β2 adrenergic receptor desensitization through chronic stimulation of Natural Killer cells

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Thesis by Martin Maria Jürgens

First Referee: Prof. Dr. Carsten Watzl

Second Referee: Prof. Dr. Silvia Capellino

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Abstract

Stress is a ubiquitous phenomenon that impacts the health of populations across all social strata. While acute stress can be beneficial, chronic stress elevates the risk for various diseases. A major part of the body's stress response operates through the Sympathetic-Adrenal-Medullary (SAM) axis, leading to the release of epinephrine. This stress mediator can impact the immune system and alter the immune defense response. Natural Killer (NK) cells play a critical role in early immune responses, defending against pathogens and malignant cells. During stressful situations, NK cells are recruited to the blood circulation within minutes. The expression of adrenergic receptors, particularly β2 adrenergic receptors (β2AR), renders them sensitive to epinephrine.

This study analyzed the response of NK cells to acute and chronic β2AR stimulation. We confirmed that acute β2AR stimulation inhibits NK cell functions *in vitro*. Epinephrine showed potent inhibitory effects, suppressing the activation of NK cells independent of the activation signal. The β2AR stimulation reduced NK cell adhesion, IFNγ secretion, degranulation, and cytotoxicity. We could show that β2AR stimulation effectively blocked the LFA-1 activity and led to the detachment from its ligand ICAM-1. The effect was rescued through the addition of Propranolol, a beta blocker, or *ADRB2* knock out implicating LFA-1 inhibition as critical role for NK cell mobilization. The metabolic analysis revealed that β2AR agonists influenced the metabolic profile upon NK cell activation, leading to a prolonged glycolysis activity displayed by Seahorse ECAR values.

In contrast, chronic β2AR stimulation nullified NK cell inhibition. After five days of β2AR treatment, NK cells were no longer responsive to a β2AR agonist. Chronic β2AR stimulation did not alter protein translation but led to receptor phosphorylation through the PKA feedback loop, initiating a G-protein switch and receptor desensitization. The β2AR treatment with long-acting β2 agonist (LABA) Indacaterol and epinephrine displayed different properties. While epinephrine inhibited NK cells only transiently as long as the β2AR agonist was abundant, Indacaterol could not be washed away and continuously stimulated the receptor. For this reason, a single LABA treatment was sufficient to induce NK cell desensitization.

However, peripheral NK cells from LABA-treated patients remained responsive to epinephrine. They did not exhibit inhibition but showed an overall correlation in NK cell fitness with asthma severity.

Taken together, the transient, inhibitory effect of epinephrine indicate that the hormone plays a crucial role in NK cell recruitment during acute stressful situations while repeated β2AR stimulation leads to desensitization.

Zusammenfassung

Stress ist ein allgegenwärtiges Phänomen, das die Gesundheit von Bevölkerungen aller sozialen Schichten beeinflusst. Während akuter Stress vorteilhaft sein kann, erhöht chronischer Stress das Risiko für verschiedene Krankheiten. Ein wesentlicher Bestandteil der Stressreaktion erfolgt über die Sympathikus-Nebennierenmark-Achse (SAM), was zur Freisetzung von Epinephrin führt. Dieser Mediator kann das Immunsystem beeinflussen und die Immunabwehrantwort verändern. Natürliche Killerzellen (NK Zellen) spielen eine entscheidende Rolle bei frühzeitigen Immunantworten und verteidigen den Körper gegen Pathogene und transformierte Zellen. In stressigen Situationen werden NK Zellen innerhalb von Minuten in den Blutkreislauf rekrutiert. Die Expression von adrenergen Rezeptoren, insbesondere β2-adrenergen Rezeptoren (β2AR), machen diese Zellen empfindlich gegenüber Epinephrin.

Diese Studie analysiert die Reaktion der NK-Zellen auf akute und chronische β2AR-Stimulation. Wir konnten bestätigen, dass akute β2AR-Stimulation die Funktionen der NK-Zellen *in vitro* hemmt. Epinephrin zeigte starke hemmende Effekte, die die Aktivierung der NK-Zellen unabhängig vom Aktivierungssignal unterdrückten. Die β2AR-Stimulation reduzierte die Adhäsion der NK-Zellen, die IFNγ-Sekretion, die Degranulation und die zytotoxische Aktivität. Wir konnten zeigen, dass die β2AR-Stimulation die LFA-1-Aktivität effektiv blockierte und zu einer Ablösung von ihrem Liganden ICAM-1 führte. Der Effekt konnte durch die Zugabe von Propranolol, einem Betablocker, oder durch *ADRB2*-Knockout gerettet werden, was darauf hinweist, dass die LFA-1-Inhibition eine entscheidende Rolle für die Mobilisierung von NK-Zellen spielt. Die metabolische Analyse zeigte, dass β2AR-Agonisten das metabolische Profil bei der Aktivierung von NK-Zellen beeinflussen und zu einem anhaltenden Anstieg der Seahorse-ECAR-Werte führen.

Im Gegensatz dazu hob die chronische β2AR-Stimulation die Hemmung der NK-Zellen auf. Nach fünf Tagen β2AR-Behandlung reagierten die NK-Zellen nicht mehr auf einen β2AR-Agonisten. Die chronische β2AR-Stimulation veränderte die Proteinsynthese nicht, führte jedoch zur Phosphorylierung des Rezeptors durch die PKA-Rückkopplungsschleife, die einen G-Protein-Schalter initiierte und eine Desensibilisierung des Rezeptors bewirkte. Die Behandlungen mit β2AR-Langzeitagonist (LABA) Indacaterol und Epinephrin zeigten unterschiedliche Effekte. Während Epinephrin die NK-Zellen nur solange hemmte wie der β2AR-Agonist vorhanden war, konnte Indacaterol nicht entfernt werden und stimulierte kontinuierlich den Rezeptor. Aus diesem Grund reichte eine einzige LABA-Behandlung aus, um eine Desensibilisierung der NK-Zellen zu induzieren.

Die peripheren NK-Zellen von mit LABA behandelten Patienten konnten jedoch immer noch auf Epinephrin reagieren. Die peripheren NK-Zellen blieben responsiv, ohne Hemmung der Funktion, aber zeigten insgesamt eine Korrelation in der NK-Zellfitness mit der Schwere des Asthmas.

Zusammenfassend deutet die transiente, inhibitorische Wirkung von Epinephrin darauf hin, dass das Hormon eine entscheidende Rolle bei der Rekrutierung von NK-Zellen bei akuten Stresssituationen spielt während wiederholte β2AR-Stimulation zu einer Desensibilisierung führt.

1 Introduction

1.1 Stress

Stress is a universal phenomenon that significantly impacts health and is designated by the World Health Organization (WHO) as the "*Health Epidemic of the 21st Century*"[1]. The latest survey of a German health insurance company identified stress load among all age groups [2]. Over the last few decades, the proportion of Germans experiencing frequent stress has surged by 30%, affecting more than a quarter of the population [2]. Although everybody experiences stress, the phenomenon is challenging to define due to its highly subjective and personal nature. Technically speaking, any stimulus can trigger a stress response, whether environmental (e.g. hurricanes, financial crises), physical (e.g. wounds, infections), or psychological (e.g. pressure, fear). Coping with stressful situations is crucial for evolutionary survival.

Walter Cannon, a pioneering figure in stress research, introduced the concept of the "fight-or-flight" response in 1915, emphasizing the body's mobilization of energy for improved escape or attack during stressful situations [3]. Cannon proposed that both physical and psychological trauma trigger the release of epinephrine and induce physical adaptations [4, 5].

Hans Selye, often regarded as the "father of stress", was the first person who identified stress as trigger for diseases. He introduced the term "General Adaptation Syndrome" in 1936, highlighting stress as a response with three stages: the alarm phase, the stage of resistance, and the stage of exhaustion [6, 7]. He concluded that stress is a non-specific response following the same pattern independent of the stressor [8, 9]. Selye's work linked the release of epinephrine and corticoids to stress-induced diseases what laid the foundation for understanding the complex stress system [10].

In the last decades the concepts of Cannon and Selye have been revised and extended by various scientist. Sterling and Eyer as well as Schulkin, McEwen and Stellar evolved the idea of homeostasis and Selye's general adaptation syndrome [11-13]. They proposed the concept of allostasis and refined that not all physiological regulations aim homeostasis. Allostasis allows the body to adapt to current stressors, leading to a new allostatic state. Mediators like epinephrine or cortisol promote these adaptations, but inadequate adaptation can result in allostatic overload and subsequent diseases [14]. It is has been shown that chronic stress and allostatic overload can lead to illness [15].

In clinical practice, semi-structed interviews (Diagnostic Criteria for Psychosomatic Research) and self-rated questionnaire (PsychoSocial Index) are used for diagnosis and assessment of stress, psychological distress, sickness behavior and quality-of-life [16, 17]. Additionally, several biological parameters including epinephrine have been recognized to describe the allostatic load with a number of limitations due to the complexity of stress [17, 18]. A stressor can affect various systems in the body, depending on timing, duration, intensity and

short- and/or long-term consequences. In general, stimuli can be categorized based on their duration into acute, repeated acute, and chronic stress. Acute stress typically lasts for minutes or hours. Repeated acute stress involves a series of stress responses interspersed with brief recovery periods. Chronic stress can persist for days, months, years or does not even have a defined ending [15].

Acute stress induces physical adaptions according to the threat like dilate pupils, constrict muscles, elevated heart rate and mobilization of the immune system [19]. Especially the innate immune system is critical in the acute phase to effectively clear pathogens and infections [20]. However, a response to acute stress can be maladaptive when the stress is too intense (e.g. stress induced fainting[21]).

Contrary, repeated acute stress and chronic stress is associated with dysfunctional immune responses and higher risk for diseases [20]. A persisting stress stimulus keeps the body in alert mode inducing symptoms like poor concentration, fatigue and exhaustion[19]. Chronic stress increases the risk of mental illnesses such as depression and burnout as well as the risk of physical diseases like circulatory diseases, infections, cancer and other illnesses [2, 22-24]. The increased susceptibility indicates that protective biological networks like the immune system may be affected by stress mediators.

The body's stress response operates through two major axes: the Sympathetic-Adrenal-Medullary and the Hypothalamus-Pituitary-Adrenal axis [25]. The released molecules may be beneficial in acute stress responses but turn into negative effectors in chronic stress scenarios [14, 26, 27].

1.1.1 The stress system: sympathetic adrenal medullary axis and hypothalamic-pituitary-adrenal axis

Key components of the stress response are the locus coeruleus (LC) - norepinephrine (NE) system, the sympathetic adrenal medullary axis (SAM) and the hypothalamic-pituitary-adrenal (HPA) axis [28, 29]. The LC-NE system, situated in the brainstem, serves as the primary source of NE in the brain, playing a crucial role in promoting and maintaining arousal, memory, complex behaviors, and an alert state [30]. It also contributes to activating the HPA axis and influences the functional state of the SAM axis through the inhibition of parasympathetic action while increasing sympathetic discharge [30, 31]. The SAM and HPA axes serve as the major stress mechanisms for signaling stress, inducing adaptations across various human body systems [25].

The SAM axis, part of the sympathetic nervous system (SNS), dominates the rapid autonomic stress response [28]. Sensory signals from inflammation or other stressors activate sympathetic preganglionic neurons, which in turn innervate different organs throughout the body, including the heart, trachea, lungs, pupillary muscles of the eye, and more (see Figure 1) [32,

33]. One neuron trajectory terminates in the adrenal medulla, where chromaffin cells release catecholamines such as norepinephrine (also known as noradrenaline) and epinephrine (also known as adrenaline) directly into the bloodstream [32, 33]. Catecholamines are neurotransmitters and play a major role during the stress response. The most important catecholamines are dopamine, norepinephrine and epinephrine. These hormones are all synthesized throughout the same pathway. Norepinephrine and epinephrine interact with adrenergic receptors and impact multiple organs, inducing changes like increased heart rate, muscle contractions, bronchodilation, gluconeogenesis, glycogenolysis, dilated eyes, and mobilization of immune cells [19, 33, 34]. These adaptations equip the body with added strength, concentration, and energy, while also preparing the immune system during stressful situations.

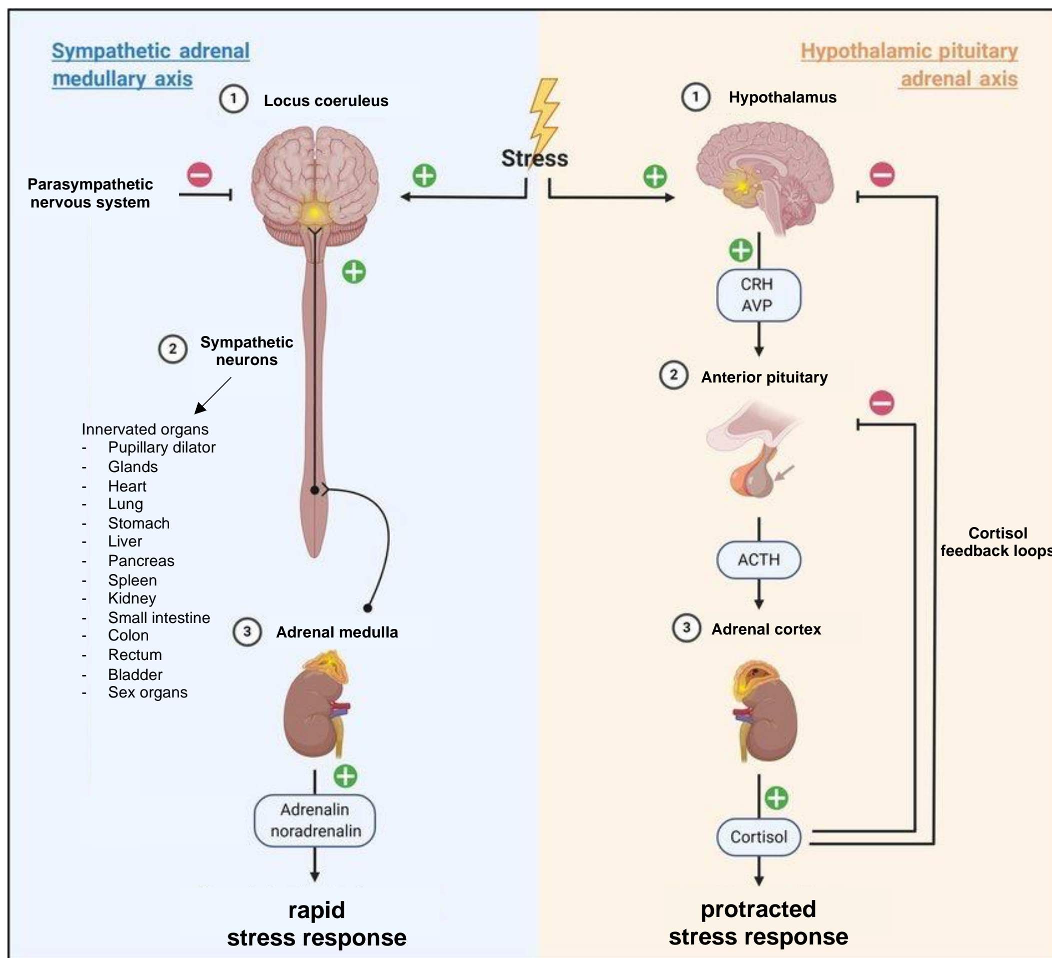

Figure 1: Stress response axes SAM and HPA

Schematic illustration of the stress reaction. Stress signal activates SAM and HPA axes which induce a rapid and a delayed, protracted stress response, respectively. (CRH= corticotropin releasing hormone, AVP= arginine vasopressin, ACTH= adrenocorticotropic hormone). Green signs indicate activating signals while red signs indicate inhibitory signals, edited from Carlton [35]

The autonomic nervous system, comprising the SNS and parasympathetic nervous systems, regulates stress responses. In acute stress reactions the SNS predominates, while the parasympathetic component facilitates relaxation during "rest and digest" conditions [33, 36].

In contrast to the rapid SNS activation, the HPA system provides a delayed, protracted stress response. Activation of the paraventricular nucleus in the hypothalamus initiates the release of arginine vasopressin (AVP) and corticotrophin-releasing hormones (CRH) [37, 38]. These hormones stimulate the anterior pituitary gland to release Adrenocorticotropic hormones (ACTH), triggering the secretion of the glucocorticoid cortisol from the adrenal cortex [39]. Cortisol interacts with various cells, inducing physiological effects based on their location and the cortisol level [40-43]. The cortisol interactions lead to an elevated cardiovascular function, suppression of insulin and mobilization of glucose, delayed wound healing and immune suppression [19, 44, 45].

The SAM and HPA systems exhibit overlapping processes, particularly in brain areas, yet they are suggested to function simultaneously and independently, with the SAM response initiating the immediate reaction, followed by the HPA axes, culminating in both non-genomic and genomic-mediated cortisol effects for long-term adaptations [25, 46, 47].

1.1.2 Stress and the immune system

The immune system comprises a network of organs, proteins, and molecules with the task of protecting the organism from diseases. The human immune system can be categorized into two sections: the natural innate and the acquired adaptive immune systems [48].

Innate immunity is the system's defense mechanism that offers immediate protection against various pathogens. It serves as the initial line of defense and does not necessitate prior exposure or sensitization. This defense system is critical for preventing the initial establishment of infections and malignant transformed cells [49]. Infections or tissue injuries activate tissue-resident macrophages, dendritic cells, and mast cells, leading to the release of inflammatory mediators, cytokines, chemokines, and the recruitment of natural killer (NK) and other innate immune cells [50, 51]. Dendritic cells and Macrophages can phagocytose pathogens and serve as antigen-presenting cells to T and B lymphocytes, bridging the innate and adaptive immune system [48].

The adaptive immune system provides a specific protection and develops tailored immune responses to each antigen [52]. Upon the entry of a pathogen into the body, the antigen presentation process specifies immune cells and induces a delayed but specific immune response [53]. If the same pathogen attempts to infect the body again, the immunological memory of each antigen enables the adaptive system to mount a stronger and faster response. Long-lived memory B- and T-cells can trigger cell proliferation and activate cytotoxic T cells and the humoral immune response [54].

Immune cells are equipped with a variety of receptors and cytokines to interact with the neuroendocrine system and stress mediators [55]. The brain and the immune system can com-

municate in both directions. The autonomic nervous system can regulate immune responses, while inflammation can trigger neural circuits, as highlighted by sickness behavior [56, 57].

In the following, this introductory section focuses on the effect of the key stress mediators, glucocorticoid and catecholamines, on the immune system.

Key mediators of the catecholaminergic SNS stress response are norepinephrine and epinephrine. Adrenergic nerves of the SNS terminate in organs and tissues of the immune system, including the spleen, thymus, and other lymphoid tissues [58]. Norepinephrine can be released by nerve terminals directly into these immunological tissues, while epinephrine is released by the adrenal medulla into the peripheral bloodstream [59]. Catecholaminergic mediators have been shown to effectively influence immune trafficking. Interaction with adrenergic receptors induces a rapid and transient increase in neutrophils, monocytes and lymphocytes in the circulating bloodstream [60, 61]. Especially cytotoxic T cells, NKT cells, NK cells and pro-inflammatory monocytes are recruited from the marginal pool by epinephrine - β2 adrenergic receptor (β2AR) interaction [34]. Additionally, acute stress elevates pro-inflammatory cytokines in the bloodstream [62]. The meta-analysis of Marsland [63] demonstrated increases in circulating inflammatory cytokines IL-2, IL-6, IL-1β, TNF-α and anti-inflammatory cytokines IL-4 and IL-10 upon acute stress. When cells/subjects are further challenged during the stress response by an additive stimulus (LPS or PHA), the cytokine composition changes to increased levels of IL-6, IL-1β, TNF-α, and especially IFNγ, as well as a decreased IL-4 level. Additionally, the chemotaxis of lymphocytes is affected under acute stress situations, leading to an increased density of cellular adhesion molecule Mac-1 and integrin LFA-1 [64, 65]. Activation of the adhesion molecule and integrin by chemoattractant induces recruitment to the site of inflammation. In summary, these effects support cellular and humoral immunity, leading to an accelerated resolution of infections and wound healing in acute stressful situations [66-68].

Although robust data about the inflammatory effect of acute stress exists, little is known about the mechanisms driving cytokine release. The secretion of IL-6 is suggested to be mediated through adrenergic receptors at brown adipose tissue [69], whereas the origin of peripheral IL-1β and TNF-α is not clear. Adrenergic receptor activation on mononuclear cells has been shown to stimulate NF-κB pathways, which are involved in cytokine production [70, 71] supporting sympathoadrenal activation as a driving force of inflammation. On the other hand, epinephrine has immunosuppressive properties. β2AR interaction inhibits TNF-α secretion in macrophages [72], and suppresses inflammatory cytokine production in dendritic cells [73]. It also inhibits NK cell activation [74] and $CD8^+$ T cell effector functions[75]. Additionally, catecholamines are able to induce CD4+ T cell differentiation. Th1 cells have been shown to profoundly express the β2AR, while Th2 cells have only limited expression [76]. β2AR stimulation on Th1 cells suppresses IFNγ secretion while increasing IL-10 secretion by dendritic cells, thus suppressing Th1 response [77]. Interestingly, B cell antigen presentation to CD4+

T cells is supported through adrenergic receptor stimulation, inducing a shift to a humoral response [78].

Glucocorticoids play a crucial role in shifting the immune response from an inflammatory (Th1/cellular) to an anti-inflammatory (Th2/humoral) state, safeguarding the organism from the consequences of an overactive immune response [79, 80]. The elevation of innate inflammatory cytokines such as IL-1, TNF-α, and IL-6 triggers the activation of the HPA axis through feedback mechanisms [79, 81]. As the HPA axis response is delayed compared to the sympathetic-adrenal response, this phase of the stress response is suggested to be immunomodulatory by shifting to a Th2 response and suppressing inflammation. Thus, the combined effects of glucocorticoids and catecholamines result in the shutdown of systemic pro-inflammatory cytokines during acute stress, protecting the organism from systemic inflammation and relocating inflammation to locally injured or infected cells.

Chronic stress can alter the immune response and impede the wound-healing process, rendering individuals more susceptible to complications and infections [82, 83]. Chronically stressed individuals may exhibit reduced antibody production and vaccination responses. For instance, those caring for a spouse demonstrated a lower immune response (humoral and cellular) to an influenza-virus vaccine [84]. Chronic stress has also been associated with chronic inflammatory disorders such as rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease [85].

Dysregulation of the HPA axis can lead to elevated cortisol concentration (hypercortisolism), promoting a state of chronic low-level inflammation associated with decreased immunocompetence and various health issues [86]. Interestingly, altered cortisol expression can also result in hypocortisolism [87]. Decreased cortisol levels have been identified after excessive, repetitive HPA activation and have been reported in patients with chronic pain or chronic fatigue syndrome [88, 89].

Although chronic stress is primarily associated with the HPA axis, it has been demonstrated that repeated acute stress stimuli lead to elevated epinephrine levels [46, 47]. In animal models, repeated stress did not habituate the transcription of genes encoding epinephrine producing enzymes after 6 or even 42 days [90, 91]. Also, chronically stressed individuals were shown to be more sensitive to acute stress, inducing an excessive sympathetic-adrenal response with prolonged decline in NK cell activity [92]. The extended release of catecholamines through the SAM axis is associated with various pathological conditions like high blood pressure, chronic pain, and cardiomyopathies [93-95]. Additionally, elevated urinary epinephrine concentrations have been observed in post-traumatic stress disorder patients who are more susceptible to infections [96]. Chronic stress and epinephrine-mediated immune suppression have also been linked to cancer. In animal models, ganglionic blocker and beta blocker were found to reduce tumor growth and metastasis due to repealed NK cell suppression [97, 98]. Consequently, the use of beta blockers during and shortly after cancer surgery

was evaluated in several trials, resulting in a variable but improved outcome for patients [99-102].

In conclusion, stress can be paradoxical. The key mediators of stress, cortisol and epinephrine, exhibit immune-suppressive properties [103], while the acute stress response has been shown to be beneficial for the elimination of infections or injuries. In contrast, chronic stress has been associated with chronic inflammatory disorders [85], while glucocorticoids are used as therapeutics to treat the same pathological conditions [104].

1.2 Natural Killer cells

NK cells are a vital component of the innate immune system and belong to the subset of white blood cells known as lymphocytes. In general, they are characterized by surface markers CD56, CD16 and the absence of CD3. They play a crucial role in the early immune response, defending the body against pathogens, virus-infected cells and transformed cells [105-107]. NK cells can be found in the peripheral blood, constituting 5-15% of all mononuclear cells, and migrate to inflamed tissues in response to chemoattractants [108]. Additionally, they reside in organs such as the spleen, liver, and lungs, with a smaller presence in the thymus and secondary lymphoid organs [109].

NK cells originate from hematopoietic stem cells in the bone marrow and undergo maturation, acquiring surface marker proteins CD56 and Killer cell immunoglobulin-like receptor (KIR). During the "NK cell education" process, NK cells develop cytotoxic and immunomodulatory functions, ensuring self-inhibition by rendering autoreactive NK cells anergic or in a hyporesponsive state [110]. Complete functional competence is achieved only with the expression of self-specific inhibitory receptors (KIR) [111]. The functional maturation process enables NK cells to identify endogenous cells by engaging with major histocompatibility complex (MHC) class I molecules [112]. NK cell subsets can be differentiated based on the expression of CD56 and CD16. Although all NK cells can perform the same functions, the subsets are described according to their preferences. The $CD56^{dim}$, $CD16^{+}$ subset is categorized as cytotoxic NK cells, capable of inducing antibody-dependent cellular cytotoxicity (ADCC) due to the expression of the Fc receptor CD16. The $CD56^{bright}$, $CD16^{-}$ NK cell subset is known as cytokine producer, releasing inflammatory IFNγ upon activation [113].

Unlike T and B cells, NK cells do not require prior exposure to an antigen to become activated. They rely on a germline-encoded receptor system to identify target cells [112]. This identification is achieved through a balance of inhibitory and activating receptors on their cell surface. Most inhibitory receptors recognize self-antigen MHC-I molecules on healthy cells, preventing NK cells from attacking them. However, when NK cells encounter cells lacking these self-antigens or if activating receptors are stimulated, they become activated[114].

1.2.1.1 NK cell activation

NK cells can be activated through various activating receptors (Figure 2), each binding to specific cellular ligands (see top row, Figure 2) [115]. Additionally, activating NK cell receptors can be grouped based on their intracellular signal mechanism. Receptors such as NKp30, NKp44, NKp46, or CD16 interact with immunoreceptor tyrosine-based activation motif (ITAM)-containing adapter proteins [116].

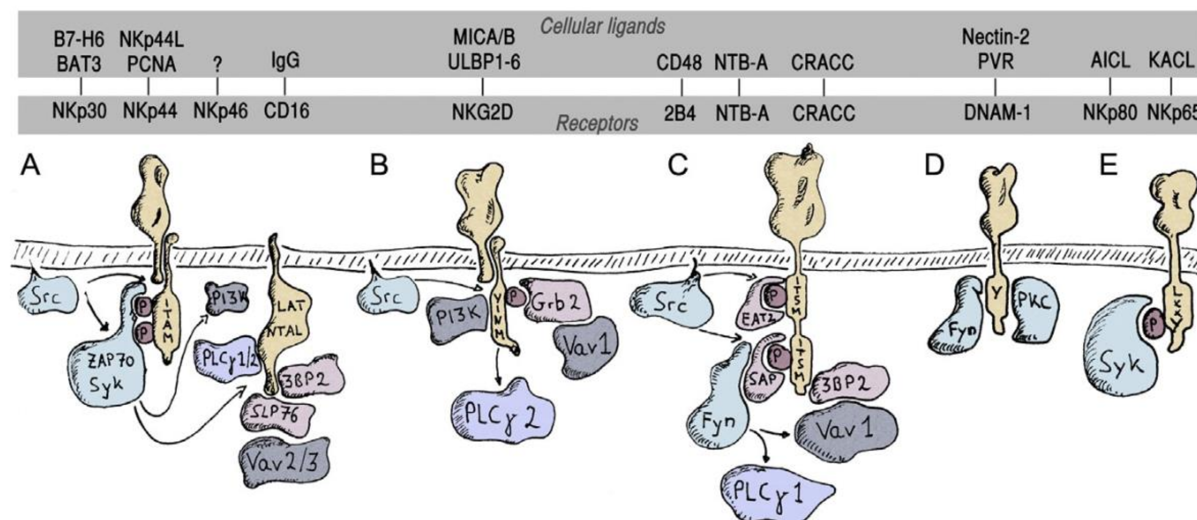

Figure 2: Human activating Natural Killer cell receptors, their ligands and cellular signals
Schematic illustration of activating receptors categorized in (A) Receptors (NKp30, Nkp44, NKp46, CD16) couple to ITAM containing proteins, (B) NKG2D receptor and adapter protein DAP10 with YINM motif (C) receptors with ITSM-signaling motifs (2B4, NTB-A, CRACC), (D) DNAM-1 receptor, and (E) NKp80 and NKp65 receptor with hemi-ITAM motif. Adopted from Watzl [108].

NKp30 can bind to several ligands derived from pathogens or tumor cells (e.g. BCL-2 associated athanogene 6 (BAG6, also known as BAT3) and B7-H6). The CD16 receptor is capable of binding the Fc domain of the immunoglobulin G (IgG) antibody [113]. Antibodies are part of the adaptive, humoral immune response directed against specific antigens, such as surface molecules of infected cells, enabling NK cells to identify antibody-coated target cells and induce ADCC. Therefore, NK cells can act at the intersection of the innate and adaptive immune system. Both receptors, NKp30 and CD16, associate with CD3ζ and FCεRIγ. Receptor engagement leads to ITAM phosphorylation, initiating a signaling cascade resulting in NK cell activation [117].

In contrast to NKp30, the NKG2D receptor is expressed on several immune cells, including macrophages, CD8+ T cells and NK cells. The homodimer NKG2D recognizes ligands (MHC class I-like ligands like MICA/B, UL16-binding proteins 1-6 (ULBP1-6)) upregulated in stressed cells due to infections or transformations [118]. Once the receptor binds to a ligand, the adapter protein DAP10 with its YINM motif couples, initiating a cascade for NK cell survival and cytotoxicity [108]. Unlike CD16, other activating receptors must act synergistically to activate resting NK cells. The NKG2D receptor shows synergistic effects in combination

with 2B4 [108]. The 2B4 receptor, a signaling lymphocyte activation molecule (SLAM)-related receptor, can bind to CD48, a GPI-anchored surface molecule expressed on various cells including all peripheral blood lymphocytes [119]. The receptor transduces the signal via an immunoreceptor tyrosine-based switch motif (ITSM) motif to initiate NK cell activation. The group D and E (Figure 2) show the activating receptors DNAM-1, NKp80, and NKp65. DNAM-1 signals via PKC and Fyn, while NKp80 and NKp65 signal via a hemi-ITAM pathway to activate NK cells[120].

In addition to cellular ligands, NK cells can be activated by cytokines. Prominent NK cell activators include interferon-α/β (IFN-α/β) and interleukins (IL)-2, -12, -15, -18 [106]. These cytokines are released during certain infections by endothelial and immune cells, resulting in NK cell activation to eliminate target cells. During NK cell cultivation, these interleukins are used to stimulate proliferation and survival of cells. In contrast to resting NK cells, these pre-activated, cultured NK cells do not require co-stimulation of activating receptors to initiate cytotoxicity and cytokine production [108].

1.2.1.2 NK cell cytotoxicity and cytokine production

Once a NK cell identifies a target cell, it adheres via integrins, such as Lymphocyte function-associated antigen 1 (LFA-1), to the target and forms an immunological synapse (IS) [121]. The integrin LFA-1 is a heterodimeric protein consisting of the subunits CD11a (αL) and CD18 (β2). In a resting state, the LFA-1 conformation is bent and has only low affinity for its ligand. However, upon an inside-out signal from other receptors, the LFA-1 conformation changes [122]. The stimulation of activating NK cell receptors opens and extends the integrin's conformation, resulting in a higher affinity state. Additional clustering of LFA-1 increases avidity, allowing the integrin to more efficiently bind to its ligand the intercellular adhesion molecule (ICAM) [123]. Thus, NK cell activation controls the adhesion capacity of the integrin. Besides its role during IS formation, LFA-1 is also involved in the migration process from the bloodstream to inflamed tissue, making the protein an important functional component of the NK cell [124].

The adhesion and the IS enable NK cells to induce apoptosis in the target cell through two main mechanisms. First, NK cell polarization directs internal granules to the contact surface. The release of cytotoxic perforin and granzymes into the synaptic cleft induces apoptosis in the target cell. Perforin forms micropores in the target membrane and granzyme activates caspases of the apotosis cascade, leading to the loss of mitochondrial membrane function [125].

The second mechanism induces target cell apoptosis by death receptor ligands. NK cells are equipped with Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL), enabling them to trigger apoptosis in the target cell. The death receptors contain an intracellular

"death domain" which, upon engagement of the receptor, activates the caspase cascade and cell death [126].

These apoptosis-inducing mechanisms allow the NK cell to kill several target cells in a row, a phenomenon known as "serial killing" [127]. In addition to their cytotoxic functions, NK cells also regulate the immune response through the production of cytokines, such as IFNγ and TNF-α. These cytokines influence the activities of other immune cells, such as the activation of T cells, dendritic cells, or neutrophils, demonstrating a regulatory role in the immune response [128].

1.2.1.3 NK cell metabolism

The upregulation of diverse metabolic pathways in NK cells is crucial for their effector functions. In general, glycolysis and oxidative phosphorylation (OxPhos) are the primary pathways for energy production. Both pathways are interconnected through the tricarboxylic acid (TCA) cycle and utilize glucose as a cellular fuel. Indeed, they differ in their energy production and speed. At rest, NK cells primarily rely on the slower but more productive OxPhos pathway. Upon activation, they upregulate both OxPhos and glycolysis to meet the increased energy demands required for effector functions. Glycolysis enables faster glucose processing, resulting in quicker ATP availability compared to OxPhos [129-131].

Moreover, glycolysis plays a vital role in generating precursor molecules essential for the biosynthesis of effector molecules and supports proliferation [131]. In a murine model, the production of IFNγ by NK cells have been shown to rely on glycolytic and oxidative metabolism when activated through NK cell receptors, while cytokine activation does not depend on these pathways. Inhibition of glycolysis leads to reduced production of effector molecules and reduced NK cell activity. $CD56^{bright}$ NK cells exhibit a higher metabolic response and upregulate nutrient receptors in response to cytokine stimulation compared to $CD56^{dim}$ NK cells [130].

As described by Picard [133], mitochondria play a pivotal role as they host both the TCA cycle and the electron transport chain. Consequently, the mitochondrial mass and depolarization can serve as marker for a cell's metabolic status, particularly since lymphocyte activation often correlate with an increase in mitochondrial mass. However, it's important to note that mitochondrial mass alone does not necessarily indicate functionality. The functionality of mitochondria is better assessed by their structural integrity, which is influenced by processes like fusion and fission known to impact mitochondrial functions[129]. Small and fragmented mitochondria are associated with diminished cytotoxicity and NK cell depletion [132, 133].

The metabolic profile of NK cells serves more than just energy production and biomolecular synthesis. It is intricately linked to the immune response. Depending on the specific needs of a cell at any given time, different pathways are utilized. Therefore, analyzing the NK cell metabolism upon β2AR is of great interest.

1.2.2 β2 Adrenergic receptor

The β2AR belongs to the adrenergic receptor family, also known as adrenoreceptors, which can be categorized into three major types: α1, α2, and β receptors, with three subclasses in each type [134]. These receptors are G protein-coupled receptors (GPCRs), consisting of seven transmembrane domains and signaling through heterotrimeric G proteins. Adrenergic receptors selectively recognize and bind to catecholamines, such as epinephrine [135]. Various cells and tissues express adrenergic receptors, with immune cells, especially lymphocytes, known to express α1, α2 and β2 adrenergic receptors [136]. The β2AR is particularly abundant on NK cells, making them the most sensitive lymphocyte subset to epinephrine [137].

The binding of a β2AR agonist can activate two different pathways, the canonical and the non-canonical pathway (Figure 3) [138].

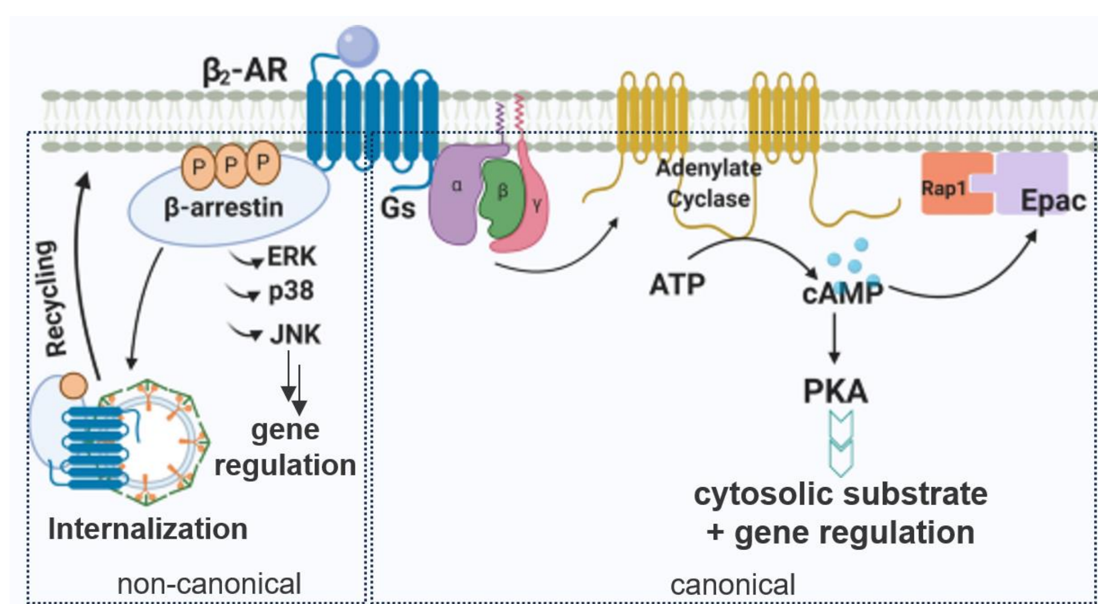

Figure 3: β2 adrenergic receptor pathways
Schematic illustration of β2 adrenergic receptor signaling. Receptor stimulation can induce a non-canonical, beta-arrestin mediated signal (left) or canonical signal transduction via Gs-Protein (right). Edited from Dorotea and Ha [139].

The canonical pathway transduces the signal through the coupled G protein. The β2AR agonist binding promotes a guanosine diphosphate to guanosine triphosphate (GTP) exchange, leading to the dissociation of the G protein trimer into Gαs-GTP and Gβγ dimer [140]. The Gαs-GTP subunit stimulates adenylate cyclase, converting adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) [136, 141]. The second messenger (cAMP) further activates protein kinase A (PKA) and induces the release of the catalytic subunits, which phosphorylate transcription factors or cytosolic substrates [142]. PKAs exist in different types. Generally, the PKA type I is anchored in the plasma membrane or subcellular structures whereas the type II is localized in the cytosol [143]. Besides PKA activation, cAMP can also regulate the function of ion channels or bind to the Exchange Protein Directly Activated

by cAMP (EPAC) [140]. The exact function of EPAC is still under investigation. The activity state of EPAC is determined by its multidomain architecture which allows specific protein-protein and protein-lipid interactions that control major biologic responses [144]. It has been shown to be involved in viral replication and cytokine/chemokine responses [145]. The β2AR signal is terminated by degradation of cAMP through phosphodiesterase [138].

The non-canonical pathway is G protein independent and involves β-arrestin activation [146]. This pathway typically opposes canonical signal transduction. GRK-mediated phosphorylation of the receptor is crucial for the non-canonical pathway. Sustained agonist stimulation leads to the phosphorylation of the β2AR by GRK2, resulting in desensitization and internalization of the receptor [146]. β-arrestin recruitment activates internalization through clathrin and its adapter protein AP2. Once endocytosed, the receptor can be recycled back to the membrane or degraded [139]. Furthermore, this pathway has been linked to mitogen-activated protein kinases (MAPK) signaling [147, 148]. High concentrations of β2AR agonists induce β-arrestin-mediated signaling, promoting GRK 5/6 kinases to phosphorylate the receptor, leading to MAPK activation followed by ERK, p38 or JNK and subsequent activation of transcription factors and gene regulation [138]. In addition to the non-canonical pathway, chronic β2AR stimulation turns off the signal through a PKA feedback loop (Figure 4).

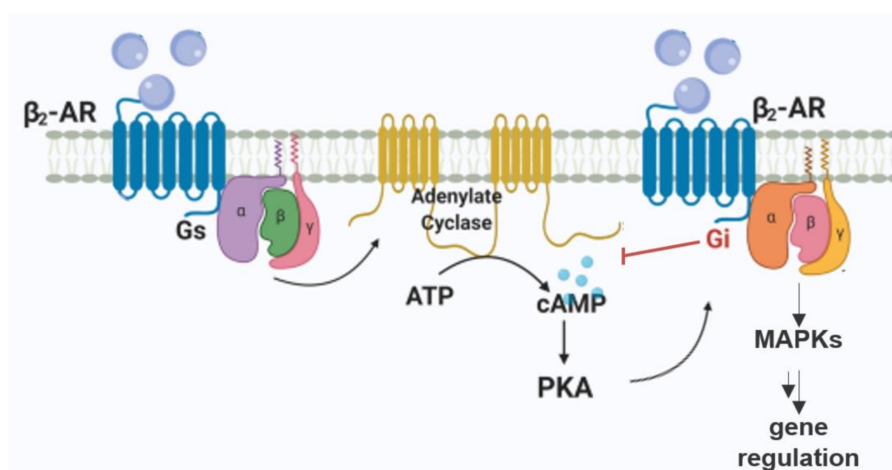

Figure 4: Chronic stimulation-initiated G protein switch of the β2AR
Schematic illustration of β2 adrenergic receptor desensitization. Chronic receptor stimulation can induce PKA feedback loop which leads to a switch of the coupled G-protein. The stimulation of a β2AR coupled to a Gi-Protein induces the inhibition of the adenylate cyclase (right). Edited from Dorotea and Ha [139].

Following chronic receptor stimulation, intracellular cAMP increases and activates PKA, phosphorylating serine/threonine target sites at the third intracellular loop of the β2AR [149]. This phosphorylation uncouples the Gs protein and increases the affinity to Gi protein [150]. The exchange in the coupling G proteins is called a G protein switch. The stimulation of the Gαi subunit inhibits adenylate cyclase [136], counteracting the Gαs-initiated pathway and inducing MAPK-mediated gene regulation [138].

Therefore, chronic stimulation or high agonist concentrations can induce different pathways, enabling the neuroimmune crosstalk to adjust the immune response to specific conditions and allowing the SNS to both inhibit and enhance immune responses.

1.2.3 Effect of stress on Natural Killer cells

Stress, whether chronic or acute, can significantly impact the function and activity of NK cells, with potential implications for overall health. NK cells, among lymphocytes, express the highest numbers of intracellular glucocorticoid receptors [151] and β2 adrenergic receptors [152]. This innate immune cell is considered the most sensitive peripheral blood mononuclear cell (PBMC) subtype to stress mediators [153] and is utilized as a biomarker for acute stress [154].

Upon acute stress, the SNS activates the release of epinephrine. In these situations, NK cell numbers rise within minutes in the bloodstream [81]. *In vivo* studies analyzing psychological stressors or exercise demonstrate that the release of epinephrine leads to a redistribution of NK cells, increasing their numbers as well as their activity [153, 155-158]. NK cell mobilization is suggested to originate from several reservoirs, such as the marginal pool, and be initiated by the blockade of adhesion molecules like CD62L and CD11a (LFA-1) [34]. The interaction between epinephrine and the β2 adrenergic receptor predominantly leads to the recruitment of cytotoxic $CD56^{dim}$, $CD16^{+}$ NK cells, preparing the organism to combat infections and malignant cells [34, 159]. Supporting this assumption, *in vivo* studies have revealed an activating effect of acute stress on NK cells [153, 157, 160, 161].

However, the general assumption is that epinephrine acts immunosuppressive on the immune system, blocking the activity and adherence of NK cells for mobilization. The immunosuppressive effect was confirmed through several *in vitro* studies showing an inhibitory effect on NK cell activity [162-164]. Furthermore, the meta-analysis of Segerstrom [156] elucidated that the increased cytotoxicity in various *in vivo* studies is caused more by the elevated number of NK cells than by an increased cytotoxicity of the NK cell itself. Supporting an inhibitory effect by epinephrine, decreased NK cell functions could also be related to acute stress after surgery and traumatic or thermal injuries [161, 165]. Additionally, the blockade of the adrenergic receptor during cancer surgery is suggested to have a beneficial effect on tumor control [101].

Indeed, epinephrine is metabolized within minutes after release, and the hormone may have a priming effect on NK cells [166]. Increased activity was shown after co-incubation of NK cells and epinephrine *in vitro* [167]. Furthermore, NK cell expansion and memory functions were impaired in MCMV-infected mice lacking *ADRB2*, suggesting a regulatory role of the β2AR in the response to viral infection [26]. Another mechanism for NK cell activation could involve IL-2 and IL-12 signaling, which are potent stimuli for NK cells, and whose gene ex-

pressions for these cytokines are upregulated in acute stress [168]. Additionally, cytokine responsiveness modulates the expression of the *ADRB2*, creating a feedback loop. *In vivo* analysis showed that IL-12 increases the expression of the receptor on NK cells [26, 169], whereas epinephrine administration decreases the numbers of β2AR [170].

Chronic stress is associated with reduced NK cell cytotoxicity and suppressed proliferation [156, 171, 172]. The shift to Th2 cytokine expressions decreases NK cell functions, as shown by individuals who lost a spouse and experienced other chronic stressors, leading to decreased NK cell proliferation and cytotoxicity [156]. Besides the immunosuppressive action of glucocorticoids, epinephrine has also been shown to reduce NK cell activity in chronic stress. Rodents exposed to chronic β2AR agonists or wet-cage exposure had an inhibited immunostimulatory response to IL-12 [173]. Also in humans, the effect of acute psychological challenges on chronically stressed individuals has been shown to alter the immune response. These chronically stressed individuals had higher peak levels of epinephrine with reduced NK cell cytotoxicity and a more pronounced NK cell distribution [92]. Additionally, relatives of breast cancer patients who experienced high levels of distress showed reduced NK cell activity along with increased concentrations of catecholamines [174]. This can result in a reduced ability to target and eliminate virally infected or cancerous cells, leaving the body more vulnerable to diseases.

1.3 Asthma and long-acting β2AR agonist

Asthma is a chronic respiratory condition that affects the airways in the lungs. Typical symptoms are shortness of breath, wheezing, coughing, and chest tightness [175]. It is a heterogeneous disease with various types which can be influenced by a combination of genetic and environmental factors [176]. A pathologic characteristic of the disease is the chronic inflammation of the airways [177]. Asthma patients have a bronchial hyperreactivity and mucus overproduction, which can be induced by a specific stimulus. The chronic inflammation is orchestrated by many immune and epithelial cells causing a type 2 immune response [175].

Diagnosing asthma involves reviewing a patient's history, looking for symptom patterns, conducting allergy tests and blood tests. Additionally, spirometry is used to measure the volume and speed of inhaled and exhaled air. The pulmonary function is an important factor in the determination of asthma severity [178].

Typically, a combination of corticosteroids and β2AR agonists are used to treat asthma [178]. Patients use inhalers to directly address the cells in the airway. The corticosteroids should help prevent symptoms and control inflammation by suppressing immune cells [175]. The β2AR agonist should provide a quick relief during asthma attacks. Generally, two types of agonists are used to treat asthma patients. Short-acting β2AR agonists (SABAs) provide instant relief and have a duration of action of approximately 6 hours, while long-acting β2AR

agonists (LABAs) are used for long-term asthma management and can remain active for more than 12 hours with some ultra-long-acting LABAs lasting up to 24 hours [179]. Examples for prominent LABAs are Formoterol, Salmeterol or Indacaterol (ultra long-acting LABA). The long duration of action of LABAs is caused by membrane interactions. LABAs are lipophilic compounds that can stay at the membrane, dissociate slower and repeatedly stimulate the receptor [179].

The mode of action of these drugs is based on adrenergic receptor stimulation. They mimic epinephrine and act as ligands on adrenergic receptors [180]. In asthma treatments, the agonist functions on the smooth muscle cells to open the constriction [169]. Activation of the β2AR at the smooth muscle cells initiates a similar transmembrane signaling cascade as in NK cells resulting in the activation of PKA (detailed mechanism in section 1.2.2 β2 adrenergic receptor) [181]. Indeed, in the smooth muscle cells PKA can phosphorylate different intracellular substrates and Gq-coupled receptors, leading to a cascade of signals, such as the reduction of intracellular Ca^{2+}. The change in the Ca^{2+} concentration leads to the inhibition of myosin light chain kinase phosphorylation, which prevents airway smooth muscle contraction [182].

However, the use of β2AR agonists, either alone or in combination with corticosteroids, can have adverse effects outside of the respiratory area. Even though this drug class is only detected in very low concentrations in the peripheral blood, these drugs can initiate cardiovascular effects [183] with increases in pulse rate, blood pressure or tachycardia [184]. Patients with diabetes should also be cautious, as β2AR stimulation in the liver can lead to glycogenolysis and increased blood sugar [185]. Additionally, β2AR agonists may cause tremors in muscle cells [183].

The catecholamine-like mode of action makes LABA treatments a model for chronic stress scenarios. LABAs, especially Indacaterol, are specific β2AR agonists and can be interpreted as stable, synthetic version of epinephrine. Asthma patients use LABA drugs in a daily rhythm. Therefore, NK cells of asthma patients were used as *ex vivo* models for chronic β2AR stimulation.

1.3.1 Role of Natural Killer cells in asthma

NK cells play a crucial role in immune responses within the lung, constituting about 10–20% of the lymphocytes in this organ, which is one of the highest percentages compared to other organs [186, 187]. The vast majority are cytotoxic, mature $CD56^{dim}$, $CD16^{+}$, $CD57^{+}$ and $NKG2A^{-}$ NK cells. Unlike some other tissues, only a minority of lung NK cells express tissue-resident markers, suggesting that the lung is mainly populated with circulating NK

cells.[188]. These cells dynamically migrate from the blood and locate in the lung parenchyma during homeostasis [187].

The airway is a main pathogenic entry site and regularly exposed to environmental toxins and pathogens. Therefore, the immune response and inflammation need to be tightly regulated to avoid permanent pulmonary inflammation and damage. In steady-state conditions, lung NK cells are described to be hypofunctional with low cytotoxicity [187]. It is suggested that TGF-β and prostaglandin E2 from alveolar macrophages might suppress NK cell functions [189, 190] while the spontaneous production of IL-15 by bronchial epithelial cells supports NK cell survival [191]. NK cells are important for the protection and represent a first line of defense [192]. During infections NK cells are recruited to the lung and actively secrete antiviral cytokines like IFNγ [193]. Although the threshold for NK cell activation might be raised, the cytotoxicity of the NK cell subset assures an effective response against pathogens as soon as the threshold is crossed [194]. The loss of NK cell functions due to genetic deficiencies are associated with decreased protection against bacterial and viral infections in the respiratory tract [195].

NK cells from patients with asthma exhibit functional and phenotypic differences compared to those from healthy donors. Peripheral NK cells from asthma patients often show an increased frequency of IL-4 producing cells, while the frequency of $IFN\gamma^+$ NK cells is reduced [196]. In severe asthma, activated $CD69^+$ NK cells have been correlated with reduced FEV_1 scores [197]. Also, the *in vitro* CCL18 response of NK cells from allergic asthma patients is impaired and NK cell cytotoxicity as well as eosinophil apoptosis induction is decreased [198-200].

The exact role of NK cells in asthma pathogenesis is still under investigation. There is evidence that NK cell activity is enhanced in PBMCs of asthma patients, but it decreases after allergen challenges [201]. In a mouse model, NK cell depletion before sensitization declined pulmonary eosinophils and cytokine levels of IL-4 as well as IL-5 in the broncho lavage fluid [202]. Therefore, NK cells may be critical for eosinophilic disease development. In contrast, a different animal model showed that allergic eosinophilic inflammation can be inhibited by NK cells. The stimulation of the airways via IL-2 and IL-18 led to upregulated IL-12 production in the lung. These cytokines elevated the frequency of $IFN\gamma^+$ NK cells which ultimately resulted in a suppressed inflammation and reduced hyperresponsiveness [203]. These findings demonstrate that the inflammatory environment might be critical for the NK cell function. An *ex vivo* analysis confirmed that NK cells produce IL-5 and IL-13 when stimulated with IL-4, but shift to an IFNγ production when the stimulation is changed to IL-12 [204-206]. Therefore, the Th2 cytokine environment in asthma may influence the phenotype and function of airway NK cells. The role of NK cells in asthma exacerbations is of particular interest. Asthma exacerbations are closely linked to respiratory viral infections and asthmatics experience more severe and longer-lasting symptoms after an infection [188]. *In vitro* exposure of NK

cells from severe asthma patients to rhinoviruses showed reduced activity, cytotoxicity, and anti-viral cytokine production, which could contribute to the increased severity and duration of symptoms in asthmatics following infections [188]. Interestingly, the impaired NK cell functions were shown to be independent of the corticosteroid treatment [207].

Overall, NK cells in the lung play a complex role in immune regulation and their dysregulation or altered function may contribute to the development and exacerbation of asthma. Furthermore, asthma treatments have been shown to suppress immune cell activity [175, 188]. The package leaflets of asthma bronchodilators list palpitations, nasopharyngitis, headache, cough and oral candidiasis as common adverse effects (1 person in 10) [208, 209]. *In vitro* analyses have been shown that NK cells are sensitive to these active substances. Corticosteroids and β2AR agonists decrease the cytotoxicity, proliferation and IFNγ production of NK cells [210-215]. As respiratory tract viruses are one of the most frequent causes of asthma exacerbations in adults and children [216], understanding the impact of LABAs on immune responses, including NK cell function, is crucial for comprehensive patient care.

2 Aim of this thesis

The aim of this thesis was to evaluate the effects of epinephrine exposure and β2AR stimulation on NK cells. Epinephrine is released in stressful situations and one of the main mediators of the stress response. While acute stress can be beneficial, chronic stress is known to increase the risk for diseases. To analyze the effect of chronic stress, former studies have focused on the interaction between corticosteroids and the immune system. Less is known about the effect of repeated β2AR stimulation. NK cells are crucial for immune surveillance and an effective immediate immune response. Therefore, we wanted to analyze, if β2AR stimulation can modulate NK cell functions in acute and chronic stress scenarios.

The goal was to analyze the effect of acute β2AR agonist exposure on NK cells and investigate the functional and metabolic properties. Subsequently, we assessed whether and how chronic, repeated acute β2AR stimulation affects the NK cell response. For this purpose, we employed Indacaterol as a stable, long-acting β2AR agonist and epinephrine treatments.

Furthermore, we aimed to evaluate the clinical relevance and examine the *in vivo* translation of those *in vitro* results. Asthma patients commonly use β2AR agonist like Indacaterol as medication. The regular application and prolonged duration of LABA treatments resembled our chronic, repeated acute β2AR treatment scenario. Therefore, we used peripheral NK cells of asthma patients as an *ex vivo* model and analyzed if the regular LABA treatment affected peripheral NK cells in a similar manner like chronically β2AR treated NK cells *in vitro*.

Understanding the effects of stress and β2AR stimulation on NK cells contributes to the knowledge of the intricate interactions between the nervous and immune systems. It also emphasizes the importance of stress management in maintaining a healthy immune system and helps to evaluate the application of synthetic β2AR agonist in asthma treatments.

3 Material and Methods

3.1 Material

3.1.1 Technical Equipment

Table 1: Technical Equipment and manufacturer.

Equipment	Model	Company
Cell counter	CASY Cell Counter & Analyzer	OLS® OMNI Life Science (Bremen, Germany)
Centrifuges	Heraeus Multifuge 3 S-R	Thermo Fisher Scientific (Waltham, MA, USA)
	5910-RI	Eppendorf (Hamburg, Germany)
	Heraeus Megafuge 40R	Thermo Fisher Scientific (Waltham, MA, USA)
	Heraeus Fresco 21 centrifuge	Thermo Fisher Scientific (Waltham, MA, USA)
Clean Benches	HERA Safe 2020	Thermo Fisher Scientific (Waltham, MA, USA)
Developer	Agfa CP 1000 LC 50	Agfa-Gevaert Group (Mortsel, Belgium)
Flow cytometer	BD LSRFortessa	BD Bioscience (San Jose, CA, USA)
Gel electrophoresis device	PowerEase 500 Power supply + Xcell SureLock	Bio-Rad Laboratories (Hercules, CA, USA)
Heating block	Thermomixer comfort	Eppendorf (Hamburg, Germany)
Incubator	HERAcell240i CO_2 Incubator	Thermo Fisher Scientific (Waltham, MA, USA)
Microscope	Axio observer 7	Zeiss (Jena, Germany)
Heating device	Heating device Humidity S1	PeCon GmbH (Erbach, Germany)
Heating insert	Heating insert P Lab-Tek™ S1	PeCon GmbH
Incubator	Incubator PM2000 RBT	PeCon GmbH
CO_2 module	CO_2 module S	PeCon GmbH
Temperature mod	Temp module S	PeCon GmbH
Camera	Axiocam 506 mono	Zeiss (Jena, Germany)
LED light source		

Objective	Colibri 7 Plan-Apochromat 20x/0.8 DIC (UV)VIS-IR	Zeiss (Jena, Germany) Zeiss (Jena, Germany)
Nucleofector	4D-Nucleofector®	Lonza (Basel, Switzerland)
Plate Reader	GloMax® Discover Microplate Reader	Promega (Madison, WI, USA)
Real-Time Cell Analyzer	xCELLigence DP	OLS® OMNI Life Science (Bremen, Germany)
Seahorse analyzer	Agilent Seahorse XFe96 Analyzer	Agilent technologies (Santa Clara, CA, USA)

3.1.2 Kits

Table 2: Kits and providing companies.

Kit	Company
Cyclic AMP ELISA kit	Cayman Chemicals (Ann Arbor, MI, USA)
Dynabeads™ Untouched™ Human NK cells Kit	Thermo Fisher Scientific (Waltham, MA, USA)
ELISA MAX™ Deluxe Set Human IFNγ	BioLegend (San Diego, CA, USA)
MitoProbe™ TMRM Assay-Kit	Thermo Fisher Scientific (Waltham, MA, USA)
P3 Primary Cell 4D-Nucleofector® Kit	Lonza (Basel, Switzerland)
Western Bright™ Sirius Western blotting detection kit	Advansta (San Jose, CA, USA)

3.1.3 Antibodies

Table 3: Primary antibodies and providing company.

Antibody (clone)	Label	Company
β_2AR (6H8)	unconjugated	Abcam (Cambridge, UK)
β_2AR (non-phospho)	Unconjugated	7TM Antibodies (Jena, Germany)
2B4 (C1.7)	Unconjugated	Beckman Coulter (Brea, CA, USA)
CD107a (H4A3)	PE-Cy5	BioLegend (San Diego, CA, USA)
CD16 (3G8)	PE	BioLegend (San Diego, CA, USA)

CD16 (3G8)	Unconjugated	BioLegend (San Diego, CA, USA)
CD16 (Leu11c)	PE	BD Bioscience (San Jose, CA, USA)
Glut-1 (202915)	PE	R&D Systems (Minneapolis, MN, USA)
Glut-2 (199017)	AF647	R&D Systems (Minneapolis, MN, USA)
Glut-3 (202017)	AF700	R&D Systems (Minneapolis, MN, USA)
NKG2D (149810)	Unconjugated	R&D Systems (Minneapolis, MN, USA)
NKp30 (p30.15)	Unconjugated	Self-made

Table 4: Secondary antibodies and providing company.

Antibody	Label	Company
F(ab)2 frag goat@human Fcγ	PE	Jackson ImmunoResearch (Baltimore, PA, USA)
Goat@mouse IgG	Unconjugated	Dianova (Hamburg, Germany)
Goat@rabbit IgG	HRP	Cell Signaling Technology (Danvers, MA, USA)
Mouse@rabbit IgG	PE	Jackson ImmunoResearch (Baltimore, PA, USA)

3.1.4 Single guide RNA

Table 5: sgRNA, sequence and providing company.

sgRNA	Sequence	Company
ADRB2 CRISPR718977_SGM	AAGAATATGGGCGGCCCCAA	Thermo Fisher Scientific (Waltham, MA, USA)
ADRB2 CRISPR718974_SGM	CAGACGCTCGAACTTGGCAA	Thermo Fisher Scientific (Waltham, MA, USA)

3.1.5 Consumables and Reagents

Table 6: Consumables, reagents and providing companies.

Consumables/ Reagent	Company
7-AAD Viability Staining Solution	BioLegend (San Diego, CA, USA)
Barbadin	MedChem Express (South Brunswick, NJ, USA)
BD FACSFlow Sheath Fluid	BD Bioscience (San Jose, CA, USA)

BD FACS™ Permeabilizing Solution 2	BD Bioscience (San Jose, CA, USA)
CASYton	OLS® OMNI Life Science (Bremen, Germany)
CellTracker Red	Thermo Fisher Scientific (Waltham, MA, USA)
Dimethyl sulfoxide (DMSO)	Thermo Fisher Scientific (Waltham, MA, USA)
DMEM	Thermo Fisher Scientific (Waltham, MA, USA)
DPBS	Thermo Fisher Scientific (Waltham, MA, USA)
E-Plate 16 PET	OLS® OMNI Life Science (Bremen, Germany)
Epinephrine	Sigma-Aldrich (St. Louis, MO, USA)
ESI 09	Tocris Bioscience (Bristol, UK)
Fetal bovine serum (FBS)	Thermo Fisher Scientific (Waltham, MA, USA)
Glycerol	Carl Roth (Karlsruhe, Germany)
H89 (hydrochloride)	Cayman Chemicals (Ann Arbor, MI, USA)
IBMX	Cayman Chemicals (Ann Arbor, MI, USA)
IMDM	Thermo Fisher Scientific (Waltham, MA, USA)
IMDM GlutaMAX™	Thermo Fisher Scientific (Waltham, MA, USA)
Indacaterol	Cayman Chemicals (Ann Arbor, MI, USA)
MitoTracker™ Deep Red FM	Thermo Fisher Scientific (Waltham, MA, USA)
Nunc® Maxisorp plates	Sigma-Aldrich (St. Louis, MO, USA)
NuPAGE gels®	Thermo Fisher Scientific (Waltham, MA, USA)
NuPAGE MOPS SDS Running Buffer (20X)	Thermo Fisher Scientific (Waltham, MA, USA)
Orthovanadate	Sigma-Aldrich (St. Louis, MO, USA)
Pancoll Human, density 1.077 g/ml	PAN Biotech GmbH (Aidenbach, Germany)
Paraformaldehyde (PFA)	Agilent technologies (Santa Clara, CA, USA)
Penicillin-Streptomycin Solution	Thermo Fisher Scientific (Waltham, MA, USA)
Pertussis Toxin	Tocris Bioscience (Bristol, UK)

Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich (St. Louis, MO, USA)
Poly-(L)-Lysine	Sigma-Aldrich (St. Louis, MO, USA)
Polyvinylidene difluoride membrane	Merck Millipore (Burlington, MA, USA)
Propranolol-Hydrochlorid	Thermo Fisher Scientific (Waltham, MA, USA)
rh ICAM-1–Fc Chimera Protein	R&D Systems (Minneapolis, MN, USA)
rh IL-12	R&D Systems (Minneapolis, MN, USA)
rh IL-15	PAN Biotech GmbH (Aidenbach, Germany)
rh IL-18	MBL Life Science (Nagoya, Japan)
rh IL-2	NIH cytokine Repository (Frederick MD, USA)
RPMI	Thermo Fisher Scientific (Waltham, MA, USA)
Seahorse XF RPMI medium	Agilent technologies (Santa Clara, CA, USA)
Seahorse xFe96 FluxPak	Agilent technologies (Santa Clara, CA, USA)
SYTOX™ Blue dead cell stain	Thermo Fisher Scientific (Waltham, MA, USA)
True Cut Cas9 Protein v2	Thermo Fisher Scientific (Waltham, MA, USA)
Zombie Yellow™	Bio-Rad Laboratories (Hercules, CA, USA)

3.1.6 Buffers and media

Table 7: Buffers, media and composition.

Buffer/ Media	**Composition**
A549 / K562 medium (CTL)	10% FBS 1% penicillin/streptomycin in DMEM
Blocking buffer	5 % milk in PBS-T
Dynal buffer	2mM EDTA 0.1% BSA in PBS
PBS (10x)	1.37 M NaCl 81 mM KCl 27 mM $Na_2HPO_4 \cdot H_2O$ 15 mM KH_2PO_4 ddH2O, pH= 7.4
FACS buffer	2% FBS in PBS

LCAA (-)	0.5% BSA in PBS
LCAA (+)	1mM $CaCl_2$ 2mM $MgCl_2$ 0.5% BSA in PBS
Digitonin lysis buffer	50 mM Tris-HCl (pH 8) 300 mM sodium chloride 20% (w/v) Glycerol 2 mM EDTA 10% Digitonin 1mM Orthovanadate 1mM PMSF ddH_20, pH=7.3
NK cell medium	10% FBS 1% penicillin/streptomycin in IMDM GlutaMax^{TM}
PBS-T	0.05 % Tween20 in PBS
PBS-T-NaCl	0.5 M NaCl 0.05 % Tween20 in PBS
Reducing sample buffer (RSB) (5x)	10 % (w/v) SDS 25 % (v/v) 2-ME 0.1 % (v/v) bromophenol blue 50 % (w/v) Glycerol 0.3125 mM Tris-HCl In ddH2O, pH= 6.8
Running buffer	5 % MOPS SDS Running Buffer (20X) in ddH_2O, pH= 7.3
Transfer buffer	20 % (v/v) MeOH 24 mM Tris 129 mM Glycin in ddH_2O, pH= 7.3

3.1.7 Software

Table 8: Software and providing company.

Software	Company
FlowJo 10.5.3	FlowJo, LLC (Ashland, USA)
GraphPad PRISM Version 9	GraphPad (La Jolla, USA)
Seahorse analytics	Agilent technologies (Santa Clara, CA, USA)
ImageJ (Fiji)	Wayne Rasband and contributors, NIH (Maryland, MD, USA)
BioRender	BioRender (Toronto, Canada)

3.1.8 Cells

Table 9: Cells and origin.

Name	Origin
Primary NK cells	Whole blood from healthy humans
Peripheral blood mononuclear cells	Whole blood from healthy humans / asthma patients
K562	Chronic Myeloid Leukemia
K562mbIL15-IL21-41BBL (Feeder cells)	Chronic Myeloid Leukemia
A549-H2Bj-GFP	Lung carcinoma

3.2 Methods

3.2.1 Cell Culture of Cell Lines

For long-term storage, cryo cultures were frozen in 10% DMSO in FBS at a concentration of $10x10^6$ cells/mL. Cells were slowly frozen in Corning® CoolCell® containers and stored at -175°C in the gas phase of liquid nitrogen until usage. After thawing, the freezing medium was diluted in PBS and quickly removed by centrifugation. Subsequently, cells were resuspended in their respective media. All cell lines were maintained in a $75cm^2$ cell culture flask and cultured at 37°C, 5% CO_2.

A549-H2Bj-GFP cells were split twice a week when reaching a 95% confluence. A549 cell media was removed, cells were washed with PBS, detached by Trypsin, and seeded at a 1:3 ratio in fresh A549 media.

K562 cells were split three times a week when reaching a density of more than $0.8\ x10^6$cells/mL. Cell count was determined by CASY cell counter. Media was replaced with fresh K562 media, and cells were seeded at a density of $0.3\ x10^6$ cells/mL.

3.2.2 Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinized blood samples of healthy donors. For this, a 15 mL Pancoll density solution (1.077 g/mL) was slowly layered with 25 mL blood and centrifuged (25 min, 1025xg, no brake). The separated PBMCs, found as the white intermediate layer, were transferred to a fresh tube and washed three times with PBS. PBMCs were either used directly for experiments or stored as cryo culture in 10% DMSO/ FBS at -175°C.

3.2.3 Isolation and Cultivation of Natural Killer Cells

NK cells were purified from PBMCs by negative isolation using the Dynabeads® Untouched™ Human NK Cells kit following the manufacturer`s instructions (Invitrogen). In brief, biotinylated antibodies were rotated with PBMCs for 20 minutes at 4°C. The cell-antibody solution was washed with Dynal buffer and rotated with streptavidin coated magnetic beads for 15 minutes at room temperature. The solution containing tube was placed in a magnet and the supernatant was transferred to a fresh collection tube. Subsequently, the purity and viability of the isolated NK cells were determined by flow cytometry. The isolation resulted in a 90-99% purity. NK cells were defined as viable (7-AAD negative), CD56+, CD3- cells.

For NK cell cultivation, isolated cells in media were seeded in 96-well round-bottom plates at a density of $1x10^6$ cells/mL. Additionally, NK cells were stimulated with irradiated feeder cells (K562-mbIL15-mbIL21-41BBL) at a ratio of 1:2 (feeder cells to NK cells) and 200 U/mL IL-2. Over the next weeks, NK cells were examined every third day, and half of the media was replaced. When the cell density was $>3x10^6$ cells / mL, NK cells were split to a density of $1.5\text{-}2x10^6$ cells/mL. NK cell media was supplemented with 100 U/mL IL-2. After three weeks, pre-activated, cultured NK cells were used for further experiments. NK cells were cultured at 37°C with 5% CO_2.

3.2.4 Acute β2AR Stimulation

In general, cultured NK cells were treated with epinephrine (solved in H_2O) or Indacaterol (DMSO dissolved) at a concentration of 1 µM. When cultured NK cells were acutely treated, the β2AR agonist was added 0.5 h before or directly when conducting a xCELLigence, LC-AA or Seahorse assay. NK cell controls to Indacaterol were treated with the same DMSO concentration (0.01% v/v) as samples.

3.2.5 Chronic β2AR Stimulation

In chronic β_2AR stimulation settings, cultured NK cells were harvested and media was replaced with fresh IL-2 supplemented media. Next, the NK cell density was adjusted to $2x10^6$ cells/mL and cells were seeded in a 96-well U-plate. Samples were treated daily with epinephrine or Indacaterol (1 µM) while controls were treated with Medium or DMSO (0.01% v/v). In total, the samples were treated for 96 h with the last addition performed directly in the assay or 0.5 h before.

3.2.6 Flow Cytometry

Flow cytometric analysis was performed by using the BD LSRFortessa flow cytometer. Samples were resuspended in 100 µL FACS buffer solution before measurement. Data were analyzed using FlowJo (version 10) software.

3.2.6.1 Protein expression and viability analysis

NK cell β_2 adrenergic receptors were stained by incubating of 0.2×10^6 cultured NK cells with anti-β_2AR antibody (6H8, 1:50) in FACS buffer at 4°C for 20 minutes. Subsequently, the cells were washed with FACS buffer and incubated with a secondary antibody mouse@rabbit-PE (1:200) in FACS buffer under the same conditions. The expression levels were assessed by flow cytometry.

To measure the influence of β2AR agonists on glucose transporter expression after CD16 activation, cultured NK cells were treated with a β2AR agonist (1µM) for 15 minutes followed by anti-CD16(3G8) antibody (1µg/mL) addition. After 2 h incubation at 37°C and 5% CO_2, NK cells were stained at room temperature for 12 minutes with anti-GLUT-1-PE (202915, 1:100), anti-GLUT2-AF647 (199017, 1:100), anti-GLUT3-AF700 (202017, 1:100), and viability dye 7AAD (1:100). Protein expression of CD16 was analyzed in a second sample by anti-CD16-PE (Leu11c, 1:100). The NK cells were washed with FACS buffer and kept in the dark at 4°C until flow cytometric measurement.

3.2.6.2 Mitochondria Mass – Mitotracker Staining

Like in the examination of the Glucose transporters, mitochondrial mass was analyzed after β2AR agonist treatment and CD16 activation. Cultured NK cells were treated with a β2AR agonist (1µM) for 15 minutes followed by anti-CD16 (3G8) antibody (1µg/mL) addition. After 2 h of incubation at 37°C and 5% CO_2, NK cells were washed with PBS and stained with zombie yellow (1:1000 in PBS), Mitotracker Deep Red® (200 nM) and anti-CD16 (Leu11c)-PE (1:100). Sample was kept for 10 minutes in the dark at room temperature, then washed, resuspended in FACS buffer and measured by flow cytometry.

3.2.6.3 Depolarization of Mitochondria – TMRM

Cultured NK cells were pretreated as in 3.2.6.2 for mitochondrial mass. The MitoProbe™ TMRM Assay-Kit was used as recommended by the manufacturer. TMRM-PE solution (20nM) was added to cultured NK cells after 1.5 h of CD16 activation. NK cells were further incubated for 30 minutes at 37°C with 5% CO_2. As a control, cultured NK cells were treated with CCCP (50µM) for 5 minutes at 37°C. CCCP (Carbonyl cyanide 3-

chlorophenylhydrazone) is a protonophore that increases the proton permeability across the mitochondrial membrane (depolarizing the mitochondria). After incubation, NK cells were washed in PBS and resuspended in zombie-yellow staining solution (1:1000 in PBS). After incubation for 10 minutes in the dark, NK cells were washed with FACS buffer and analyzed by flow cytometry.

3.2.6.4 Degranulation Assay

The degranulation of cultured NK cells was analyzed by evaluating the surface protein CD107a expression. Cultured NK cells were activated by immobilized antibodies. Nunc Max-iSorp plates were prepared for this purpose and antibodies anti-NKG2D(149810)+ anti-2B4(C1.7), anti-NKp30(p30.15) or anti-CD16(3G8) were added to the wells, incubated over-night at 4°C at a concentration of 1 µg/mL in PBS. The next day, the plate was washed with PBS and $0.2x10^6$ cultured NK cells were transferred to the antibody-coated wells. Additional-ly, anti-CD107a-PE-Cy5 (H4A3, 1:100) was added. After 3h at 37°C with 5% CO_2, CD107a surface expression was determined by flow cytometry.

3.2.7 Ligand Complex Adhesion Assay

The protocol was adapted from Urlaub et al. 2017 [124]. ICAM-1-Fc complexes were pre-pared by mixing 50 ng/mL recombinant human ICAM-1-Fc chimera and F(ab)2 fragments of goat anti-human IgG, Fcγ fragment specific (80 ng/mL PE labeled) in LCAA(-)buffer for at least 20 minutes in the dark. $0.2x10^6$ cultured NK cells were seeded in a conical 96- well plate, washed and incubated for 10 min with 1 µg/mL of the primary antibodies NKG2D(149810)+2B4(C1.7). Subsequently, cells were washed with PBS again and resus-pended in 28µL LCAA(+)buffer containing Indacaterol (1µM), DMSO (0.01% v/v) or epi-nephrine (1µM). 2µL ICAM-1–Fc complexes were added (dilution 1:20 f.c.). NK cells were transferred to 37 °C. 10 µL of a goat anti-mouse IgG solution was added to a final concentra-tion of 2.3 ng / mL for antibody cross-linking. Stimulation control was performed by adding 10 µL Mg^{2+} / EGTA (10 mM / 1 mM) instead of antibodies. After 10 min at 37 °C, NK cells were fixed by adding 40 µL paraformaldehyde to a final concentration of 2% for 10 minutes. In the last step, NK cells were washed and resuspended in FACS buffer. Data were acquired by flow cytometry. The LFA-1 activity is measured by PE intensity of ICAM-1-Fc bound to LFA-1 and displayed as geometric mean fluorescence intensity (gMFI).

When indicated (figure 6), cultured NK cells were additionally pre-treated with inhibitors (10 µM H89 or 25 µM ESI09) for 0.5 h before the assay, and the inhibitors were also kept during the assay. So, after washing, the inhibitors were added simultaneously with β_2AR agonist.

The LFA-activity was normalized to the control and is displayed as normalized geometric mean fluorescence intensity (n gMFI).

In re-stimulation experiments (figure 11, E), inhibitors (10 µM H89 or Barbadin 50µM and its solvent control DMSO (1% v/v)) were kept in media for 24 h and were washed out before LC-AA and second β_2AR stimulation. The LFA-activity was normalized to the control and is displayed as normalized geometric mean fluorescence intensity (n gMFI).

3.2.8 Interferon-γ Detection ELISA

As in the Degranulation assay (3.2.6.4), 96-well plates were prepared by antibody immobilization. On the next day, $0.2x10^6$ cultured NK cells were treated with epinephrine, Indacaterol (1µM) or DMSO for 0.5 h and transferred to the antibody-coated plate. NK cells were stimulated for 5 h at 37°C, 5% CO_2. Alternatively, cultured NK cells were stimulated by cytokines IL-12/18 (0.5 ng/mL; 2.5 ng/mL) or K562 target cells (E:T = 1:2). After incubation, NK cells were centrifuged at 500xg for 5 min and the supernatant was stored at -20°C. The secreted IFNγ concentration was determined by human ELISA MAX™ Deluxe Set Human IFNγ from BioLegend. The absorbance was measured at 450 nm according to manufacturer's instructions, using the GloMax® plate reader from Promega.

3.2.9 RTCA-based Detachment Assay

Detachment of cultured NK cells from ICAM-1 was determined by xCELLigence® real-time cell analysis. In the first step, E-plates were coated with 50 µL of a 7 ng/mL goat anti-mouse antibody (in PBS) solution and incubated for 1h at 37°C. Next, the plate was washed with PBS and 50 µL of a 2 ng/mL solution of recombinant human ICAM-1–Fc chimera (in PBS) was incubated for 1 h at 37°C. The E-plates were washed again and ready for the assay. Cell index background was measured with 50 µL NK cell media before $0.1x10^6$ cells/well were added in a 50 µL volume. The impedance was measured for 2 h in 10 min intervals until the cell index reached a plateau. The experiment was paused and 10 µL Propranolol (1µM f.c.) or medium was added quickly. The assay was resumed and measured in a 2 min interval for 0,5 h. Next, the experiment was paused again and 10 µL epinephrine (1µM f.c.) or Indacaterol (1µM f.c.) or medium, DMSO (0.01% v/v f.c.) were added to the respective wells. Subsequently, the cell index was followed up in 2 min intervals. For the analysis, the cell index was normalized to the point of β_2AR stimulation and quantified 40 min after β_2AR agonist addition.

3.2.10 CRISPR-Cas9-based Knockout of *ADRB2*

The material (cuvettes, media, rack, reaction tubes, PBS and Pasteur pipettes) was prewarmed to 37°C. The Nucleofector was set to solution P3 and pulse code DK100. For each attempt, $4x10^6$ cultured NK cells were harvested and centrifuged at 100xg for 10 minutes. NK cells were washed with prewarmed PBS and a second time centrifuged at 100xg, 10 minutes. Subsequently, the supernatant was adequately aspirated and cells were resuspended in 100µL Lonza P3 buffer (buffer was prepared according to manufacturer's instructions). TrueCut Cas9 protein v2 and sgRNA were kept on ice and mixed in a 1:1 ratio (1 µL of each *ADRB2* sgRNA (1,5 nM) + 2µL Cas9 protein solution (1 µg/µL)). The mixture was incubated for 10 minutes at room temperature. The control set was prepared in the same way, but did not include sgRNA. After incubation, the Cas9/sgRNA mixture was added to the NK cells and carefully resuspended in cuvettes. The cuvettes were transferred to the Nucleofector and pulsed. After nucleofection, 500µL RPMI medium without serum was slowly added and solution was incubated for 10 minutes at 37°C. The transfected cell suspension was slowly transferred to prewarmed reaction tubes by Pasteur pipette. Cuvettes were washed with 700 µL NK cell medium and the unified solution was incubated for 1 h at 37°C with 5% CO_2. In the last step, 100 U/mL IL-2 and 2.5 ng/mL IL-15 were added and cell suspension was seeded in a 96-U-well plate. After two days, the medium was exchanged and experiments with the *ADRB2* KO NK cells were conducted after 5 days. The knock out was verified functionally and by western blot analysis.

3.2.11 Western Blot

$2x10^5$ cultured NK cells were transferred to reaction tubes and pelleted by centrifugation (500x*g*, 5 min, 4°C). The supernatant was aspirated and NK cells were lysed with 16 µL Digitonin lysis buffer for 20 min on ice. The lysate was centrifuged (16,000x*g*, 20 min, 4°C) and supernatant was transferred to a fresh tube. Proteins were denatured by adding 4 µL 5xRSB and boiled for 5 minutes at 95°C. SDS-PAGE and Western blot were performed as described by Picard et al. 2022[217]. Briefly, samples were loaded onto a 4-12% SDS-NuPAGE gel and 150 V for 1,25 h in MOPS buffer was applied. Proteins were transferred from the SDS-gel to a polyvinylidene difluoride membrane for 1.5 h at 200mA. The membrane was blocked with 5% milk powder in PBS-T for 1 h at room temperature and then washed with PBS-T. The primary antibody (β_2AR (non-phospho), 1:1000) was incubated with the membrane overnight at 4°C with gently rocking. The membrane was washed three times before being incubated with a goat@rabbit HRP-conjugated secondary antibody (1:10000) for 1 h at room temperature. The detection was carried out using a Sirius detection kit and Agfa developer. The relative expression was evaluated by densitometry using ImageJ.

3.2.12 Microscopy-based Killing Assay

The microscopy-based killing assay was employed to assess NK cell cytotoxicity. This assay was conducted by Elizabeth Hennes at the Max-Planck Institute for Molecular Physiology in Dortmund. Cultured NK cells were pre-treated with Indacaterol (1µM) or DMSO (0.01% v/v), serving as a solvent control (as described in the sections acute and chronic b_2AR stimulation).

In a brief overview of the procedure, A549-H2Bj-GFP cells were cultured in RPMI1640 media overnight at a density of $3.5x10^3$ cells per well. Subsequently, pretreated NK cells were added in a 1.5:1 Effector-to-Target (E:T) ratio. The entire assay was conducted at 37°C with 5% CO_2, and live-cell analysis was performed using an IncuCyte S3 system at 10x magnification. The cells were incubated for a duration of 8 hours. Following incubation, the plates were carefully washed with PBS, and quantitative analysis of GFP fluorescence was carried out using IncuCyte 2019B Rev Software. Live cells could be detected by fluorescence measurement of the histone 2B-GFP tag. For reference, A549-H2Bj-GFP cells without NK cells (referred to as A549 control) were utilized to calculate the specific lysis. Specific lysis, representing the percentage of target cells killed by NK cells, can be calculated based on the GFP fluorescence measurements.

$$\%\, specific\ lysis = 100 * \frac{(A549 + NK) - (A549\ control)}{0 - (A549\ control)}$$

A549-H2Bj-GFP cells were kindly provided by Dr. Slava Ziegler and Prof. Herbert Waldmann.

3.2.13 Serial Killing Assay

The serial killing assay, as described by Prager et al. in 2019[218], involved the following procedure: First, a Zeiss Axio Observer Z1 7 microscope equipped with a 20×/0.8 Plan-Apochromat objective and an incubation chamber with environmental control (maintaining 37°C, 5% CO_2, and humidity using device S1) was prewarmed. Next, a 4-chamber microchip was equilibrated with 200 µL sterile filtered CTL media per well at 37°C. Cultured NK cells were stained with CellTracker Red (5µM) for 0.5 h at 37°C and subsequently washed with sterile filtered CTL media. The NK cell count was adjusted to $0.233x10^6$ NK cells/mL. Before initiating the experiment, either Indacaterol (1 µM) or DMSO (0,01 %v/v) was added 0.5 h before commencing the assay. K562 cells, suspended in CTL media, were added to the microchip with 10,000 cells/chamber along with SYTOX™ blue dead cell stain (1µM). After allowing the K562 cells to settle at the bottom of the well (10-15 minutes after addition), 15 µL of NK cells (3,500 cells) were added dropwise. Time-lapse live-cell microscopy was initiated, capturing images every 3 min over a 16 h period. SYTOX™ blue and CellTracker red

were excited using the Colibri 7 LED-module 475 (filter set 38 HE LED) and 567 (filter set 91 HE LED), while brightfield images were acquired with the TL LED module. Subsequently, the images were merged and analyzed using ImageJ.

3.2.14 Metabolic Extracellular Flux Assays

To observe the metabolic profile of cultured NK cells after β2AR stimulation, we used the Seahorse XFe96 Analyzer. We measured NK cell glycolysis via the extracellular acidification rate (ECAR) and oxidative phosphorylation by measuring the oxygen consumption rate (OCR). All measurements were performed in triplicates. The day before the assay, we hydrated sensor cartridges with sterile filtered water at 37°C overnight. On the day of the assay, sensor cartridges were calibrated by replacing the water with pre-warmed XF Calibrant solution. These cartridges were incubated for an hour at 37°C without CO_2. The cell culture plates were coated with Poly-(L)-Lysine (PLL; 10% v/v in H_2O). A 30µL PLL solution per well was incubated for 10 minutes at room temperature followed by washing the cell culture plates with PBS, leaving them to air-dry under sterile conditions. A NK cell solution of $2.5x10^6$ cells/mL was resuspended in Seahorse RPMI medium (1mM glucose, 100µM Pyruvate, 200 µM glutamine) and 250,000 cells per well were immobilized for 30 min at 37°C without supplemented CO_2. Simultaneously, injection ports of sensor cartridges were loaded with 10x concentrated substances as indicated in result graphics. When Propranolol (f.c. 1µM), H89 (f.c. 10 µM) were applied, these substances were loaded in the first port, followed by β2AR agonist epinephrine (f.c. 1µM) Indacaterol (f.c. 1µM) or DMSO/Medium controls in the second port. The next port was used for NK cell activation. NK cells were either activated by anti-CD16 antibody (f.c. 1 µg/mL) or cytokines IL-12/15/18 (f.c. 50 ng/mL / 250 ng/mL / 250 ng/mL). When injection ports were loaded, the sensor cartridge was equilibrated in the Seahorse XFe96 Analyzer. The cell culture plate wells were filled with Seahorse RPMI media to a volume of 180 µL, and the measurement run was started. The measurement intervals were set to a 3 min mixing followed by 3 min of measurement. OCR and ECAR levels were analyzed over 160 minutes. Results were normalized to the timepoint of β_2AR agonist addition and analyzed using Seahorse analytics software.

3.2.15 Proteomics

Our collaboration partner, Jörg Reinders, and his colleagues from the analytical chemistry department of the IfADo conducted the analysis of over 1100 proteins. The samples were prepared by Sabine Wingert and myself. For this purpose, $1x10^6$ cultured NK cells per sample were chronically stimulated with epinephrine (1µM), Indacaterol (1µM) or DMSO (0.01% v/v), (as described in 3.2.4). Media treatment were used as reference. After 96 h of stimula-

tion, NK cells were centrifuged at 500xg for 5 minutes and the supernatant was aspirated. The cell pellets were frozen at -80°C.

The frozen pellets were resuspended in 80% methanol and lysed by ultrasonication at 4°C. Subsequently, the lysates were centrifuged (21000g, 5 min, 4°C) and proteolytic digested by Trypsin. Samples were prepared according to Fischer and Kessler [219] using Gel-aided sample preparation. The peptides were then separated based on hydrophobicity in a reversed-phase column and a pre-column trapping. The MS/MS analysis was performed as described by Gillet [220]. Firstly, a MS library was generated using the TOP30-method and secondly, the SWATH method was used for sample analysis. The data were analyzed using PeakView and MarkerView.

3.2.16 cAMP ELISA

The cAMP concentration of chronically stimulated NK cells was evaluated using the cAMP kit from Cayman chemicals. For sample preparation, $2x10^6$ cultured NK cells/sample were chronically stimulated with Indacaterol (1µM) or DMSO (0.01 % v/v) (see section 3.2.6). The media, supplemented with IL-2 (100 U/mL) and IL-15 (2.5 ng/mL), with either DMSO or Indacaterol was replaced daily for four days. When indicated, NK cells were additionally pre-treated with Pertussis toxin (100 ng/mL) for 30 min at 37°C before each addition of DMSO or Indacaterol. Just before the last DMSO or Indacaterol treatment, NK cells were incubated with 100µM IBMX (3-Isobutyl-1-methylxanthin), a nonspecific inhibitor of phosphodiesterase, for 0.5 h. Subsequently, NK cells were counted and centrifuged for 5 minutes at 500xg. The supernatant was removed and the cell pellets were lysed in 100 µL of 0,1 M HCl per 1 million cells. After a 20 min incubation at room temperature, the lysate was centrifuged for 10 minutes at 1000xg. The supernatant was stored at -20°C until cAMP analysis. The following ELISA was conducted according to the manufacturer's instructions and absorption was determined, using GloMax® plate reader from Promega.

3.2.17 Asthma Patients and Healthy Subjects

Blood samples were obtained from asthma patients (n=10) through our collaboration with Prof. Dr. Michaela Schedel from the Department of Pulmonary Medicine at the University Medical Center-Ruhrlandklinik, Essen, Germany. These asthma patients were recruited during routine visits and 7.5 mL of heparinized blood samples were collected. An age and sex-matched healthy control group was recruited from the Ruhrlandklinik Essen (n=7) and the Leibniz Research Centre for Working Environment and Human Factors, Dortmund, Germany (n=3). The study was approved by the Westdeutsche Biobank - University Medical Center Essen, and for the healthy control samples, by the Human Ethics Committee of the Leibniz

Research Centre for Working Environment and Human Factors, Dortmund. The blood samples were analyzed on the same day as sampling.

PBMCs were isolated using Pancoll solution as described (3.2.2). These PBMCs were then functionally analyzed through IFNγ ELISA, LC-AA and degranulation assay (as descripted in the assay description above). Additionally, the composition of the PBMC's was determined through flow cytometry by Dr. Maren Claus.

3.2.18 Statistics

Statistical analysis was performed using GraphPad Prism version 9. The respective statistical analysis methods are indicated in the result legends.

4 Results

4.1 Acute β2AR stimulation of NK cells

4.1.1 β2AR expression and epinephrine titration

In the first step, we wanted to confirm that NK cells can interact with epinephrine. Natural Killer cells are primarily known to express the β2 adrenergic receptor (β2AR) [136]. Consequently, we evaluated the β2AR expression levels in cultured, pre-activated NK cells (Figure 1).

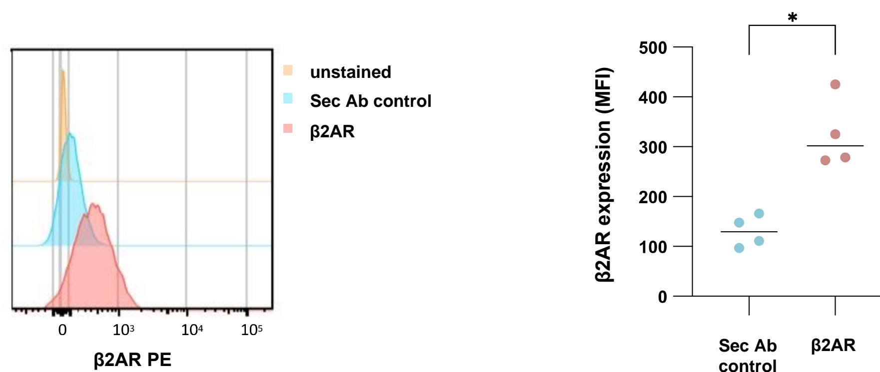

Figure 5: β2 adrenergic receptor expression.
The β2 adrenergic receptor (β2AR) expression analysis of cultured NK cells (median, n=4). Result was statistically analyzed by paired t-test (*, $P<0.05$).

Our analysis, conducted via flow cytometry, confirmed the presence of β2AR on cultured NK cells (see Figure 5). However, it is important to note that the results exhibited inconsistency. Despite using various buffers and antibodies targeting different epitopes, we were unable to consistently detect receptor expression levels. Notably, our findings align with previous work conducted by our colleagues [136, 142, 164].

Additionally, we conducted a titration of epinephrine to determine the optimal concentration for functional analysis (Figure 6). The physiologic epinephrine plasma concentration has been described in the low nanomolar range for non-stressed conditions and can increase by a factor of 300 in stressful situations [221, 222]. Thus, we titrated epinephrine in a range from 1nM to 10 µM and examined the IFNγ secretion by ELISA and the LFA-1 activity by ligand complex adhesion assay (LC-AA). It became evident that when cultured NK cells were pre-treated with varying concentrations of epinephrine and subsequently activated via cytokines (IL-12 and IL-18), the IFNγ secretion of cultured NK cells was reduced starting from a concentration of 10nM (Figure 6A). The inhibitory effect could be identified in a dose-dependent manner with the strongest effect observed at an epinephrine concentration of 1µM.

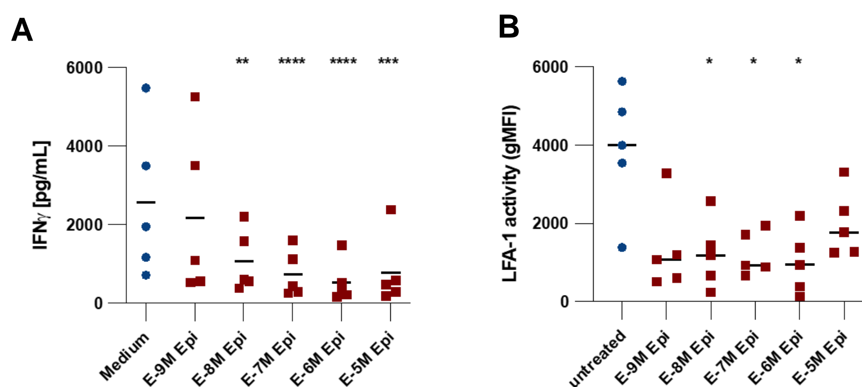

Figure 6: Titration of epinephrine.

(A) NK cells were pretreated with epinephrine as indicated and stimulated by IL-12/IL-18 (6h). Supernatant was measured for IFNγ secretion by ELISA (mean, n=5). (**B**) The LFA-1 activity of cultured NK cells was determined by LC-AA. NK cells were treated with epinephrine with indicated concentrations. Cells were stimulated by crosslinking NKG2D/2B4 antibodies (median, n=5). Results were statistically analyzed by one-way ANOVA test (****, P<0.0001; ***, P<0.001; **, P<0.01; *, P<0.05).

Furthermore, the ligand complex adhesion assay revealed the most consistent impact of epinephrine at the micromolar level (Figure 6B). The LC-AA is based on inside-out signaling, where the activation of receptors results in a conformational change in the integrin LFA-1, affecting its affinity and avidity for its ligand ICAM-1. In our assay a labeled recombinant human ICAM-1 complex was used to gauge LFA-1 activity. Depending on the affinity and avidity state, the labeled ICAM-1 complex attached to the integrin which was detectable by flow cytometry. The LFA-1 activity measurement revealed that epinephrine reduced the integrin activity, potentially impeding NK cell adhesion.

In previous experiments the working group of Prof. Carsten Watzl investigated whether NK cell cultivation influenced the inhibitory effect of epinephrine or the expression of the β2AR. Remarkably, NK cell cultivation had no significant impact on either variable. Neither β2 adrenergic receptor expression nor the functional inhibition of IFNγ secretion or LFA-1 activity by epinephrine was affected by the state of NK cell cultivation (unpublished). Consequently, for all subsequent analyses, we utilized cultured NK cells, hereafter referred to simply as "NK cells."

The inhibitory *in vitro* effect of β2AR stimulation on NK cells has been described in previous studies [163, 164]. Our initial findings confirm these studies and underscore that epinephrine is a potent inhibitor of NK cells, with the most pronounced effect observed at a concentration of 1µM.

4.1.2 Acute epinephrine and Indacaterol stimulation inhibits NK cells

Next, we aimed to validate that the inhibitory effect of epinephrine results from specific interaction with β2AR. We employed the β2AR agonist Indacaterol as alternative stimulus. We

used Indacaterol in the same concentration as epinephrine (1 µM) and functionally analyzed the NK cells response.

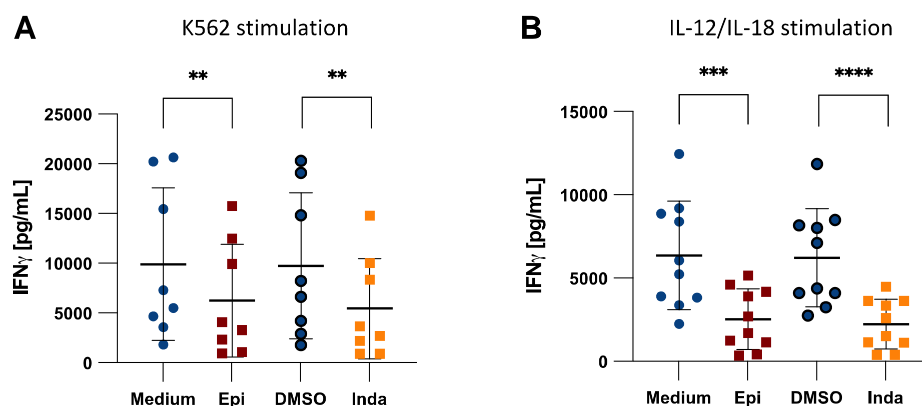

Figure 7: Acute β2AR stimulation inhibited IFNγ release.

Cultured NK cells were pretreated with 1µM epinephrine or Indacaterol and stimulated by K562 (**A**) or IL-12/IL-18 (**B**) for 6h. Supernatant was measured for IFNγ secretion by ELISA (mean, SEM; K562 n=8, IL-12/IL-18 n=10). Statistical analysis was performed using ordinary one-way ANOVA test (****, $P<0.0001$; ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$)

The analysis of secreted IFNγ of NK cells, pre-treated with either epinephrine or Indacaterol, showed a similar inhibitory effect of both agonists. Regardless of the NK cell activation, K562 target cells or cytokine (IL-12, IL-18) stimulated, Indacaterol and epinephrine significantly inhibited the secretion of IFNγ (Figure 7A, B). Since Indacaterol was solved in DMSO, the solvent was used as a control to Indacaterol.

We could verify the specific inhibitory effect by employing Propranolol, a beta blocker, to rescue the binding activity in a LC-AA (Figure 8A). As mentioned above, NK cells needed to be activated before measuring the LFA-1 activity. Therefore, controls correlated to the highest LFA-1 activity possible when activated through NKG2D and 2B4 receptors. Both β2AR agonists significantly inhibited the activation of the LFA-1 integrin. This inhibitory effect could be reversed by the antagonist Propranolol (Figure 8A, green triangle). NK cells pre-treated with the beta blocker no longer responded to epinephrine or Indacaterol and showed similar LFA-1 activity as controls.

The blockade of the LFA-1 activation by epinephrine and Indacaterol could potentially hamper NK cells from attaching to other cells. To elucidate the kinetics of the inhibitory effect on NK cells and gain further insights how the adhesion is affected, we conducted the impedance-based xCELLigence method. The method is based on a E-plate with a gold electrode studded surface and the application of a current. This electron flow is impeded (extended concept of resistance) by immobilization of cells. Using the cell analyzer, we could monitor the NK cell detachment from immobilized ICAM-1 ligands in real-time. We coated the xCELLigence E-plate with rhICAM-1, which served as a ligand for the integrin LFA-1, and added NK cells. The NK cell adhesion to the ligand induced a change in the impedance, resulting in an increase of the cell index (Figure 8B).

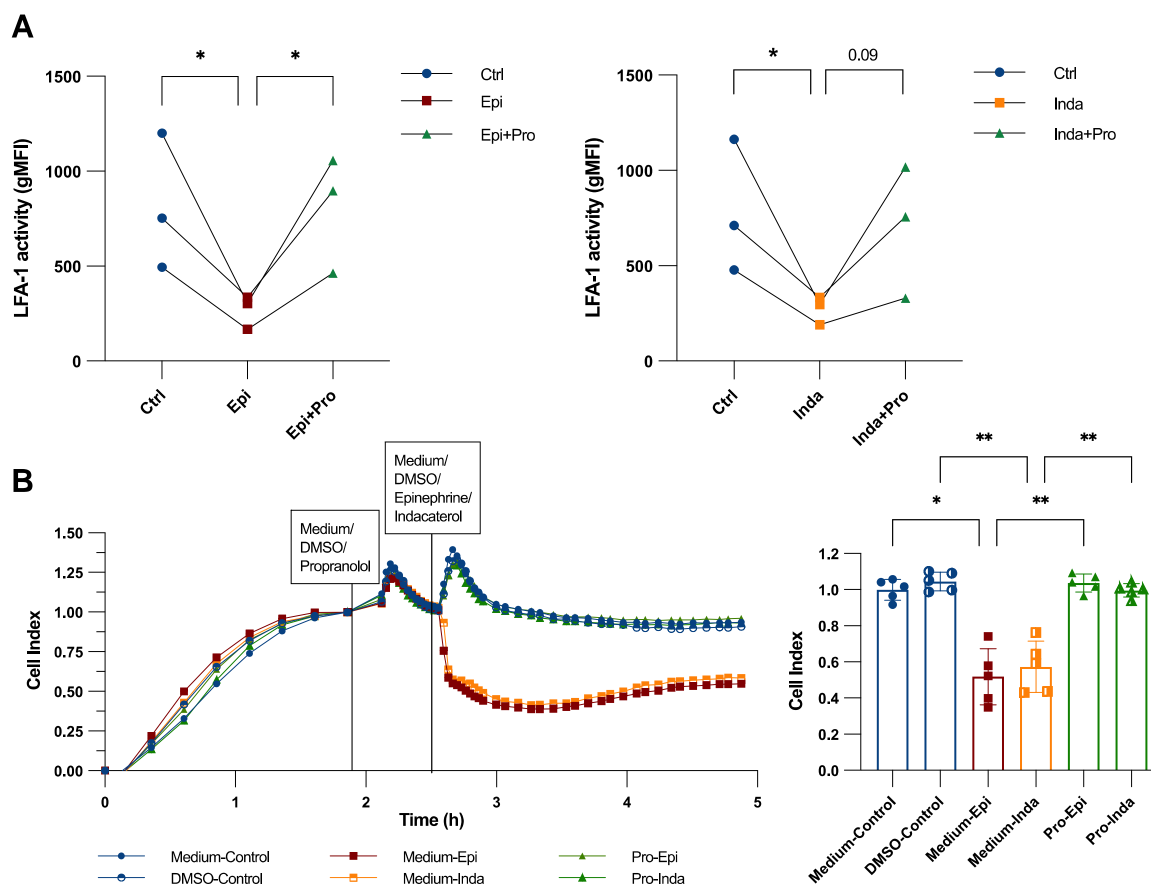

Figure 8:Acute β2AR stimulation inhibited LFA-1 activation.

(**A**) The LFA-1 activity of cultured NK cells was measured by ligand complex adhesion assay (LC-AA). NK cells were treated with epinephrine ± Propranolol (left) or Indacaterol ± Pro (right) and stimulated by NKG2D/2B4 crosslinking. DMSO, as solvent control to Inda, or medium treatments were used as controls (n=3). In (**B**), the detachment of cultured NK cells from plate-bound rhICAM-1 was measured by xCELLigence RTCA. NK cells were first treated with Medium, DMSO or Propranolol (betaAR blocker) and secondly with medium, DMSO or epinephrine, Indacaterol. The cell index was normalized to the first addition timepoint. A representative curve is shown on the left. The normalized cell index was quantified 40 minutes after β2-AR agonist addition (mean, SEM; n=5, right panel). Statistical analysis was performed using ordinary one-way ANOVA test (**, P<0.01; *, P<0.05)

However, upon addition of epinephrine or Indacaterol a rapid decrease of the cell index occurred through the detachment of the NK cells. The quantification of the data revealed that within minutes the NK cells have significantly detached (Figure 8B, red and orange squares). This indicated that not only the attachment of NK cells might be affected, but also the detachment of NK cells was initiated by epinephrine. The detachment was blocked by the beta blocker Propranolol demonstrating the specific β adrenergic receptor interaction.

To further confirm the β2AR specificity, we employed the CRISPR-Cas9 technique to knock out the gene (*ADRB2*) encoding the β2 adrenergic receptors. The western blot analysis confirmed a successful knockout in most of the cells and showed a substantial reduction of the β2AR protein (Figure 9).

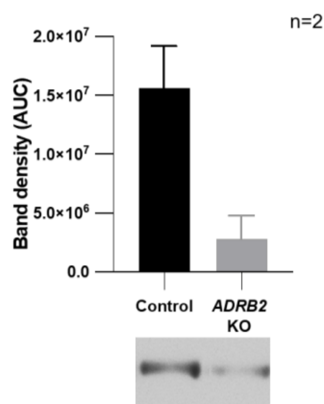

Figure 9: Verification of CRISPR-Cas9 mediated *ADRB2* knock out.
Quantification of β2AR expression by densitometry of western blot analysis. Cultured NK cells (nucleofected without sgRNA) as control vs. *ADRB2* KO NK cells (mean, SEM; n=2).

Unfortunately, along with flow cytometric analysis (4.1.1), the western blot results could not be verified by flow cytometry. Indeed, the β2AR knockout was conclusively confirmed through functional analysis. We assessed the adhesion of *ADRB2*-KO NK cells using the xCELLigence (Figure 10).

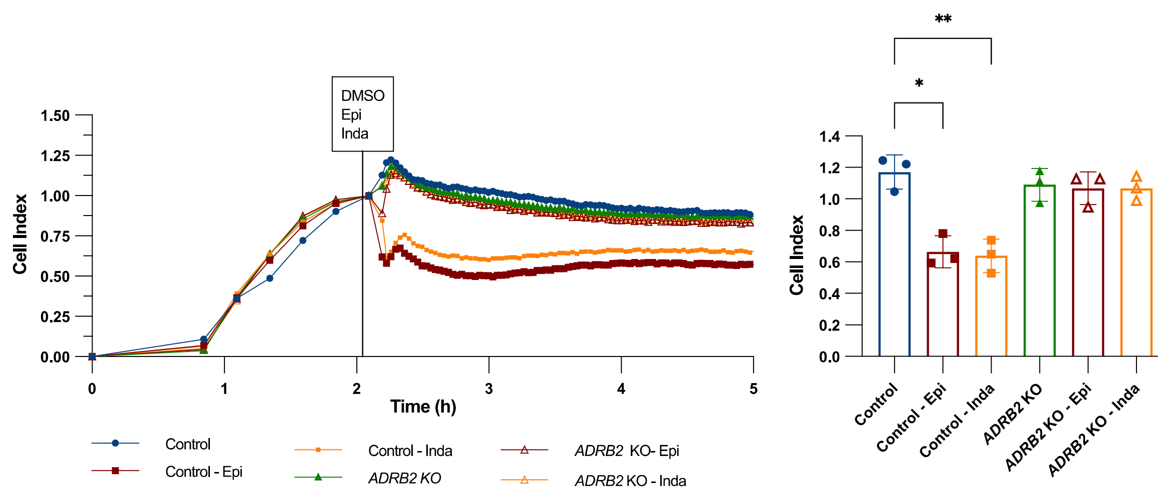

Figure 10: *ADRB2* KO NK cells do not react to β2AR agonist.
The detachment of cultured NK cells and *ADRB2* KO NK cells from plate-bound rhICAM-1 was measured by xCELLigence RTCA. The cell index was normalized to timepoint of addition. A representative curve is shown on the left. The normalized cell index of 3 independent experiments was quantified 10 minutes after β_2-AR agonist addition (mean, SEM; right panel). Statistical analysis was performed using ordinary one-way ANOVA test (**, P<0.01; *, P<0.05)

The *ADRB2* knockout cells yielded similar results as the blockade of beta-adrenergic receptors (Figure 8). The *ADRB2* knockout completely abolished the inhibitory effect of the β2AR

agonist, mirroring the results of the beta blocker Propranolol. Control NK cells treated with epinephrine (red square) or Indacaterol (orange square) initiated a rapid detachment of the cells. In contrast, *ADRB2*-KO NK cells did not react to the β2AR agonist, further confirming the successful β2AR knockout and the epinephrine-β2AR specific interaction.

In vivo, the hormone epinephrine transiently increases the number of NK cells in the peripheral blood. Multiple studies have shown that stress, exercise, or medication can result in a release of epinephrine, leading to a rapid increase of NK cells [222-224]. Our results show that epinephrine-mediated LFA-1 inhibition is β2 adrenergic receptor specific, potentially contributing to the recruitment of NK cells in the blood stream *in vivo*.

4.1.3 Indacaterol inhibits NK cell cytotoxicity

In addition to NK cell activity, we were also interested in understanding whether β2AR stimulation resulted in a decrease in NK cell cytotoxicity. We analyzed the killing ability of NK cells targeting A549 lung cancer cells or K562 lymphoblast cells.

In the first killing assay, NK cells and A549-H2Bj-GFP were co-incubated for 8 hours. Following careful washing, the living cells were quantified by the GFP tag and fluorescence microscopy. The experiment was conducted by our collaboration partner at the Max-Planck institute for molecular physiology in Dortmund. The analysis revealed that reduced NK cell activation also resulted in reduced NK cell cytotoxicity. Specifically, NK cells pre-treated with β2AR agonist Indacaterol showed a more than 50% reduction in specific lysis compared to control treated NK cells (Figure 11A).

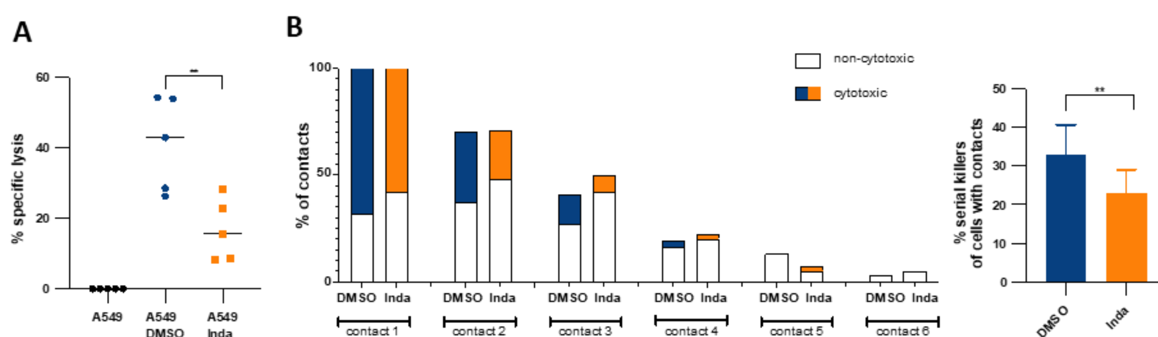

Figure 11: Indacaterol inhibits NK cell cytotoxicity and reduces serial killing.
(A) The specific lysis of cultured NK cells against A549-H2Bj-GFP (E:T, 1.5:1) were analyzed by Incucyte S3 microscopy. NK cells were acutely treated with DMSO or Indacaterol. Target cells without NK cells were used as control and set to 0% specific lysis (median, n=5). (**B**) Serial killing of cultured NK cells treated with Indacaterol or DMSO was analyzed by live cell fluorescence microscopy. K562 and NK cells (E:T, 1:3) were co-incubated for 16 h. 100 NK cells out of four independent experiments were followed up and analyzed for non-cytotoxic and cytotoxic contacts. NK cells with more than one kill were categorized as serial killers and quantified (right panel). Statistical analysis was performed using ordinary one-way ANOVA test or paired t test in (B) (**, P<0.01; *, P<0.05)

In the second killing assay, we analyzed the serial killing capability of NK cells by live cell fluorescence microscopy. NK cells were either treated with β2AR agonist Indacaterol or solvent control DMSO and co-incubated with K562 target cells on a microarray slide. These cells were monitored for 16 h, and dead cells were identified by a dead cell marker. For each condition, we tracked 100 NK cells and quantified cytotoxic or non-cytotoxic contacts. The analysis of serial killing data revealed that control NK cells and Indacaterol treated cells had similar numbers of cells which got in contact with a target cell, 63 to 64 NK cells with contacts out of 100 NK cells. However, when these contacts were further differentiated in cytotoxic and non-cytotoxic, we could show that Indacaterol treatment resulted in fewer cytotoxic contacts (Figure 11B, left panel). Displayed from the first contact to the fourth contact, NK cells treated with solvent control DMSO showed higher proportions of cytotoxic contacts (Figure 11B, left panel, blue bars). Particularly, the serial killing events (more than 1 kill) were significantly reduced in NK cells treated with Indacaterol (Figure 11B, right panel).

Thus, acute β2AR agonist Indacaterol treatment effectively reduced NK cell killing. NK cell cytotoxicity was reduced independently of the target cells, whether adherent A549-H2Bj-GFP or suspension K562 cells.

4.1.4 β2AR signals via cAMP-PKA pathway

Following the identification of the inhibitory β2AR agonist effect, we wanted to investigate the responsible signal transduction pathway of β2AR in NK cells. The β2AR is a G-coupled receptor, and binding of a β2AR agonist to its receptor results in the activation of the enzyme adenylate cyclase (AC) [225]. This membrane-bound enzyme converts ATP, leading to an increase of the intracellular cAMP concentration. The second messenger cAMP then binds to Protein Kinase A (PKA), which can phosphorylate transcription factors or cytoplasmic enzymes [18]. In addition to PKA activation, cAMP can regulate ion channel function and bind to the Exchange Protein Directly Activated by cAMP (EPAC) [142]. To identify the proteins involved in the β2AR mediated inhibitory effect, we blocked specific pathways and examined if the LFA-1 activity was restored by the blockade.

Using the LC-AA as functional readout, we analyzed the roles of the cAMP downstream molecules PKA and EPAC (Figure 12). NK cells were treated with ESI09 (EPAC inhibitor), H89 (PKA inhibitor), or a combination of both. Then the NK cells were activated and analyzed for LFA-1 activity. The inhibitors were present throughout the assay. While ESI09 had no significant rescuing effect on the epinephrine-initiated inhibition, H89 significantly restored LFA-1 activity (Figure 12). The combination of both inhibitors showed minimal additional increase in LFA-1 activity, indicating that PKA was the primary player responsible for the inhibited LFA-1 activity.

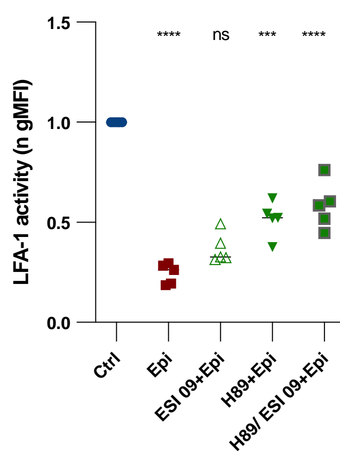

Figure 12: β2AR stimulation acts via cAMP-PKA pathway.
The LFA-1 activity was measured by LC-AA. NK cells were pre-incubated with PKA inhibitor (H89, 10µM) and/or EPAC inhibitor (ESI09, 25µM). NK cells were treated with epinephrine (1µM) ± inhibitor (inhibitor were kept throughout the whole experiment.) Statistical analysis was performed using ordinary one-way ANOVA test (****, P<0.0001; ***, P<0.001). Data were normalized to control.

Taken together, the β2AR primarily operates through the cAMP-PKA pathway, resulting in a disruption of the activating and LFA-1 stimulating inside-out signal.

4.1.5 Metabolic profile of β2AR stimulated and activated NK cells

As acute β2AR stimulation resulted in the inhibition of NK cells, we sought to investigate whether β2AR agonists disrupt the bioenergetics of activated NK cells. Two major energy pathways exist for generating ATP: oxidative phosphorylation and glycolysis. To measure these energy pathways, we employed the Seahorse analyzer with its extracellular flux rate technology. The Seahorse analyzer monitors the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) which are described to be proportional to mitochondrial respiration (OCR) and glycolysis (ECAR) [226].

To analyze NK cell metabolism, we immobilized pre-activated, cultured NK cells and examined their metabolic profile when treated with β2AR agonist (with or without beta blocker Propranolol pre-treatment) and additionally activated by CD16 or cytokines. In general, the stimulation of NK cells via anti-CD16 antibody led to a robust NK cell activation. Both energy pathways got activated resulting in a sharp increase of OCR and ECAR levels upon antibody stimulation (Figure 13, blue lines).

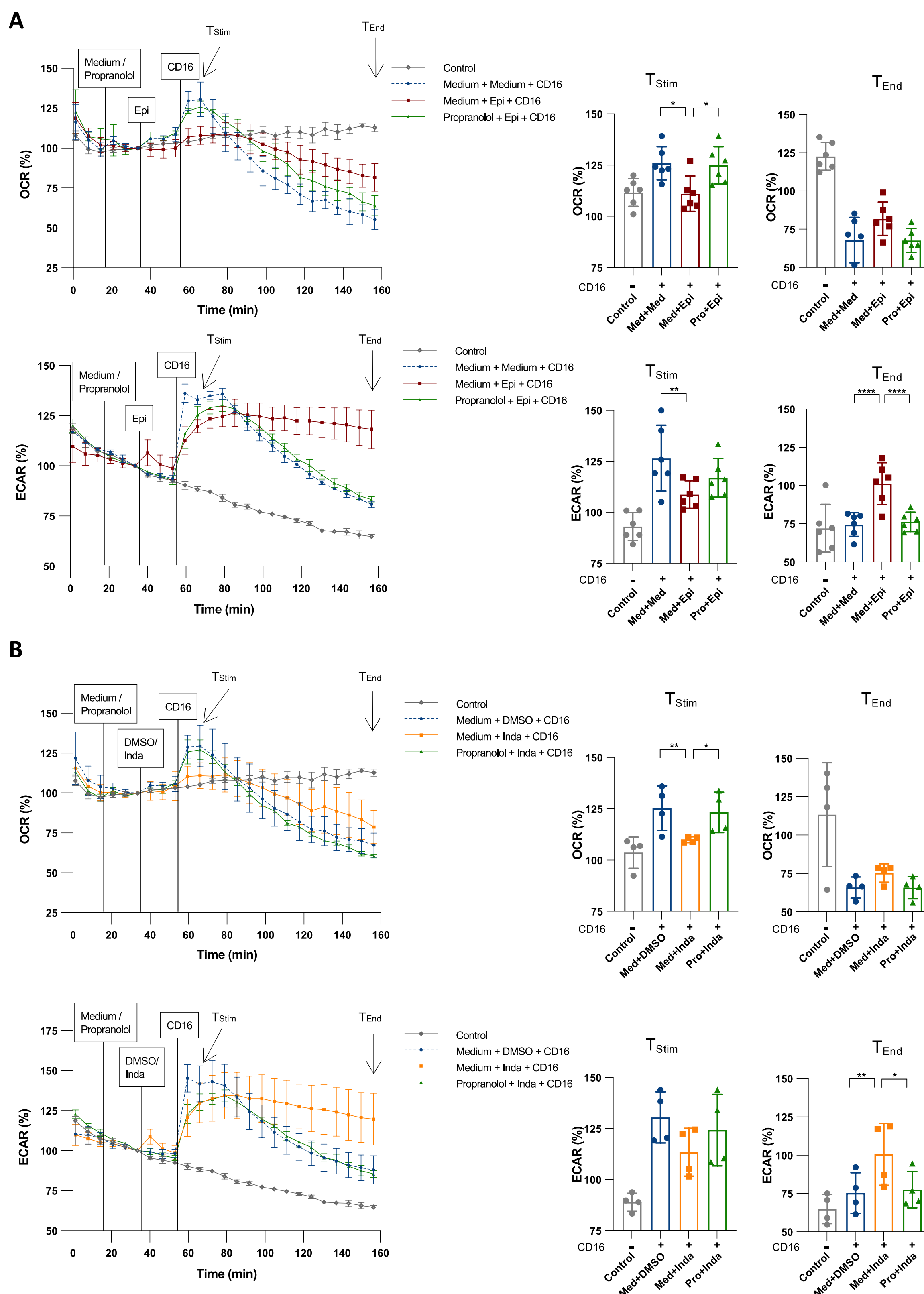

Figure 13: β2AR stimulation changes the metabolic profile of CD16 activated NK cells

The metabolic profile of cultured NK cells was evaluated by Seahorse technology. Representative measures are shown for oxygen consumption rate (OCR, top) and extracellular acidification rate (ECAR, bottom). Each condition was analyzed in triplicates. NK cells were treated in three injection steps. Firstly, with Propranolol or Medium, in the second step with Medium, epinephrine (**A**) or DMSO, Indacaterol (**B**) before activation in last step via CD16 by antibody addition. The oxidative phosphorylation (OCR) and glycolysis (ECAR) measurements were normalized to the point of the β_2AR agonist addition and quantified twelve minutes after stimulation (T_{Stim}) or at the end (T_{End}) (right panels, n=6 in A, n=4 in B). Statistical analysis was performed using ordinary one-way ANOVA test (****, P<0.0001; ***, P<0.001; **, P<0.01; *, P<0.05).

However, after an active 15-20 minutes period, OCR and ECAR levels started decreasing. Surprisingly, OCR levels dropped below baseline (Figure 13, grey lines), indicating a reduced mitochondrial energy production post CD16-mediated activation. ECAR levels remained above baseline, but steadily declined over time.

In β2AR treatment analysis, NK cells showed to be metabolically sensitive to β2AR agonists. The injection of epinephrine (see Figure 13A, red line) or Indacaterol (Figure 13B, orange line) resulted in a direct, transient increase of the ECAR level.

Furthermore, β2AR agonist treatment, followed by NK cell activation via CD16, slightly reduced the CD16 initiated ECAR increase. Interestingly, Indacaterol and epinephrine treatment prolonged the increased ECAR levels caused by the activating receptor (Figure 13A, B, bottom panel, red or orange line). In contrast to declining ECAR levels after CD16 activation of control NK cells, ECAR levels of β2AR agonist treated NK cells remained significantly higher. A similar result could be observed when β2AR agonist treated NK cells were activated with cytokines (refer to Supplement 1), demonstrating that the prolonged increase of the extracellular acidification rate was independent of the NK cell activation stimulus and choice of β2AR agonist. Meanwhile, β2AR agonists blocked the CD16-mediated increase of OCR values (Figure 13A, B top panels) and the OCR values remained near to baseline levels upon CD16 activation. Indeed, OCR levels of NK cells treated with β2AR agonist declined slower after CD16 activation as well.

The Seahorse analyses were quantified at the timepoint of stimulation through CD16 and at the end of the assay (almost 2h after β2AR agonist addition). The result highlighted the significant impact of β2AR agonists on the metabolism of activated NK cells. Especially, the blockade of the CD16 mediated increase at OCR T_{Stim} and the prolonged increase of ECAR values quantified at ECAR T_{End} demonstrated the influence of β2AR agonist on the NK cell metabolism.

To demonstrate that the metabolic effect is specifically induced by the β2AR, we employed the beta blocker Propranolol. The addition of the beta blocker abolished the metabolic effects of β2AR stimulation (Figure 13, green line) resulting in similar OCR and ECAR courses like control NK cells.

Propranolol confirmed that β2AR agonists influence NK cell functions as well as metabolism.

To further evaluate the metabolic effects of β2AR agonist and CD16 activation on NK cell oxidative respiration, we assessed mitochondrial parameters. We wanted to elucidate why OCR levels dropped below baseline upon antibody-mediated CD16 activation and if β2AR agonists influenced this effect.

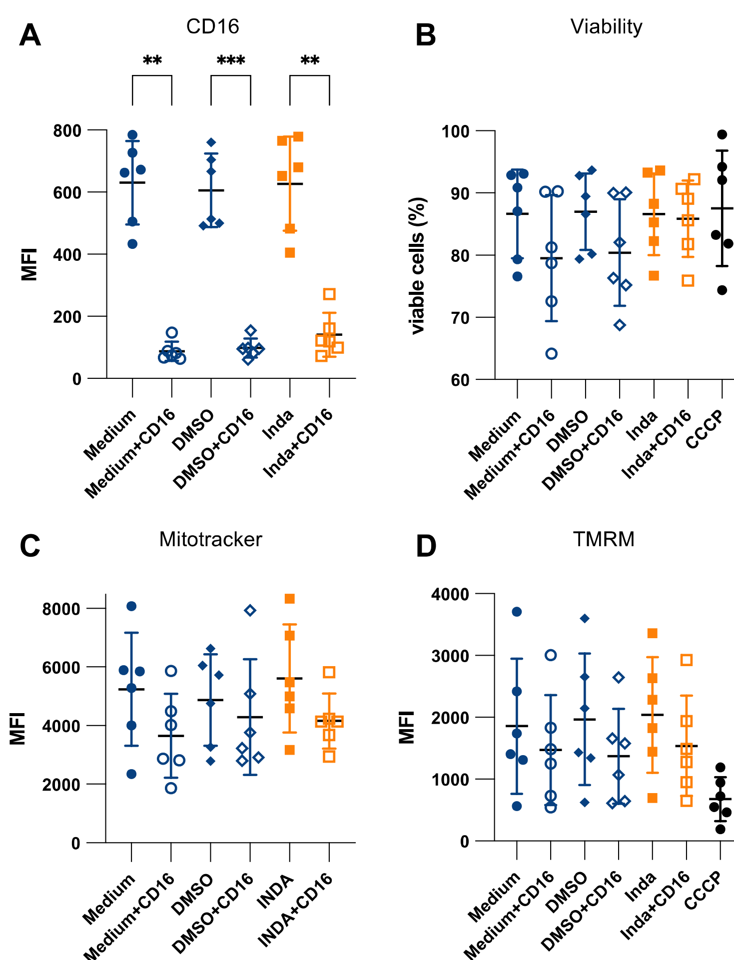

Figure 14: CD16 activation reduced mitochondrial capacity independent β2AR stimulation. NK cells were stimulated via CD16 ± Indacaterol (1µM) for 2h, subsequently stained and analyzed via flow cytometry. Cells were analyzed for CD16 expression (**A**), viability (**B**), size of mitochondria (mitotracker) (**C**), and depolarization (TMRM) (**D**). Statistical analysis was performed using ordinary one-way ANOVA (***, P<0.001; **, P<0.01; *, P<0.05).

We examined the CD16 expression, viability, and mitochondrial properties 2 h after β2AR agonist treatment and CD16 activation through flow cytometry (Figure 14). The antibody-mediated activation of NK cells resulted in a reduction of all measured parameters. Especially, CD16 surface proteins were drastically reduced 2 h after antibody stimulation (Figure 14A). Also, the viability of NK cells, mitochondrial mass (mitotracker) and the mitochondrial depolarization (TMRM) were reduced 2 hours after CD16 receptor activation (Figure 14B-D). The reduced size and depolarization of the mitochondria were indicators for the affected mitochondrial working capability, demonstrating a reduced ATP generation via oxidative phosphorylation which likely contributed to the OCR value reduction below baseline (Figure 13). The β2AR agonist treatment did not impact the mitochondrial working capability, although Indacaterol slightly increased the frequency of viable NK cells after CD16 activation (Figure 14B).

Given that PKA is described to modulate and increase glycolysis [227], we examined if the PKA inhibitor H89 could block the sustained ECAR increase (Figure 15).

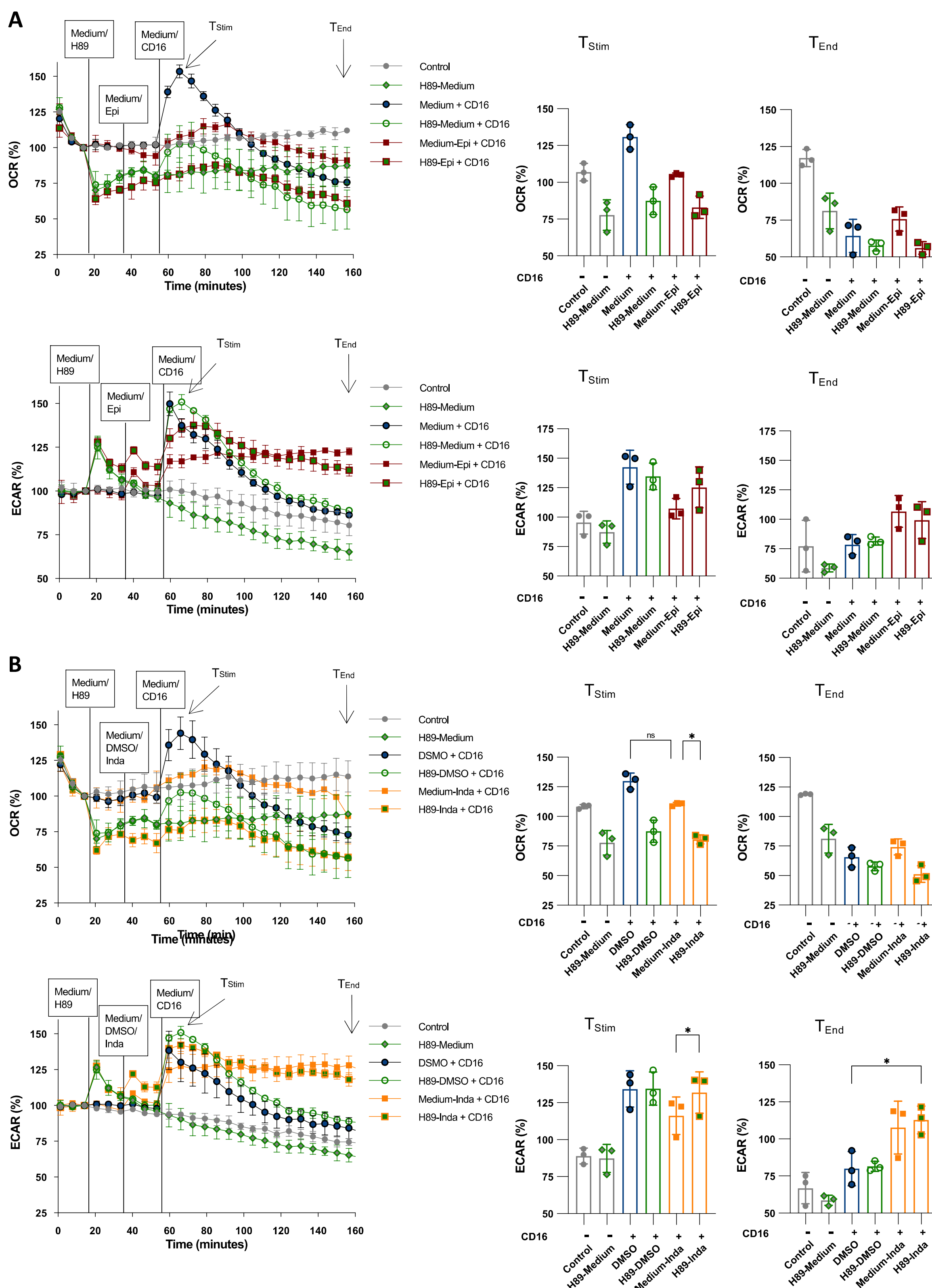

Figure 15: β2AR agonist induced prolongation of ECAR values upon CD16 activation is independent of PKA.

Representative curves of the metabolic profile measured by Seahorse technology. Each condition was analyzed in triplicates. The OCR (top) and ECAR (bottom) measurements were normalized to the point of the H89 (10µM) PKA inhibitor addition. NK cells were firstly treated with H89 or Medium, in the next injection step with Medium or epinephrine (**A**) or with DMSO or Indacaterol in (**B**) before activation via CD16. The timepoints after stimulation (T_{Stim}) and at the end (T_{End}) were quantified of 3 independent experiments (right panels). Statistical analysis was performed using one-way ANOVA (*, $P<0.05$).

Unfortunately, the PKA inhibitor by itself had an effect on the NK cell metabolism (Figure 15, green lines). Post H89 injection, the OCR values shifted to a lower level. Also, the ECAR measurements reacted transient and sensitive to the addition. Notably, despite these effects, the PKA inhibitor did not influence the pattern of the ECAR course of the curve or OCR course of the curve. The H89 treated samples showed similar reactions to the β2AR agonist as respective control groups of epinephrine treated NK cells (Figure 15A, red square and green filled triangle with red border) or Indacaterol treated NK cells (Figure 15B, yellow square and green filled triangle with yellow border).

Due to the PKA effect by itself, the quantification of the inhibitor treated NK cells did only partially match the control treated NK cells. However, the OCR and ECAR levels showed the same trend. The comparison of the differences between NK cells treated with or without a β2AR agonist and NK cells treated with H89 and with or without β2AR agonist demonstrated a similar reaction. Importantly, the quantification of the ECAR endpoint (ECAR T_{end}) showed similar results and no effect of the PKA inhibitor.

Therefore, the β2AR agonist effect on the metabolism could be interpreted as PKA independent.

In contrast to the literature, the PKA inhibitor did not affect glycolysis or alter the prolongation of increased ECAR. Indeed, the influence on the measurement of the inhibitor by itself makes the interpretation of the result difficult.

Furthermore, we explored whether PKA activation increased glycolytic capacity by raising glucose transporter expression. As another feature of the metabolic modulation by PKA, the glucose uptake should increase due to an increased number of glucose transporters [227]. Because of this reason and in addition to the Seahorse measurements, we analyzed the expression levels of glucose transporters 2 h after β2AR treatment and CD16 stimulation (Figure 16) through flow cytometry.

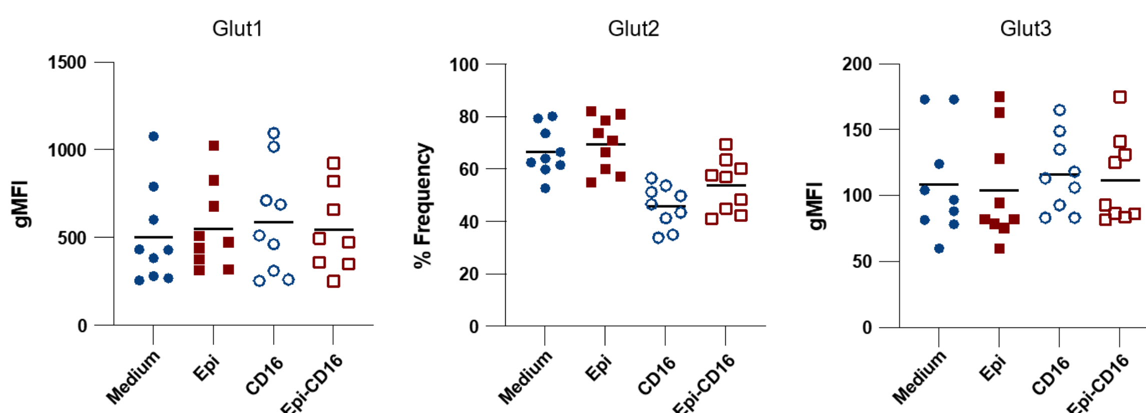

Figure 16:Epinephrine treated NK cells did not increase Glucose transporter expression. Expression levels of Glucose transporters (Glut1, Glut2 and Glut3) after 2h CD16 stimulation ± epinephrine (1µM) were analyzed by flow cytometry.

In general, no significant alteration of the glucose transporter expression was observed. Only the Glut2 transporter showed a non-significant decrease after CD16 stimulation. Therefore, we could not confirm that PKA activation increased the glycolytic capacity by raising the quantity of glucose transporters.

In summary, β2AR agonism did not block either of the two energy pathways. Instead, β2AR agonists prolonged glycolytic ECAR values upon CD16 activation in a PKA independent manner, while CD16-mediated NK cell activation led to reduced mitochondrial working capacity, affecting OCR levels.

4.2 Chronic β2AR stimulation of NK cells

After we identified and confirmed the inhibitory effect of epinephrine and Indacaterol on NK cells, we investigated if repeated β2AR stimulation extended NK cell inhibition. In chronic stress, repeated elevated concentrations of epinephrine have been measured and associated with negative outcomes of cancer treatments and post-surgery recovery [228, 229].

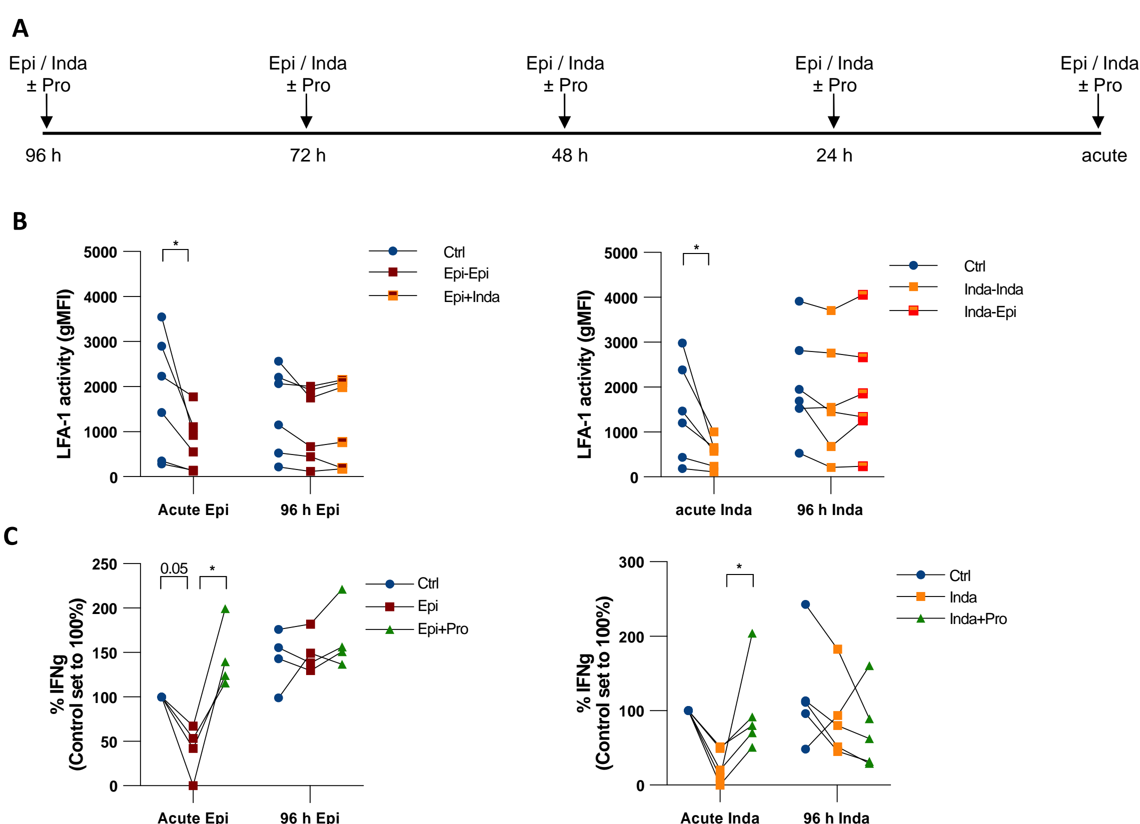

Figure 17: Chronic stimulation canceled β2AR induced inhibition.
(**A**) Cultured NK cells were either treated chronically every 24 h for five consecutive times or acutely with 1µM epinephrine/ Indacaterol. In (**B**), The LFA-1 activity was analyzed by LC-AA (n=6). NK cells were either acutely (left) treated with epinephrine (left) or Indacaterol (right). After chronic treatment with β2AR agonist, NK cells were treated with epinephrine or Indacaterol in the last step. In (**C**), the IFNγ secretion was measured by ELISA. NK cells were stimulated for 6 h with plate-bound CD16 antibody (n=4-5). Statistical analysis was performed for acute stimulation using paired t-test in (B) and ordinary one-way ANOVA in (C) (*, P<0.05).

To simulate a repeated stress scenario, we stimulated NK cells on five consecutive days (see Figure 17A). After the fifth addition of a β2AR agonist, we analyzed the functional impact and compared acute and chronic β2AR treatment effects (Figures 17-20).

Surprisingly and in contrast to acute stimulation, the repeated addition of β2AR agonists did not show an inhibitory effect on NK cells. Using the LC-AA as functional readout, we compared the LFA-1 activity of acutely and chronically β2AR agonist treated NK cells (Figure 17B). While acute treatment led to an inhibition of the LFA-1 activity, chronic treatment showed no effect. The LFA-1 activity remained on a similar level as the LFA-1 activity of control treated NK cells. Also, the choice of the β2AR agonist had no influence on the non-responsiveness of chronic treated NK cells. NK cells which were treated with epinephrine first and then with Indacaterol, or vice versa, showed the same result as NK cells treated with a single β2AR agonist. In all cases, NK cells did not react to the β2AR agonist treatment after the fifth addition (Figure 17B, orange or red border of filled squares).

Similar results were obtained when NK cells were activated and the IFNγ secretion was measured by ELISA (Figure 17C). We observed that the acute β2AR agonist treatment blocked IFNγ secretion, which could be rescued by the addition of Propranolol, while chronic treatment had no impact on IFNγ secretion. So, NK cell activation was no longer significantly inhibited when NK cells were stimulated via β2AR for 96 hours (Figure 17C).

Analysis of the xCELLigence detachment assay confirmed the results (Figure 18). Acute treatment with Indacaterol led to an immediate release of the NK cell from immobilized rhICAM-1. In contrast, chronically stimulated NK cells showed no response to the β2AR agonist addition (Figure 18, yellow filled square with green border).

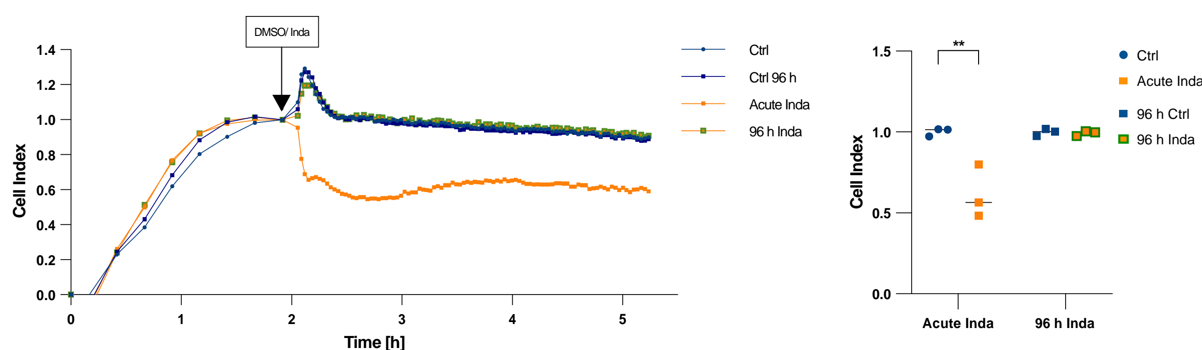

Figure 18:Chronic β2AR stimulation blocked detachment.
The detachment of chronically or acutely treated NK cells from plate-bound rhICAM-1 was measured by xCELLigence RTCA. The cell index was normalized to the timepoint of addition. A representative curve is shown on the left. The right panel shows the quantification 40 minutes after normalization of three independent experiments (median, n=3). Statistical analysis was performed using ordinary one-way ANOVA in (*, P<0.05; **, P<0.01).

The result showed that not only the activation of NK cells was no longer blocked by chronical treatments, but also the detachment was no longer triggered by β2AR stimulation. Therefore, chronic epinephrine or Indacaterol treatments made NK cells non-responsive to the β2AR agonists.

The result was further verified by flow cytometric degranulation assay (Figure 19). The degranulation measurement was based on the activation of NK cells, using plate-bound antibodies, and the flow cytometric analysis of the CD107a (LAMP-1) expression. The protein is typically found on the membrane of lytic granules. Upon NK cell activation and the release of cytotoxic granules, the fusion of the granule with the cytoplasmic membrane results in the surface exposure of CD107a [230].

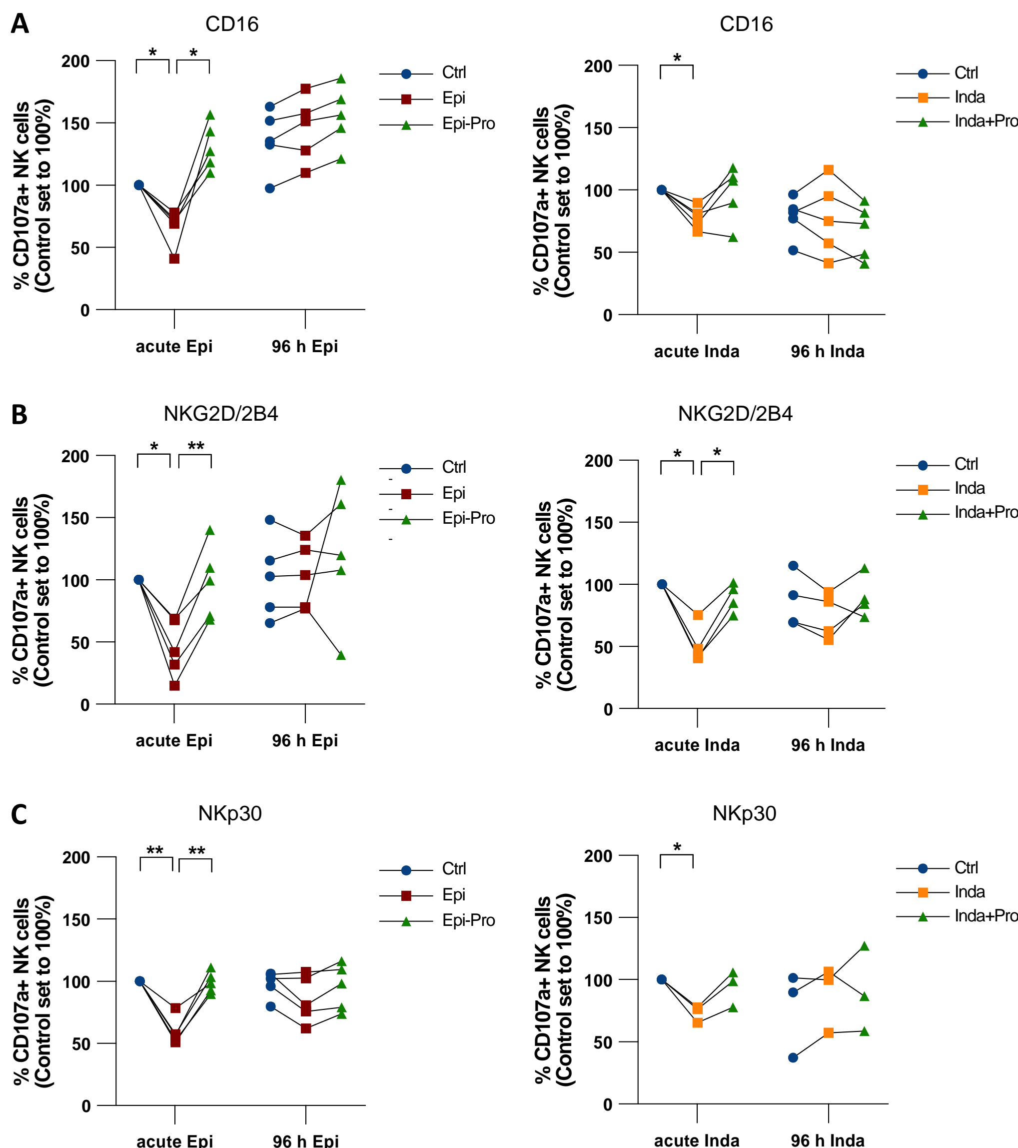

Figure 19: Chronic stimulation cancels β2AR inhibition independent of NK cell activation.
The degranulation was measured by flow cytometric CD107a expression analysis. NK cells were stimulated for 3 h with plate-bound antibodies, anti-CD16 in (**A**), anti-NKG2D+2B4 in (B) and anti-NKp30 in (**C**) (n=3-5). Statistical analysis was performed using two-way ANOVA test (*, P<0.05; **, P<0.01).

As seen in the analysis of the LFA-1 activity and IFNγ secretion, degranulation was primarily affected by the acute addition of a β2AR agonist. This effect was independent of the activating receptors and signaling pathways as activation via CD16, NKG2D/2B4 and NKp30 were affected in similar manner (Figure 19A-C). Also, the non-inhibitory effect of the β2AR agonist through chronic application was sustained independent of the activating receptor. These

cases displayed that the regulation through the β2AR globally affects NK cell activation and is not restricted to a specific activation mechanism.

As the primary task of Natural Killer cells is to kill transformed and infected cells, we also examined the effect of chronic β2AR treatment on NK cell cytotoxicity (Figure 20). In line with LFA-1 activity, degranulation, IFNγ secretion and detachment of NK cells, the cytotoxicity of chronically β2AR stimulated NK cells was no longer inhibited. Using A549-H2bj-GFP target cells and Incucyte® fluorescence microscopy (like in 4.1.3), the cytotoxicity of chronically stimulated NK cells remained the same as of control treated NK cells. In contrast, acute β2AR stimulated were significantly inhibited in comparison to chronically treated NK cells.

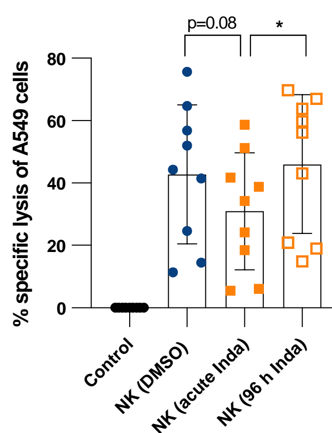

Figure 20: Chronic stimulation does abolished inhibition of NK cell cytotoxicity.
The cytotoxicity of chronic stimulated NK cells was evaluated by Incucyte® microscopy against A549-H2Bj-eGFP. The specific lysis was determined after 8 h co-incubation in a ratio of 1.5:1 (E:T). Results were normalized to A549 control (mean, n=8). Statistical analysis was performed using ordinary one-way ANOVA (*, $P<0.05$).

To summarize, NK cells were non-responsive to the repeated β2AR agonist stimulation. The chronic treatment resulted in an abolished inhibitory effect of β2AR agonists, having no longer an effect on LFA-1 activity, degranulation, IFNγ secretion, detachment or cytotoxicity of NK cells. NK cells behaved like untreated cells, showing full NK cell functions.

4.2.1 Metabolic extracellular flux analysis after chronic β2AR stimulation

Given that acute β2AR treatment led to a metabolic change in NK cells (probably independent of PKA), we sought to understand how chronic β2AR treatment affects NK cell bioenergetics.

We treated NK cells chronically with Indacaterol and analyzed the OCR and ECAR levels by Seahorse analyzer (Figure 21).

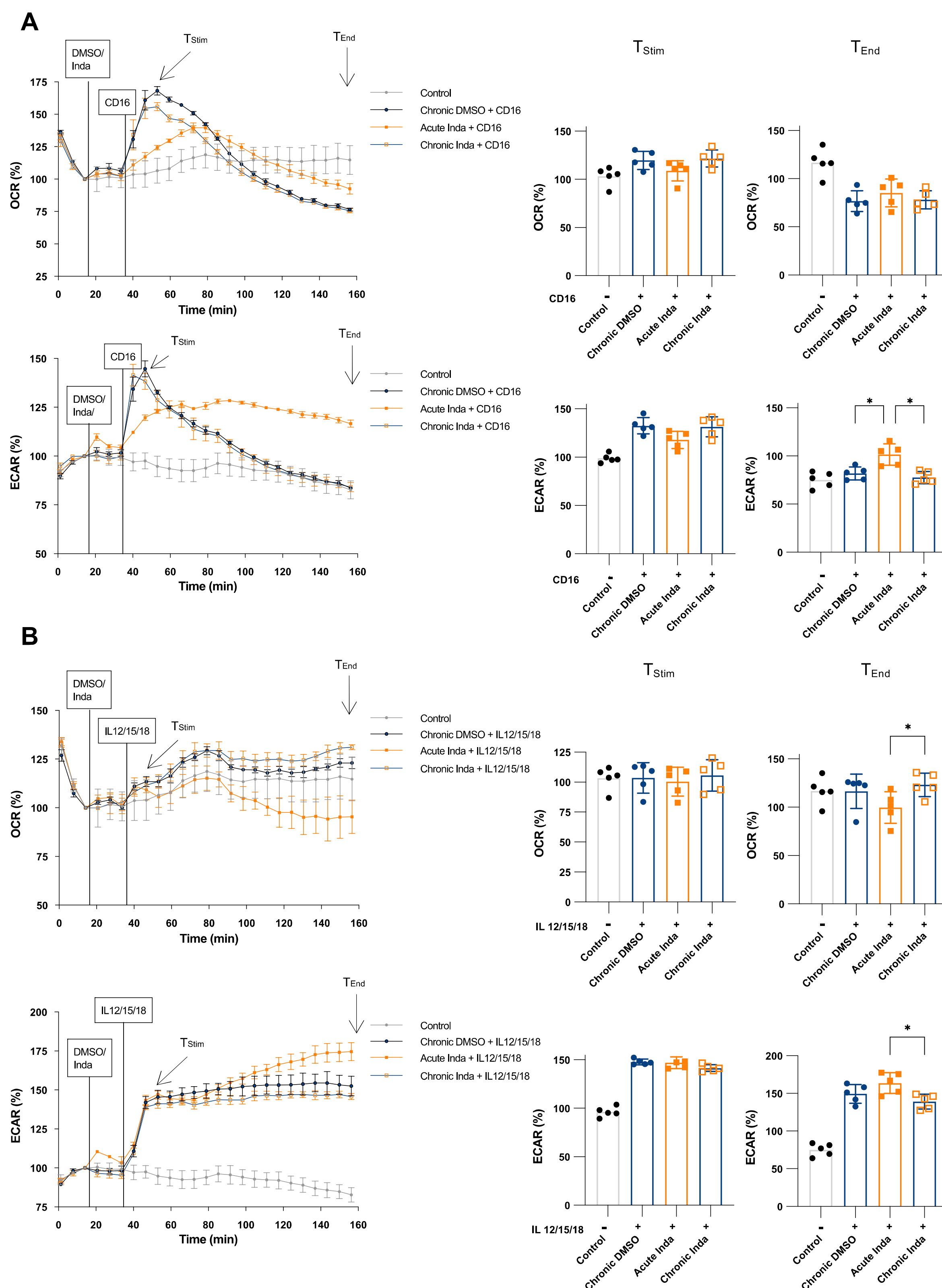

Figure 21: Chronic stimulation blocks metabolic shift of activated NK cells.
Cultured NK cells were either treated chronically (empty yellow square, blue line) or acutely (yellow square and line) with Indacaterol. The metabolic profile of cultured NK cells was evaluated by Seahorse technology. Representative measures are shown for oxygen consumption rate (OCR, top) and extracellular acidification rate (ECAR, bottom). The measurements were normalized to the point of the β2AR agonist addition. NK cells were treated with medium, DMSO as solvent control or Indacaterol (1µM) before activation via CD16(**A**) or cytokines IL-12/IL-15/IL-18 (**B**). The timepoints after stimulation (T_{Stim}) and at the end (T_{End}) were quantified of 5 independent experiments (right panels). Statistical analysis was performed using ordinary one-way ANOVA test (*, $P<0.05$).

Like in the functional analysis, chronic β2AR treatment did not affect the metabolic profile and NK cells were no longer sensitive to β2AR stimulation, implying a complete lack of response to the fifth β2AR agonist addition. Chronically treated NK cells (Figure 21, blue line with yellow empty square) showed the same course as DMSO control treated NK cells (Figure 21, blue line). In contrast, acute treatment with Indacaterol (Figure 21, yellow line with filled square) prolonged the increase of ECAR values, and acute β2AR treatment resulted in a transient and short-lived ECAR reaction, while chronically treated NK cells remained unresponsive to Indacaterol. As in the degranulation analysis (Figure 19), the non-inhibition was independent of the way NK cells were activated. The metabolic profile of NK cells activated by CD16 (Figure 21A) and the profile of NK cells activated through cytokines (IL-12, IL-15, IL-18) (Figure 21B) showed similar courses of chronically treated NK cells in comparison to the control NK cells.

The quantification confirmed similar results between control NK cells and chronically treated NK cells. In contrast, the endpoint ECAR values of acutely treated NK cells were significantly higher as from NK cells chronically treated or control treated.

In conclusion, chronic β2AR treatment rendered NK cells non-responsive to β2AR stimulation and their metabolic profile remained unaltered.

4.2.2 Chronic stimulation desensitizes β2R signaling

To investigate the mechanisms underlying the canceled response of NK cells caused by repeated β2AR stimulation, we initiated a comprehensive analysis. Initially, we analyzed the β2AR expression levels of chronically stimulated NK cells. In previous results, the Watzl research group verified that NK cells, whether untreated or chronically treated with a β2AR agonist, expressed the β2 adrenergic receptor to the same extent (unpublished). Therefore, chronic stimulation did not lead to an internalization of the receptor. Additionally, they analyzed the gene transcription levels of NK cells to elucidate whether specific genes were involved. The results did not show differentiated levels and an unchanged transcriptome (unpublished).

In the next step to identify potential factors responsible for the altered responsiveness, we conducted a protein translation analysis and compared chronically stimulated NK cells to control NK cells. In preparation for the proteomic analysis, NK cells were chronically treated with a β2AR agonist and cell pellets were frozen. Subsequently, the samples were lysed, digested and the peptide fragments were analyzed through LC MS/MS, performed by our collaboration partner Dr. Jörg Reinders. More than 1100 proteins were identified and compared to the protein expression of control NK cells (Figure 22A, B).

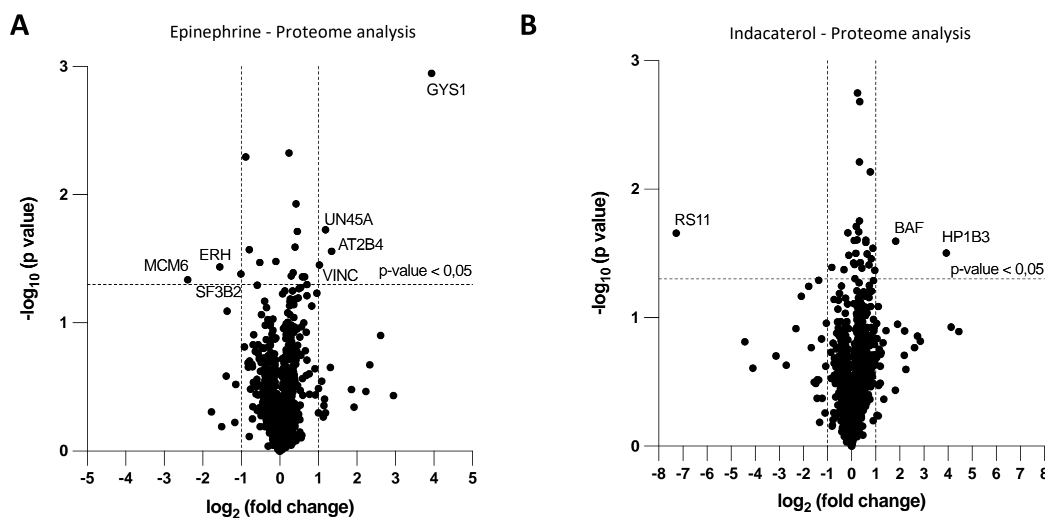

Figure 22:Chronic β2AR stimulation does not alter protein translation.
NK cells were chronically (96 h) treated with epinephrine (**A**) or Indacaterol (**B**) and analyzed for proteomic differentiations. Representative volcano plots are shown of three independent experiments.

The following proteins were significantly differentially expressed (fold change > 2, p-adjusted< 0.05):

- Chronic epinephrine treatment (Figure 22A): downregulated MCM6 (DNA replication licensing factor), ERH (Enhancer of rudimentary homolog), SF3B2 (Splicing factor 3B subunit 2); upregulated VINC (Vinculin; Actin filament), UN45A (Protein unc-45 homolog A; co-chaperone), AT2B4 (Plasma membrane calcium-transporting ATPase 4), GYS1 (Glycogen synthase)
- Chronic Indacaterol treatment (Figure 22B): downregulated RS11 (Ribosomal protein S11); upregulated BAF (Barrier-to-autointegration factor), and HP1B3 (Heterochromatin protein 1-binding protein 3).

None of the differentially expressed proteins could be associated to the canceled inhibition. Most of the proteins affect general processes like DNA replication or protein translation. Similar to the transcriptional analysis, there was no overlap between the effects of epinephrine and Indacaterol treatment. Also, STRING-based network analysis (STRING is a database of known and predicted protein-protein interactions) did not yield further insights. Therefore, changes in gene transcription or protein expression levels are unlikely to be the cause for the canceled response of repeated β2AR stimulation.

Since both gene and protein expression levels remained unaltered and β2AR expression was unaffected, we proceeded to analyze the downstream molecules of the β2AR response. Using a cAMP ELISA, we observed a decrease in intracellular cAMP concentration in chronically treated NK cells compared to control and acutely β2AR-stimulated NK cells (Figure 23). This decline in cAMP concentration led us to hypothesize that chronic β2AR stimulation resulted in a G-protein switch, altering the receptor's affinity towards its subunit and promoting interactions with inhibitory G-protein subunits that inhibit cAMP production. To investigate this

hypothesis, we employed the Gαi protein inhibitor Pertussis toxin (PTX), preventing the inhibitory Gα subunit from interacting with the β2AR. The addition of the inhibitor led to increased cAMP concentration and abolished the significant difference between acute and chronic treatment of the β2AR.

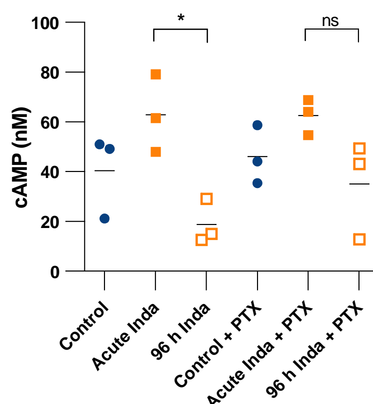

Figure 23: Cyclic AMP concentration decreases in chronically β2AR stimulated NK cells.
The cAMP concentration was measured of chronically or acutely stimulated (Indacaterol) NK cells ± Gi protein inhibitor (Pertussis Toxin, PTX). The cAMP concentration was analyzed by ELISA of lysed NK cells (mean, n=3). Statistical analysis was performed using ordinary one-way ANOVA (*, P<0.05).

The result indicated that chronic β2AR agonist treatment led to a G-protein switch of the adrenergic receptor which interfered with the cAMP supporting signal of acute β2AR stimulation.

To investigate the kinetic and identify the timepoint when the G-protein switch is initiated, we treated NK cells with Indacaterol, incubated the cells and treated these NK cells at different timepoints a second time with Indacaterol (Figure 24A).

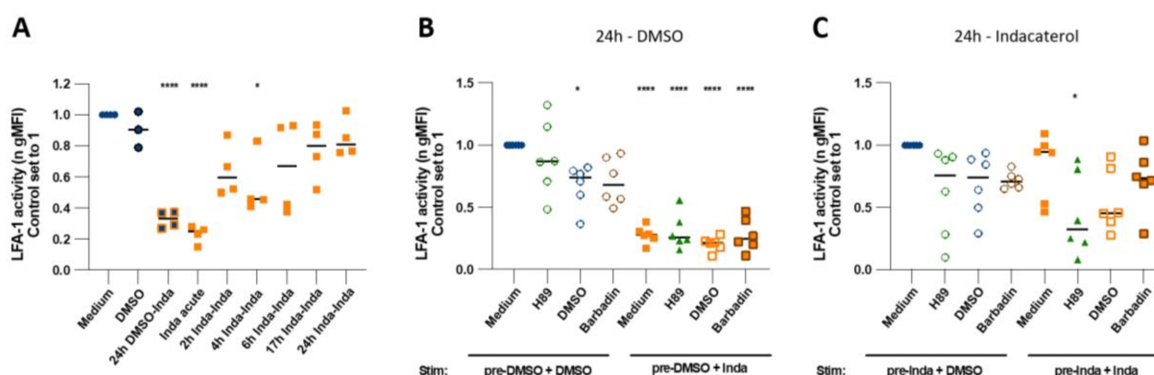

Figure 24: PKA feedback loop desensitizes β2AR and abolishes β2AR agonist inhibition.
In (**A**), NK cells were treated with Indacaterol before restimulation at indicated timepoints. The LFA-1 activity was analyzed by LC-AA. In (**B, C**), NK cells were pretreated with medium, PKA inhibitor H89 or DMSO solvent control, β-arrestin/β2-adaptin interaction inhibitor Barbadin. Subsequently, NK cells were treated with DMSO (B) or Indacaterol (C). After 24h pre-treatment, NK cells were washed and (re-)stimulated with DMSO or Indacaterol. LFA-1 activity was measured by LC-AA. Medium control was set to 1 and compared to samples (median, n=6). Statistical analysis was performed using ordinary one-way ANOVA (****, P<0.0001; ***, P<0.001; **, P<0.01; *, P<0.05).

The examination of the LFA-1 activity revealed that already after 2 h of Indacaterol treatment NK cells started to be partially unresponsive. After 24 h of Indacaterol treatment, NK cells were almost completely unresponsive to a second Indacaterol treatment, indicating a relative fast G-protein switch of the β2AR by Indacaterol.

To elucidate the mechanism of desensitization, we used the PKA inhibitor, H89, and beta-arrestin - adaptin interaction inhibitor, Barbadin, to block specific desensitization pathways. The PKA inhibitor was expected to block the PKA feedback loop which leads to phosphorylation of the receptor and initiates the G-protein switch, whereas Barbadin was intended to block the beta-arrestin pathway downstream of β2 adrenergic receptor phosphorylation by G-coupled receptor kinase (GRK).

We pretreated NK cells with these inhibitors and subsequently added DMSO (Figure 24B) or Indacaterol (Figure 24C) for 24 h. Following the treatment, we washed the NK cells and administered a second treatment with either DMSO or Indacaterol directly in a LC-AA. The LFA-1 activity showed that NK cells, which were treated with DMSO first and were treated with Indacaterol second, reacted similarly to acutely treated NK cells, with an inhibited LFA-1 activity (Figure 24B). In contrast, NK cells that were treated twice with Indacaterol showed no inhibition of the LFA-1 activity, similar to chronically treated NK cells (Figure 24C, yellow filled squares). Indeed, when NK cells were pretreated with PKA inhibitor H89 before the first Indacaterol treatment, the LFA-1 activity was inhibited again when NK cells were treated a second time with Indacaterol (Figure 24C, green filled triangles). This implies that H89 could block the PKA feedback loop and render β2AR sensitive to a second Indacaterol treatment. In contrast, Barbadin did not significantly impact the LFA-1 activity. The treatment slightly reduced the LFA-1 activity, but the reduction was independent of the second treatment, DMSO control or second Indacaterol treatment (Figure 24C, right panel, brown borders). This indicates that the inhibitor itself impacted the LFA-1 activity. Additionally, it is worth noting that the DMSO control relative to Barbadin (Figure 24C, right panel, blue circle and yellow empty square) itself already exhibited an effect on the LFA-1 activity which was probably due to the high DMSO concentration (1% v/v).

Taken together, chronic treatment did not affect the transcription or protein translation in NK cells, nor did it affect the β2AR expression on NK cells. Instead, the repeated treatment with a β2AR agonist initiated a PKA-mediated G-protein switch and blocked the inhibitory signal of the β2 adrenergic receptor for further β2AR treatments.

4.3 Differences between the properties of epinephrine and Indacaterol

As Indacaterol induced a G-protein switch within 24 h of NK cell treatment, we wanted to know which differences between the kinetics of Indacaterol and epinephrine exist. Epineph-

rine is described to be metabolized within minutes after release whereas Indacaterol has a half-life of 40 h [157, 179].

Therefore, we treated NK cells only for two minutes with a β2AR agonist and subsequently washed the NK cells. After the two-minute treatment, we employed a LC-AA with a second β2AR agonist treatment to examine how LFA-1 activity was affected (Figure 25A).

Interestingly, NK cells treated two minutes with epinephrine and with medium in the LC-AA, fully recovered and behaved like untreated NK cells. Medium treated control cells and epinephrine-medium treated NK cells showed similar LFA-1 activity whereas NK cells treated acutely with epinephrine and NK cells which were treated twice with epinephrine showed inhibited LFA-1 activity (Figure 25A, left panel).

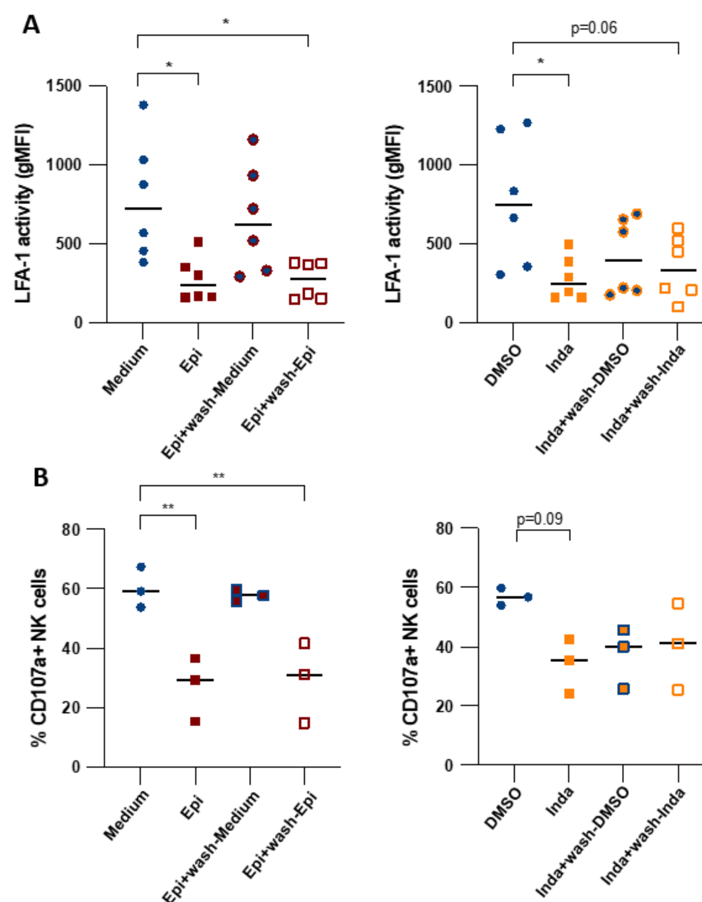

Figure 25: Epinephrine acts transiently at the β2AR.
(A) NK cells were pretreated for 2 min with Epi (left) or Inda (right), washed and repeatedly treated with β2AR agonists or Medium/DMSO. The LFA-1 activity of NK cells was analyzed by LC-AA (median, n=6). In (**B**) NK cells were pretreated like in (A) and stimulated by plate-bound anti-CD16 antibodies (3 h). The CD107a+ frequency (degranulation) was analyzed by flow cytometry (median, n=3). Statistical analysis was performed using ordinary one-way ANOVA (**, P<0.01; *, P<0.05).

In contrast, Indacaterol treated NK cells did not recover. NK cells were inhibited to almost the same level as acutely Indacaterol treated NK cells, independent of the second treatment in the LC-AA (Figure 25A, right panel). The same effects were visible when NK cells were ana-

lyzed for degranulation (Figure 25B). Epinephrine inhibition was very transient and lasted only as long as epinephrine was abundant.

In contrast, Indacaterol inhibition remained, even when NK cells were washed. The inhibition also remained even in a 3 h incubation assay, like the degranulation assay. Indacaterol is a long-acting β2AR agonist (LABA) with a lipophilic structural component, the amino substituent. Consequently, the β2AR agonist is believed to dissociate more slowly from the membrane, remaining in close proximity to the β2AR and continuously stimulating the receptor [231, 232]. Therefore, we analyzed how long NK cells were affected by the long-acting β2AR agonist. NK cells were treated with epinephrine or Indacaterol for 0.5 h, then washed and incubated for 3 days. At the third day, NK cells were treated a second time with a β2AR agonist and LFA-1 activity was determined (Figure 26A). NK cells treated first with epinephrine showed a similar inhibition between the acute and the second β2AR treatment. In contrast, NK cells, treated first with Indacaterol, showed no inhibition independent of the choice of the second β2AR agonist treatment. These results demonstrate that Indacaterol desensitized NK cells and this effect lasts for more than three days *in vitro*.

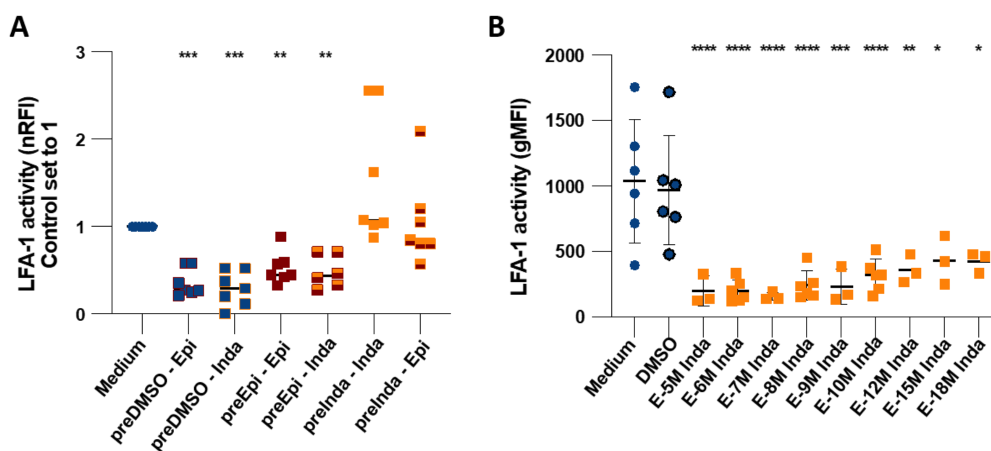

Figure 26: Indacaterol effects are persisting.
(A) NK cells pretreated with DMSO (blue), epinephrine (red) or Indacaterol (yellow), washed and incubated in media for 3 days. After incubation, cells were treated a second time with Medium/DMSO or β_2-AR agonists. The LFA-1 activity of NK cells was analyzed by LC-AA (median, n=8). In (**B**), Indacaterol was titrated and LFA-1 activity was measured by LC-AA. (n=3-6). Statistical analysis was performed using ordinary one-way ANOVA (****, P<0.0001; ***, P<0.001; **, P<0.01; *, P<0.05).

Additionally, NK cells are very sensitive to Indacaterol. A titration of the β2AR agonist showed that even at very low concentrations like 10^{-12} M to 10^{-18} M, Indacaterol was able to significantly inhibit NK cell LFA-1 activity (Figure 26B).

Therefore, NK cell functions like degranulation and adhesion can be long-term affected in very low concentrations of long-acting β2AR agonist even when only treated for a single time.

4.4 Peripheral NK cells from asthma patients are not desensitized

As Indacaterol demonstrated a persistent stimulatory effect on NK cells, we sought to investigate whether long-acting β2AR agonist treatment led to NK cell desensitization *in vivo*. In lung diseases such as asthma or COPD, patients use inhalers with long-acting β2AR agonists (LABA) as daily therapeutic to relax airway smooth muscles [185].

To examine the effect of LABA *ex vivo*, we took blood samples from 10 asthma patients and 10 healthy control subjects to identify if these cells show desensitized functions.

Table 10: Characteristics of asthma patient and healthy subject study cohort.

A

	Healthy	Asthma
Sex	m: 4 W: 6	m: 2 W: 8
Age (years)	54 ± 18	54 ± 15
LABA SABA	0/10 0/10	10/10 10/10
Biologics	0/10	7/10

B

Asthma Patient ID	1	2	3	4	5	6	7	8	9	10
Duration (years)	>10	1-5	>10	>10	N.A.	N.A.	N.A.	>10	N.A.	5-10
FEV_1 (%)	69,2	34	70	35	76	33,1	39,1	88,4	81	62

The study cohorts predominantly consisted of women with an average age of 54 years, as indicated in Table 10A. All asthma patients in the study were prescribed both long-acting β2AR agonist and short-acting β2AR agonists for use as needed. The prescribed LABA therapeutics were Salmeterol (3 patients) or Formoterol (7 patients). It's worth noting that Salmeterol and Formoterol are both long-acting β2AR agonists, much like Indacaterol. Like most LABAs, these agonists feature lipophilic structures as amino substituents, and their effects are known to last for more than 12 hours [233, 234].

Moreover, 70% of the patients in the study were using biologics directed against IL-4, IL-5, or IgE, as outlined in Table 10A. While the exact durations of the diseases were not available for all cases, it is worth mentioning that all patients had been under treatment for several years, with their conditions falling within the moderate to severe stages, as shown in Table 10B.

The forced expiratory volume exhaled in the first second (FEV_1%) is a physiological measurement, typically assessed through spirometry, that evaluates an individual's ability to inhale and exhale air over time. The examination of FEV_1% is a crucial component of the asthma diagnosis process and provides insights into the progression of lung conditions and asthma. In general, a mild asthma classification corresponds to a FEV_1% score equal to or greater than 80%, while lower scores are associated with moderate (60-80%) or severe (below 60%) classifications [235, 236].

The blood samples from asthma patients were provided by our collaboration partner at the Ruhrlandklinik Essen. All samples were drawn during routine visits and analyzed within the same day. In the first step of analysis, my colleague Dr Maren Claus evaluated the NK cell frequency and expression levels of activating NK cell receptors. Patients and healthy controls showed similar results (data not shown).

Next, we examined the IFNγ secretion of the PBMCs and degranulation of NK cells. Therefore, the cells were stimulated through plate-bound antibodies targeting either CD16 or NKG2D and 2B4 or NKp30 and treated with epinephrine and with or without Propranolol. In both functional analyses, NK cells of healthy controls and asthma patients behaved similar (Figure 27A, B, supplement 2). In all cases, the addition of epinephrine led to an inhibition of the NK cells.

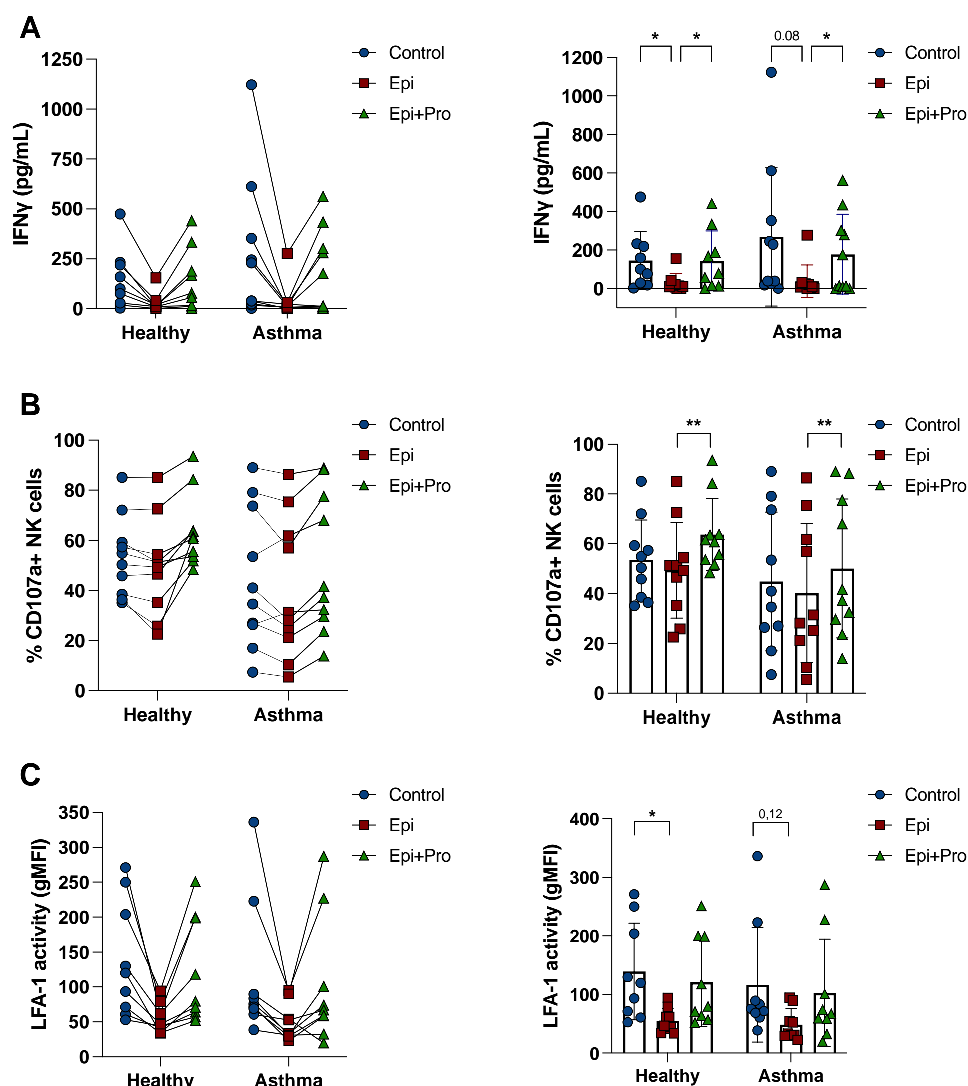

Figure 27:Peripheral NK cells from Asthma patients do still react to epinephrine.
PBMCs of cohorts were isolated and further analyzed by functional assays. (**A**) PBMCs were treated with epinephrine and stimulated by plate-bound CD16 antibody (6h). IFNγ secretion was measured by ELISA (n=10). Results are shown for single donors (left panel) and statistically analyzed (right panel). (**B**) Like in (A), PBMCs were treated with epinephrine and stimulated via CD16 (3h). Degranulation frequency (CD107a+) of NK cells was analyzed by flow cytometry (n=10). (**C**) The LFA-1 activity of NK cells was determined by LC-AA. PBMCs were treated with epinephrine(1µM) and stimulated by crosslinking NKG2D/2B4 antibodies (n=9). Results were statistically analyzed with two-way ANOVA test (**, P<0.01, *, P<0.05)

A similar result was obtained in a LC-AA as NK cells were inhibited independent of LABA treatments (Figure 27C). This suggests that peripheral NK cells from asthma patients using long-acting β2AR agonists as daily therapy did not exhibit desensitized β2AR responses. The NK cells retained their ability to respond to epinephrine.

Interestingly, even though LABA treatment had no effect on NK cell functions, the overall NK cell performance of asthma patients correlated with their FEV_1 (%) value. To determine the NK cell performance, the results of the untreated asthma NK cells were compared and ranked for the LC-AA, IFNγ ELISA and degranulation assay. The ranking positions of the NK cells for each assay were then summed to obtain an overall NK cell performance score for each asthma patient. The overall performance score can be interpreted as a measure of NK cell fitness within the asthma patient group. A high score indicated low NK cell performance (as these NK cells ranked last), and a small overall NK cell performance score indicated good NK cell fitness (as these NK cells ranked first). It's important to note that the data set included only 8 patients due to one incomplete patient data set and one outlier. The correlation was not significant when the outlier was included (see Supplement 3). However, the FEV_1 (%) from the remaining 8 asthma patients correlated significantly with the overall NK cell performance score (R^2=0.75), indicating a potential connection between NK cell fitness and asthma severity (Figure 28).

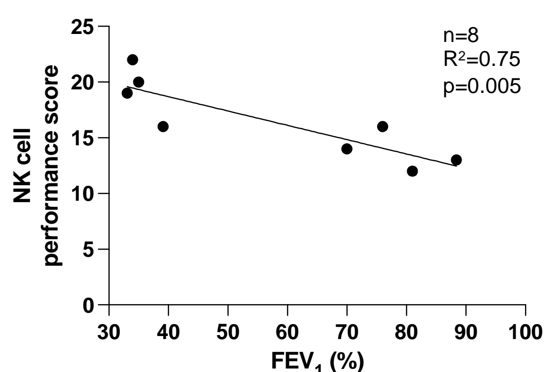

Figure 28: NK cell performance of asthma patients correlates with FEV_1 (%).
NK cell performances of functional analyses were ranked and sum up as NK cell performance score (low value indicates a good NK cell performance). The NK cell performance of 8 patients were correlated to their FEV_1 (%) value. Statistical analysis was performed using linear regression parameters R square and significance p calculations.

Taken together, NK cells from asthma patients retained their ability to respond to epinephrine. LABA treatments did not desensitize peripheral NK cells, even though the fitness of NK cells from asthma patients correlated with the severity of the disease, suggesting a possible link between NK cell fitness and the disease. However, this correlation should be interpreted with caution and verified with a larger cohort analysis.

To summarize all results:

Acute β2AR stimulation inhibited NK cell functions, including IFNγ secretion, degranulation, adhesion, and cytotoxicity. Additionally, β2AR agonists influenced the metabolic profile upon NK cell activation, leading to a prolonged increase in Seahorse ECAR values.

In contrast, chronic β2AR stimulation canceled NK cell inhibition. After five days of β2AR treatment, NK cells were no longer responsive to a β2AR agonist. Chronic β2AR stimulation

led to the phosphorylation of the receptor through the PKA feedback loop, initiating a G-protein switch and desensitization of the receptor. The β2AR agonists Indacaterol and epinephrine displayed different properties. Epinephrine inhibited NK cells only transiently as long as the β2AR agonist was abundant, whereas Indacaterol persisted at the NK cell membrane and continuously stimulated the receptor. For this reason, a single LABA treatment was sufficient to induce NK cell desensitization. Importantly, peripheral NK cells from patients using LABA as daily therapy could still react to epinephrine and did not show inhibited or desensitized NK cell function.

5 Discussion

Epinephrine is an important mediator of stress, affecting various cells and organs in our body. The aim of this thesis was to evaluate the influence of epinephrine exposure and β2 adrenergic receptor stimulation on NK cells. The analyses should help to understand if and how NK cells could possibly be affected during stress responses and repeated β2AR treatments.

NK cells have been described to be sensitive to epinephrine due to β2AR interactions [153]. Unfortunately, we were not able to consistently detect the receptor expression by western blot or flow cytometry. Besides technical problems like stability, buffer solution or dilution factors as possible pitfalls, the accessibility of the epitopes is an important factor which could have influenced the expression detection. However, we could functionally demonstrate the specific epinephrine-β2AR interaction through a beta receptor blockade by Propranolol and *ADRB2* knockout. In both cases, the epinephrine effect was canceled in adhesion assays which confirmed the epinephrine-β2AR specificity.

Acute epinephrine treatment inhibited NK cells. We were able to show that epinephrine strongly reduced LFA-1 activity. Importantly, as LFA-1 activity is induced by an activating inside-out signal, the research group of Prof. Carsten Watzl investigated where the activating signal was disrupted. They demonstrated through western blot analysis that epinephrine blocks the activating signal by reducing the phosphorylation right at the activating receptor itself (unpublished). In addition, we were able to show that epinephrine inhibition sets in very quickly. We analyzed the detachment of preactivated, cultured NK cells by real-time cell analysis. The experiment revealed that the immune cell detached within minutes from the ICAM-1 ligand. Our results are in line with previous studies which showed that the release of epinephrine upon stress is responsible for NK cell mobilization [34, 237, 238]. *In vivo*, acute stress or physical exercise mobilizes leukocytes in the bloodstream within minutes [239]. We could confirm that the catecholamine suppresses the integrin LFA-1 and is potentially the reason that NK cells are flushed out of the marginal pool in the circulation.

Besides LFA-1 activity, epinephrine inhibited IFNγ secretion. The secretion is an important marker for NK cell activity. The inhibition of IFNγ secretion was independent of the signaling pathway. Epinephrine blocked the cytokine and target cell contact-mediated NK cell activation. The cytokines IL-12 and IL-18 are well known to stimulate IFNγ secretion [240, 241]. During inflammation these cytokines are produced by antigen presenting cells and are important in the early control of infections. To the same extent as cytokine treated NK cells, activation with target cells was reduced. K562 target cells are described to powerfully stimulate NK cells by high expression of ligands for NKG2D and NCR [242, 243]. These results imply a global inhibition of activating signals by epinephrine and demonstrate the strength of the inhibitor. Furthermore, the stimulation of the β2AR by Indacaterol resulted in reduced NK cell cytotoxicity. Upon β2AR stimulation, the specific lysis of adherent and suspension target cells was decreased. The live cell microscopy analysis revealed that especially NK cell serial

killing was reduced. An important factor for NK cell killing is the building of a stable immunological synapse (IS). During this process the integrin LFA-1 accumulates at the contact interface and drives IS formation [244, 245]. Blockade of the LFA-1 activating inside-out signal by β2AR stimulation could potentially reduce NK cell effectiveness in IS formation, ultimately reducing the killing capacity.

The immunosuppressive effect confirmed previous *in vitro* studies which showed an inhibitory effect of epinephrine on NK cell activity and cytotoxicity [162-164]. We could not observe an activity stimulating effect of epinephrine at lower doses as described by Hellstrand [167]. The titration of epinephrine revealed that all tested concentrations, ranging from 10^{-9} M (1 nM) to 10^{-5} M (10 μM), reduced NK cell activity. The physiological plasma concentration of epinephrine in extreme acute stress situations has been described to reach up to 196 nM (35.9 ng/ml) [222]. Therefore, our results indicate that acute stress leads to an inhibition of NK cells under physiological acute stress conditions. Interestingly, *in vivo* studies showed the opposite. These studies observed an increase in NK cell cytotoxicity [157, 158, 223]. Segerstrom and Miller concluded in a meta-analytic study that most increased activity measurements *in vivo* were based rather on the increased NK cell numbers than on the cytotoxic strength of the single cell [156].

Mechanistically, the β2AR is a G protein-coupled receptor and its pathways have been widely studied in immune cells. In principle, the stimulation can act via a canonical or a non-canonical G protein independent pathway. We could show that the blockade of PKA could significantly rescue LFA-1 activity and only a minor effect was visible when EPAC was inhibited. This result demonstrates that the acute β2AR stimulation primarily initiates the canonical cAMP-PKA signaling pathway. PKA has been shown to be involved in many NK cell processes. In β2AR stimulation, it serves as a critical regulator of NK cell function, contributing to the fine-tuning of stress response.

To promote complete NK cell activity, the energy supply is a crucial parameter. Therefore, we analyzed the effect of epinephrine on the NK cell metabolism. Two major energy pathways exist for generating ATP: OxPhos and glycolysis. These parameters were analyzed using Seahorse technology. At steady state, epinephrine did not alter either of these pathways. This changed when NK cells were further activated through CD16.

Generally, NK cell activation without epinephrine pretreatment resulted in a transient increase in OCR values. Following this brief rise, the OCR values decreased below the initial baseline. Pretreatment with β2AR blocked the elevation of OCR levels, albeit surprisingly, it only marginally mitigated the decline below baseline. Flow cytometric analyses of CD16 receptor expression on NK cells revealed a significant reduction post-activation. This finding aligns with existing literature, which describes NK cells shedding the receptor upon activation [246]. Further analyses discovered that the CD16 activation decreased the size and depolarization of the mitochondria in CD16-activated NK cells. The β2AR pretreatment did not show an effect on these mitochondrial parameters. Additionally, cell viability decreased upon CD16 activation.

Interestingly, β2AR pretreatment showed no observable reduction in viability. Activated human NK cells have been documented to undergo rapid apoptotic cell death following Fc receptor stimulation. This mechanism is believed to play a crucial role in regulating inflammatory NK cells [247]. Consequently, the activation of the apoptotic pathway reduced mitochondrial capacity, leading to a decrease in OCR values upon CD16 activation. The sustained viability of NK cells pretreated with a β2AR agonist likely accounts for the slight increase in terminal OCR values. Given that β2AR treatment has been demonstrated to globally inhibit NK cell activation, the variation in NK cell viability could potentially be attributed to a reduced CD16 signal in NK cells.

In addition to the altered OCR values, ECAR values were also affected by β2AR pretreatment. Upon activation by CD16, NK cells exhibited elevated glycolysis, as indicated by elevated ECAR levels, throughout the remainder of the assay, whereas NK cells without pretreatment returned closer to baseline values. A similar effect was observed when NK cells were activated by the cytokine mix IL-12, IL-15, and IL-18. ECAR values increased and remained significantly higher than those of NK cells without β2AR treatment.

The ECAR measurement relies on pH changes resulting from the secretion of lactic acid into the media. This secretion lowers the pH, which should correlate with an increase in glycolytic ATP production. The cAMP-PKA pathway has been described as modulating glycolysis and glucose transport [227]. We were unable to block the prolonged increase in ECAR values with PKA inhibition. Additionally, the evaluation of glucose transporters GLUT-1, GLUT-2, and GLUT-3 did not reveal any specific upregulation initiated by epinephrine. Hence, it is plausible that epinephrine affects the metabolic pathway independent of PKA. However, it's important to note that the PKA inhibitor itself influenced NK cell metabolism during measurements, complicating the interpretation of the Seahorse results. Furthermore, we were unable to functionally demonstrate whether the increase in glycolysis is accompanied by heightened activation at later time points following epinephrine stimulation. Additionally, we cannot exclude the possibility that other acidic substances besides lactic acid may have influenced ECAR levels. Further investigation into proton channels or the secretion of amino acids could potentially shed light on their impact on pH measurements.

Besides repeated acute stress, chronic stress is known to negatively impact our health and cancer progression [14, 25, 82, 229, 248-251]. Chronic stress has been shown to alter the immune response and suppress immunoprotective responses [15, 84, 252]. However, the mechanism how chronic stress increases disease susceptibility remains unclear.

We simulated the condition by stimulating NK cells with β2AR agonists for five consecutive days. Most studies on NK cells show a reduced activation and cytotoxicity upon sustained stimulation. In animal models, chronic epinephrine led to progression of leukemia or to an increased risk to MCMV infection [74, 253]. In humans, a reduced responsiveness to cytokines in chronically stressed persons resulted in reduced cytotoxicity[254].

Our NK cell analyses yielded results contrary to those described in the literature. NK cells treated five times with epinephrine or Indacaterol did not exhibit significant reductions in their activity. In a chronic setting, β2AR stimulation completely lost its inhibitory effect. Both chronically β2AR-treated NK cells and untreated NK cells displayed similar levels of LFA-1 activity and IFNγ secretion. To determine whether the repealed inhibitory effect is independent of the activating receptor, we activated NK cells via different receptor pathways (CD16, NKG2D/2B4, or NKp30) and measured degranulation. The results demonstrated that the inhibitory effect of β2AR stimulation was globally negated. The results were confirmed by cytotoxicity and metabolic analyses. In all cases, NK cells did not respond to the β2AR agonist treatment after the fifth addition. Instead, the inhibitory β2AR treatment effect was repealed, and NK cells behaved similar to untreated cells.

A common reaction of chronically treated GPCRs is the internalization of the receptor. The β2AR is described to internalize upon high concentrations of epinephrine or sustained activation [140, 147, 255]. In contrast to the literature, the Watzl research group verified that NK cells, whether untreated or chronically treated with a β2AR agonist, expressed the same amount of β2 adrenergic receptors (unpublished). Therefore, chronic β2AR stimulation did not result in receptor internalization. Additionally, they analyzed the gene transcription levels of chronically treated NK cells to elucidate whether specific genes were involved. The transcriptome analysis did not reveal differentiated levels (unpublished). Similarly, the proteome analysis did not identify any potential candidate which could be involved in the altered chronic β2AR response.

Another mechanism to deactivate the β2AR response is receptor desensitization [255]. This process does not require receptor internalization but rather modifies the functioning of the β2AR. We observed a decrease in the concentration of the downstream molecule cAMP in chronically treated NK cells compared to control and acutely β2AR-stimulated NK cells. The decline in cAMP concentration led us to hypothesize that chronic β2AR stimulation resulted in a G-protein switch. It has been described that following chronic receptor stimulation, intracellular cAMP increases, activates PKA and can lead to phosphorylation of the β2AR itself [149]. This phosphorylation uncouples the Gs protein and increases the affinity to Gi protein [150]. The increased cAMP concentration after the Gαi protein inhibitor PTX treatment further strengthened the hypothesis.

In addition to the G protein switch, an alternative desensitization pathway exists. Sustained β2AR stimulation has been shown to trigger the non-canonical pathway as well. The G protein independent pathway activates β-arrestin, resulting in desensitization and internalization of the receptor [146]. The majority of research demonstrating the activation of non-canonical signaling pathways by adrenergic receptors has been conducted using *in vitro* techniques with diverse cell lines. These studies have primarily concentrated on β2ARs in non-immune cells. Therefore, it remains unclear to what degree the activation of non-canonical signaling pathways through β2-ARs are stimulated in immune cells [138].

We were able to demonstrate that chronic β2AR stimulation induces a PKA-mediated G-protein switch in NK cells. To block specific desensitization pathways, we utilized a PKA inhibitor and a β-arrestin-adaptin interaction inhibitor. Treatment with the PKA inhibitor resulted in NK cells that remained sensitive to β2AR stimulation. Consequently, the G protein switch was blocked, and upon a second β2AR stimulation, LFA-1 activity was inhibited instead of experiencing the repealed β2AR stimulation effect. However, inhibition of β-arrestin showed no effect in this context. The result confirmed our hypothesis that chronic β2AR stimulation led to a G protein switch induced by a PKA feedback loop.

We further analyzed the desensitization kinetics by utilizing the long-acting property of the LABA Indacaterol. Kinetic analysis showed that the β2AR agonist continuously stimulated the β2AR, leading to receptor desensitization within 24 h. This kinetic analysis functionally supported that LABAs, as lipophilic compounds, can persist at the membrane and repeatedly stimulate the receptor [179].

The actual purpose of LABAs is to support people with lung diseases like asthma or COPD. These patients use inhalers with the β2AR agonists as daily therapeutic to relax airway smooth muscles [185]. We could show that NK cells are very sensitive to even very low concentrations (10^{-18}M) of the LABA Indacaterol. Interestingly, the vast majority of NK cells in the lung are cytotoxic, mature $CD56^{dim}$, $CD16^{+}$, $CD57^{+}$ and $NKG2A^{-}$ NK cells suggesting that the lung is mainly populated with circulating NK cells [188]. These cells dynamically migrate from the blood and locate in the lung parenchyma during homeostasis [187]. Additionally, pathologic conditions like asthma are linked to an imbalance in β2AR desensitization [140].

The analysis of peripheral NK cells from asthma patients revealed that daily LABA treatment did not affect the β2AR response *ex vivo*. We functionally analyzed if peripheral NK cells could still react to epinephrine. In all analyzed cases, NK cells from healthy subjects and asthma patients behaved similar. Epinephrine was able to inhibit the LFA-1 activity, the degranulation independent of the activation receptor and reduced IFNγ secretion. The results were comparable to acute β2AR stimulated NK cells *in vitro*. Therefore, the result of chronically stimulated NK cells could not be translated to an *ex vivo* setting. One possible explanation for the contrary result could lie in the source of NK cells and the distinct LABAs utilized by the asthma patients in comparison to those in the *in vitro* experiments. The asthma cohort used Formoterol and Salmeterol which are administered twice daily with a duration of 12 h. In contrast, Indacaterol is an ultra-long-acting beta2 agonist (ULABA) with a longer duration of action lasting 24 h [232]. Despite some differences, all three LABAs are high-efficacy β2AR ligands with Indacaterol and Formoterol exhibiting a higher intrinsic efficacy than salmeterol [256]. These drugs possess an acceptable safety profile and demonstrate similar systemic side effects [257]. Moreover, they are only detectable in very low plasma concentrations after the inhalation of therapeutic doses [258]. In the blood, Formoterol is to 61-64% bound to plasma proteins [259]. Consequently, the systemic effect of these LABAs may be too low to affect peripheral NK cells. However, it's important to note that we cannot exclude

the possibility of β2AR desensitization in lung NK cells, considering their higher exposure to β2AR treatment. Scientifically regrettable, obtaining lung NK cell samples is challenging.

Although, the role of NK cells in asthma is still inconclusive [260], they can produce Th2 type cytokines and induce eosinophil migration which could contribute to immunopathogenesis of asthma [261]. A loss of β2AR responsiveness could inhibit a possible NK cell deactivation and promote eosinophilic inflammation. Additionally, the airway is a main pathogenic entry site which is controlled by NK cells defense [192]. Sustained Gi-β2AR activation could potentially dysregulate NK cell mobilization leading to uncontrolled battlefields.

Despite their efficacy, LABAs are not recommended as standalone therapies for asthma. Monotherapy with LABAs has been associated with an increased risk of life-threatening exacerbations and asthma-related deaths [262]. The desensitization of the β2AR on airway smooth muscle cells resulted in the loss of drug effectiveness and induced tolerance to the rescue short acting β2AR agonist treatments which led to decreased asthma control and hospitalization [182, 185, 262, 263]. To address this concern, the FDA issued black box warnings, recommending the prescription of LABAs only in combination with inhaled corticosteroids (ICS) as part of a comprehensive asthma management strategy [262]. The combination therapy aims to enhance efficacy through molecular interactions. The β2AR stimulation facilitates glucocorticoid receptor translocation to the nucleus and in turn increases the number of β2AR due to increased transcription [264, 265]. Thereby, downregulation of β2AR due to long-term β2AR agonist usage is counteracted by glucocorticoids in lymphocytes [141, 266].

In NK cells, we could not observe a downregulation of the β2AR. Instead, a single Indacaterol treatment induced a G-protein switch within 24 h. In line with our results, also *in vivo* desensitization and tolerance to this β2AR agonist is reached within the first days of therapy [183].

The analyzed patient's cohort had been under asthma treatment for several years, with their conditions falling within the moderate to severe stages. Interestingly, the overall NK cell performance of asthma patients significantly correlated with their FEV_1 (%) value. The NK cell performance can be interpreted as a kind of NK cell fitness demonstrating that a low NK cell fitness correlates with asthma severity. This data confirms previous findings that could show a negative correlation between activated $CD69^+$ NK cells in peripheral blood and FEV1% scores in asthmatic patients [260]. Determining whether the correlation between NK cell activity and asthma severity corresponds to higher susceptibility requires further investigation. Asthma patients undergo immunosuppressive treatment, which could also contribute to the correlation observed. However, the low number of analyzed NK cells necessitates further expansion for a more comprehensive interpretation. Therefore, additional studies with larger sample sizes are warranted to elucidate the relationship between NK cell activity and asthma severity accurately.

In contrast to Indacaterol, epinephrine was not able to induce a G-protein switch with a single treatment. Also *in vivo*, epinephrine is described to be metabolized within minutes after release whereas Indacaterol has a half-life of approximately 40 h [157, 179]. Benshop et al. ana-

lyzed the increase of NK cells after the release of epinephrine. They could show that NK cell-endothelial cell interactions were only reduced for the duration of β2AR stimulation [267]. We confirmed that the inhibitory effect of epinephrine is very transient and NK cells repetitively react to epinephrine in short intervals. This transient inhibitory effect of epinephrine could explain the contrary *ex vivo* results in previous studies, which described the catecholamine as enhancer of NK cell activity [157, 158, 223]. To accurately analyze NK cell activity, cells need to be isolated from the blood and its containing substances. Therefore, exposure to epinephrine and its inhibitory effect could have been missed in these studies.

Importantly, the short inhibitory duration of epinephrine in the circulation indicates that the primary role of the hormone is not to inhibit effector functions, but rather to recruit cytotoxic cells for immunological defense. This hypothesis is further supported by the epinephrine-induced upregulation of LFA-1 expression on lymphocytes [268]. LFA-1 is essential for immune cell recruitment to the target site. The rapid release of epinephrine during acute stress flushes NK cells into the circulation and primes them for migration to local infections. Therefore, the rapid SAM response induces immune activation and provides immediate protection against threats. This effect holds great potential and is utilized in medicine. Exercise-mediated tumor immunotherapy is considered part of cancer therapy strategy. In addition to the general benefits of physical activity, Pederson and colleagues demonstrated that running suppressed tumor growth through epinephrine and IL-6-dependent NK cell mobilization in mouse models [269]. Similarly, in humans, exercise training has been reported to increase NK cell cytotoxicity in breast cancer and stomach cancer patients [270-272].

On the other hand, prolonged psychological stress during cancer treatment and surgery can negatively influence the outcome [273]. In the transition from acute to chronic stress, repeated β2AR stimulation by epinephrine can inhibit NK cell cytotoxicity and potentially affect treatments and clinical outcome for patients. Additionally, acute stress in chronically stressed people induced the release of higher epinephrine levels and protracted decline in NK lysis [92]. The value of the blockade of epinephrine-mediated NK cell inhibition has also been evaluated by other studies. In animal models, ganglionic blocker and beta blocker were found to reduce tumor growth and metastasis due to the repealed NK cell suppression [97, 98]. Consequently, the use of beta blockers during and shortly after cancer surgery was evaluated in several trials resulting in a variable, but enhanced outcome for patients [99-102].

In contrast, our *in vitro* analysis of chronic β2AR stimulation resulted in desensitization of the receptor without a loss of NK cell effector functions. Indeed, the examination of the specific β2AR-epinehrine *in vitro* evaluates just a small component of the complex human stress response, which involves various substances and cells in addition to epinephrine. Consequently, further investigations are needed to elucidate if specific factors interfere with desensitization *in vivo*.

Another possible reason for the contrary result to the studies mentioned above could be the usage of a relative high epinephrine concentration. In stressed people, the peak of plasma epi-

nephrine concentration was detected in the low nanomolar range while we saw the most profound result at a concentration of one micromolar. Nevertheless, we observed the same trend along all concentrations down to the nanomolar range. Another factor could be the fast metabolization of epinephrine *in vivo*. A reduced concentration and duration of epinephrine exposure could potentially allow NK cells to recover *in vivo* whereas *in vitro* sustained activation led to a switch of the signaling. Therefore, chronic stress or repeated β2AR stimulation *in vivo* could result in inhibition of NK cells.

In cases when epinephrine exposure reaches desensitization levels, NK cells with a desensitized receptor could remain at the endothelium as LFA-1 activity is increased by Gi-coupled GPCRs [238]. Due to those reasons, repeated epinephrine exposure could either result in functionally active NK cells which remain at the endothelium or inhibited NK cells in the blood circulation. Both cases could potentially contribute to higher infection susceptibility in chronically stressed people.

In summary, this comprehensive examination of β2AR-NK cell interactions provides nuanced insights into the balance between acute immune activation and chronic desensitization. The study underscores the dynamic nature of these interactions, emphasizing their relevance for therapeutic interventions. Understanding the implications of acute and chronic β2AR stimulation on NK cell functions offers valuable perspectives for conditions involving chronic stress or prolonged β2 agonist usage, contributing to the broader comprehension of stress-related immune responses and potential therapeutic strategies.

6 References

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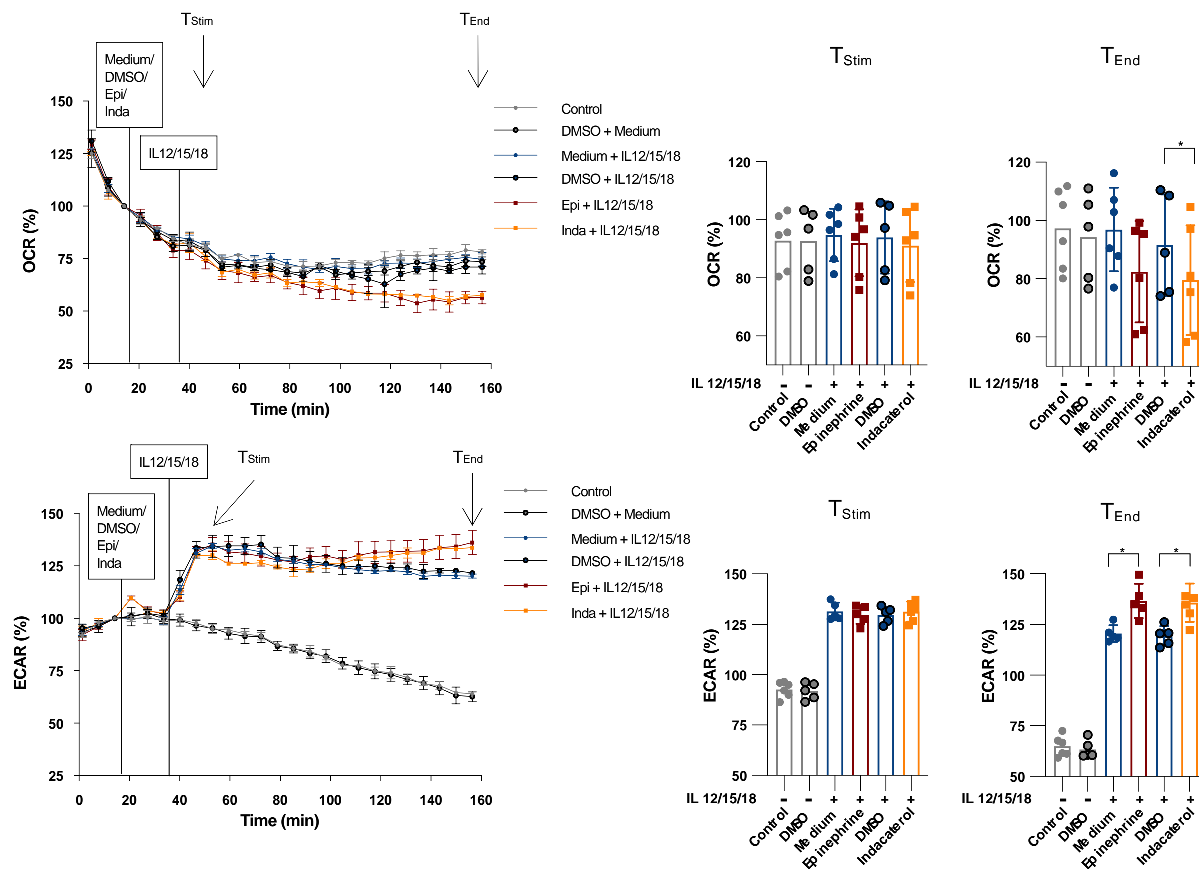
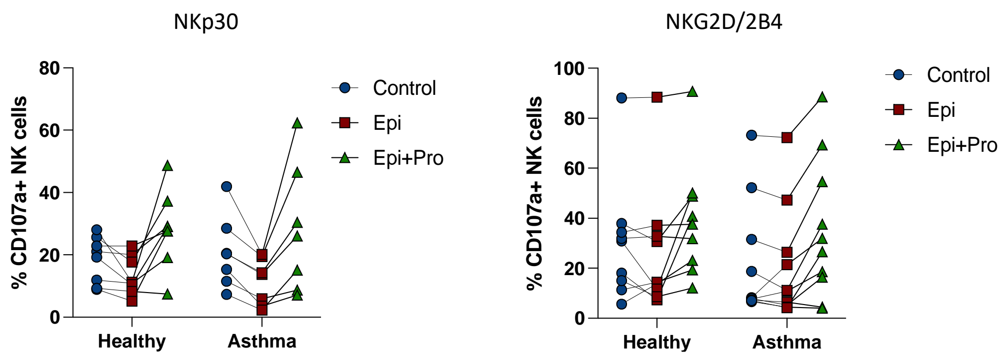
7 Supplement

7.1 Metabolic profile of cytokine stimulated NK cells



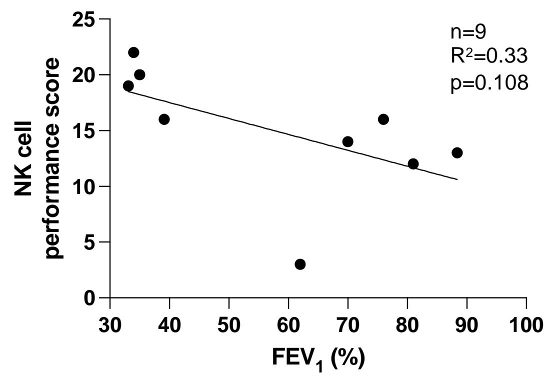
Supplement 1: β2AR stimulation increased ECAR values upon cytokine activation.
Representative curves of the metabolic profiles measured by Seahorse technology. Each condition was analyzed in triplicates. The OCR (top) and ECAR (bottom) measurements were normalized to the point of the β2AR agonist addition. NK cells were treated with Medium, DMSO as solvent control, epinephrine or Indacaterol (1µM) before activation via IL12/15/18. The timepoints after stimulation (T_{Stim}) and at the end (T_{End}) were quantified of 5 independent experiments (right panels). Statistical analysis was performed using ordinary one-way ANOVA (*, $P<0.05$).

7.2 Peripheral NK cells from asthma patients are not desensitized.



Supplement 2: Flow cytometry expression analysis of NK cell composition of Asthma patients and healthy subjects. Degranulation assay.

PBMCs of asthma patients or healthy controls were treated with epinephrine and stimulated by plate-bound NKp30 (left) or NKG2D/2B4 (right) antibodies (3h). Degranulation frequency (CD107a+) of NK cells was analyzed by flow cytometry (NKp30 n=6, NKG2D/2B4 n=9).



Supplement 3: Correlation of NK cell performance score against FEV_1 (%).

NK cell performances of functional analyses were ranked and summed up as NK cell performance score (low value indicates a good NK cell performance). The NK cell performance of 9 patients were correlated to their FEV_1 (%) value.

8 Abbreviations

β2AR	beta-2 adrenergic receptor
A549-H2Bj-GFP	A549 cells expressing histone H2B fused to green fluorescent protein
ACTH	Adrenocorticotropic hormones
ADCC	Antibody-dependent cell-mediated cytotoxicity
ANS	Autonomic nervous system
AP2	Adaptor protein 2 complex
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
cAMP	Cyclic adenosine monophosphate
CCCP	Carbonyl cyanide m-chlorophenyl hydrazine
CCL18	Chemokine (C-C motif) ligand 18
CD	Cluster of differentiation
CRH	Corticotrophin-releasing hormones
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
ECAR	Extracellular Acidification Rate
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPAC	Exchange factor directly activated by cAMP
Epi	Epinephrine
ERK	Extracellular signal-regulated kinases
ESI 09	EPAC Inhibitor
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum

FCεRIγ chain	Fc epsilon receptor I gamma chain
FDA	Food and Drug Administration
FEV1	Forced expiratory volume in 1 second
FSC	Forward scatter
GC	Glucocorticoid
GLUT	Glucose transporter
GPCR	G protein-coupled receptors
GPI	Glycosylphosphatidylinositol
GR	Glucocorticoid receptor
GRK	G protein-coupled receptor kinase
HCl	Hydrochloric acid
HPA	Hypothalamic-pituitry-adrenal axis
HRP	Avidin-horseradish peroxidase
IBMX	3-Isobutyl-1-methylxanthin (PDE inhibitor)
ICAM	Intercellular Adhesion Molecule
ICS	Inhaled corticosteroid
IfADo	Leibniz Research Centre for Working Environment and Human Factors
IFNγ	Interferon γ
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
ILC	Innate lymphoid cells
IMDM	Iscov's Modified Dulbecco's Medium
Inda	Indacaterol
IS	Immunolgical synapse
ITAM	Immunoreceptor tyrosine-based activation motif
JNK	c-Jun N-terminal kinases
KIR	Killer-cell immunoglobulin-like receptors

LABA	Long-acting β2-adrenergic agonists
LC	Locus coeruleus
LC-AA	Liquid chromatography amino acid analysis
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complex
MS/MS	Tandem Mass Spectrometry
n gMFI	normalized geometric mean fluorescence intensity
NE	Norepinephrine
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cells
OCR	Oxygen Consumption Rate
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PFA	Paraformaldehyde
PHA	Polyhydroxyalkanoates
PKA	Proetin kinse A
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl Fluoride
Pro	Propranolol
PTSD	Post-Traumatic Stress Disorder
PTX	Pertussis toxin
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute Medium
RTCA	Real-Time Cell Analysis
SABA	Short-acting β2-adrenergic agonists

SAM	Sympthatic adrenal medullary axis
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SNS	Sympathetic nervous system
Th1	Th1 helper cells
TMRM	Tetramethylrhodamine Methyl Ester
TNF-α	Tumor necrose factor
TRAIL	TNF-related apoptosis-inducing ligand
WHO	World health organization

Overview of aids

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