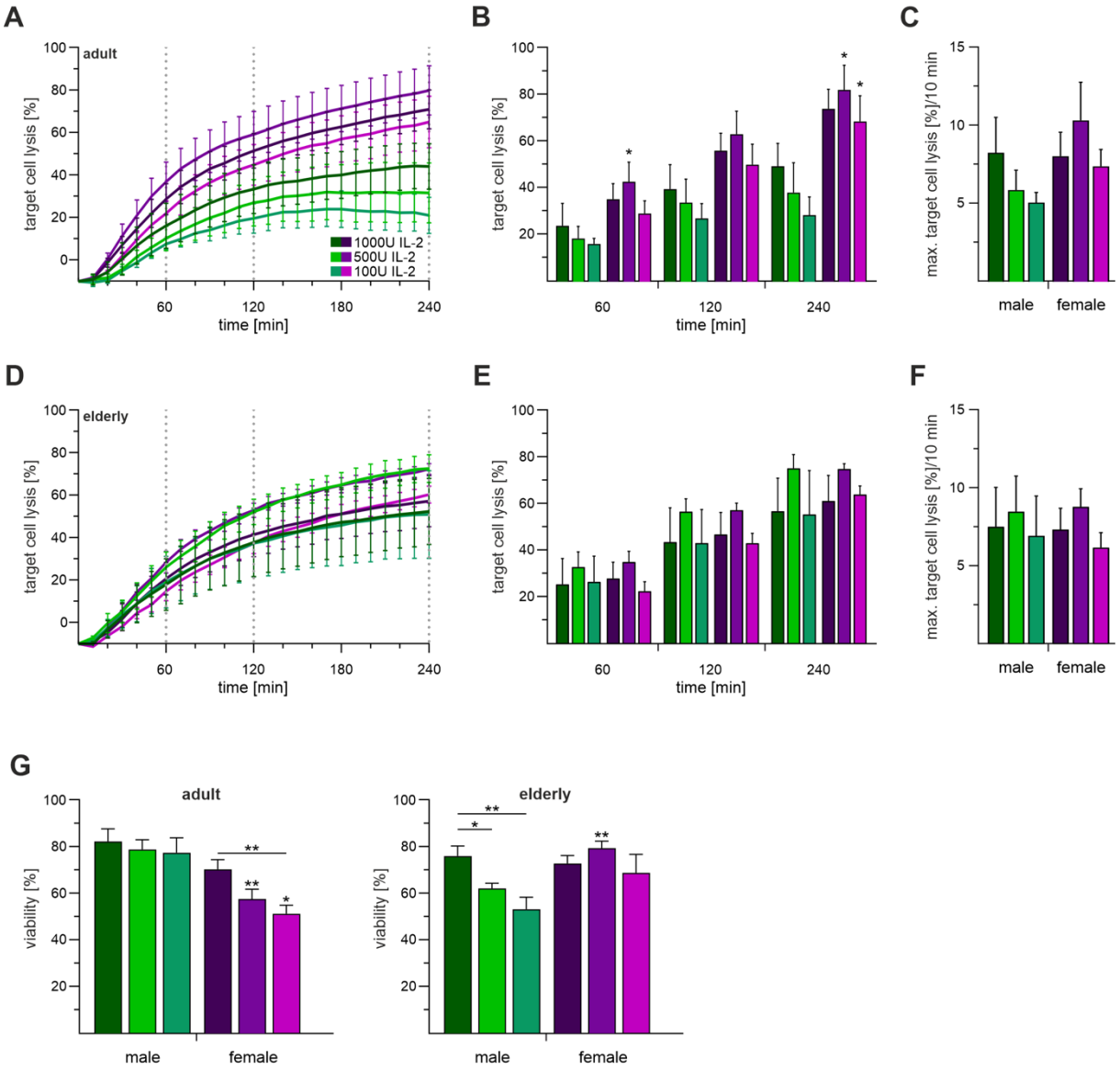


Figure 9: Adult female mice NK cells exhibit higher cytotoxicity compared to male mice NK cells. Real-time killing assay from adult (**A-C**) and elderly (**D-F**) male (green) and female (purple) mice NK cells on day one of stimulation. NK cells were stimulated with varying IL-2 concentrations (1000U, 500U, or 100U) as depicted in shades of green and purple. YAC-1 were utilized in a 5:1 effector-to-target cell ratio. Figures **A** and **D** depict killing kinetics over the time course of four hours. Figures **B** and **E** depict mean target cell lysis at 60 min, 120 min, and 240 min. Figures **C** and **F** depict maximal target cell lysis per 10 min or killing rate. Viability of murine NK cells is depicted in figure **G**. Significance depicted above bars indicate differences between sexes. Significance between conditions was calculated using unpaired ordinary one-way ANOVA and differences between sexes using unpaired Student's t-test. Data presented as mean ± SEM. Significance levels are indicated as * $p < 0.05$, ** $p< 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. *n = 3-6 per group.*

Understanding that cell viability plays a crucial role in the effectiveness in cytotoxicity, viability was quantified (Figure 9G). Interestingly, only NK cells from adult female mice demonstrated a significant IL-2-dependent decrease in viability from 70.2% to 51.2% aligning with findings from previously published research (G. Wang et al., 2017). Conversely, the viability of NK cells from adult male mice remained relatively stable, fluctuating slightly between 82.1% to 77.2%. In the case of elderly mice, a distinct pattern emerged: male NK cells showed a clear dependence on IL-2 for maintaining viability, which gradually diminished from 75.9% to 53.1%. In contrast, elderly female NK cells exhibited a robust viability that was not significantly

influenced by IL-2 levels. Notably, their viability reached its maximum at 500U IL-2, increasing from 72.7% (with 1000U) to 79.7% (with 500U), before decreasing to 68.7% (with 100U). Therefore, to preclude the influence of viability changes, killing assays were performed using the living cell count.

Taken together, these observations suggest a 500U IL-2 concentration as optimal for stimulating murine splenic NK cells, with no significant IL-2-dependent changes in cytotoxicity after accounting for viability. The underlying reasons for the observed enhanced cytotoxicity in adult female mice warrant further investigated.

3.2.2 Subtype and NK Receptor Distribution of Splenic NK Cells

In a previous study, we discovered that murine CTLs' memory and effector T cells share similar cytotoxic capacities (Zöphel et al., 2022). However, the impact of NK cell subtypes on cytotoxicity remains unexplored, as does the effect of IL-2 in modulating murine NK cell subtype distribution. Another key property of NK cells is the expression of activating and inhibitory NK cell receptors to control activation of NK cells. A study also found a relationship between IL-2 supplementation and expression of various NK cell receptors (Sharma & Das, 2018). However, these studies only utilized one IL-2 concentration to test against absence of IL-2. Therefore, the influence of IL-2 concentration was investigated.

In freshly isolated NK cells, the subtype distribution, as determined by CD27 and CD11b expression, significantly differed between male and female mice across both age cohorts (Figure 10A/B/E). In adult mice, comparable amounts of less mature $CD11b^{low}CD27^{high}$ NK cells were found, with portions of 15.5% and 15.1% in male and female mice, respectively (Figure 10B). Notably, male mice's NK cells contained significantly more $CD11b^{high}CD27^{high}$ cells compared to female mice (34.9% vs. 25.6%). However, female mice exhibited a significantly higher portion of matured $CD11b^{high}CD27^{low}$ NK cells, with 58.5% compared to 48.7% in adult male mice.

Surprisingly, adult female mice exhibited significantly lower expression of activating receptors NK1.1 and NKp46 compared to their male counterparts (Figure 10C). In adult female mice, 94.4% of NK cells were positive for NK1.1 and 1.5% for NKp46, compared to 98.3% and 63.9% in adult male mice. Additionally, NKG2D expression slightly decreased from 91% (male) to 85.6% (female).

Furthermore, adult female mice NK cells displayed significantly higher expression of the inhibitory receptor killer cell lectin-like receptor G1 (KLRG1) with 51.8% compared to 32.7% in male mice (Figure 10D). However, Ly49A and NKG2A expression was comparable between

sexes with 15.1% and 18.4% for Ly49A and 30.8% and 23.9% for NKG2A in male and female mice, respectively.

Similar to adult mice, elderly female mice had comparable proportions of $CD11b^{low}CD27^{high}$ NK cells, with percentages ranging from 36.8% in males and 34.7% in females (Figure 10E). Additionally, there was a noticeable difference in the distribution of NK cell subtypes; elderly female mice had a significantly lower ratio of $CD11b^{high}CD27^{high}$ NK cells (21.1%) compared to elderly male mice (32.6%). Conversely, a higher percentage of mature $CD11b^{high}CD27^{low}$ NK cells was observed in elderly female mice (42.4%) compared to their male counterparts (29.3%).
The expression of activating receptors NK1.1 and NKp46 was also significantly lower in female mice (Figure 10F), with only 90.1% of NK cells being $NK1.1^{+}$ and 48.5% being $NKp46^{+}$, as opposed to 95.1% $NK1.1^{+}$ and 66.4% $NKp46^{+}$ in male mice. Furthermore, NKG2D expression was slightly lower in female mice (78.8%) than in male mice (85.8%).
Unlike in adult mice, no differences were found in the expression of inhibitory receptors between elderly. KLRG1 expression was found within a range from 23.8% to 29.3%, Ly49A expression varied from 12.8% to 15.4%, and NKG2A expression was between 34.2% and 28.1% for male and female mice, respectively.

To investigate the influence of IL-2 on NK cell subtype distribution and NK cell receptor expression, flow cytometry analysis was conducted on day one of stimulation (Figure 11). The ratio of $CD11b^{low}CD27^{high}$ NK cells remained relatively constant, ranging from 9.9% to 13.3% in males and from 10.3% to 14.4% in females with decreasing IL-2 concentrations (Figure 11A). A notable trend emerged across both sexes; as IL-2 concentrations decreased, $CD11b^{high}CD27^{high}$ NK cells diminished, whereas the proportion of $CD11b^{high}CD27^{low}$ NK cells increased. Specially, the portion of $CD11b^{high}CD27^{high}$ NK cells dropped from 61% to 47% in adult males and significantly from 50.1% to 31.8% in adult females. Remarkably, female mice had significantly fewer $CD11b^{high}CD27^{high}$ NK cells than male mice when stimulated with 1000U and 500U IL-2. Conversely, the ratio of $CD11b^{high}CD27^{low}$ NK cells rose from 28.6% to 39.3% in males and from 39.3% to 53% in females as IL-2 concentrations decreased.
At least 96.3% of all tested NK cells were positive for NK1.1, showing no variation due to sex or IL-2 concentration (Figure 11B). The expression of NKG2D was similarly high, above 98.3% in all cases except in NK cells from adult female mice stimulated with 100U IL-2, where 96.4% were $NKG2D^{+}$. Consistent with the day of isolation, adult female mice's NK cells exhibited a significantly lower proportion of NKp46 expressing cells across all tested IL-2 levels compared to their male counterparts. In adult male mice at least 80.2% of all NK cells are $NKp46^{+}$.

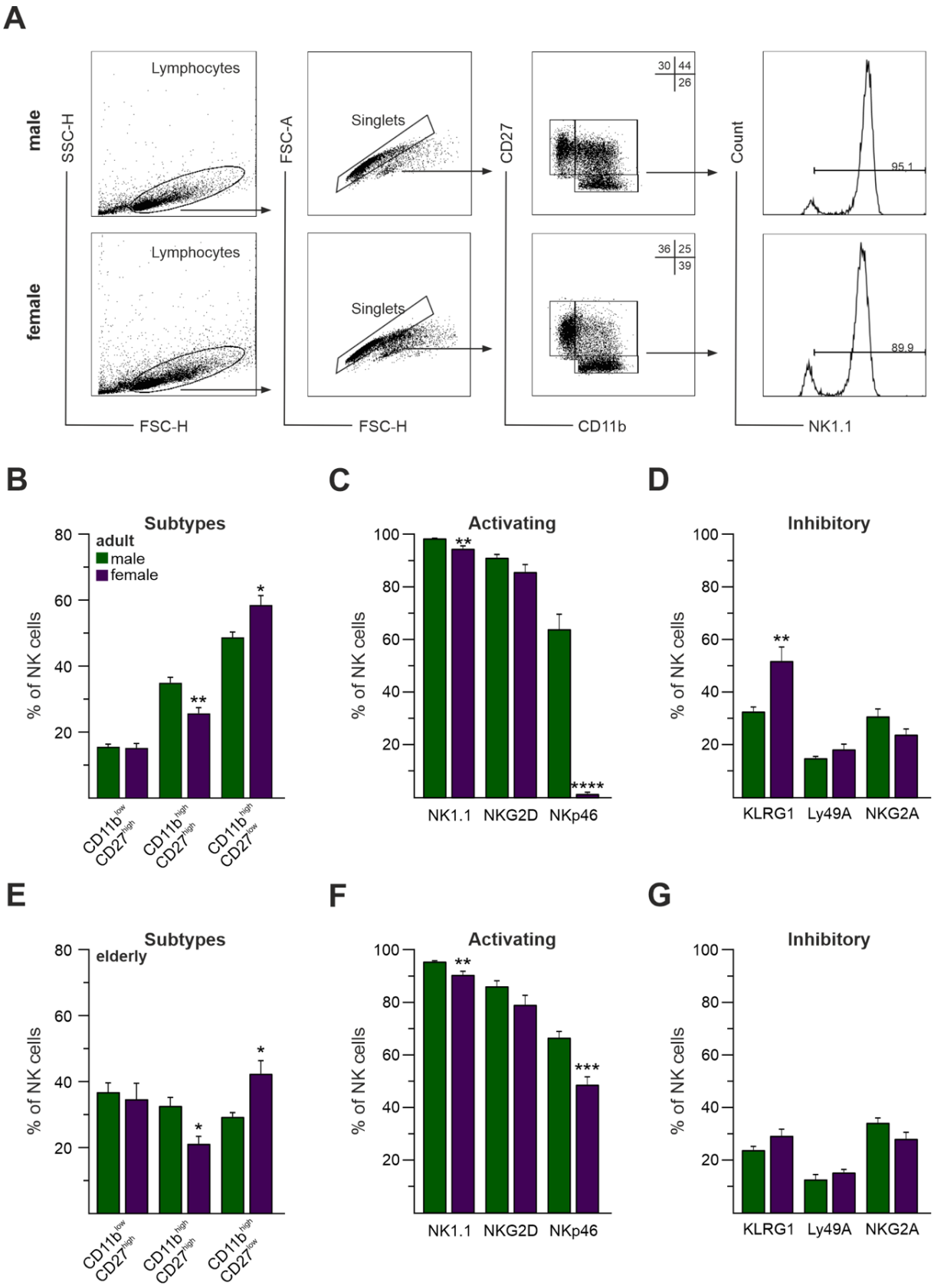


Figure 10: Female mice NK cells display more mature NK cells but significantly less activating receptors. Flow cytometry stainings to quantify subtype distribution as determined by expression of CD11b and CD27 (**B**, **E**) and expression of activating (**C**, **F**) and inhibitory (**D**, **G**) NK cell receptors of adult and elderly mice NK cells, respectively. Male mice NK cells are depicted in green and female mice NK cells in purple. Analysis was performed on the day of isolation. Representative gating is shown in figure **A**. Significance depicted above bars indicate differences between sexes. Significance between sexes was tested using unpaired Student's t-test. Data presented as mean ± SEM. Significance levels are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. *n = 5-9 per group.*

However, the percentage of NK cells from adult female mice dropped to 38.2% with 1000U IL-2, 18.7% with 500U IL-2, and only 11.3% with 100U IL-2 underscoring a pronounced IL-2-dependence for NKp46 expression in these cells.

The expression of inhibitory receptors was markedly lower than that of activating receptors (Figure 11C). Expression of KLRG1 significantly increased from 26.8% to 39.8% in male mice and from 41.1% to 50.9% in female mice. Furthermore, female mice displayed a higher portion of KLRG1$^+$ cells than male mice, especially when stimulated with wither 1000U or 500U IL-2. Ly49A expression remained relatively constant, with a slight decrease observed, ranging between 17.4% and 21.9%. Though the expression of NKG2A remained stable across different IL-concentrations, NK cells from adult female mice consistently showed significantly lower levels of NKG2A$^+$ cells compared to their male counterparts. Specifically, 36.6% and 39.6% of male mice NK cells were NKG2A$^+$ in contrast to only 16% to 26.4% were NKG2A$^+$ in female NK cells.

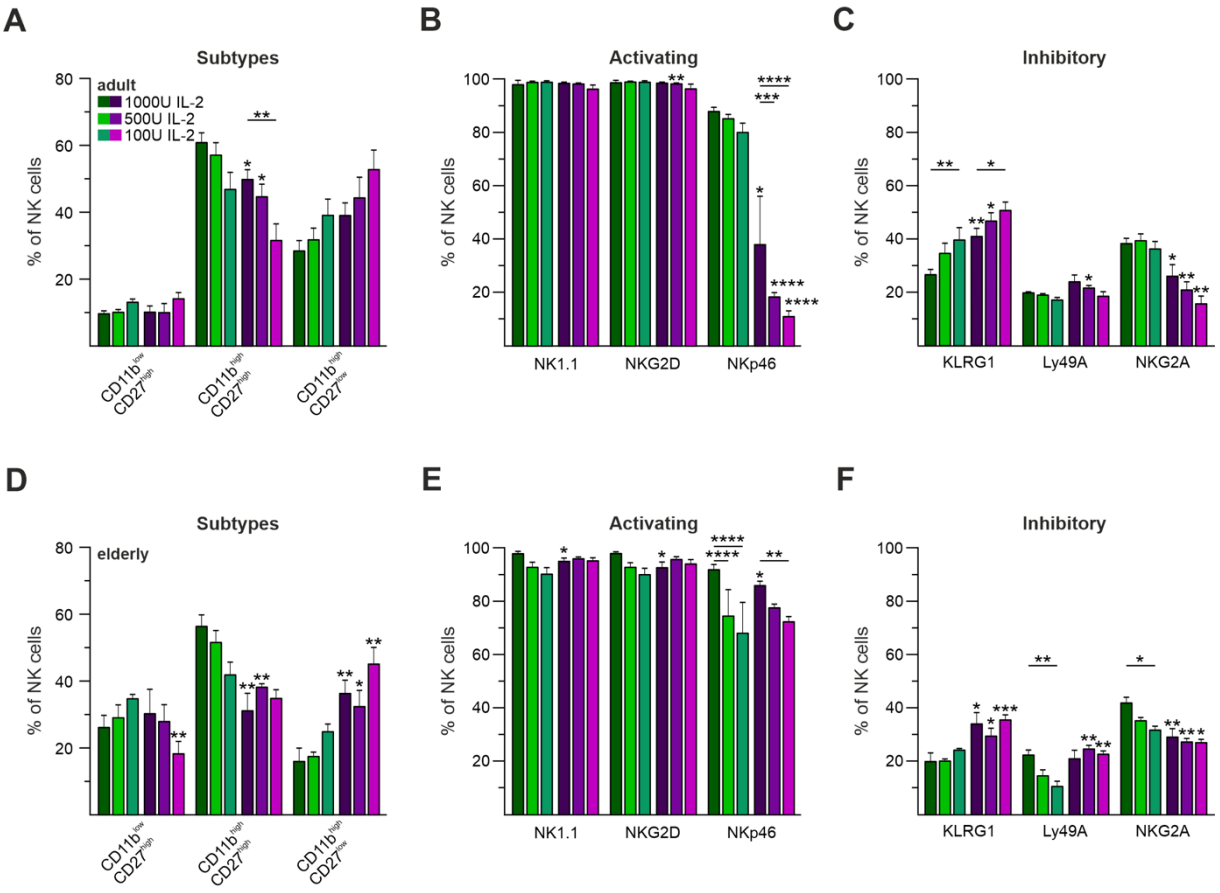


Figure 11: Female mice NK cells display more mature NK cells especially under decreasing IL-2 concentrations. Flow cytometry stainings to quantify subtype distribution (**A**, **D**) and expression of activating (**B**, **E**) and inhibitory (**C**, **F**) NK cell receptors of adult and elderly mice NK cells, respectively. Stimulation conditions of male mice NK cells are depicted in shades of green and in shades of purple for female mice. Analysis was performed on day one of stimulation. Significance depicted above bars indicate differences between sexes. Significance between sexes was tested using unpaired Student's t-test. Data presented as mean ± SEM. Significance levels are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. *n = 4-6 per group.*

In elderly mice, subtype distribution showed no IL-2-dependent differences, although some trends were observed (Figure 11D). Notably, as IL-2 concentrations decreased, the percentage

of $CD11b^{low}CD27^{high}$ NK cells in male mice increased from 26.3% to 35% but decreased from 30.5% to 18.5% in female mice. This sex difference became significant under 100U IL-2 stimulation. Moreover, female NK cells had significantly fewer $CD11b^{high}CD27^{high}$ NK cells than males when stimulated with either 1000U or 500U IL-2. This suggests a more pronounced impact of IL2 on the $CD11b^{high}CD27^{high}$ NK cells population in males, which decreased from 56.6% to 42.1%, compared to a more sustained range between 31.4% and 38.4% in females. Conversely, elderly female mice consistently showed a significantly higher portion of mature $CD11b^{high}CD27^{low}$ NK cells across all IL-2 treatments (1000U: 36.6% vs. 16.2%; 500U: 32.6% vs. 17.7%; 100U: 45.4% vs. 25.1%) compared to male mice.

Similar to findings in adult mice NK cells, the expression of activating receptors NK1.1 and NKG2D in elderly mice remained consistently higher, exceeding 90.2%, irrespective of IL-2 concentrations (Figure 11E). However, under 1000U IL-2 stimulation, female mice NK cells exhibited significantly lower expression of all tested receptors compared to male mice NK cells. Unlike in adult mice, NKp46 expression was IL-2 dependent, decreasing from 92.1% to 68.3% in males and from 86.1% to 72.6% in females.

The expression of inhibitory receptors was largely IL-2 independent, except for Ly49A and NKG2A in elderly male mice (Figure 11F), where the proportion of receptor positive cells decreased from 22.6% to 10.9% for Ly49A and from 42.1% to 32% for NKG2A. In contrast, elderly female mice maintained consistent expression levels of Ly49A between 21.3% and 24.9%, significantly higher than in their male counterparts, and NKG2A between 29.4% and 27.3%, which was significantly lower than in male mice. Moreover, female mice consistently had a higher percentage of $KLRG1^{+}$ cells across all tested conditions, with 34.3% to 35.8% of NK cells expressing KLRG1, compared to 20.2% to 24.5% in male mice.

In summary, the subtype distribution analysis of splenic NK cells revealed distinct differences between the sexes, with female mice predominately displaying a higher proportion of mature $CD11b^{high}CD27^{low}$ NK cells. This pattern was consistent both immediately after isolation and following overnight stimulation. While no substantial changes were found in response to decreasing IL-2 concentrations, certain trends emerged, notably that reduced IL-2 levels seem to favor the age- and sex-independent development of mature $CD11b^{high}CD27^{low}$ NK cells. Both adult and elderly mice showed a high prevalence of NK cells expressing activating receptors NK1.1, NKG2D, and NKp46. Nevertheless, female mice in both age cohorts demonstrated reduced NKp46 expression, potentially affecting receptor-mediated signaling. Conversely, female mice NK cells tend to express more of the inhibitory receptors KLRG1 and Ly49A but less NKG2A. The impact of these differences on activation and cytotoxicity, requires further analyses, particularly through the quantification of ligand expression on the used target cell line YAC-1 target cell line.

3.2.3 NK Cell Abundance in Mouse Spleen and Blood

Research indicated significant variability in the percentage of NK cells across different mouse tissues (Beli et al., 2014; Grégoire et al., 2007). In both studies, blood and spleen contain similar or a slightly higher ratio of NK cells in blood. Notably, when comparing absolute cell numbers, the spleen harbor up four times as many NK cells as the blood in adult mice, and this ration increases to approximately five times in elderly mice. However, findings on NK cell abundance between sexes has been inconsistent. One study highlighted a greater abundance of NK cells in spleen of adult female mice, while reported higher counts in males (Cheng et al., 2023; Menees et al., 2021). The sex-specific NK cell abundance in murine blood remains to be explored. Given the discrepancies, this study aimed to evaluate NK cell abundance in both male and female mice across both age groups, focusing on blood and spleen samples to clarify previous conflicting results.

Contrary to earlier findings, this study revealed that splenocytes from adult male and female mice exhibited similar proportions of NK cells, as characterized by the expression of NK1.1 and NKp46 markers, at 3.98% and 3.6%, respectively (Figure 12A/B). However, in elderly mice NK cell abundance was significantly higher in females at 4% compared to only 2.82% in males (Figure 12C).

Unlike previous studies, NK cell abundance in blood was at least twice as high as in the spleen. Specially, in both adult and elderly mice, NK cell prevalence in females slightly declined from 12.4% to 9.4% in adults (Figure 12D) and from 11% to 8.5% in elderly mice (Figure 12E).

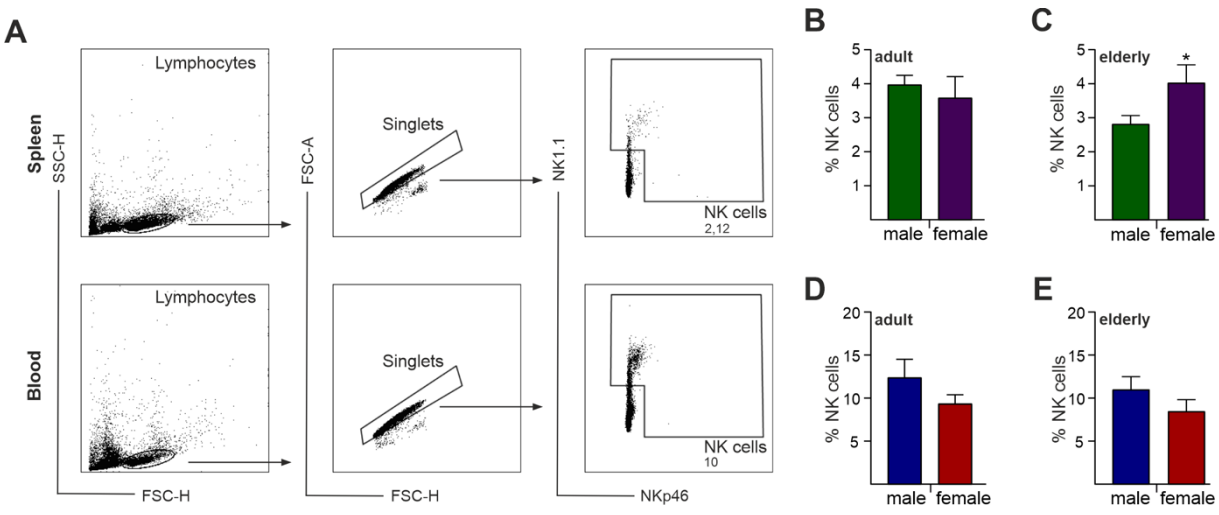


Figure 12: Murine blood contains a higher percentage of NK cells compared to spleen. Flow cytometry analysis of proportion of NK cells in spleen and blood of adult (**B**, **D**) and elderly (**C**, **E**) mice, respectively. NK cells were characterized by expression of NK1.1 and NKp46 (**A**). Analysis was performed on the day of isolation. Significance depicted above bars indicate differences between sexes. Significance between sexes was tested using unpaired Student's t-test. Data presented as mean ± SEM. Significance levels are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. *n = 3-15 per group.*

Overall, these findings indicate that murine blood contains a higher percentage of NK cells compared to the spleen. Furthermore, except in the spleen of elderly mice, female mice tend to have fewer NK cells than their male counterparts. While this study highlights variations in NK cell abundance between spleen and blood, it underscores the need for further investigation into the functional capabilities of blood NK cells.

3.2.4 Cytotoxicity of Blood NK cells

Unstimulated murine blood NK cells are characterized by higher expression of granzyme A compared to those in the spleen NK cells, but expression of granzyme B and perforin are comparably low in both tissues (Fehniger et al., 2007). The cytotoxic activity of murine blood NK cells, however, has not been extensively studied. To address this, the cytotoxicity of murine blood NK cells was evaluated following overnight stimulation with IL-2. Due to the fact that murine blood contains a very low number of below half a million of NK cells (Beli et al., 2014; Grégoire et al., 2007), cells were only stimulated with 100U IL-2.

In adult mice, NK cell analysis did not reveal any significant sex-specific differences in cytotoxicity (Figure 13A-C). End point lysis and maximal target cell lysis per 10 min were marginally higher in females with 33.7% (vs. 25.7%) and 4.95% (vs. 3.51%), respectively.

Notably, NK cells from elderly male mice exhibited higher cytotoxicity (Figure 13D-F). End point lysis after 240 min was higher in males at 36.9% compared to 24.8% in females. Similarly, maximal target cell lysis per 10 min increased from 3.02% in females to 3.86% in males.
Cell viability across both sexes and age cohorts remained consistent (Figure 13G), with high levels observed at 81.4% and 86.2% in adult males and females, respectively, and 78.3% and 80% in their elderly counterparts. Though, no differences were found, cytotoxicity assays were performed using living cell count.

In summary, while in female adult mice tend to exhibit higher cytotoxicity this is reversed in elderly mice. Here, cytotoxicity of male mice was higher compared to their female counterpart. However, if this is due to changes in subtypes distribution or expression of NK cell receptors remains ambiguous and was therefore investigated further.

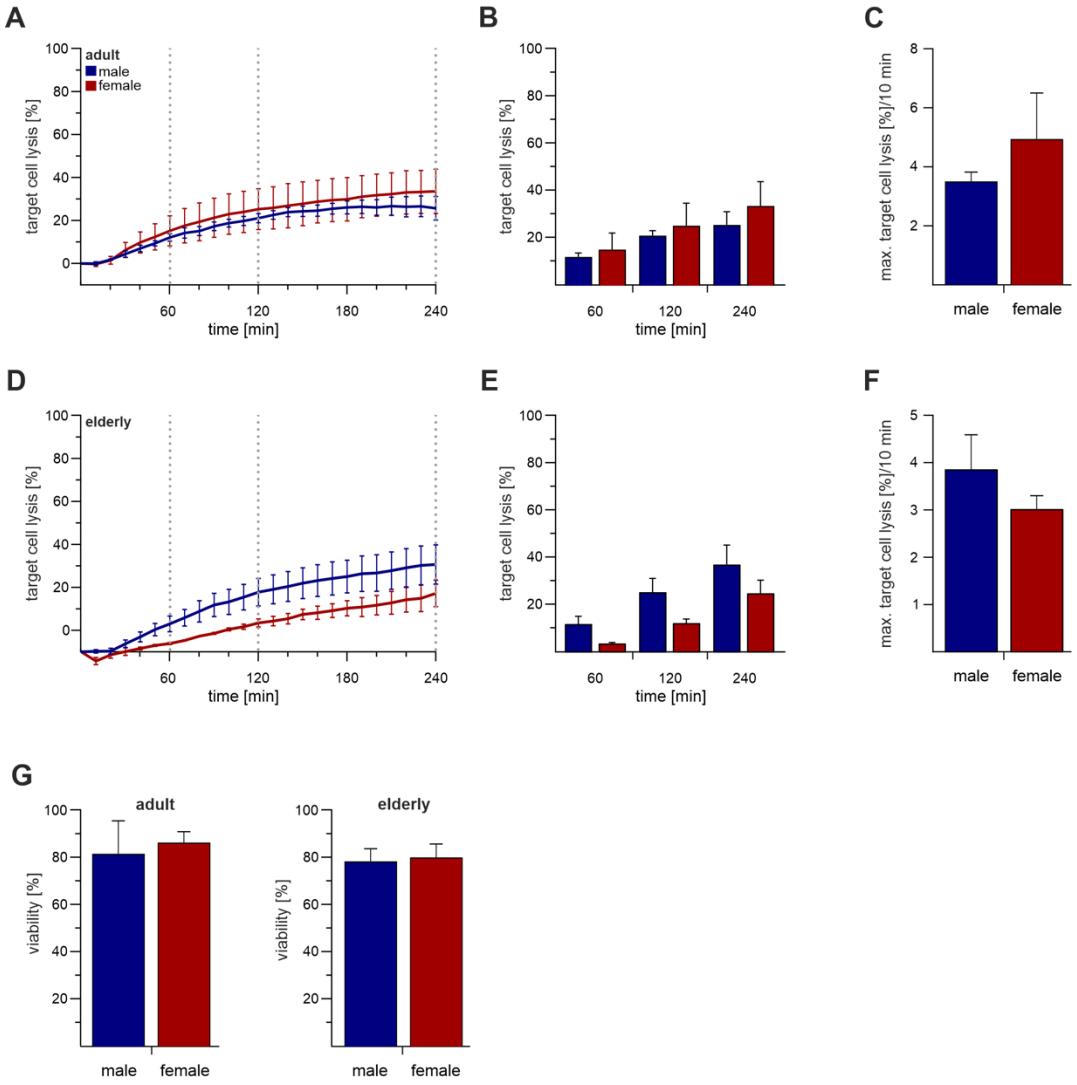


Figure 13: Blood NK cells from elderly male mice tend to be more cytotoxic compared to female mice NK cells. Real-time killing assay from adult (**A-C**) and elderly (**D-F**) male (blue) and female (red) mice NK cells on day one of stimulation. NK cells were stimulated with 100U IL-2. YAC-1 were utilized in a 5:1 effector-to-target cell ratio. Figures **A** and **D** depict killing kinetics over the time course of four hours. Figures **B** and **E** depict mean target cell lysis at 60 min, 120 min, and 240 min. Figures **C** and **F** depict maximal target cell lysis per 10 min or killing rate. Viability of murine NK cells is depicted in figure **G**. Significance depicted above bars indicate differences between sexes. Significance between sexes was tested using unpaired Student's t-test. Data presented as mean ± SEM. Significance levels are indicated as * $p < 0.05$, ** $p< 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. *n = 3-8 per group.*

3.2.5 Subtype and NK Receptor Distribution of Blood NK Cells

Previous studies have documented variations in subtype distribution of NK cells between the spleen and blood in both mice and rats (Banh et al., 2012; Beli et al., 2014; Inngjerdingen et al., 2012), revealing a lower prevalence of less mature $CD11b^{low}CD27^{high}$ NK cells and a higher proportion of mature $CD11b^{high}CD27^{low}$ NK cells compared to spleen NK cells. Despite these findings, the expression of NK cell on blood NK cells remains largely unexplored. To address this gap, blood NK cells were analyzed immediately after isolation and following overnight stimulation with 100U IL-2.

Aligning with earlier findings, blood NK cells consisted mostly of mature CD11b^{high}CD27^{low} NK cells with a very low abundance of CD11b^{low}CD27^{high} NK cells in both adult and elderly mice (Figure 14A/B/E). Among adult mice, the subtype distribution was remarkably similar between males and females with 6.14% and 6.67% of NK cells being CD11b^{low}CD27^{high}, 31.3% and 29.2% being CD11b^{high}CD27^{high} NK cells, and 62% and 62.4% being CD11b^{high}CD27^{low}, respectively (Figure 14A).

The expression of activating receptors was similar across sexes with a notable trend of slightly lower expression in female mice NK cells (Figure 14B). NK1.1 expression remained high, with a minimum of 89.6% NK1.1^{+} cells in females and 97.8% in males. NKG2D expression was generally lower, at 86.2% for males and 82% for females. Notably, NKp46 expression was substantially lower compared to NK1.1 and NKp46, with only 48.4% of male and 19.2% of female NK cells being NKp46^{+}, highlighting a pronounced difference in the prevalence of this receptor between sexes.

Unlike their splenic counterparts, blood NK cells exhibited a higher expression of inhibitory receptors, particularly KLRG1 and NKG2A (Figure 14C). A significant proportion of NK cells, namely 57.9% and 61.6%, were KLRG1^{+}. While Ly49A expression in blood NK cells was comparable to splenic NK cells, adult female mice displayed significantly higher expression rate of 16.5% compared to 12% in male mice. Conversely, NKG2A expression was significantly lower in adult female mice, with only 24.2% of NK cells being NKG2A^{+}, in contrast to 45.6% in adult male mice, indicating a significant sex difference in the expression of this inhibitory receptor.

In elderly mice, the distribution of NK cell subtypes showed that CD11b^{low}CD27^{high} NK cells were similarly represented between sexes, with 10.9% in males and 7.75% in females (Figure 14E). However, a significant difference was observed in the other subtypes; female mice had fewer CD11b^{high}CD27^{high} NK cells (27.6% vs. 35.6%) but a higher percentage of CD11b^{high}CD27^{low} NK cells (63.7% vs. 53%) compared to males.

The pattern of activating receptors was consistent between sexes, except for NKp46 (Figure 14F). NK1.1 expression remained high, with 90.8% in elderly male mice and 86.9% in females. NKG2D expression was somewhat lower, at 78.5% in males and 70.7% in females. Notably, a significant sex difference was observed in NKp46 expression; 45.5% of male NK cells were NKp46^{+}, compared to only 21.6% of female NK cells.

As with in adult mice, the expression of inhibitory receptors on blood NK cells was enhanced compared to splenic NK cells (Figure 14G). KLRG1 was expressed in 46.2% of male NK cells and 55.8% of female NK cells. The expression levels for Ly49A and NKG2A were similar between sexes, with 13.1% and 12.6% of cells expressing Ly49A and 44% and 43% of cells expressing NKG2A, respectively, indicating no significant difference for these receptors.

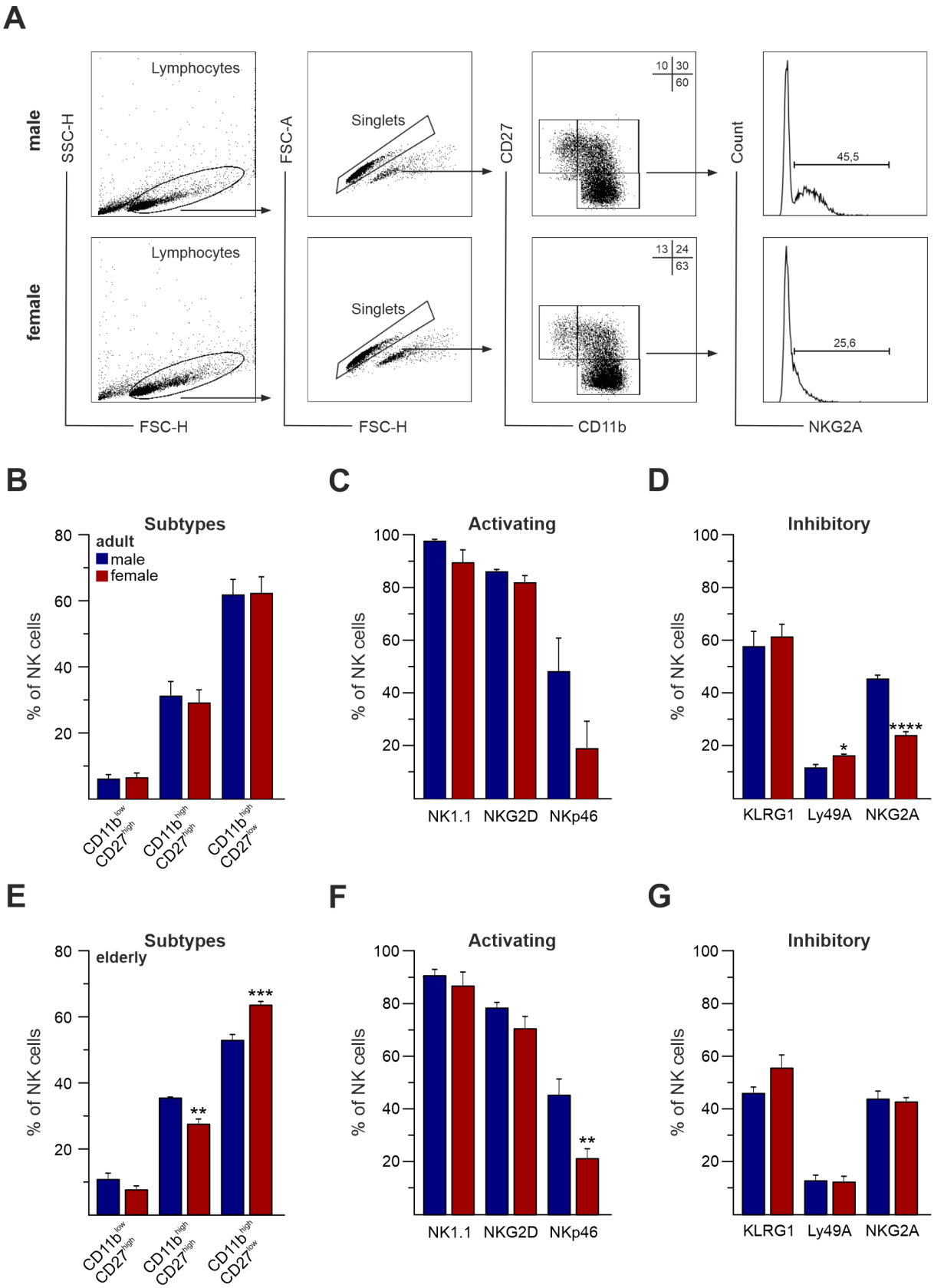


Figure 14: Elderly female mice exhibit higher abundance mature CD11b^{high}CD27^{low} NK cells in blood but less activating receptors. Flow cytometry stainings to quantify subtype distribution as determined by expression of CD11b and CD27 (**B**, **E**) and expression of activating (**C**, **F**) and inhibitory (**D**, **G**) NK cell receptors of adult and elderly mice NK cells, respectively. Male mice NK cells are depicted in green and female mice NK cells in purple. Analysis was performed on the day of isolation. Representative gating is shown in figure **A**. Significance depicted above bars indicate differences between sexes. Significance between sexes was tested using unpaired Student's t-test. Data presented as mean ± SEM. Significance levels are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. *n = 3-6 per group.*

Following overnight stimulation with 100U IL-2, subtype distributions and NK cell receptor expression was repeatedly assessed to investigate the impact of IL-2 on these parameters.
These findings post-stimulation mirrored those observed immediately after isolation, with mature $CD11b^{high}CD27^{low}$ NK cells remaining the predominant NK cell subtype among both adult and elderly mice. Notably, no sex-specific differences were found in NK cell subtype distribution of adult mice (Figure 15A). The proportions of $CD11b^{low}CD27^{high}$ NK cells were between 9.36% in males and 6.22% in females. The remaining NK cells were at a more advanced stage of maturation, with 24.1% and 29.4% of NK cells being $CD11b^{high}CD27^{high}$, and 65% and 62.9% being $CD11b^{high}CD27^{low}$ in male and female mice, respectively.
The expression of activating receptors post-stimulation revealed no sex-specific differences (Figure 15B), aligning with observations in splenic NK cells. Consistent with splenic NK cell data, NK1.1 expression was notably high, present on at least 91.6% of all NK cells, with male mice showing an even higher percentage at 98.8%. NKG2D expression was also substantial, measured at 98.1% in males and 94.2% in females. However, NKp46 expression remained lower in comparison; 72.4% of male NK cells expressed NKp46 and only 29.5% of female NK cells.
The expression of inhibitory receptors, apart from Ly49A, did not display sex-dependent variations (Figure 15C). For adult mice, 59.6% of male NK cells were $KLRG1^{+}$, compared to a higher proportion of 68.4% in female NK cells. In contrast, Ly49A expression presented a noticeable sex difference: only 15% of male NK cells expressed Ly49A, whereas a significantly higher percentage of 20.6% was observed in female NK cells. Additionally, the expression of NKG2A in adult female mice showed a slight reduction, dropping from 29.1% in males to 18.6% in females.

In elderly mice, NK cell subtype distribution was aligning between male and female mice compared initial observations on the day of isolation (Figure 15D). While the proportion of mature $CD11b^{high}CD27^{low}$ NK cells remained higher in female mice (67.6% vs. 56.9%), the difference was not statistically significant. Additionally, the percentage of the less mature $CD11b^{low}CD27^{high}$ and $CD11b^{high}CD27^{high}$ NK cells reached comparable levels across sexes. Specifically, elderly male mice's NK cells contained between 18.1% and 22.6% of these less mature subtypes, whereas in NK cells from female mice, the range was between 12.5% and 17.9%.
Elderly female mice's NK cells exhibited a significant reduction in the expression of activating receptors NK1.1, NKG2D, and NKp46 compared to their male counterparts (Figure 15E). The portion of NK1.1 expressing cells decreased from 94.8% in males to 74% in females. Similarly, the prevalence of $NKG2D^{+}$ cells fell from 93.6% in male mice NK cells to 70.1% in females. The most striking sex disparity was found in NKp46 expressing cells, decreasing from 76.1%

in males to 20.7% in females, indicating that male mice had over 3.5 times more NKp46 expressing cells than females.

In contrast, expression patterns of inhibitory receptors showed no sex differences, mirroring observations made at the day of isolation (Figure 15F). The proportion of KLRG1+ NK cells was nearly identical among male and female mice, with 46.8% and 46.9%, respectively. Ly49A expression was similarly low across both sexes, found on 12.9% and 14.2% of NK cells, while NKG2A was expressed on 36.2% of male and 30.8% of female NK cells, showing no significant sex difference in the expression of these inhibitory receptors.

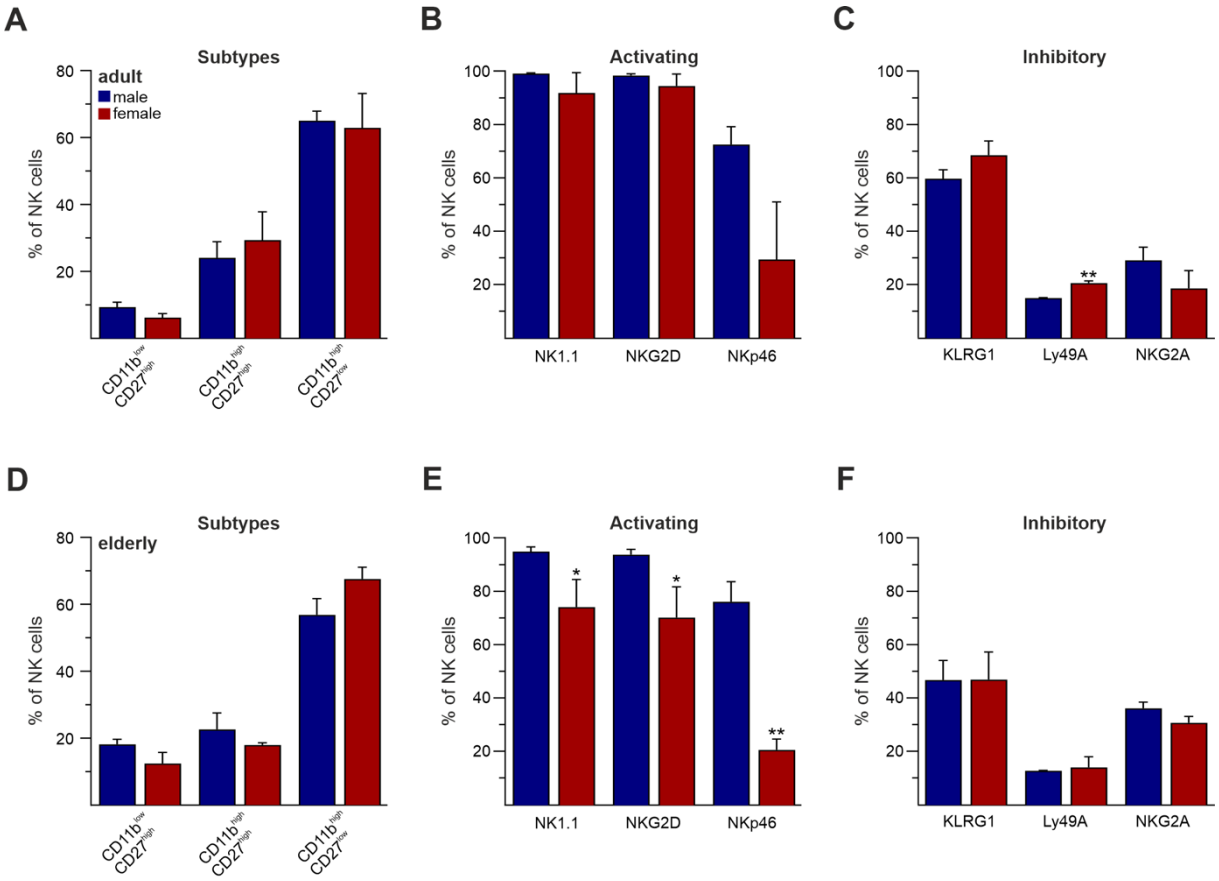


Figure 15: Blood NK cells from elderly female mice express significantly less activating receptors compared to those from male mice. Flow cytometry stainings to quantify subtype distribution (**A**, **D**) and expression of activating (**B**, **E**) and inhibitory (**C**, **F**) NK cell receptors of adult and elderly mice NK cells, respectively. Male mice NK cells are depicted in blue and female mice in red. Analysis was performed on day one of stimulation. Significance depicted above bars indicate differences between sexes. Significance between sexes was tested using unpaired Student's t-test. Data presented as mean ± SEM. Significance levels are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. *n = 3-5 per group.*

Taken together, murine blood NK cells consistently demonstrated a higher proportion of mature $CD11b^{high}CD27^{low}$ NK cells than their splenic NK cells counterparts at both times of analysis. Elderly female mice, in particular, showed a tendency towards increased presence of mature NK cells, whereas in adult mice, the subtype distribution was comparable across sexes. Similar to observations in splenic NK cells, overnight IL-2 stimulation enhanced the expression of activating receptors. Notably, blood NK cells from female mice, especially in the elderly group, exhibit significantly lower expression of all activating receptors, including a pronounced

reduction in NKp46. This diminished expression might account for the observed enhanced cytotoxicity in elderly male mice NK cells. In general, murine blood NK cells exhibited higher expression of inhibitory receptors compared to mouse spleen, without showing a clear sex bias. The only exception was the consistently higher expression of Ly49A in adult female mice NK cells compared to males.

3.3 Interleukin-2 Dependence of Human NK Cells across Age and Sex

Given the inherent limitation in NK cell numbers in mice and to facilitate a direct comparison with murine NK cells from blood, NK cells were isolated from human blood samples. In alignment with the analysis performed on murine NK cells, human NK cells were examined across a spectrum of parameters including cytotoxicity, subtype distribution, and NK receptors expression. Furthermore, the investigation was broadened to assess exhaustion and the expression of effector molecules, enriching our understanding of NK cell functionality. Understanding NK cell function during aging in both sexes, alongside the considerable therapeutic possibilities in immunotherapy, highlights the importance of recognizing these variations.

3.3.1 NK Cell Abundance in Healthy Human Donors

Natural killer cells, constituting approximately 5% to 10% of human blood lymphocytes, play an important role in the body's innate immune response (Angelo et al., 2015; Dogra et al., 2020). The impact of age on NK cell abundance has been a subject of mixed findings, with some studies reporting no changes across different ages, while others observed an increase in NK cell numbers with advancing age (Agarwal et al., 2022; Almeida-Oliveira et al., 2011; Chidrawar et al., 2006; Gounder et al., 2018; Le Garff-Tavernier et al., 2010; Lutz et al., 2005, 2011; Phan et al., 2017; Przemska-Kosicka et al., 2018). Furthermore, the question whether sex influences NK cell abundance has also yielded inconclusive results, with discrepancies noted across different studies (Al-Attar et al., 2016; Dogra et al., 2020; Gounder et al., 2018; Graydon et al., 2023; Z. Huang et al., 2021; Phan et al., 2017). In an effort to clarify these disparities, this study quantified NK cell abundance in healthy female and male donors across a broad age spectrum using flow cytometry.

NK cells were defined based on their expression of as $CD45^{+}CD3^{-}CD56^{+}$ lymphocytes (Figure 16). Consistent with the findings of several studies, NK cell abundance slightly increased with age, particularly among female participants. However, this increase from 10.5% to 12% in male and from 8% to 12.2% in female participants did not reach statistical significance (p=0.3577).

Moreover, the analysis revealed no difference in NK cell abundance between sexes (p=0.1878).

These findings suggest that both age and sex have minimal impact on NK cell abundance in healthy individuals. This age- and sex-independence highlights the robust consistency in the proportion of NK cells within the human immune system.

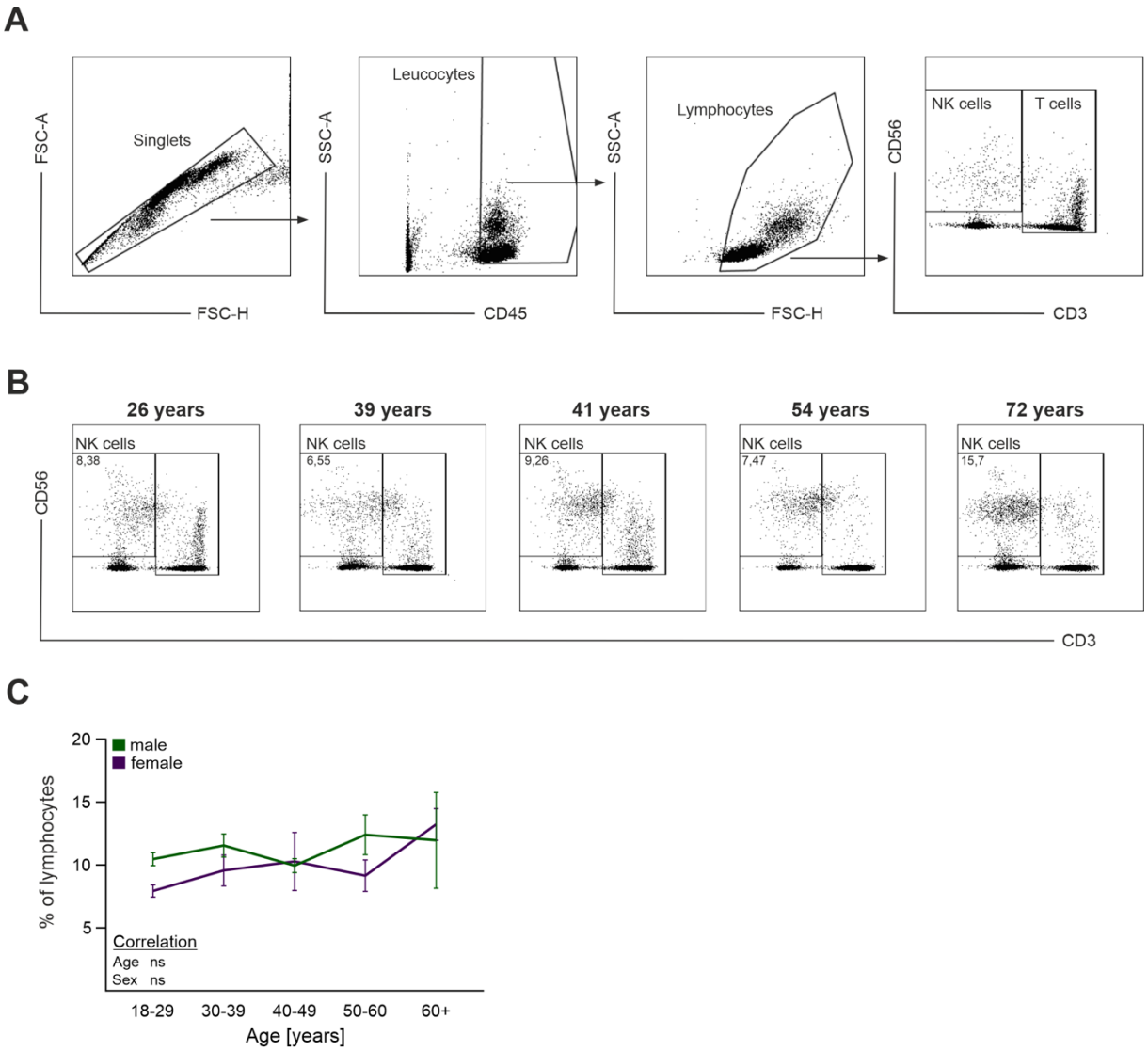


Figure 16: NK cell abundance in human PBMCs is age- and sex-independent. Flow cytometry analysis of NK cell abundance in PBMCs of healthy male and female donors after the age of 18 years. Figure **A** depicts a representative gating strategy to identify NK cells as $CD45^{+}CD3^{-}CD56^{+}$. Representative quantification of NK cell abundance in female donors of each age cohort is depicted in figure **B**. Statistical quantification and analysis of NK cell abundance of male (green) and female (purple) donors in all age cohorts is shown in figure **C**. Analysis was performed on the day of isolation. Significance was tested using two-way ANOVA. Data presented as mean ± SEM. Significance levels are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. *n = 3-16 per group.*

3.3.2 Cytotoxicity of Human NK cells under the Influence of IL-2

Like NK cell abundance, alterations in cytotoxicity due to aging are not clear. Some studies have found a decrease in cytotoxicity with age (Facchini et al., 1987; Sansoni et al., 1993; Vitale et al., 1992), while others found no changes (Almeida-Oliveira et al., 2011; Le Garff-

Tavernier et al., 2010), and yet others reported an increase. (Krishnaraj & Svanborg, 1992; Kutza & Murasko, 1994). Interestingly, IL-2 supplementation appear to boost NK cell cytotoxicity across age groups, with a more pronounced effect observed in the younger individuals (Konjevic et al., 2010; Kutza & Murasko, 1994). However, another study only found a marginal decrease in cytotoxicity of IL-2-activated NK cells with age (Le Garff-Tavernier et al., 2010). The influence of sex on NK cell cytotoxicity is less well-researched with one study finding no difference in cytotoxicity between men and women (Phan et al., 2017). However, in children, the sex-specific difference was age-dependent (Horikoshi, 1985). Many of these cytotoxicity assays were conducted using whole PBMC populations rather than isolated NK cells. Given these gaps, a detailed analysis of the influence of IL-2 on both age and sex is still required. Therefore, isolated NK cells from healthy men and women of all ages were analyzed regarding cytotoxicity in the absence or presence of IL-2.

The cytotoxicity of human NK cells decreased with age, but it was notably enhanced by the supplementation with 50U IL-2 (Figure 17). While certain age groups revealed substantial differences in end point lysis between male and female participants (Figure 17A), the overall analysis showed that sex did not significantly affected NK cell cytotoxicity ($p=0.1006$) (Figure 17B). Nonetheless, age negatively impacted NK cell cytotoxicity across all conditions ($p=0.0471$), with end point lysis decreasing from 55% to 29.4% in male participant NK cells without IL-2 and from 73.3% to 36.6% in male participant NK cells supplemented with 50U IL-2. Similarly, in females, end point lysis decreased from 69.1% to 34.8% without IL-2 and from 85% to 48.6% when supplemented with 50U IL-2, moving from the youngest to the oldest participants. Strikingly, the most influential parameter was supplementation of IL-2 during overnight stimulation, enhancing NK cell cytotoxicity for both sexes ($p<0.0001$).

In contrast to end point lysis, maximal target cell lysis per 10 min was not altered with age ($p=0.1538$), ranging from 4.44% to 6.14%% in males and from 3.56% to 9% in females without IL-2 supplementation (Figure 17C). However, the addition of IL-2 markedly enhanced maximal target cell lysis, increasing it from 8% to 10.1% in male participants and from 7% to 13.6% in female participants ($p<0.0001$). The participants' sex did not significantly influenced these outcomes ($p=0.1783$).

Viability, considered a crucial parameter, was comprehensively assessed (Figure 17D). NK cell viability of NK cells remained high and stable across all age cohorts, with a minimum observed viability of 97.6% ($p=0.5031$). In male participants, NK cell viability ranged from 98.5% to 99.5% without IL-2 supplementation and from 98.2% to 99.6% with IL-2. Conversely, NK cells from female participants displayed a significantly lower viability, ranging between 98.3% and 98.7% without IL-2 and dropping to between 97.6% and 98.2% with 50U IL-2 ($p= 0.001$). This supports previous findings, with IL-2 supplementation leading to a significant increase in viability for both

sexes (p=0.0027). Nevertheless, to exclude the possibility that viability could affect cytotoxicity, all killing assays were performed using a count of living cells.

In summary, even short-term IL-2 stimulation of human NK cells increased both their cytotoxicity and viability. Age impacts only the end point lysis. Notably, NK cells from female donors showed reduced viability in comparison to those from male donors, with viability being the only parameter to reveal sex related differences.

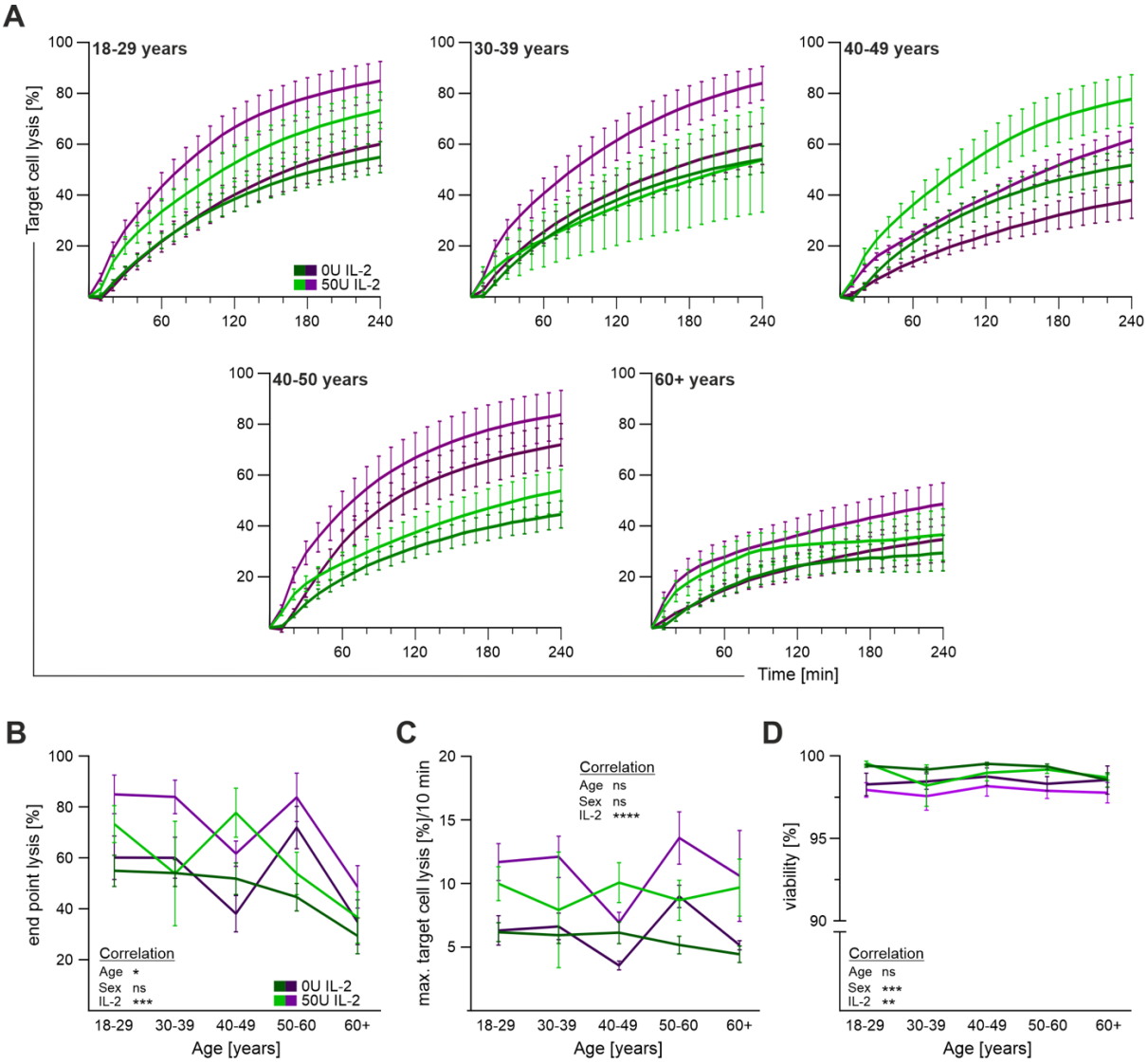


Figure 17: IL-2 significantly increases cytotoxicity and viability of human NK cells in all age cohorts. Real-time killing assay of male and female human donor NK cells in the presence or absence of IL-2 as depicted in shades of green and purple, respectively (**A**). Quantification of mean end point lysis (**B**) and maximal target cell lysis per 10 min or killing rate (**C**). K-562 were utilized in a 5:1 effector-to-target cell ratio. Viability of human NK cells is depicted in figure **D**. NK cells were stimulated either without IL-2 or with 50U IL-2 as depicted in shades of green and purple. Analysis was performed on day one of stimulation. Significance was tested using three-way ANOVA. Data presented as mean ± SEM. Significance levels are indicated as * $p < 0.05$, ** $p< 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. *n = 3-25 per group.*

3.3.3 Subtype and NK Receptor Distribution of Human NK Cells

Changes in human NK cells subtype distribution and the expression of various NK cell receptors during aging have been extensively investigated, yet conclusions remain indecisive. While some studies indicated no age-related changes in the distribution of $CD56^{bright}$ to $CD56^{dim}$ NK cells (Dogra et al., 2020), the majority of studies observed an age-associated increase in $CD56^{dim}$ together with a decrease in $CD56^{brigh}$ NK cells (Almeida-Oliveira et al., 2011; Bigley et al., 2015; Chidrawar et al., 2006; Hayhoe et al., 2010; Le Garff-Tavernier et al., 2010; Lutz et al., 2011; Phan et al., 2017; Przemska-Kosicka et al., 2018). However, little is known about sex-specific differences in NK cell subtype distribution. Phan and colleagues reported a significant increase in $CD56^{bright}$ NK cells in women, contrasting with Dogra and colleagues, who observed an opposite trend, although another study aligned with Phan's findings (Al-Attar et al., 2016). Therefore, subtype distribution and NK cell receptors expression were assessed using flow cytometry in both sexes across all age cohorts.

In healthy human donors, the ratio of $CD56^{bright}/CD56^{dim}$ NK cells significantly decreased with age, indicating an increase in $CD56^{dim}$ NK cells, verifying earlier findings (p=0.0091) (Figure 18A). For male participants, the $CD56^{bright}/CD56^{dim}$ ratio dropped from 0.07 to 0.039, and for female participants, it decreased from 0.067 to 0.029. Thus, the distribution of this subtypes showed no sex-specific differences (p=0.089).

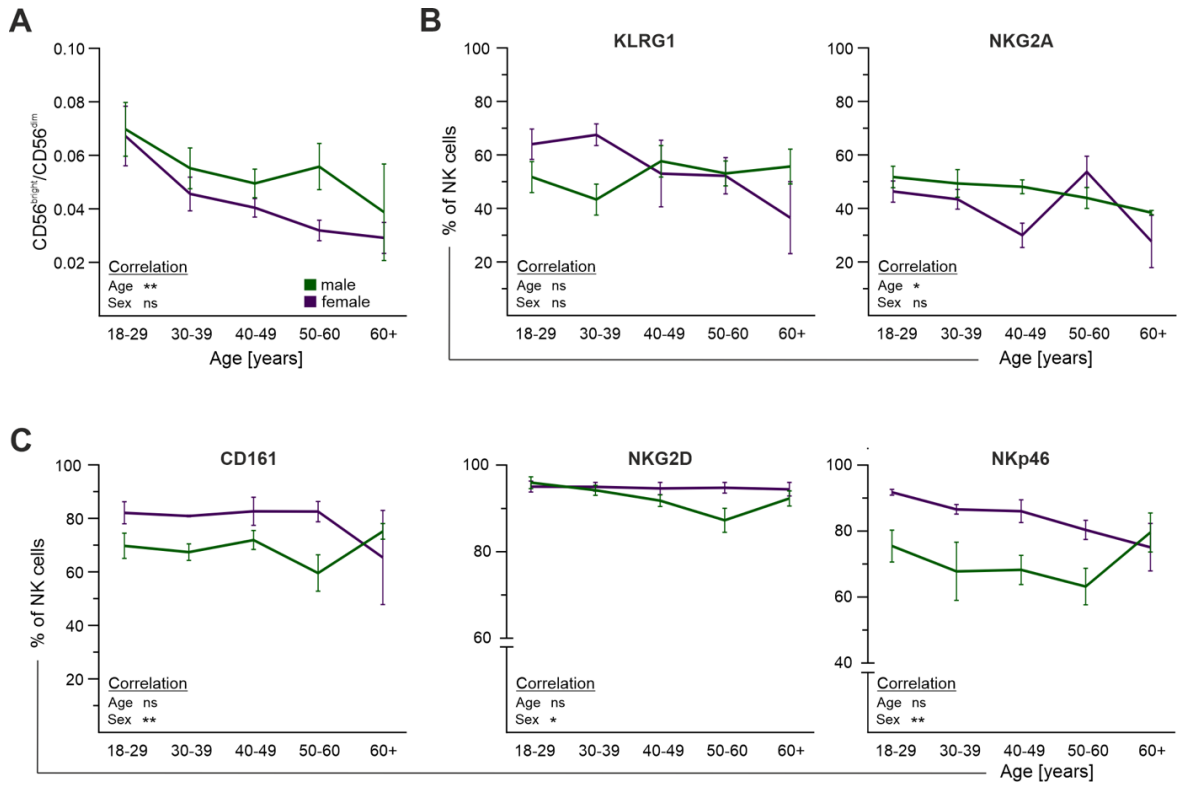


Figure 18: Human NK cell subtype and NK cell receptor distribution are age- and sex-dependent. Flow cytometry analysis of NK cell subtype distribution (**A**), inhibitory receptor expression (**B**), and activating receptor expression (**C**) on human NK cells of healthy men (green) and women (purple) after the age of 18 years. Analysis was performed on the day of isolation. Significance was tested using two-way ANOVA. Data presented as mean ± SEM. Significance levels are indicated as * p < 0.05, ** p< 0.01, *** p < 0.001 and **** p < 0.0001. *n = 3-11 per group.*

Findings on age-related changes in expression of inhibitory receptor KLRG1 among NK cells are inconsistent. Some research indicates that KLRG1 expression diminishes in NK cells (Hayhoe et al., 2010), particularly among cytomegalovirus (CMV)-seronegative donors (Bigley et al., 2015), while other studies report an increase in $KLRG1^{+}$ NK cells with age (Müller-Durovic et al., 2016). Unlike the clear patterns seen in subtype distribution, the variations in KLRG1 expression related to sex remain unexplored.

Immediately following isolation, NK cells showed no significant differences in KLRG1 expression attributable to either age or sex (p=0.6243/p=0.5915) (Figure 18B). Among male donors, KLRG1 expression remained stable, ranging from 51.8% in the youngest and 55.7% in the oldest age group. Conversely, in female donors, KLRG1 expression decreased from 64% to 36.6%, respectively.

Another inhibitory receptor, NKG2A, showed variable expression changes with age, either decreasing or maintaining stability (Le Garff-Tavernier et al., 2010; Lutz et al., 2005, 2011; Phan et al., 2017). Notably, NKG2A expression does not vary based on sex (Al-Attar et al., 2016; Phan et al., 2017).

Aligning with prior findings, a significant decrease in NKG2A expression with age was observed (p=0.0281) (Figure 18B), dropping from 51.8% to 38.5% in male and from 64.4% to 27.7% in female donors. No significant sex-specific differences were detected (p=0.0666).

While it has been documented that the expression of the activating receptor CD161 decreases with age (Kurioka et al., 2018; Lopez-Sejas et al., 2016), insights into sex-specific differences in CD161 expression are still lacking.

Contrary to previous findings, this study observed no age-related changes in CD161 expression (p=0.6644) (Figure 18C). Nevertheless, significant differences between male and female participants were noted (p=0.0052). Among the male donors, CD161 expression remained relatively stable, ranging from 69.8% and 75.2%. Conversely, in female donors, while generally higher, CD161 expression experienced a decrease from 82.1% in the youngest cohort to 65.5% in the eldest cohort.

Similar to CD161, the expression of the activating receptor NKG2D either decreases or remains stable as individuals age (Almeida-Oliveira et al., 2011; Le Garff-Tavernier et al., 2010; Phan et al., 2017; Sundström et al., 2007; J. Wang et al., 2017). There are no significant differences in NKG2D expression based on sex (Phan et al., 2017).

During aging, NKG2D expression was found to be stable, albeit with a slight tendency to decrease (p=0.057) (Figure 18C) (Hazeldine et al., 2012). Nonetheless, a minor sex-bias favoring female donors was observed (p=0.036), with NKG2D expression levels being relatively constant between 94.4% and 95.1%, while, in male donors the range was from 87.3% to 95.9%.

Activating receptor expression of NKp46 on human NK cells tends to decline from adulthood to the older age, stabilizing before adulthood, as observed in several studies (Almeida-Oliveira et al., 2011; Hazeldine et al., 2012; Le Garff-Tavernier et al., 2010; Phan et al., 2017; Sundström et al., 2007). While Phan et al. reported no significant sex differences in NKp46 expression (Phan et al., 2017), in this study, a pattern of decreasing NKp46 expression with age was noted, particularly in female donor NK cells, although not reaching statistical significance (p=0.2994) (Figure 18C). Contrary to findings by Phan et al., a significant sex-bias in NKp46 expression favoring females was observed (p=0.0012). In female donors, NKp46 expression diminished from 91.8% in those aged 19 to 29 to 75.1% in individuals older than 60 years. Conversely, in male donors, NKp46 expression varied from 63.2% to 79.6%.

3.3.3.1 Influence of IL-2 on Subtype and NK Cell Receptor Distribution

To further investigate the impact of IL-2 on subtype distribution and expression of NK cell receptors, the analysis was conducted again one day post-isolation. This time, NK cells underwent overnight stimulation with 50U IL-2 or were left untreated.

Surprisingly, nothing is known about the influence of IL-2 on NK cell subtype distribution. However, a study performed following highly purified donor NK cell immunotherapy revealed an increase in the $CD56^{dim}$ population upon IL-2 treatment, leading to a decreased $CD56^{bright}$/ $CD56^{dim}$ ratio (Brehm et al., 2011).

Consistent with the findings on the day of isolation, the $CD56^{bright}$/ $CD56^{dim}$ ratio of NK cells decreased with age (p=0.0308) with no difference between sexes (p=0.3489) (Figure 19A). Additionally, treatment with IL-2 did not alter $CD56^{bright}$/ $CD56^{dim}$ ratio (p=0.1146). For male donor NK cells not treated with IL-2, the $CD56^{bright}$/ $CD56^{dim}$ ratio varied from 0.111 to 0.053 and for those treated with 50U IL-2 it ranged from 0.122 to 0.68. Among the female donors, the ratio shifted from 0.139 to 0.045 in untreated cells and from 0.128 to 0.035 upon IL-2 treatment.

Overall, the expression of NK cell receptors was not altered during overnight culture. Reflecting the findings from the day of isolation, NK cells from female participants demonstrated a decline in KLRG1 expression moving from the youngest to the eldest age cohort without IL-2 supplementation dropping from 53.5% to 29.7%. In male participants, it remained stable between 43.3% and 48% and therefore, age-related changes were not statistically significant (p=0.3414) (Figure 19B). In contrast to the previous finding, there was no sex-bias in KLRG1 expression observed (p=0.8825). Despite increased in transcriptional expression of KLRG1 following IL-2 treatment (Dybkaer et al., 2007), no corresponding increase was found at the cell surface level (p=0.6694). Specifically, in male donors, KLRG1 expression ranged from 39.4% to 51.5%, and in women from 15.4% to 61.6%.

The expression of NKG2A showed a trend towards an age-related decline, although this was not statistically significant (p=0.058) (Figure 19B). Unlike observations on the day of isolation, there was a pronounced sex-bias favoring male donor NK cells (p=0.0012). IL-2 treatment increased NKG2A expression (p=0.0186) (Hromadnikova et al., 2013). Among male donors, NKG2A expression rose from a range of 50% and 54.9% to between 51.9% and 62.6% following IL-2 stimulation. For female donors, the increase was from 27.8% and 50.9% to a range of 32.9% and 55.4%.

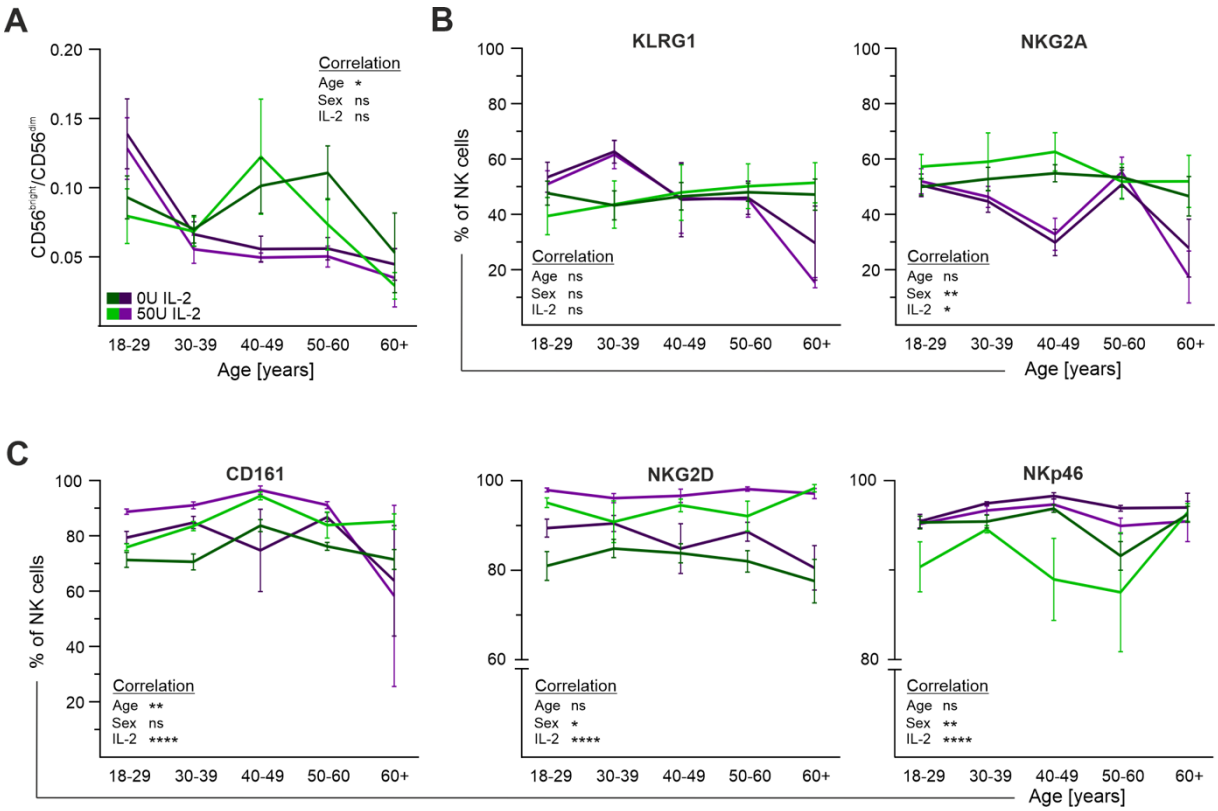


Figure 19: Supplementation of IL-2 significantly increases expression of activating NK cell receptors. Flow cytometry analysis of NK cell subtype distribution (**A**), inhibitory receptor expression (**B**), and activating receptor expression (**C**) on human NK cells of healthy male (green) and female (purple) donors after the age of 18 years. NK cells were stimulated either without IL-2 or with 50U IL-2 as depicted as shades of green and purple. Analysis was performed on day one of stimulation. Significance was tested using three-way ANOVA. Data presented as mean ± SEM. Significance levels are indicated as * p < 0.05, ** p< 0.01, *** p < 0.001 and **** p < 0.0001. *n = 2-18 per group.*

CD161 expression significantly decreased with age (p=0.0028), yet there was no difference between sexes (p=0.2401) (Figure 19C). In male participants, 70.7% to 83.8% of NK cells were $CD161^+$ and in females this ranged between 74.9% and 86.9%. However, IL-2 treatment significantly increased CD161 expression on human NK cells (p<0.0001) (Dybkaer et al., 2007; Hromadnikova et al., 2013; Konjevic et al., 2010), with expression levels ranging from 76% to 94.5% and from 58.4% and 91.2% in male and female participants, respectively.

NKG2D expression remained constant across age groups (p=0.9616) (Figure 19C). Furthermore, female donors exhibited a higher percentage of $NKG2D^+$ NK cells compared to males, independent of treatment (p=0.0315). IL-2 treatment notably enhanced NKG2D expression (p<0.0001). In the absence of IL-2, 77.6% to 84.9% of male and 80.5% to 90.5%

of female NK cells were NKG2D$^+$. Following 50U IL-2 stimulation, the proportion of NKG2D$^+$ NK cells increased to 90.8% to 98.3% in male and from 96.1% to 98.1% in female participants. Although transcriptional analysis revealed an increase in NKG2D expression, surface staining did not show a difference between untreated and IL-2 treated NK cells, except after stimulation (Dybkaer et al., 2007; Hromadnikova et al., 2013; Konjevic et al., 2010; Sun et al., 2021).
Similar to NKG2D, the expression of NKp46 was not influenced by age (p=0.2066), but it exhibited a clear dependency on sex (p=0.0062) (Figure 19C). Female donor NK cells consistently showed higher NKp46 expression levels, ranging from 95.5% to 98.3% in untreated cells, whereas in male donors, expression varied from 91.6% to 96.9%. Furthermore, IL-2 treatment significantly decreased NKp46 expression in both sexes (p<0.0001), with a reduction from 95% to 97.3% in females and from 87.5% to 96.5% in males. This aligns with various studies indicating that IL-2 treatment can lead to decreased expression of NKp46 at both the transcriptional level and on the cell surface (Dybkaer et al., 2007; Hromadnikova et al., 2013).

In summary, while the subtype distribution of NK cells undergoes significant changes with age, only a few NK cell receptors display age-related variations in expression levels. No sex-specific differences are observed in the expression of inhibitory receptors. However, NK cells from female donors are characterized by significantly higher expression of activating receptors, especially evident on the day of isolation. Strikingly, IL-2 supplementation does not compensate for the loss of CD56^{dim} population in any age group, but it does significantly boost the expression of activating receptors such as CD161 and NKG2D, while decreasing NKp46 expression. Furthermore, following overnight stimulation, NK cells from female participants demonstrate an increased expression of inhibitory receptor NKG2A, indicating a regulatory mechanism to prevent excessive activation.

3.3.4 Exhaustion of Human NK Cells

Exhaustion drastically impacts immunomodulation. Analogous to murine cytotoxic T lymphocytes, PD-1 serves as a marker to identify exhausted cells. In healthy human samples, PD-1 expression typically remains below 1.5% with a maximum of 10% and only in a single instance among 200 donors displaying approximately 50% PD1$^+$ NK cells (Pesce et al., 2017). The effects of age and sex on PD-1 expression are less understood. Research indicates an increase in PD-1 expression among Malian children up to 18 years, with no apparent difference when compared to the youngest cohort compared of US adults (Moebius et al., 2020). While another study found increasing PD-1 expressing with age in both CD4$^+$ and CD8$^+$ T cell populations but NK cells were not specifically examined (Fu et al., 2023). Other markers indicative of exhaustion including TIGIT and TIM-3. The expression of TIGIT on NK cells from

healthy human donors shows great variability across different studies, with most studies identifying between 30% and 40% TIGIT$^+$ NK cells (Hasan et al., 2023; Yin et al., 2018; X. Zhang et al., 2021), while another study reported about 60% TIGIT$^+$ NK cells (F. Wang et al., 2015). TIM-3 expression is noted on at least 75% of freshly isolated peripheral blood NK cell (W. Jiang et al., 2022; Ndhlovu et al., 2012; Y. Wang et al., 2017). The influence of age and sex on the expression of those exhaustion markers remains unexplored, prompting their analysis using flow cytometry across males and females of all age cohorts.

Upon isolation, PD-1 expression remained stable across all age cohorts (p=0.8547), aligning with the findings of a previous study (Figure 20). In male donors, PD-1 expression slightly increased from 0.72% to 1.27% while in female donors, there was a slight decrease from 1.06% to 0.55% moving from the youngest to the oldest age cohorts. Consequently, no sex-specific differences were observed (p=0.2547).

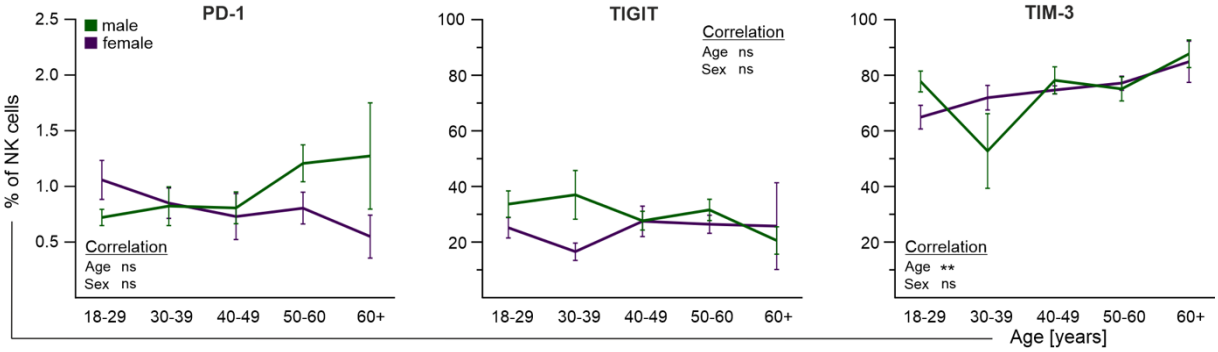


Figure 20: Exhaustion is not sex-dependent and only partially influenced by age. Flow cytometry analysis of exhaustion markers PD-1, TIGIT, and TIM-3 (left to right) on human NK cells of healthy male (green) and female (purple) donors after the age of 18 years. Analysis was performed on the day of isolation. Significance was tested using two-way ANOVA. Data presented as mean ± SEM. Significance levels are indicated as * p < 0.05, ** p< 0.01, *** p < 0.001 and **** p < 0.0001. *n = 3-13 per group.*

Similarly, TIGIT expression did not vary significantly with (p=0.8141) or sexes (p=0.0853), with expression levels in male participants ranging from 20.6% to 37% and female participants from 16.6% to 27.5%.

In contrast, TIM-3 expression uniquely showed a significant increase with age (p=0.0047), increasing from 77.8% to 87.7% in males and from 65% to 84.9% in females across the age spectrum. Nonetheless, this increase did not exhibit a sex-bias (p=0.9078).

3.3.4.1 Influence of IL-2 on Exhaustion

Following overnight stimulation, PD-1 expression remained comparable if no IL-2 was added (Figure 21). However, if cells were treated with 50U IL-2, PD-1 expression significantly increased in both male and female participants (p<0.0001). For males, PD-1 expression increased from 0.8% to 1.2% and doubled to 1.1% to 1.7% upon IL-2 treatment. For females, the proportion of PD-1$^+$ NK cells increased from 0.7% to 0.94% and to 1.2% to 1.9% after IL-2

supplementation. This mirrors findings from a previous study where PD-1 expression in human NK cells doubled after stimulation with 1000U IL-2 for three days (Judge et al., 2020). Nevertheless, the increase in PD1 expression exhibited neither a sex-bias (p=0.981) nor dependence on age (p=0.9812).

Similar to PD-1, TIGIT expression did not differ following overnight stimulation in the absence of IL-2. However, IL-2 supplementation significantly elevated TIGIT expression, increasing it from 30.2% and 42.3% without IL-2 to 49.1% and 55% with 50U IL-2 in male donor NK cells and from 29.9% and 34.6% without IL-2 to 47.7% to 65.5% with 50U IL-2 female donor NK cells ($p<0.0001$). This aligns with findings from a previous study that also reported a significant increase in TIGIT expression after stimulation with 1000U IL-2 overnight (Hasan et al., 2023). Moreover, the increase in TIGIT expression was not affected by either age (p=0.7772) or sex (p=0.9275).

TIM-3 expression remained relatively stable to a ratio between 35.3% and 39.3% in males and 32% and 44.5% in females in the absence of IL-2. Strikingly, only with IL-2 supplementation did TIM-3 expression increase to approximately 77.5% to 90% in male and 77.1% and 88% in female donors ($p<0.0001$). The decrease observed in TIM-3 expression from the day of isolation and following culture has been previously observed by several groups (So et al., 2019; Zheng et al., 2019). Moreover, TIM-3 expression was not affected by both age (p=0.6127) and sex (p=0.9305).

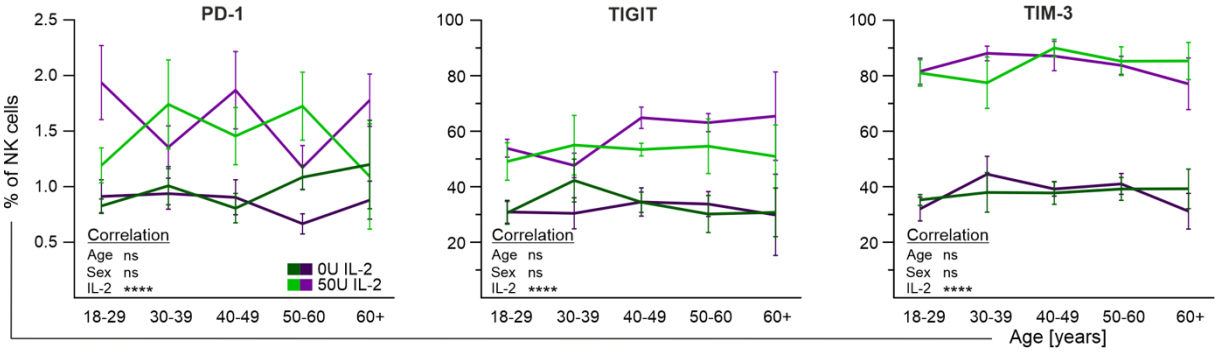


Figure 21: IL-2 supplementation significantly increases exhaustion marker expression on human NK cells of all ages. Flow cytometry analysis of exhaustion markers PD-1, TIGIT, and TIM-3 (left to right) on human NK cells of healthy male (green) and female (purple) donors after the age of 18 years. NK cells were stimulated either without IL-2 or with 50U IL-2 as depicted as shades of green and purple. Analysis was performed on day one of stimulation. Significance was tested using three-way ANOVA. Data presented as mean ± SEM. Significance levels are indicated as * p < 0.05, ** p< 0.01, *** p < 0.001 and **** p < 0.0001. *n = 3-12 per group.*

Taken together, the findings suggest that exhaustion does not significantly influence the age or sex differences in human NK cells. The expression of exhaustion markers appears primarily in response to overnight stimulation with 50U IL-2.

3.3.5 Expression of Effector Molecules in Human NK Cells

Similar to cytotoxic T lymphocytes, granule-mediated cytotoxicity plays a crucial role in the functionality and effectiveness of NK cells. To achieve this, human NK cells utilize a variety of

effector molecules, including granzymes, perforin, and also granulysin (Clayberger et al., 2012). It has been observed, that the expression of all these effector molecules is increased following IL-15 stimulation (Macedo et al., 2022). Surprisingly, the influence of sex on the expression levels of these crucial effector molecules remains largely unexplored. To address this gap, intracellular staining was performed on NK cells cultured overnight in the presence or absence of IL-2, across both sexes and all age groups.

Granulysin, a cytolytic protein exclusive to human immune cells (Clayberger et al., 2012; Peña et al., 1997), functions similarly to perforin, by disrupting the cell membrane of the target cells, causing an extreme ion influx and subsequent cell death (Kaspar et al., 2001). Post-overnight stimulation, granulysin expression did not differ across age cohorts ($p=0.3689$) or between sexes ($p=0.9102$) (Figure 22). Interestingly, research has shown serum levels of granulysin increasing until the age of 40, after which they remain stable (Ogawa et al., 2003). Nonetheless, IL-2 treatment significantly increased granulysin levels in both male and female participants ($p<0.0001$), with expression in untreated male NK cells ranging from 56.7% to 86.5% and from 83.1% to 94.5% in IL-2-treated cells. In females, granulysin expression rose from 61.3% to 79.8% without IL-2 and from 82.3% to 88.3% with 50U IL-2 supplementation. Although the specific role of IL-2 is less understood, NK cell stimulation with IL-15, in the absence or presence of IL-2, has been found to enhance granulysin expression (Crespo et al., 2020; Saini et al., 2011).

Granzyme B expression remained consistent with age ($p=0.9839$) and sex ($p=0.9703$) (Hazeldine et al., 2012). This finding aligns with studies reporting no changes in granzyme B expression levels following IL-15 stimulation, in human serum, or in unstimulated NK cells (Macedo et al., 2022; Mahapatra et al., 2017; Mellor-Heineke et al., 2013; Verschoor et al., 2023). Additionally, the immortalized human NK-92 cell line exhibited increased granzyme B expression at both mRNA and protein levels after IL-2 treatment (C. Huang et al., 2006; B. Zhang et al., 2008). Primary human NK cells also showed a significant increase in granzyme B expression post-overnight stimulation in both male and female participants ($p<0.0001$) (Figure 22). In the absence of IL-2, male donor NK cells expressing granzyme B ranged from 96.3% to 99.1%, which increased to a minimum of 99.2% following IL-2 stimulation. In female donor NK cells, the proportion of granzyme B expressing cells increased from between 96.9% and 98.9% to a minimum of 99.4%.

Whole perforin expression, detected using the antibody clone B-D48 (Hersperger et al., 2008), was not influenced by age ($p=0.6117$) or sex ($p=0.1531$), aligning with previous findings (Mahapatra et al., 2017). Nonetheless, the specific effect of IL-2 on whole perforin regulation had not been previously investigated. In human primary NK cells, IL-2 significantly increased whole perforin levels ($p=0.001$). In the absence of IL-2, the expression of perforin varied from

96.7% to 99.4% in male and from 98.3% to 99.4% in female participants. Following IL-2 supplementation, perforin expression increased to a range of 99% to 99.6% in males and

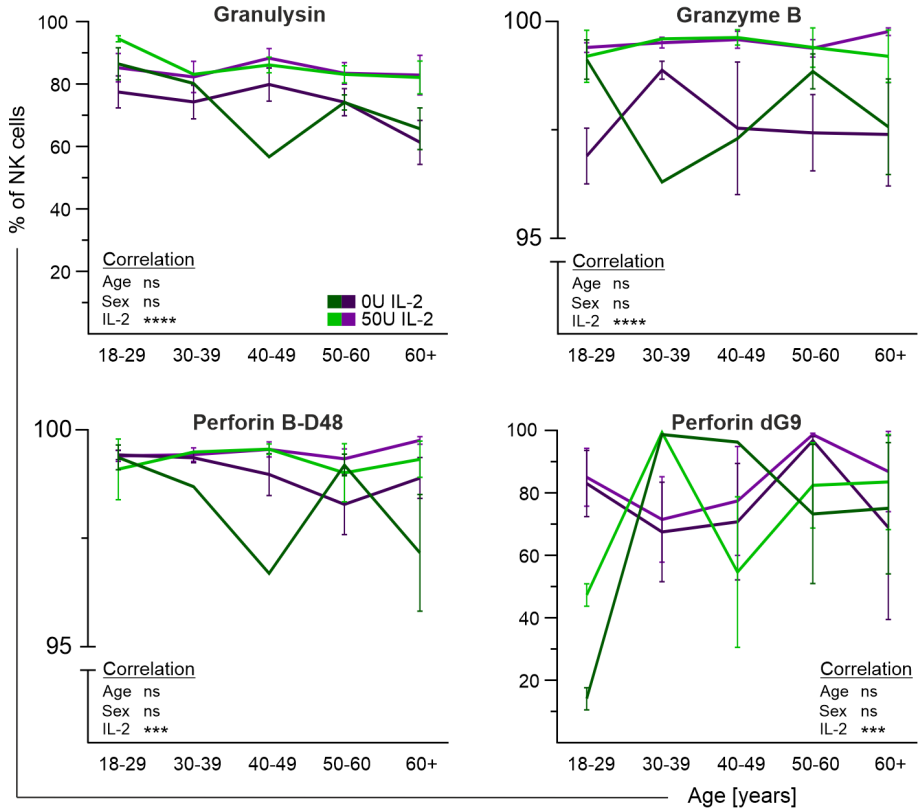


Figure 22: IL-2 supplementation significantly increases expression of effector molecules granulysin, granzyme B, and perforin. Flow cytometry analysis of effector molecules granulysin, granzyme B (top panel), and perforin (bottom panel) on human NK cells of healthy male (green) and female (purple) donors after the age of 18 years. NK cells were stimulated either without IL-2 or with 50U IL-2 as depicted as shades of green and purple. Analysis was performed on day one of stimulation. Significance was tested using three-way ANOVA. Data presented as mean ± SEM. Significance levels are indicated as * $p < 0.05$, ** $p< 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. *n = 1-12 per group.*

99.3% to 99.8% in females.

The impact of age and sex on granule-associated perforin, identified by the clone dG9 (Hersperger et al., 2008), has been more thoroughly examined compared to whole perforin. Research findings in this subject are inconclusive: one study observed an increase in expression up to adulthood followed by a significant decrease in the elderly population, another reported no age-related changes, and a third documented an increase in perforin expression in the elderly (Hazeldine et al., 2012; Mahapatra et al., 2017; Mariani et al., 1996; Rukavina et al., 1998). Furthermore, Rukavina et al. noted a marked reduction in perforin expression among female NK cells. However, no significant sex-specific differences were detected in isolated and overnight-cultured NK cells (p=0.2797), nor did age influence the expression patterns (p=0.1403). IL-2 stimulation was the only factor that significantly altered expression of granule-associated perforin expression (p=0.0003). In cells not treated with IL-2, perforin levels ranged from 14.1% to 98.6% in males and from 67.5% to 96.7% in females. Following IL-2 supplementation, expression levels increased to between 47.3% and 99.3% in males and between 71.5% and 98.6% in females.

In summary, the expression levels of all key effector molecules in NK cell appear to be independent of both age and sex. Notably, the addition of IL-2 significantly increases the expression of these molecules, which could account for the enhanced cytotoxicity capabilities observed in NK cells stimulated with IL-2.

3.3.6 Secretion Profile of Human NK Cells and Correlation with Cytotoxicity

Analyzing the expression of effector molecules alone does not fully elucidate the nuances of cytotoxicity changes. The secretion profile of stimulated primary human NK cells, particularly differences between males and females, remains unexplored. To address this, supernatants from killing assays were analyzed to assess the secretion levels of a range of effector molecules and cytokines across both sexes. The killing assay supernatants, collected immediately after the assay, were analyzed using the LegendPlex™ system by BioLegend, offering insights into how secretion dynamics might correlate with NK cell cytotoxic capabilities.

Initially, the influence of sex and IL-2 treatment on secretion profile were analyzed (Figure 23A). Similar to the expression analysis, granzyme B secretion significantly increased with IL-2 stimulation overnight for both male ($p=0.0025$) and female ($p=0.0095$) NK cells, yet no difference based on sex was detected whether without IL-2 ($p=0.7724$) or with 50U IL-2 ($p=0.2089$). For males, granzyme B secretion levels increased from 0.159 pg/mL to 1.09 pg/mL, while for females, the increase was from 0.14 pg/mL to 2.38 pg/mL.
In parallel, the secretion of TNF-α was also significantly increased in IL-2 treated cells compared to untreated controls, with no observed differences between sexes (0U: $p=0.0619$/50U: $p=0.1172$). Specifically, TNF-α levels rose from 0.119 pg/mL to 0.292 pg/mL in male ($p=0.0233$) and from 0.162 pg/mL to 0.436 pg/mL ($p<0.0001$) female participants. This findings are supported by cell culture studies, which have consistently shown that IL-2 stimulation enhances TNF-α secretion in human NK cells (De Sanctis et al., 1997; Jewett et al., 1996; Jewett & Bonavida, 1993).
Similarly, IFN-γ secretion showed a boost upon IL-2 treatment; however, only the increase was significantly pronounced in NK cells from female ($p=0.0058$) compared to male donors ($p=0.0563$) (De Sanctis et al., 1997; Jewett et al., 1996; Krishnaraj & Bhooma, 1996). In male donors, IFN-γ secretion levels increased from 0.256 pg/mL to 1.607 pg/mL, while in female donors, the increase was 0.353 pg/mL to 2.847 pg/mL. Nevertheless, no sex-specific differences were observed in either untreated ($p=0.306$) or IL-2 treated cells ($p=0.3081$).
Soluble Fas ligand (sFasL) can trigger apoptosis in target cells through the Fas receptor (CD95) in a dose-dependent manner (N.-L. Li et al., 2004). In this context, NK cells from male donors exhibited an increase in sFasL secretion from 0.043 pg/mL without IL-2 to 0.063 pg/mL with 50U IL-2 ($p=0.0068$), while female donors displayed an increase from 0.056 pg/mL without

IL-2 to 0.088 pg/mL with 50U IL-2 ($p<0.0001$). Consequently, female NK cells were found to secrete significantly more sFasL than male NK cells in both untreated ($p=0.0004$) and IL-2 treated ($p=0.0032$) NK cells. However, since K-562 cells which were used as the target cell line in the killing assay, lack CD95 expression on their surface (Munker et al., 1997; Wilson et al., 1998), sFasL does not contribute to the cytotoxicity of human NK cells in this specific assay. The secretion of perforin mirrored its intracellular expression profile, showing no significant variance between male and female donor NK cells whether untreated ($p=0.6132$) or treated with IL-2 ($p=0.1102$). Nonetheless, IL-2 supplementation markedly elevated perforin secretion in both male ($p=0.0021$) and female participants ($p<0.0001$).
IL-6, an anti-inflammatory cytokine, is known to reduce cytotoxicity through the downregulation of granzyme A expression in human NK cells (Y.-J. Kang et al., 2014; Mazzoni et al., 2020). Following overnight stimulation of primary NK cells, either in the absence or presence of IL-2, no differences were found in the secretion of IL-6 between male ($p=0.1055$) and female donors ($p=0.0838$) NK cells. Furthermore, there were no notable sex-specific differences in IL-6 secretion levels either without ($p=0.8019$) or with 50U ($p=0.2191$) of IL-2 treatment.

Forthermore, the correlation between secretion levels of nine different analytes (perforin, granzymes A and B, granulysin, TNF-α, IFN-γ, sFasL, IL-17A, and IL-6) and the endpoint lysis of the respective killing assay was analyzed regarding their correlation factor r. This analysis enables the identification of effector molecules and cytokines influencing the cytotoxicity, revealing potential targets for enhancing the cytotoxicity of human NK cells. It encompassed all age groups and both sexes, without distinguishing between conditions of IL-2 treatment.
The secretion levels of key effector molecules such as perforin ($r=0.5992$; $p<0.0001$), granzyme A ($r=0.5118$; $p<0.0001$), granzyme B ($r=0.3697$; $p=0.0014$), and granulysin ($r=0.6067$; $p<0.0001$) demonstrated a significant positive correlation with endpoint lysis in NK cell-mediated killing assays (Figure 23B). These findings align with prior research, underscoring the critical role these molecules play in the cytotoxic ability of NK cells. It is noteworthy that, unlike in NK cells, granzyme A expression did not show a correlation with cytotoxicity in CTLs (Chung et al., 2008; Harari et al., 2009), highlighting a potential difference in the mechanisms of action between these two cell types. Moreover, increased cytotoxicity is attributed not only to the elevated expression of these effector molecules but also to enhanced degranulation processes (Cohnen et al., 2013; Ferraz et al., 2017).
Contrastingly, the secretion of neither TNF-α ($r=0.05616$; $p=0.623$) nor IFN-γ ($r=0.1694$; $p=0.1462$) correlated with cytotoxicity of human NK cells. This observation diverges from the expected positive correlation commonly reported between TNF-α concentration and cytotoxicity suggesting that contribution of these cytokines to NK cell-mediated killing may be context-dependent (Machuca et al., 2006; Petrova et al., 2006; Romanova et al., 2020; J. Wu et al., 2023).

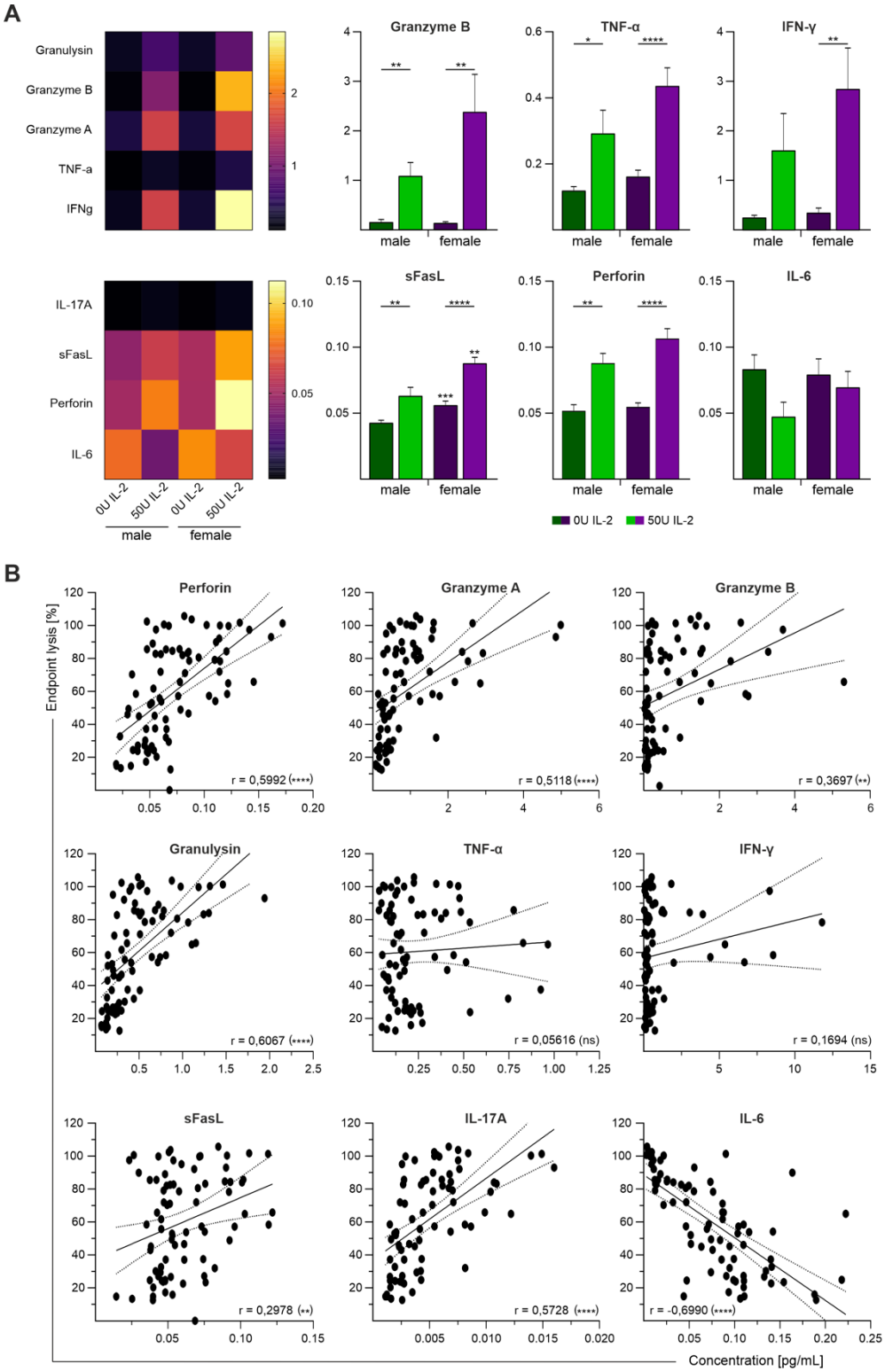


Figure 23: Cytotoxicity of human primary NK cells positively correlates with secretion of effector molecules and is enhanced upon IL-2 supplementation. LegendPlex™ analysis of killing assay supernatants after overight stimulation in the absence or presence of 50U IL-2 as depicted in shades of green (male) and purple (female). (**A**) Secretion of and effector molecules granzyme B, TNF-α, IFN-γ, soluble Fas ligand (sFasL), perforin, and anti-inflammatory cytokine IL-6 in an IL-2-dependent manner as a heat-map (left panel) and statistical analysis (right panel). (**B**) Correlation analysis of analyte secretion and endpoint lysis as quantified via killing assay. Correlation is identified by r as the correlation factor. Effector molecules perforin, granzymes A and B, granulysin, TNF-α, IFN-γ, and sFasL were analyzed. Additionally, cytokines IL-17A and IL-6 were analyzed. Significance depicted above bars indicate differences between sexes. Significance between sexes was tested using unpaired Student's t-test. Significance between conditions was tested using paired one-way ANOVA. Correlation was tested using correlation analysis. Data presented as mean ± SEM. Significance levels are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. *n = 11-27 per group. Number of pairs in correlation: 72-79.*

Similarly, IFN-γ secretion has been found to positively correlate with cytotoxicity in the NK-92 cell line and keratinocytes (Arnold et al., 1999; X. Song et al., 2020).
Soluble FasL secretion similarly exhibited a positive correlation with cytotoxicity of human NK cells ($r=0.2978$; $p=0.0077$), although previous research has yielded mixed results. While one study observed an increase in cytotoxicity corresponding to higher sFasL concentrations, another found no such correlation (Chung et al., 2008; Xiao et al., 2002), indicating that the role of sFasL in cytotoxicity might be context-dependent.
Interestingly, IL-17A displayed a significant positive correlation between the secretion level and the cytotoxicity against K-562 ($r=0.5728$; $p<0.0001$). Although, the direct impact influence of IL-17A on cytotoxicity remains unclear, research suggests it may increase migration of triple negative breast cancer cells while potentially enhancing survival in head and neck cancer (Tsai et al., 2021; M. Yu et al., 2022). Furthermore, IL-17A levels have been associated with TNF-α concentration following cerebral infarction (Xu et al., 2022), hinting at its involvement in inflammatory processes that could affect immune cell functions.
Notably, IL-6 as an anti-inflammatory cytokine, was the only analyte to exhibit a negative correlation with cytotoxicity ($r=-0.699$; $p<0.0001$), underscoring its potential role as an inhibitor of NK cell function through the suppression of granzyme B expression (Y.-J. Kang et al., 2014; J. Wu et al., 2019).

In summary, IL-2 stimulation of NK cells markedly enhances the secretion of key effector molecules, including perforin, granzymes, and granulysin, which are directly linked to increased cytotoxic abilities. Interestingly, IL-2 treatment tends to reduce IL-6 secretion, mitigating the effects of this anti-inflammatory cytokine known to adversely affect cytotoxic functions. This suggest that IL-2 not only boosts the NK cells' cytotoxic repertoire but also modulates the cytokine environment to favor effective immune responses. Moreover, this highlights the complex interplay of pro-inflammatory and anti-inflammatory cytokines in regulating NK cell activity and suggest that balancing these signals is crucial for optimal NK cell cytotoxicity.

4 Discussion

In this study, the influence of sex on effector immune cells was investigated with a special focus on IL-2 regulation across different ages. Analyses were performed on murine splenic cytotoxic T lymphocytes (CTLs) as well as murine and blood natural killer (NK) cells. Despite the well-established roles of IL-2 and its signaling pathways in these cells, limited research has been conducted on how sex impacts IL-2 dependency and how such effects evolve over an organism's lifespan. In the first section, the impact of IL-2 on the cytotoxic immune cells will be discussed in an age-dependent manner, followed by a discussion of the influence of sex on immune cell function and the interplay between innate and adaptive immunity. Lastly, the technical and methodical limitations will be briefly discussed.

4.1 IL-2-Dependency of Immune Cell Function in the Context of Aging

4.1.1 Activation and Effector Function

Across all tested immune cell types in both species, alteration in IL-2 availability showed the most pronounced effects on the immune function in different age groups. Supplementation with IL-2 notably enhances both the expression of effector molecules, as quantified by intracellular flow cytometry staining, and cytotoxicity in a dose-dependent manner for murine CTLs and human NK cells. This suggests a similar regulatory mechanism for effector molecule expression in these cells. Both perforin and granzyme B expression are regulated by IL-2, involving pathways that include STAT5B and the transcription factor Eomesodermin (Eomes) with the latter also being controlled by IL-2 signaling (Fehniger et al., 2007; Hinrichs et al., 2008; Imada et al., 1998; Janas et al., 2005; Pipkin et al., 2010). Interestingly, IL-2 and IL-15 can induced granzyme B expression without TCR-mediated stimulation, acting via CD122 (Tamang et al., 2006).

CD122, together with CD25 and CD132, form the high-affinity IL-2R complex (Beltra et al., 2016; S.-H. Lee et al., 2012). CD25 is rapidly induced following T cell activation, but is absent on naïve T cells, (Beltra et al., 2016). Initially triggered by TCR signaling, CD25 expression is maintained by a positive feedback loop with IL-2 (Malek, 2008). Our study observed a gradual loss in CD25 expression alongside decreasing IL-2 concentrations in murine CTLs. However, since CD25 expression persist even when IL-2 is neutralized, it suggests that basal CD25 expression levels can be obtained through TCR signaling alone, with enhanced expression maintained by the positive feedback loop mediated by IL-2.

Due to this loss in CD25 expression, differentiation of CTLs might be impaired as enhanced and prolonged expression of CD25 reinforces STAT5 signaling and thereby favors the generation of terminally differentiated effector CTLs (Beltra et al., 2016; Kalia et al., 2010; Pekalski et al., 2013). Analysis of subtype distribution of murine CTLs did not reveal significant

differences under varying IL-2 conditions, though terminally differentiated CTLs were not specifically examined. Therefore, it is possible that with increasing IL-2 concentration and therefore more pronounced CD25 expression, more terminally differentiated CTLs are present, particularly in elderly female mice known for their enhanced cytotoxicity. Terminally differentiated effector T cells, also known as short-lived effector T cells, are characterized by the expression of the NK cell receptor KLRG1, which is already upregulated within three days of stimulation and increases with age (Joshi et al., 2007; Panwar et al., 2020; Sarkar et al., 2008). This observation suggests that CTLs, particularly from aged individuals, may adopt the expression of NK cell receptors forming a novel immune cells subset known as NKT or NK-like T cells. This adaptation grants them the ability to eliminate their target cells in an antigen-independent manner (Kakuda et al., 2023; Michel et al., 2016). The generation of these NK-like T cells with age have been hypothesized to compensate for the loss of TCR diversity due to decreasing naïve T cells and NK cells numbers with age, suggesting a deliberate rather than incidental process during healthy aging (Vallejo et al., 2011).

The present study indicate that NK cells generally require more IL-2 for activation without achieving cytotoxicity levels or kinetics comparable to CTLs, particularly in the elderly mice. Previous studies have suggested that IL-2 production and secretion decline with age in both mice and humans, though all these studies utilized mixed cell populations or investigated $CD4^+$ T cells (Adolfsson et al., 2001; Haynes et al., 1999; Kirman et al., 1996; Rabinowich et al., 1985; Thoman & Weigle, 1981). Coupled with our results, this suggests that diminished IL-2 production and secretion is primarily caused by $CD4^+$ T cells rather than CTLs, which maintain comparable IL-2 levels across age groups (Engwerda et al., 1996). Thereby, elderly CTLs might attempt to counterbalance the declining function of NK cells due to decreasing IL-2 availability.

Similar to CTLs, NK cell cytotoxicity is primarily mediated by secretion of perforin and granzyme B, with both IL-2 and IL-15 stimulation boosting the expression of these effector molecules and the secretion of IFN-γ (Bihl et al., 2010; Fehniger et al., 2007; Hallett et al., 2008; Hong et al., 2016). Upon activation, murine NK cells express CD25, enabling IL-2 signaling through the high-affinity IL-2 receptor, thus enhancing cytotoxicity by modulating calcium signaling and conjugate formation (Gasteiger et al., 2013; Hallett et al., 2008; Müller et al., 2014; Reichlin & Yokoyama, 1998). However, prolonged stimulation and expansion creates a $CD25^+$ NK cell population with compromised efficiency in target cell elimination but heightened cytokine production (Müller et al., 2014). In this study, murine NK cells stimulated with 1000U IL-2 tend to display lower cytotoxicity compared to those stimulated with only 500U IL-2, despite higher expression of activating receptors. One could hypothesize that high IL-2 concentration can induce a $CD25^+$ NK cell population with impaired cytotoxicity. Yet, enhanced CD25 expression post-activation is crucial, as it positively effects effector molecule expression

via IL-2 signaling (Kedia-Mehta et al., 2019). Another possible explanation is that NK cells may experience activation-induced cell death (AICD), a process that IL-2 can initiate in both murine T and human NK cells (Dai et al., 1999; Marks-Konczalik et al., 2000; Refaeli et al., 2012; Rodella et al., 2001). However, viability measurement in this study did not show a consistent decline with increasing IL-2 concentrations, showing instead an inconsistent relationship influenced by age and sex. This variability indicates that AICD may not play a role in this context. Consequently, the underlying cause remains ambiguous, as this pattern does not align with the subtype distribution or the expression of activating and inhibitory receptors. Nonetheless, it is conceivable that changes in receptors not investigated in this study are crucial in IL-2-dependent cytotoxicity. Furthermore, while quantifying effector molecules could provide valuable insights, such analysis was not feasible due to very low absolute numbers of cells, as previously noted (Beli et al., 2014; Grégoire et al., 2007).

Contrary to the effects seen in murine NK cells, IL-2 supplementation significantly influenced nearly all tested parameters in human NK cells, notably increasing cytotoxicity. This enhancement in cytotoxicity due to IL-2, reported previously without age consideration (Konjevic et al., 2010; Kutza & Murasko, 1994), was observed in this study not just as an enhancement in endpoint lysis but also in accelerated kinetics of the response. This acceleration might be due to increased expression of activating receptors CD161 and NKG2D, alongside a reduction in NKp46 expression with IL-2 treatment. In previous studies, CD161, NKG2D, and NKp46 expression positively correlated with lysis of K-562 target cells, with a particularly strong correlation for NKG2D (Hood et al., 2019; Konjević et al., 2007; Sivori et al., 1999). Thus, the increase in CD161 and NKG2D expression may be able to compensate for the partial loss of NKp46 induced by IL-2. The enhanced cytotoxicity is likely also a result of increased expression of the effector molecules granulysin, perforin, and granzyme B, as demonstrated in both this and other studies (Harari et al., 2009; Hazeldine et al., 2012; Hodge et al., 2006; C. Lehmann et al., 2000; Nogusa et al., 2012; C.-H. Wu et al., 2019). However, the interplay between activating receptor expression and effector molecule levels remains only partially understood, with one investigation reporting enhanced perforin and granzyme B expression in $CD161^{+}$ NK cells (Jeffery et al., 2018). Beyond the levels of intracellular expression, degranulation capabilities are critical for cytotoxicity, as impaired degranulation can hinder target cell lysis and worsens disease progression (Jun et al., 2019; Shabrish et al., 2019). Nevertheless, several studies identified a positive correlation between IL-2 supplementation and the degranulation of human NK cells (Juliá et al., 2018; Millard et al., 2013), further augmenting their cytotoxic potential.

4.1.2 Exhaustion

Unlike CD25, CD122 expression appears to be independent of IL-2 levels, as only neutralization of IL-2 resulted in a significant loss of $CD122^+$ CTLs. It has been observed, that prolonged and enhanced expression of CD122 predisposes T cells to exhaustion by upregulating exhaustion markers as lymphocyte-activation gene 3 (LAG-3), Tim-3, and PD-1 (Beltra et al., 2016; Kalia et al., 2010). While PD-1 is commonly associated with exhaustion (Y. Jiang et al., 2015; Veluswamy & Bruder, 2018), it is also transiently expressed following T cell activation through various transcription factors (Bennett et al., 2003; Mathieu et al., 2013; Oestreich et al., 2008; Tsushima et al., 2007). During acute infection, PD-1 levels decline in later stages, contrasting with their sustained high levels during chronic infections, a process mediated by the transcription factor forkhead box protein O1 (FoxO1) (Barber et al., 2006; Staron et al., 2014). However, maximum PD-1 expression was observed when IL-2 was neutralized, potentially indicating an inability to downregulate PD-1, or its maintenance might be essential for priming PD-1-mediated apoptosis in T cells that are not fully activated through the inhibition of the survival factor B-cell lymphoma extra-large (Bcl-xL) (Chemnitz et al., 2004; Parry et al., 2005). Interestingly, this study found that CTLA4 expression in the murine system and human T cells decreased with diminishing IL-2 concentrations (M. L. Alegre et al., 1996; X.-B. Wang et al., 2001). However, considering CTLA4 and PD-1 are among several inhibitory markers of exhausted cells and are also expressed during T cell activation, they alone are insufficient for identifying exhausted cells. Therefore, additional properties have to be considered when investigating the effects of exhaustion.

Exhaustion is further characterized by impaired cytokine secretion such as TNF-α and IFN-γ (da Fonseca-Martins et al., 2019; Ingram et al., 2011; Wherry et al., 2007). Yet, in elderly mice, the secretion of TNF-α and IFN-γ remained relatively stable even with IL-2 neutralization and elevated PD-1 levels. Notably, elderly female mice CTLs secreted significantly higher amounts of nearly all tested cytokines despite generally higher CTLA4 and PD-1 expressions. Similarly, human NK cells treated with IL-2 exhibited a significant increase in exhaustion markers expression (Hasan et al., 2023; Judge et al., 2020; So et al., 2019; Zheng et al., 2019), alongside enhanced secretion of TNF-α and IFN-γ. Taken together these data suggest that the CTLs and NK cells studied are not truly exhausted. Instead, the high expression of exhaustion markers might serve as a regulatory mechanism preventing overstimulation, potentially averting activation-induced cell death (AICD) driven by IL-2 (Dai et al., 1999; Marks-Konczalik et al., 2000; Refaeli et al., 2012).

In human study, increased PD-1 expression on tumor-infiltrating lymphocytes has been linked to improved disease-free survival in colorectal cancer patients (Al-Mterin et al., 2023). Similarly, heightened expression of TIGIT and TIM-3 was observed, but a strong LAG-3 expression

correlated with lower disease-free survival and overall survival in various diseases (Al-Mterin et al., 2023; Rakova et al., 2021). Conversely, in hepatitis B virus-related hepatocellular carcinoma (HBV-HCC) patients, a high co-expression of TIGIT and TIM-3 negatively affected progress-free survival due to NK cells exhaustion (M. Yu et al., 2022). Interestingly, TIM-3 levels decrease post-target cell interaction, indicating minimal impact on NK cell functionality (Dao et al., 2020). Additionally, enhanced TIM-3 expression in acute myeloid leukemia (AML) patients was associated with upregulated effector molecules such as perforin, granzymes A and B, and IFN-γ, alongside enhanced cytotoxicity (Rakova et al., 2021).

4.2 Influence of the Sex on Immune Cell Function and IL-2 Response

This study highlights distinct sex-related differences in immune functions, particularly under varying IL-2 conditions. In murine CTLs, elderly female mice exhibited less dependency on external IL-2 compared to their male counterparts, as evidenced by differential expression of activation markers, effector molecules, and even exhaustion markers. Notably, CTLs from elderly female mice CTLs were the only group exerting effector function when autocrine IL-2 was neutralized, indicating a compensatory mechanism for IL-2 deficiency. Analysis of cytokine secretion revealed a significant increase in IL-2 production among females, suggesting a possible reliance on enhanced autocrine IL-2 production to counteract the external IL-2 shortfall. It raises the possibility that female mice CTLs might employ an alternate cytokine, with IL-15 being a likely substitute due to two shared receptor subunits with IL-2 (Bamford et al., 1994; Leonard et al., 2019). Given that many signaling pathways are mediated via the common gamma chain, cytokines like IL-4, IL-7, IL-9, and IL-21 might also act as partial substitutes for IL-2 (Leonard et al., 2019). Another possibility is that the TCR signaling of elderly female mice CTLs can be initiated independently of IL-2. As described earlier, activation via the TCR signaling itself can be initiated without IL-2, but the positive feedback loop created by IL-2 enhances TCR signaling by decreasing the signaling threshold (Au-Yeung et al., 2017; Busse et al., 2010; Malek, 2008). Therefore, elderly female mice CTLs might bypass the need for IL-2 due to an inherently lower TCR threshold, easier facilitating the expression of activation markers compared to their male counterparts. This theory aligns with our findings that CD25 and CD69 expressions are significantly higher in CTLs from elderly female mice under limited IL-2 availability. The significant increase in exhaustion marker expression observed in elderly females compared to males could be due to improved T cell activation or serve as a protective mechanism against AICD resulting from overstimulation, as discussed in the previous section., However, this protective mechanism suggests that IL-2 deprivation could also lead to AICD, likely due to the disruption of IL-2-induced pro-survival signaling pathways (Castedo et al., 2002; Mori et al., 2014; Nandagopal et al., 2014; Sinclair et al., 2008).

Sex-specific differences in CTL response were predominantly seen in the elderly, whereas differences in NK cell activity from spleen were observed in the adult mice, with females displaying higher cytotoxicity under decreasing IL-2 concentrations. Interestingly, this increase in cytotoxicity among adult females occurred despite significantly decrease in activating receptors expression, including NKp46, at the time of isolation and during the experiment. NKp46, known to be expressed on mature NK cells and acquired during the latter stages of maturation in the bone marrow (Ma et al., 2022; Walzer et al., 2007), which can induce IFN-γ production and NK cells degranulation, albeit not as effectively as NK1.1 engagement (Walzer et al., 2007). Loss of NKp46 signaling results in a decrease in cytotoxicity against YAC-1 cells but does not influence cytotoxicity against Daudi cells. Additionally, NKp46 regulates TRAIL expression, but this does not impact the cytotoxic response to TRAIL-resistant YAC-1 cells, even after overnight stimulation (Kayagaki et al., 1999; Sheppard et al., 2018). Thus, while reduced NKp46 expression would be expected to impair cytotoxicity against YAC-1 target cells, these target cells are identified not solely through NKp46 but also via NKG2D, which did not show sex differences in expression levels except under 500U IL-2 stimulation. Notably, NK cells lacking NKG2D display significantly higher perforin mRNA and protein abundance but loss of the activating receptor results in impaired cytotoxicity against YAC-1 target cells (Ogasawara et al., 2003; Prinz et al., 2020). Therefore, it is possible that both NKG2D together with NK1.1 can compensate for the loss of NKp46 in murine NK cells. This idea gains further support as NK cells from blood of elderly female mice show overall reduced expression of all activating receptors and consequent cytotoxicity against YAC-1. Conversely, expression of the inhibitory receptor KLRG1, which marks terminally differentiated immune cells, was generally higher across all tested cohorts, potentially serving as a crucial regulatory signal in the immune response by limiting IFN-γ secretion and proliferation (Alvarez et al., 2019; Kamimura & Lanier, 2015; Robbins et al., 2002). Interestingly, IL-15, and potentially IL-2, can induce KLRG1 expression, a phenomenon observed in the adult murine NK cells within this study. However, this pattern was not replicated in the human NK cells, where supplementation with 50U IL-2 did not increase KLRG1 levels, indicating a species-specific response of IL-2's effect on KLRG1 expression related to sex.

NK cells from human donors exhibited fewer sex-specific differences compared to their murine counterparts, with a noted tendency towards enhanced cytotoxicity in females following IL-2 supplementation. The augmented cytotoxicity of female NK cells is strengthened by elevated levels of activating receptors NKG2D and NKp46, while all other parameters remain similar between sexes. Notably, viability in NK cells from female donors was significantly reduced, but since cytotoxicity assessments were based on living cell counts, the effect of viability on cytotoxicity was excluded in this study. This might only play a minor role in the body due to the generally high overall viability of human NK cells. Although there was no significant change in

the levels of granulysin, perforin and granzyme B between sexes, a slight tendency towards increase perforin expression was observed in NK cells from female donors. This trend might contribute to the observed sex differences in cytotoxicity, as estradiol levels have been linked to increased NK cells degranulation in response to K-562 target cells (Al-Attar et al., 2016; Macek Jilkova et al., 2017). However, cytotoxicity among fertile and pregnant women significantly drops compared to non-pregnant women or men, despite the rise in estradiol levels during pregnancy (Boyle et al., 2024; Deng et al., 2022; Mikhailova et al., 2019), indicating additional factors at play. It's conceivable that NK cells from female donors may exhibit heightened cytokine sensitivity, as evidenced by significantly increased IFN-γ expression following cytokine stimulation (Cheng et al., 2023).

4.3 Interplay and Compensatory Mechanisms between Innate and Adaptive Immunity

This study has elucidated that murine effector immune cells from both the innate and adaptive immune system show distinct responses to IL-2, varying by age and sex, indicating their potential crucial role in compensating for each other's loss of effector functionality. Notably, sex-specific differences in CTL responses were observed in the elderly, while such differences in NK cell cytotoxicity were exclusively present in the adult mice. In both scenarios, females exhibited increased cytotoxicity, possibly offering them an advantage in tumor elimination as they age. The concept of exhaustion within murine NK cells remains underexplored, particularly concerning the effects of age and sex. However, evidence from murine T cells and human NK cells indicate that exhaustion increases with both advancing age and chronic stimulation (K. Lee et al., 2016; A. Merino et al., 2019; Parks et al., 2023). Therefore, it could be hypothesized that adult female NK cells might tend towards an exhausted phenotype as a counterbalance to their enhanced cytotoxicity. With age, a slight reduction in NK cell cytotoxicity in females is observed alongside an increase in CTL function, possibly to compensate for the partial loss in NK cell cytotoxicity. This hypothesis is supported by observations of CTLs adopting NK cell receptor expression, enabling them to eliminate their target cells in an antigen-independent manner (Kakuda et al., 2023; Michel et al., 2016), however, this theory is moderated by only a minor decline in NK cell cytotoxicity with age. Additionally, NK cell numbers remain relatively stable with age, as opposed to decreasing CTL numbers in female mice (Decman et al., 2012; Menees et al., 2021), which might be compensated for by increased CTL cytotoxicity.

Alternatively, it is also possible that the enhanced CTL-mediated cytotoxicity leads to a decrease in NK cell cytotoxicity to prevent an overactive immune response in females, which could cause autoimmunity or conditions alike to cytokine storm, or cytokine release syndrome,

observed in humans. Such cytokine storm, characterized by an excessive immune response, have been extensively investigated during the COVID-19 pandemic, illustrating that patients experiencing cytokine storm faced severe infections and an elevated risk of developing acute respiratory distress syndrome (ARDS), thereby increasing mortality rates (Montazersaheb et al., 2022; Zanza et al., 2022). However, the exact relationship between CTL and NK cell cytotoxicity, in female mice, and how these dynamics contribute to immune system balance or imbalance, warrants further detailed investigation.

4.4 Technical and Methodical Limitations

Within the scope of this study, a calcein-based bulk cytotoxicity assay was utilized to evaluate the effector function of CTLs and NK cells (Kummerow et al., 2014). In this assay, calcein is used to label the target cells and lysis of target cell is identified by loss of fluorescence compared to the live control. This method does not detect early phases of apoptosis since fluorescence loss, indicated by membrane rupture, occurs in apoptosis's final stages (Kroemer et al., 2009). Yet, it effectively identifies necrosis, characterized by rapid and eventually uncontrolled cell membrane rupture (Y. Zhang, Chen, et al., 2018). Given the early detection of cytotoxic activity in the first hour of the assay, it is most likely that granule-mediated necrosis was the primary mechanism observed. Necrosis is triggered by a high concentration of perforin in the immunological synapse, while granzyme B alone is unable to induce necrosis or even apoptosis (Backes et al., 2018). This assay offers advantages over traditional methods like ^{51}Cr-release, enabling both the quantification of endpoint lysis and the visualization of the killing kinetic, without employing harmful substances to tag the target cells (Kummerow et al., 2014; Lichtenfels et al., 1994; Neri et al., 2001). However, a decline in calcein fluorescence over time in various target cell lines limits its recommendation to short-term measurements up to four hours (Kummerow et al., 2014; Miles et al., 2015).

The secretion of various cytokines was measured using BioLegend's LegendPlex™, allowing the simultaneous quantification of up to 13 analytes per sample using a flow cytometer (J. S. Lehmann et al., 2019). Compared to the gold standard method of enzyme-linked immunosorbent assay (ELISA), the LegendPlex™ showed good correspondence (Y. Song et al., 2021), and thereby is a good alternative to ELISA.

Another consideration is the impact of different housings on the murine immune system. Our mice were housed under specific-pathogen-free (SPF) conditions (Zöphel et al., 2022). However, several studies found fundamental differences in various physiological properties of wild or even pet store mice compared to SPF laboratory mice ranging from differences in physical properties, immune cell subsets, expression of cell surface marker, and secretion of

immune effective molecules (S. Abolins et al., 2017; S. R. Abolins et al., 2011; Beura et al., 2016; Devalapalli et al., 2006). Especially NK cells from laboratory mice exhibited impaired expression of granzyme B, CD69, NKp46, and KLRG1 together with impaired CD25 and IFN-γ expression upon IL-2 stimulation compared to wild mice (Boysen et al., 2011), which might explain the impaired cytotoxicity of murine blood NK cells compared to their human counterparts observed in this study. Yet, vaccinating laboratory mice can partially mimic the gene expression profiles of pet store mice and even humans (Reese et al., 2016), suggesting that SPF mice might not be the accurate model for adult human immune system studies (Beura et al., 2016). Nevertheless, animal models, particularly mouse models, play a crucial role in biomedical research investigating various diseases and physiological responses such as diabetes or tumor response among others, as their genome is well-established, making them suitable for genetic modifications (Mukherjee et al., 2022; Vandamme, 2014). Additionally, the environment is more easily controlled for mice, limiting the effects of infections and injuries which play a key role in research around age, but also sex-differences as the effects of gender are very limited in mice as opposed to humans.

4.5 Concluding Remarks and Future Prospects

IL-2 is one of the most well investigated cytokines of the immune system and is a frequently used molecule in immunotherapy and therefore its impact on various immune cells is crucial to prevent adverse effects (Hernandez et al., 2022; T. Jiang et al., 2016; Raeber et al., 2023). While this study enables a deeper understanding of IL-2 regulation of T and NK cell immunity in the context of sex and age there are still plenty things unknown that require further investigation. A critical area for future research is the mechanism underlying the observed differential IL-2 activation requirements between male and female CTLs. This question is initial for advancing our knowledge of sex-based immune response differences. Additionally, the physiological properties of murine NK cells, including IL-2R subunit expression of exhaustion markers together with effector molecule expression, require comprehensive investigation. Moreover, the interplay between innate and adaptive immune cells must be investigated because these cells do not act independently and might be able to compensate for functional defects of the other side of the immune system as indicated in this study.

Employing advanced methodologies such as RNA sequencing (RNA-Seq) and epigenetic analysis, including chromatin immunoprecipitation (ChIP), could provide vital information on the transcriptome and histone modification of T and NK cells subjected to various treatments in both mouse and human. These approaches promise to reveal critical insights into the regulatory mechanisms at play. However, also more affordable and practical techniques such

as quantitative real-time PCR and flow cytometry can also provide essential information on the mRNA expression levels and the presence of crucial key molecules, whether on the cell surface or intracellularly. Additionally, analysis of post-transcriptional modifications might be necessary, particularly when differences between mRNA and protein levels are found. Moreover, selecting an appropriate model organism is critical, especially in immunological studies. As demonstrated in this study, NK cells derived from murine and human blood show significant disparities in certain parameters, even when homologs in both species exist, likely attributable to differences in lifestyle and environmental circumstances previously discussed.

Finally, the impact of sex on both T cell and to a lesser extent on NK cell function seems to be an important contributing factor. Many studies, particularly those involving mice, often rely on a single sex, thus neglecting and therefore overlooking intrinsic biological variances. While human research often includes age-and sex-matched controls, it tends to overlook the deeper implications these factors may introduce on study outcomes. This oversight is especially critical in pharmaceutical research, where sex-specific responses to vaccines and a heightened susceptibility to adverse effects in females compared to males have been documented (Flanagan et al., 2017; Hendriksen et al., 2021; Shan et al., 2023; Su et al., 2019). Hence, a thorough exploration of these sex-specific differences is essential, not only in basic scientific inquiry but also in the development and evaluation of pharmaceutical interventions. Acknowledging and addressing these disparities is crucial for advancing treatment quality and safety for both sexes.

5 Appendix

5.1 Supplementary Tables

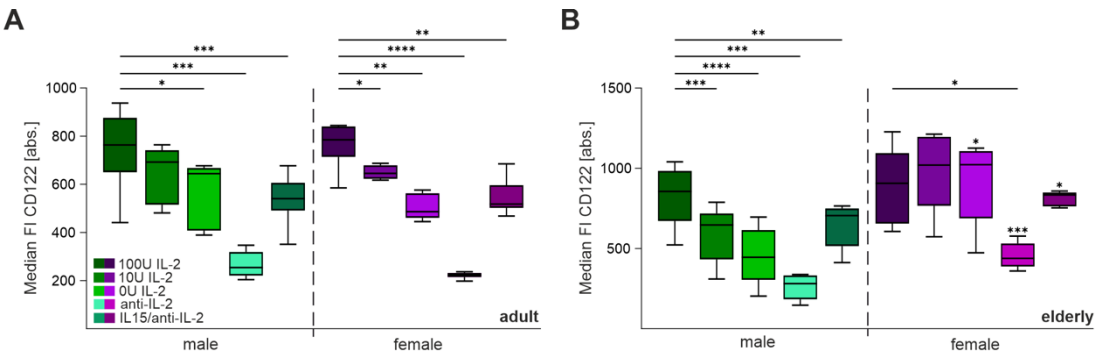
Supplementary table 21: Significance levels of CTL proliferation. Significance (p-values) in divisions 0 to 7+ (d0 to d7+) between male and female mice. Significant differences between sexes are highlighted. Significant differences compared to 100U IL-2 are depicted as symbols + p<0.05, § p<0.01, # p<0.001, ~ p<0.0001 in green (male) and purple (female).

	male vs. female									
	adult					elderly				
	100U IL-2	10U IL-2	0U IL-2	anti-IL-2	IL-15/ anti-IL-2	100U IL-2	10U IL-2	0U IL-2	anti-IL-2	IL-15/ anti-IL-2
d0	0.1858	**0.017**	0.0313	0.0667	0.3306	**0.0462**	0.0861	**0.0485**	0.0511	**0.0489**
d1	0.1914	0.1613	0.1533	0.1685	0.4634 §	**0.0179**	**0.0228**	**0.0168**	**0.0054** +	**0.0177**
d2	0.2160	0.1843	0.1632 +§	0.3076	0.3431 +	**0.0331**	**0.0392**	**0.0446** §	**0.0097** §	**0.0181**
d3	0.7198	0.2956 +	0.3665	0.4706+	0.4145	0.2842	0.3086	0.2351	**0.0493** §	**0.0339** §
d4	0.1912	0.8288	0.7469+	0.8505 §+	0.4314 §	**0.0445**	0.4400	0.3702	0.6208 §	0.0917
d5	0.4915	0.2111 +	0.3037	0.3509	0.9956 +	0.0849	0.1146	0.1081	**0.0066** §	**0.0134** §
d6	0.9990	0.4109	0.3350	0.5194 §§	0.8041 #	**0.0071**	**0.0198**	**0.0309**	**0.0124** ~+	**0.0023**
d7+	0.3628	0.4172	0.4425	0.4537	0.2725	0.2513	0.3399	0.2230	0.0894	0.1958

Supplementary table 22: Significance levels of CTL subtype distribution. Significance (p-values) between central memory (CM), effector memory (EM), naïve, and double negative (DN) was tested in male against female mice. Significant differences between sexes are highlighted. No significant differences were found compared to 100U IL-2.

	male vs. female									
	adult					elderly				
	100U IL-2	10U IL-2	0U IL-2	anti-IL-2	IL-15/ anti-IL-2	100U IL-2	10U IL-2	0U IL-2	anti-IL-2	IL-15/ anti-IL-2
CM	0.9982	0.1701	0.7621	0.6028	0.2486	0.0588	0.2375	0.1134	0.6614	**0.0040**
EM	**0.0212**	0.2431	0.4855	0.2839	0.7366	0.0706	0.3519	0.1139	**0.0019**	**0.0006**
Naïve	0.0810	0.7747	0.3582	0.9880	0.2240	**0.0295**	0.2984	0.0610	**0.0009**	**0.0011**
DN	0.2374	0.4913	0.8383	0.9207	0.3229	0.1178	0.0944	0.3356	0.2028	**0.0007**

5.2 Supplementary Figures



Supplementary Figure 24: Elderly female mice exhibit higher median fluorescence intensity of CD122 under decreasing IL-2 concentrations. Flow cytometry analysis of CD122 median fluorescence intensity (MFI) in adult (**A**) and elderly (**B**) mice, respectively. All stimulation conditions were analyzed in both sexes with male mice CTLs depicted in shades of green and female mice in shades of purple. Analysis was performed on day three of stimulation. Significance depicted above bars indicate differences between sexes. Significance between conditions was calculated using paired ordinary one-way ANOVA and differences between sexes using unpaired Student's t-test. Data presented as mean ± SEM. Significance levels are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. *n = 5-6 per group.*

5.3 Abbreviations

Akt	protein kinase B
AICD	activation induced cell death
AM	acetoxymethyl ester
AML	acute myeloid leukemia
APC	antigen-presenting cell allophycocyanin
ARDS	acute respiratory distress syndrome
Bcl	B cell lymphoma
Bcl-xL	B cell lymphoma-extra large
BD	Becton Dickinson
BSA	bovine serum albumin
CAR	chimeric antigen receptors
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl ester
ChIP	chromatin immunoprecipitation
CM	central memory
CMV	cytomegalovirus
COVID-19	coronavirus disease 2019
CTL	cytotoxic T lymphocyte
CTLA	cytotoxic T-lymphocyte associated protein
DC	dendritic cell
DD	death domain
DMSO	Dimethyl sulfoxideDN
(D)PBS	(Dulbeccos) phosphate buffer solution
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EM	effector memory
Eomes	Eomesodermin
ERK	extracellular signal-regulated kinase
FACS	fluorescence
FADD	FAS-associated death domain
FCS	fetal calf serum
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
FMO	fluorescence minus one
FoxO1	forkhead box protein O1
FoxP3	forkhead box P3
GAS	gamma interferon–activated sequence
GD	Graves' disease
GzB	granzyme B
HBSS	Hanks' balanced salt solution
HBV-HCC	hepatitis B virus-hepatocellular carcinoma
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IFN	interferon
IL	interleukin
IL-2R	interleukin-2 receptor
ILC2	type 2 innate lymphoid cells

ITAM	immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
KLRG1	killer cell lectin-like receptor G1
LAG-3	lymphocyte-activation gene 3
Lck	lymphocyte-specific protein tyrosine kinase
LRS	leukocyte reduction system
MAPK	mitogen-activated protein kinase
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MNC	mononuclear cells
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	nuclear factor of activated T-cells
NIH	National Institutes of Health
NK	natural killer
P/S	penicillin/streptomycin
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PD-1	programmed cell death 1
PE	phycoerythrin
PerCP	peridinin-chlorophyll-protein
PFA	paraformaldehyde
PI3K	phosphoinositide 3-kinases
RNA Seq	RNA sequencing
RT	room temperature
sFasL	soluble Fas ligand
SLE	systemic lupus erythematosus
SOCS	suppressor of cytokine signaling
SPF	specific pathogen-free
STAT	signal transducer and activator of transcription
TCR	T cell receptor
TIGIT	T cell immunoreceptor with Ig and ITIM
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAIL	tumour necrosis factor-related apoptosis-inducing ligand
U	units
ZAP-70	zeta-chain-associated protein kinase 70

5.4 List of Figures

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5.6 References

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5.7 Publications

Planned:

Hof C, Janku S, Jarzembowski L, Lis A. Elderly female CTLs maintain their initial activation and cytotoxicity indepent of IL-2 (in preparation).

Published:

Zöphel D, Angenendt A, Kaschek L, Ravichandran K, **Hof C**, Janku S, Hoth M, Lis A (2022). Faster cytotoxicity with age: Increased perforin and granzyme levels in cytotoxic CD8+ T cells boost cancer cell elimination. Aging Cell, 21, e13668. doi: 10.1111/acel.13668.

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Curriculum Vitae

Aus datenschutzrechtlichen Gründen wird der Lebenslauf in der elektronischen Fassung der Dissertation nicht veröffentlicht.