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Pilot *in vitro* study of dexamethasone-induced immunomodulation in murine splenic T cells

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Abstract

Glucocorticoids (GCs) are key hormones in stress response and chronic elevations from chronic stress or prolonged therapeutic use accelerates immune aging and dysfunction. To dissect their direct, time- and dose-dependent effects on T cells, we established an *in vitro* model in which murine splenocytes were cultured with graded concentrations of dexamethasone (a potent, synthetic GC) and analyzed by 22-color spectral flow cytometry over 24, 48, and 72 hours. Dexamethasone caused a progressive, dose-dependent decline in overall viability and absolute $CD4^+$/$CD8^+$ T cell counts, transiently increased proliferation (Ki-67) at 48 hours before inducing collapse and apoptosis, and modulated activation (CD69, CD25) and exhaustion (PD-1) markers. Memory and naïve T cell subsets were especially vulnerable, whereas low-dose dexamethasone selectively enriched regulatory ($CD4^+Foxp3^+$) and Th1 ($CD4^+Tbet^+$) subsets. Effector cytokine production (IFN-γ, TNF-α) was markedly suppressed in a dose- and time-dependent manner. This platform thus provides a robust tool for exploring GC-driven immunosenescence, GC resistance mechanisms, and strategies to restore T cell function after chronic GC exposure.

Keywords:

Glucocorticoids, dexamethasone, splenocytes, T cells, spectral flow cytometry

CERCS:

B500 Immunology, serology, transplantation

Institute name: Institute of Biomedicine and Translational Medicine

Research group: Molecular Pathology Research Group

Pilootuuring in vitro: deksametasooni põhjustatud immunomodulatsioon hiirte põrnas T-rakkudes

Kokkuvõte

Glükokortikoidid (GK-d) on stressivastuses võtmehormoonid ning nende nende pikaajaline kõrgenenud tase kroonilise stressi või pika ravi tõttu kiirendab immuunsüsteemi vananemist ja funktsioonihäireid. Selleks, et uurida GK-de ajast- ja doosistõltuvatd mõju T-rakkudele, lõime *in vitro* mudeli, kus hiirte põrnarakke kasvatati deksametasooni (tugev, sünteetiline GK) kasvavas kontsentratsioonis ning analüüsiti 22-värvilise spektraalse voolutsütomeetriaga 24, 48 ja 72 tunni järel. Deksametasoon põhjustas progressiivse, doosist sõltuva languse üldises elujõulisuses ja absoluutsetes $CD4^+$ ja $CD8^+$ T-rakkude arvudes, ajutise proliferatsioonitõusu (Ki-67) 48 tunnil enne aktiivsuse langust ja apoptoosi ning mõjutas aktiveerumise (CD69, CD25) ja väsimuse (PD-1) markereid. Mälu- ja naiivsed T-raku alamgrupid olid eriti vastuvõtlikud, samal ajal kui madal deksametasoonidoos rikastas selektiivselt regulatoorseid ($CD4^+Foxp3^+$) ja Th1 ($CD4^+Tbet^+$) alamgruppe. Efektortsütokiinide (IFN-γ, TNF-α) tootmine vähenes selgelt doosist ja ajast sõltuvalt. See platvorm pakub seega võimsat tööriista uurimaks GK-dest tingitud immuunsüsteemi vananemist ja GK-resistentsuse mehhanismening strateegiate testimiseks T-rakkude funktsiooni taastamiseks pärast kroonilist glükokortikoididega kokkupuudet.

Märksõnad:

Glükokortikoidid, deksametasoon, splenotsüüdid, T-rakud, spektraalne voolutsütomeetria

CERCS:

B500 Immunoloogia, seroloogia, transplantoloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

11β-HSD 11β-hydroxysteroid dehydrogenase

ACK Ammonium-Chloride-Potassium lysis buffer

ACTH adrenocorticotropin hormone

APC antigen-presenting cell

AVP arginine vasopressin

CBG corticosteroid-binding globulin

CD cluster of differentiation

cDC conventional dendritic cell

CM central memory

CMI cell mediated immunity

CNS central nervous system

CRH corticotropin-releasing hormone

CXCR CXC chemokine receptor

DC dendritic cell

Dex dexamethasone

DNA deoxyribonucleic acid

EM effector memory

FACS Fluorescent Activated Cell Sorting buffer

FMO Fluorescence Minus One

GABA γ-aminobutyric acid

GCs glucocorticoids

Gilz GC-induced leucine zipper

GR glucocorticoid receptor

HPA hypothalamic–pituitary–adrenal axis

IL Interleukin

IFN-γ Interferon Gamma

KLRG1 Killer Cell Lectin Like Receptor G1

nGRE negative glucocorticoid responsive elements

NK natural killer cell

NOD nucleotide oligomerization domain

MHC major histocompatibility complex

MKP MAPK phosphatase

MMM marginal metallophilic macrophage

MZ marginal zone

MZM marginal zone macrophage

PALS periarteriolar lymphoid sheath

PBS phosphate buffered saline

POMC Proopiomelanocortin

PVN hypothalamic paraventricular nucleus

RP red pulp

RPM red pulp macrophage

RT room temperature

SASP senescence-associated secretory phenotype

TCGF T cell growth factor

TCR T cell receptor

Th helper T cell

TNF-α tumor necrosis factor α

Treg regulatory T cell

WP white pulp

INTRODUCTION

The immune system plays a significant role in maintaining physiological homeostasis by protecting the host against infection, disease onset, and preserving self-tolerance (Abbas et al., 2022). This delicate balance can, however, be disrupted by chronic stress, which has well-known immunomodulatory effects on the immune system. Exposure to chronic stress triggers the hypothalamic-pituitary-adrenal (HPA) axis and leads to persistent elevation of circulating glucocorticoids (GCs) which are the primary endocrine mediators of stress (Bellavance & Rivest, 2014). GCs modulate immune function by altering leukocyte development, proliferation, cytokine production, and survival, often skewing immune responses toward a suppressed or dysregulated state (Seiler et al., 2020; Zhang et al., 2020).

One of the lymphoid sites affected by stress-induced immune modulation is the spleen, which is involved in the induction of immune responses to blood-borne pathogens and in the maintenance of immune homeostasis (Bronte & Pittet, 2013). Chronic stress has been shown to disrupt splenic architecture, decrease cellularity, and modify the distribution and function of key immune cell subsets, particularly T and B lymphocytes (Hernandez et al., 2013; Irwin & Cole, 2011; Wang et al., 2007). These changes can compromise systemic immune competence and increase susceptibility to infection and disease.

To investigate the immunomodulatory mechanisms of chronic stress, synthetic GCs such as dexamethasone (Dex) provide a useful tool with which to study stress-induced immune dysregulation both *in vivo* and *in vitro* (Giles et al., 2018; Skupio et al., 2015; Snyder-Mackler et al., 2019). Dex is a potent GC receptor agonist that mimics the physiological effects of endogenous GCs but with greater receptor affinity and metabolic stability. The immunosuppressive actions of Dex are therapeutically beneficial in certain inflammatory and autoimmune disorders (Jevnikar et al., 1992; Verhoef et al., 1999; Yao et al., 2022).

Chronic stress has been associated with accelerating aging (Epel & Lithgow, 2014; McEwen, 2012; Polsky et al., 2022). Aging is known to alter immune cell composition and function, a phenomenon referred to as immunosenescence (Pawelec, 2018). Since both chronic stress and aging independently affect immune homeostasis, understanding their combined influence is of growing importance, especially in the context of age-related diseases. With this in mind, the aim is to standardize an *in vitro* platform of GC exposure to further study GC-induced immunosenescence. In the current thesis, we conducted a pilot study to investigate how varying concentrations of Dex affect splenic T cell dynamics *in vitro* over time. We examined the cells at 24, 48, and 72 hours post treatment, specifically looking at markers of activation, growth,

exhaustion, effector functions, and survival. This would aid in understanding better how long-term exposure to GCs changes immune cell function and help identify potential differences in the patterns of stress-related immune modulation in various T cell subsets.

1 LITERATURE REVIEW

1.1 CHRONIC STRESS AND IMMUNE DYSREGULATION

Stress is defined as any internal or external stimulus that triggers a biological response (Yaribeygi et al., 2017). It disrupts the body's homodynamic balance, also referred to as homeostasis. This disruption occurs in response to a variety of intrinsic or extrinsic challenges or stimuli, which may be real or perceived and are collectively termed stressors (Agorastos & Chrousos, 2022). The effect of stress strongly depends on duration and intensity. Consequently, two types of stress are distinguished. Acute stress is referred to as stress that lasts minutes to hours, whereas chronic stress remains for several hours per day for weeks or months (Dhabhar & Mcewen, 1997). Acute stress enhances immune function by temporarily redistributing immune cells, leading to immune surveillance and readiness. In contrast, chronic stress has a suppressive effect on immunity (Dhabhar & Mcewen, 1997).

Chronic stress has severe deleterious consequences for human health, including reduced life expectancy (Härkänen et al., 2020; Pardon, 2007). Chronic stress exposures may lead to lasting alterations in emotional, physiological, and behavioral reactions, which can affect susceptibility to and the progression of diseases (Cohen et al., 1995; McEwen, 1998). One of the most severe consequences of chronic stress is its impact on cardiovascular health, significantly increasing the risk of coronary heart disease and atherosclerosis (Golbidi et al., 2015; Kivimäki & Steptoe, 2018; Lagraauw et al., 2015). Chronic stress in occupational or domestic settings correlates with approximately a twofold elevation in myocardial infarction risk (Rosengren et al., 2004), and elevated levels of perceived stress at a time of myocardial infarction increase the mortality risk within the subsequent two years by 40% (Arnold et al., 2012).

Chronic stress also affects brain function, particularly in the hippocampus, which is involved in verbal memory and contextual memory, which includes the time and place of events with strong emotional connections (Eichenbaum et al., 1992). In response to stress, the body releases steroid hormones GCs that, in turn, influence the immune function. These hormones can cause immune suppression or increased inflammation, resulting in greater susceptibility to infections, autoimmune diseases, and cancer (Alotiby, 2024).

1.1.1 Effect of chronic stress on immunity

Chronic stress alters immune parameters associated with cell-mediated immunity (CMI), such as leucocyte development (Domínguez-Gerpe & Rey-Méndez, 2001; Jiang et al., 2017),

antibody production (Dragoş & Tănăsescu, 2010; Silberman, 2003), natural killer (NK) cells activity (Capellino et al., 2020; Katz et al., 2025), virus specific T and NK cell responses (Bonneau et al., 1991), and macrophage activity (Brown & Zwilling, 1994).

Chronic stress modifies cytokine release patterns, which enhances and suppresses the immune response simultaneously (Marshall et al., 1998). Cytokines are small proteins secreted by white blood cells that regulate the balance between humoral and cell-mediated immune responses. $CD4^+$ T helper cells are the primary source of cytokine synthesis and regulation, and they are classified into two subtypes: helper T cells, type 1 (Th1) and type 2 (Th2) (Hanson et al., 2007). Th1 cells, which produce proinflammatory cytokines such as interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), IL-2, IL-6, IL-8, and IL-1β, stimulate CMI, phagocyte-dependent inflammation, and worsen disease symptoms. On the contrary, Th2 cells provoke robust humoral-mediated immunity by forming antibodies and the accumulating eosinophil through the release of anti-inflammatory cytokines such as IL-1ra, IL-4, IL-5, and IL-10 (Shinkai et al., 2002).

Prolonged release of GCs shifts the Th1-Th2 equilibrium towards Th2 (Assaf et al., 2017; Chiappelli et al., 1994; Segerstrom & Miller, 2004). The suppression of Th1 leads to weakening cellular immunity, and the active promotion of Th2 increases susceptibility to allergic and autoimmune disorders. The anti-inflammatory effect of GCs is widely used to treat inflammatory diseases such as rheumatoid arthritis (Verhoef et al., 1999). However, constant exposure to GCs induces a prolonged, non-resolving inflammation state in the central nervous system (CNS) by gradually developing reduced GC sensitivity or resistance, which further stimulates inflammation (Ramamoorthy & Cidlowski, 2013).

1.1.2 Effect of chronic stress on aging

With aging, the immune system undergoes a progressive decline known as immunosenescence, which modifies the structure of immune organs as well as alters the abundance and functionality of immune cells (Pawelec, 2018).

In parallel with this decline, older individuals develop a persistent and low-grade inflammatory condition called inflammaging (Ray & Yung, 2018; Y. Zhu et al., 2014). This chronic inflammation is closely linked to the accumulation of molecular byproducts from damaged or dying cells, including mitochondrial components, nucleic acids, and other proteins that act as endogenous danger signals for innate immune receptors such as toll-like receptors, NOD

(nucleotide oligomerization domain)-Like Receptors and cGMP-AMP synthase. Their activation results in a chronic release of pro-inflammatory cytokines (Biagi et al., 2010). Further aging immune cells – particularly T and B lymphocytes – can adopt a senescence-associated secretory phenotype (SASP), which can induce prolonged inflammation (Sanada et al., 2018).

Chronic stress influences immune cell aging by altering telomere length and telomerase activity (Lin & Epel, 2022). Blood lymphocytes and monocytes from women experiencing chronic stress exhibit significantly shorter telomeres than those from low-stress individuals (Epel et al., 2004). Telomerase activity, important for telomere length maintenance, is also reduced in high-stress women, indicating that stress impairs the cells' ability to regenerate shortened telomeres (Epel et al., 2004). The rate of telomerase shortening is associated with a predisposition to various diseases, including pancreatic cancer or dementia (Blackburn & Epel, 2012).

Additionally, immunosuppressive effects of chronic stress studied in the context of cancer development (Ben-Eliyahu et al., 2007) have shown that chronically stressed mice develop tumors faster, reach 50% to 100% tumor incidence sooner than controls, and have poor tumor regression (Saul et al., 2005). Chronic stress elevates tumorigenesis by inducing degradation or loss of p53 function, disrupting its roles in DNA repair, cell cycle arrest, aging, and apoptosis, which are related to tumor suppression and effective cancer treatment (Vazquez et al., 2008). Overall, chronic stress dysregulates the immune system by accelerating immunosenescence and disease progression.

1.2 ROLE OF SPLEEN IN CHRONIC STRESS

1.2.1 Structure and function of the spleen

The spleen is a secondary lymphoid organ located in the left upper quadrant of the abdomen (Abbas et al., 2022). In contrast to lymph nodes, which filter bacteria and cellular debris carried by lymph, the spleen directly examines antigens from the bloodstream (Noble et al., 2018). It is functionally and physically separated into two parts: the white pulp (WP), with a high concentration of lymphocytes, and the red pulp (RP), which primarily consists of vascular sinusoids filled with blood (Abbas et al., 2022; Chadburn, 2000; Mebius & Kraal, 2005). The zone between them is called the marginal zone (MZ) (**Figure 1**) (Mebius & Kraal, 2005). A fibrous capsule composed of connective tissue covers the organ, giving rise to trabeculae that provide structural support to the larger blood vessels (Chadburn, 2000; Mebius & Kraal, 2005).

Arterial blood enters MZ and flows into the RP, where RP macrophages (RPMs) remove aged erythrocytes, apoptotic cells, and pathogens (Haan et al., 2012; Kurotaki et al., 2015). The RP also houses immune effector cells such as neutrophils, monocytes, and γδ T cells, which respond to inflammatory stimuli (Borges Da Silva et al., 2015; Bronte & Pittet, 2013; Mebius & Kraal, 2005). The WP is a site of lymphocyte activation and antigen-specific immune responses. Its structure is maintained by chemokines that guide B and T cells into distinct zones (Nolte et al., 2003, 2004), enabling efficient surveillance of blood-borne antigens. The MZ acts as a key transition area between the blood and WP, containing specialized macrophages – marginal zone macrophages (MZMs) and marginal metallophilic macrophages (MMMs) – that phagocytose pathogens and apoptotic cells (Bronte & Pittet, 2013). It also contains conventional dendritic cells (cDC) and marginal zone B (MZB) cells, which contribute to rapid antigen recognition and humoral defense (Lewis et al., 2019; You et al., 2011).

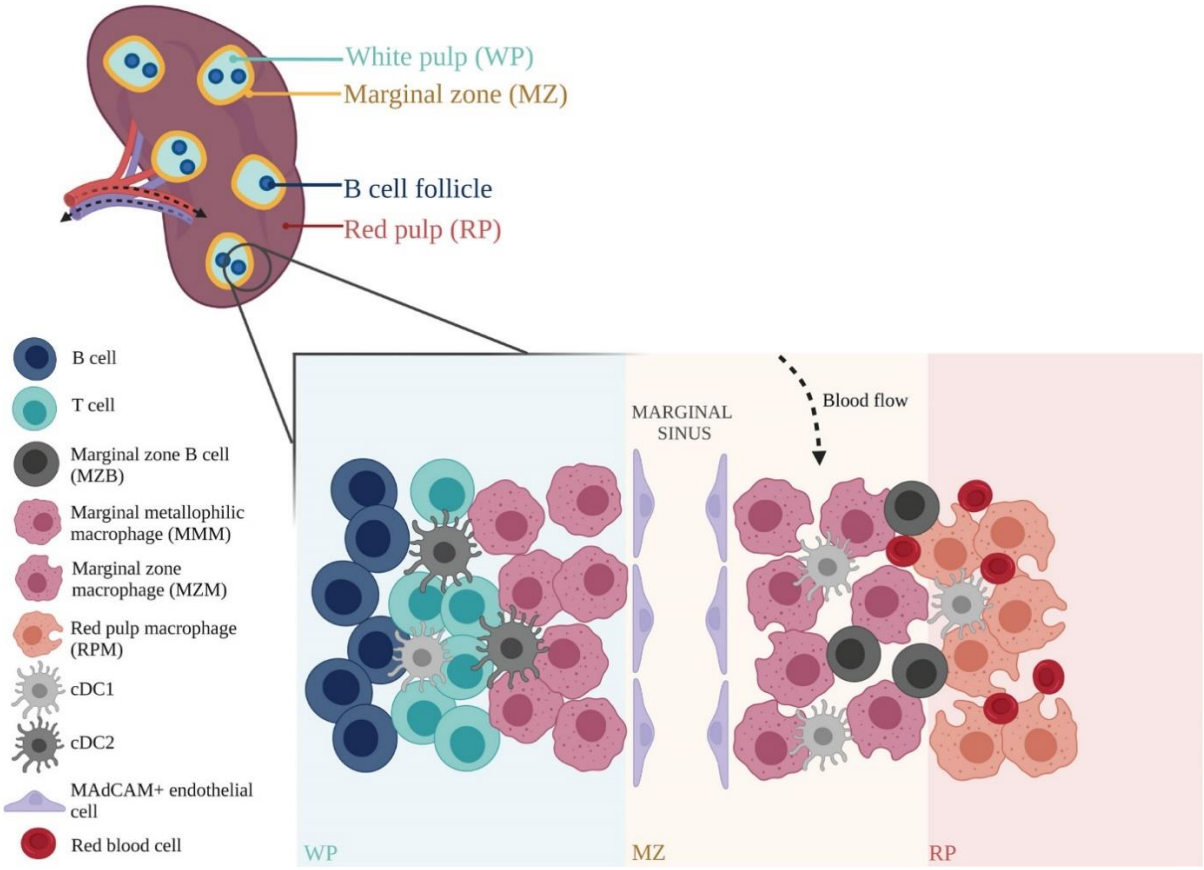


Figure 1. The mouse spleen anatomy. Arterial blood enters marginal zone (MZ) and moves through the red pulp (RP) cords, where red pulp macrophages (RPMs) scan for blood-borne antigens. Following this, the blood is gathered in sinuses and exits the spleen via the efferent vein, returning to systemic circulation. The white pulp (WP) is the site where adaptive immune reactions to systemic antigens are triggered, primarily involving T and B lymphocytes.

Circulating lymphocytes can exit the bloodstream and access the WP through the MZ (Zheng et al., 2022).

1.2.2 Splenocyte populations and their role in immunity

The spleen hosts various immune cells that coordinate both innate and adaptive responses. DCs and macrophages detect pathogens using pattern recognition receptors and initiate immune activation by producing cytokines and presenting antigens to T cells (Cerutti et al., 2013; Davies et al., 2013; Den Haan & Kraal, 2012; Nolte et al., 2003). NK cells reside mainly in the RP but migrate to the WP during infection to secrete IFN-γ and support T cell responses in the spleen (Bekiaris et al., 2008; Kang et al., 2008). Monocytes in the spleen act as precursors for inflammatory and regulatory myeloid cells (Geissmann et al., 2010). They can differentiate into effector cells that help clear infections or produce anti-inflammatory cytokines like IL-10 and TGF-β (Bronte & Pittet, 2013). The spleen also serves as a reservoir for monocytes that can be rapidly mobilized during inflammation.

B and T lymphocytes, the leading players of adaptive immunity, are strategically organized in the spleen. B cells form germinal centers where they mature and differentiate into antibody-secreting plasma cells (Coffey et al., 2009; Pereira et al., 2010). T cells are primarily located in the WP, particularly in the periarteriolar lymphoid sheath (PALS), which surrounds the central arterioles (Bronte & Pittet, 2013). $CD4^+$ T (or Helper T (Th)) cells support other immune cells by secreting cytokines and providing co-stimulatory signals. Within the spleen, $CD4^+$ T cells help activate B cells in nearby follicles to produce high-affinity antibodies (Qi, 2016). A specialized subset, T follicular helper (Tfh) cells, is crucial for guiding B cell maturation in germinal centers (Eisenbarth, 2019). $CD8^+$ T (or Cytotoxic T) cells recognize and kill infected or abnormal cells. In the spleen, naïve $CD8^+$ T cells are initially activated by antigen-presenting cells in the PALS (N. Sharma et al., 2015). Once activated, they migrate to the bloodstream to target infected cells (Khanna & Lefrançois, 2008). Some of these cells later differentiate into memory T cells, which persist in both the WP and RP and enable quicker responses upon re-exposure to the same pathogen (J. K. Hu et al., 2011; Jung et al., 2010; Khanna & Lefrançois, 2008; N. Sharma et al., 2015).

T cells constantly recirculate through the spleen, allowing them to scan for antigens delivered via the bloodstream. Their strategic positioning and ability to interact with DCs and B cells make the spleen a key site for initiating and regulating systemic immune responses. Together,

these splenocyte populations contribute to immune surveillance, pathogen clearance, and long-term immunity.

1.2.3 Changes in the spleen under chronic stress

Chronic stress impacts spleen morphologically as well as functionally. Prolonged stress exposure has been shown to reduce spleen size and disrupt its histological architecture, wherein there is a loss of GCs and disorganization of the WP (Domínguez-Gerpe & Rey-Méndez, 2001; Lei et al., 2024). These structural changes reflect underlying alterations in immune cell homeostasis and trafficking.

Functionally, chronic stress alters the distribution of leukocyte populations in spleen and peripheral blood. Several studies report that it leads to a significant reduction in the numbers of B and T lymphocytes, particularly affecting the $CD4^+/CD8^+$ T cell ratio, thereby disturbing adaptive immunity (Sterzer et al., 2004; K. X. Wang et al., 2007). Notably, chronic stress also increases the proportion of $CD4^+$ Th17, suggesting a potential dysregulation of helper T cell responses (Shi et al., 2022).

Together, these findings indicate that chronic stress not only leads to immune cell loss and altered composition in the spleen but also drives a shift toward immune dysfunction, which may predispose individuals to infections, impaired vaccine responses, and age-related immune decline.

1.3 GLUCOCORTICOIDS

GCs are lipid-soluble steroid hormones derived from the adrenal glands that circulate in the blood in response to physiological cues and stress (Miller & Auchus, 2011). GCs play a crucial role in maintaining homeostasis and regulating immune responses in a healthy state. However, prolonged exposure to these hormones due to chronic stress can lead to immune dysregulation (Dallman, 1993). Various systems regulate the body's response to stress, either individually or collectively, such as the CNS, the HPA axis, and the immune system. Brain regions such as the amygdala and hippocampus process sensory and emotional inputs, while the autonomic nervous system – particularly the sympathetic branch – rapidly mobilizes resources (McEwen, 2007). This coordination enables the CNS to initiate defense mechanisms before physical harm occurs. An organism's perception of physical or emotional stress triggers the HPA axis to secrete GCs

from adrenal cortex, such as cortisol in humans and corticosterone in rodents (Timmermans et al., 2019).

1.3.1 GCs and HPA axis

The HPA axis regulates adrenal GC production**.** GCs are secreted from the adrenal glands in the bloodstream in a circadian and ultradian rhythm under basal, unstressed conditions. The peak levels are reached during the active phase, which occurs in the morning for humans and at the beginning of nighttime for nocturnal animals like mice (Begemann et al., 2025; Spiga et al., 2014).

The activation of the HPA axis is initiated in limbic regions, including direct projections from the central nucleus of the amygdala or indirect pathways through the bed nucleus of the stria terminalis. These regions transmit signals to the hypothalamic paraventricular nucleus (PVN), where corticotropin-releasing hormone (CRH; previously known as corticotropin-related factor) and arginine vasopressin (AVP) are produced and subsequently released to interact with the anterior pituitary. CRH and AVP promote the release of adrenocorticotropin hormone (ACTH) into the bloodstream. ACTH targets the adrenal cortex, triggering the production and release of GCs (Arborelius et al., 1999; Spiga et al., 2014).

1.3.2 GCs and GC Receptors

GCs exhibit their functions by binding to glucocorticoid receptors (GRs), an intracellular ligand-dependent transcription factor. GR is found in almost all cell types and tissues. When GCs bind to GR, they translocate to the nucleus, which leads to a change in the production of several metabolic, immune and inflammatory proteins through transactivation or transrepression. This interaction allows GCs to be involved in a variety of physiological processes including metabolism (Vegiopoulos and Herzig, 2007), the immune response (Cruz-Topete & Cidlowski, 2015; De Bosscher & Haegeman, 2009), electrolyte balance (Hawkins et al., 2012), cardiovascular function (Nussinovitch et al., 2010), growth (Donatti et al., 2011), mood and cognitive functions (Farrell & O'Keane, 2016; Joëls, 2011; Tatomir et al., 2014), reproduction (Whirledge & Cidlowski, 2017) and development (Fowden & Forhead, 2015).

One of the mechanisms via which GCs control the activity of the HPA axis is facilitated by the binding of GCs to GRs at the PVN and pituitary gland levels to downregulate the *CRH*, *CRH-R1,* and the *POMC* gene (**Figure 2**). The proopiomelanocortin prohormone, the precursor of

ACTH, is encoded by *POMC*. The binding of GR to negative glucocorticoid-responsive elements (nGREs) suppresses the expression of the *CRH*, *CRH-R1,* and the *POMC* genes (Malkoski & Dorin, 1999); Drouin et al., 1989). GCs coordinate the activity of the HPA axis non-genomically by releasing endocannabinoids from CRH neurons that inhibit glutamate release from presynaptic excitatory synapses (Di et al., 2003) or by releasing γ-aminobutyric acid (GABA) at inhibitory synapses of CRH neurons (Di et al., 2009; Tafet & Nemeroff, 2016).

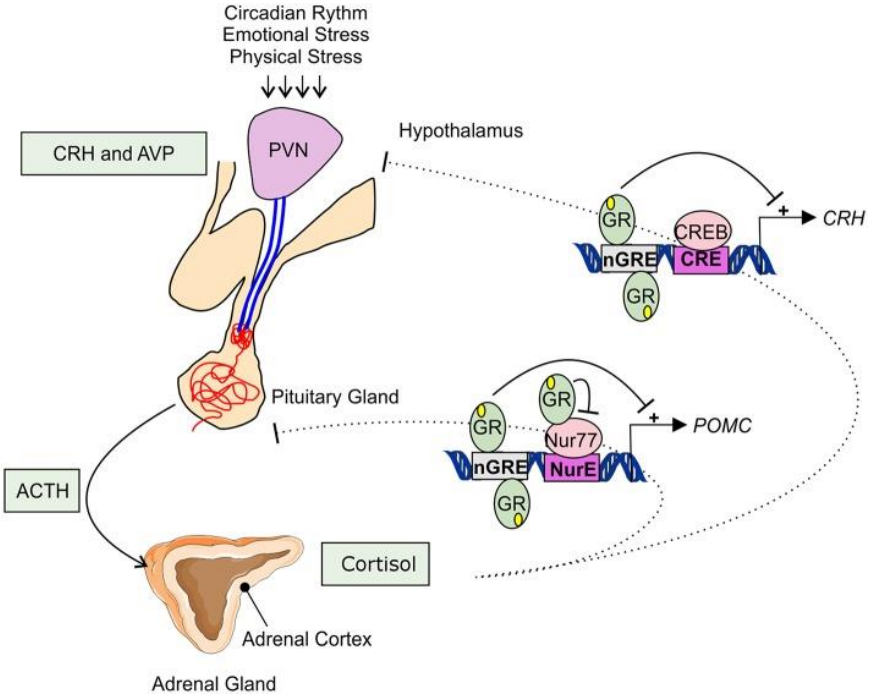


Figure 2. Regulation of GC-GR signaling. The hypothalamic-pituitary-adrenal (HPA) axis activity is controlled by the circadian rhythm and can be induced by physiological and emotional stress. When activated, corticotrophin-releasing hormone (CRH), and arginine vasopressin (AVP) are released from the hypothalamic paraventricular nucleus (PVN). This induces the release of adrenocorticotrophic hormone (ACTH) from the pituitary gland into the systemic circulation. ACTH will activate cortisol synthesis in the cortex of the adrenal gland. Cortisol negatively regulates the HPA-axis activity, e.g., by repressing the transcription of *CRH* and *POMC* by binding to nGRE or by binding to the transcription factor Nur77 involved in the *POMC* expression (Timmermans et al., 2019).

1.3.3 Negative feedback mechanism of GCs

To keep the GCs inactive, plasma proteins bind and transport them in the bloodstream. Corticosteroid-binding globulin (CBG) is the primary GC-binding protein in the plasma, binding approximately 80-90% of all GCs (Hammond, 2016). To release GCs, CBG is targeted

by several proteases, such as neutrophil elastase, at the sites of infection (J. G. Lewis & Elder, 2014). Free GCs permeate through the cell membrane to perform their function. Two enzymes reach the balance between active and inactive forms of GCs. 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) coverts inactive cortisone (or 11-dehydrocorticosterone in mice) into the active cortisol (or corticosterone, in mice) while 11β-HSD2 reverses the reaction (Seckl, 2004).

1.3.4 Physiological and clinical effects of GCs

GCs are released in response to stress and have numerous physiological effects, including protein catabolism, glucose metabolism regulation, and inflammation suppression. These hormones are widely used in clinical settings as anti-inflammatory and immunosuppressive agents, particularly in treating autoimmune diseases, allergies, and transplant rejection. Dex, a synthetic GC, is often prescribed to treat acute exacerbations of multiple sclerosis, allergies, cerebral edema, inflammation, and shock. Due to the anti-inflammatory effects of GCs, Dex was widely used during the COVID-19 pandemic (A. Sharma, 2021). In clinical research involving hospitalized COVID-19 patients, Dex administration reduced 28-day mortality in individuals receiving either mechanical ventilation or supplemental oxygen (RECOVERY Collaborative Group et al., 2021). Thus, GCs are being widely used for multiple treatment strategies.

1.4 EFFECT OF GCs ON T CELL IMMUNITY

Endogenous GCs are crucial in modulating T-cell immunity, particularly during the preparatory and early phases of immune responses. Since GC secretion follows circadian rhythms, these fluctuations are also expected to influence immune cell dynamics. Lymphocyte trafficking between the blood and lymphoid tissues exhibits a circadian pattern in both mice and humans (Besedovsky et al., 2014; Shimba et al., 2018). GCs such as Dex stimulate interleukin-7 receptor α (IL-7Rα or CD127) and chemokine receptor CXCR4, facilitating T-cell relocation to $CXCL12^{+}$ lymphoid niches (Shimba et al., 2018).

In murine models, T cells preferentially accumulate in secondary lymphoid organs during the active (nocturnal) phase, when antigen exposure is most likely, and return to the bloodstream during the sleep phase (Shimba et al., 2018). In humans, a similar GC-CXCR4 mechanism promotes the circadian redistribution of T cells, supporting immune surveillance when a

pathogen encounter is most probable (Besedovsky et al., 2014). Disrupting GC-CXCR4 signaling leads to increased T cell retention in circulation and impaired lymphoid organ homing (Besedovsky et al., 2014; Shimba et al., 2018). These stress-induced shifts may lead to immune dysregulation.

1.4.1 Effect of GCs on T cell differentiation

The differentiation of $CD4^+$ and $CD8^+$ T cells into various effector phenotypes depends on their cytokine environment and transcriptional programming and is crucial for coordinating different types of immune responses (Sun, 2016; Taniuchi, 2018; J. Zhu et al., 2010). Although the GR is expressed by all T cells, the sensitivity to GC treatment varies significantly between subsets. Therefore, GCs favor specific T helper (Th) cell responses over others. Th1 cell responses are strongly suppressed, while Th2 cell responses exhibit moderate suppression by GCs. In contrast, Th17 cell responses remain largely unaffected, allowing their activity to persist even under GC treatment. This differential sensitivity to GCs shapes the immune response, influencing the balance between pro-inflammatory and regulatory mechanisms.

Th1 cells: Dendritic cells and macrophages exposed to GCs, including Dex, exhibit a reduced capacity to present antigens and produce IL-12 and IFN-γ, both of which are essential for Th1 differentiation (Li et al., 2015; Oh et al., 2017). Dex suppresses IL-12-induced STAT4 phosphorylation (Franchimont et al., 2000), STAT1 gene expression (X. Hu et al., 2003), as well as expression of *Tbx21*, which encodes T-bet protein, and *Ifng* (Banuelos et al., 2016; De Castro Kroner et al., 2018; Liberman et al., 2007). They are key transcriptional programs required for Th1 differentiation.

Th2 cells: Th2 differentiation is also altered by GCs, though to a lesser extent than Th1. While GCs do not significantly affect IL-4-induced STAT6 phosphorylation (Franchimont et al., 2000), they regulate Th2 differentiation through other mechanisms. GC-induced MKP-1 (MAPK phosphatase-1) expression inhibits p38 MAPK activation, leading to decreased GATA3 expression (Liberman et al., 2007; Maneechotesuwan et al., 2009). The downregulation of GATA3 limits the production of IL-4, IL-5, and IL-13 (Banuelos et al., 2016; Martin et al., 2013; Peek et al., 2005), thereby modulating Th2 responses. In comparison to the suppression of Th1 differentiation, Th2 responses are relatively favored by GCs.

Th17 cells: GCs such as Dex support Th17 differentiation by increasing the rate of expression of the IL-1 and TGF-β receptor on T cells (Galon et al., 2002) and by working synergistically

with STAT3 activated by IL-6 (Z. Zhang et al., 1997). In addition, GCs promote the expression of RORγt (De Castro Kroner et al., 2018) and the production of IL-17 (Banuelos et al., 2016; Schewitz-Bowers et al., 2015), while inhibiting IL-22 (Banuelos et al., 2016; De Castro Kroner et al., 2018) and GM-CSF (Martin et al., 2013). These effects collectively favor a partially skewed but functional Th17 response under GC influence.

T regulatory cells (Tregs): In contrast to Th cells, GC signaling significantly enhances Treg differentiation. Elevated differentiation and functionality of Tregs correlate with increased expression of TGF-β receptors (Galon et al., 2002), FOXP3 (Karagiannidis et al., 2004; Schmidt et al., 2018) and, IL-10 (De Castro Kroner et al., 2018; Galon et al., 2002). During Treg cell differentiation, the expression of GR is increased (Schmidt et al., 2018). GC-regulated gene, GC-induced leucine zipper (*Gilz*), stimulates Treg differentiation (Bereshchenko et al., 2014). The excessive transgenic production of GR leads to a reduction in the Th population. However, it does not suppress Treg cell numbers and may even increase them, as transgenic mice show a higher proportion of Tregs than wild-type mice (Yakimchuk et al., 2015). Conversely, GR deletion in Tregs results in exacerbated colitis and resistance to GC therapy in models of autoimmune encephalitis and allergic airway inflammation (Kim et al., 2020; Rocamora-Reverte et al., 2019), underscoring the importance of GR in Treg-mediated immunoregulation.

Memory T cells: The differentiation of memory T cells depends on GC signaling. Memory precursor effector cells express low levels of Killer Cell Lectin Like Receptor G1 (KLRG1; typically found on terminal effector T cells) and high levels of IL-7Rα (a characteristic marker of memory precursors and low in terminal effector cells) (Kaech et al., 2003). IL-7 phosphorylates STAT5 via IL-7Rα, leading to increased production of anti-apoptotic BCL-2 and BCL-XL, which are significant for the survival of memory T cells in the long-term (Kaech et al., 2003). The activation of BCL-2 via GR–IL-7Rα is essential for the sustained preservation of memory T cells, as the population of memory cells significantly decreases without T cell GR expression (Shimba et al., 2018; B. Yu et al., 2017). GCs increase T cell production of IL-7Rα (Franchimont et al., 2002; Lee et al., 2005), indicating a potential way for improving T cell memory. Moreover, GC signaling showed a link to the transcriptional regulation of memory T cell differentiation. *Nr3c1*, which encodes the GR, is identified as a key regulator of the memory T cell transcriptional program (Tehseen et al., 2024). GCs enhance the expression of genes required for memory T cell survival, such as *Il7ra*, *Cxcr4*, *Tgfbr1*, *Tgfbr2,* and *Foxp1* (Shimba et al., 2018). Knocking down GR in mature T cells using short hairpin RNA led to a shift towards terminal effector differentiation and a significant drop in IL-7Rα^{+}KLRG1^{-} memory precursor cells at the top of the reaction (B. Yu et al., 2017). The same result was achieved when

Ncor1 (GR co-activator) was deleted (B. Yu et al., 2017). These findings highlight the role of GCs in memory T-cell differentiation.

1.4.2 Effect of GCs on T cell activation and proliferation

GCs exert suppressive effects on T cells by inhibiting their activation and proliferation. The expansion of activated T cells relies on T cell growth factor (TCGF), also known as interleukin-2 (IL-2), which is synthesized by activated T cells. This cytokine is transcriptionally regulated and plays a key role in clonal expansion following TCR engagement. The synthetic GC, Dex, strongly suppresses the transcription of IL-2 mRNA and IFN-γ mRNA (Bianchi et al., 2000), thereby suppressing both proliferative and effector functions of T cells.

GCs suppress effector T cell proliferation by selectively inhibiting of T cells with low-affinity TCRs while allowing T cells with high TCR affinity to persist (Tokunaga et al., 2019). This selection is crucial for shaping the memory T cell pool, ensuring that only the most effective clones survive. This mechanism is reminiscent of GC effects in the thymus, where they antagonize TCR signaling to promote a strongly reactive TCR repertoire. However, unlike thymic selection, GC-mediated inhibition of weak TCR clones reduces TCR diversity in the memory response, which may limit future immune adaptability.

The expression of surface and intercellular markers such as CD25, CD69, PD-1 and Ki-67 can assess T cell activation and functional state. Dex reduces the percentage of early activated ($CD69^+$) $CD4^+$ and $CD8^+$ T cells in activated populations, both *in vitro* and *in vivo* (Iorgulescu et al., 2021; Meyer-Heemsoth et al., 2023). At the same time, Dex increases the percentage of $CD4^+CD25+^+$ cells in the thymus and spleen (X. Chen et al., 2004).

Dex increases the expression of PD-1 during T cell activation. Central memory (CM; $CD44^+CD62L^+$) and effector memory (EM; $CD44^+CD62L^-$) T cells showed a stronger Dex-induced upregulation of PD-1 in comparison to naïve T cells (Xing et al., 2015). PD-1 is a cell-surface receptor that reduces T cell activity when bound to its ligand, PD-L1 or PD-L2 (Keir et al., 2007). PD-1 is extensively expressed in tumor-infiltrating lymphocytes, and high PD-L1 levels are frequently observed in various tumor types, suppressing local anti-tumor T cell responses (Chiou et al., 2005). GC-induced PD-1 upregulation may therefore contribute to immune exhaustion and further limit cytotoxic T cell responses in chronic inflammatory states or the tumor microenvironment.

Dex promotes Treg activation and proliferation by increasing Ki-67 expression on Tregs, which supports immunosuppressive function (Cook et al., 2016). Dex does not significantly affect Ki-67 expression in tumor microenvironment (Aston et al., 2019).

1.4.3 Effect of GCs on T cell function

The primary function of GCs in clinical settings is immunosuppression. Synthetic GCs such as Dex, betamethasone, and prednisone are prescribed to reduce autoimmunity, inflammation, or rejection of transplant. In the absence of adrenal GC synthesis, the removal of pathogens can be significantly faster (Jamieson et al., 2010; Roggero et al., 2006; Ruzek et al., 1999); however, the T cell mortality is increased because of unregulated T cell responses, cytokine storm, and vascular shock (Brewer et al., 2003; Roggero et al., 2006; Ruzek et al., 1999). In antigen-presenting cells, GCs impair antigen processing and presentation, reduce co-stimulatory signals, and suppress the production of pro-inflammatory cytokines essential for T cell activation (Elftman et al., 2007; Li et al., 2015; Oh et al., 2017; Yang et al., 2019). This downstream suppression of innate immunity significantly dampens T cell priming and effector responses.

Transcriptionally, GCs, including Dex, influence the expression of genes associated with T cell viability and functionality (Franchimont et al., 2002). Dex upregulates the pro-survival receptor *Il7r*, which encodes the IL-7Rα in murine and human activated T cell (Franchimont et al., 2002; Lee et al., 2005). This receptor is highly expressed in naïve and CM T cells. In contrast, its expression is relatively low on effector memory T cells, suggesting that GCs may support the survival and homeostasis of early-stage or memory-precursor T cell subsets (Tehseen et al., 2024). Phenotypically, Dex treatment showed a dose dependent decrease in multiple T cell subsets, including $CD3^+$, $CD4^+$, $CD8^+$, $CD44^+$, and $CD8^+CD122^+$ T cells (L. Chen et al., 2018). In contrast, $CD4^+CD25^+$ Tregs, are selectively enriched following Dex exposure (L. Chen et al., 2018). This shift likely contributes to an immunosuppressive environment by stimulating the regulatory T cells while suppressing effector T cell activity.

Collectively, these findings highlight the ability of GCs to fine-tune T cell responses, preserving regulatory and memory-supportive pathways while limiting excessive effector functions that may lead to immunopathology.

2 AIMS

GCs are key mediators of the physiological stress response and are known for their profound role in immunomodulation (Cain & Cidlowski, 2017). Persistent stress conditions are characterized by chronic exposure to GCs, which has been implicated in accelerated aging of the immune system and compromised T cell function (Graham et al., 2006). Dex, a synthetic GC analog, is frequently used to model GC-induced immune alterations (Giles et al., 2018). While *in vivo* studies have demonstrated the systemic immunosuppressive effects of GCs, a controlled *in vitro* model is essential to investigate the temporal and dose-dependent impact of GC exposure on immune cells in isolation.

In this study, we aimed to standardize an *in vitro* platform to model GC-induced immune cell changes, focusing on splenic T cell responses to Dex treatment over time. A 22-color flow cytometry panel was employed to capture the phenotypic and functional shifts in T cells under different Dex treatment concentrations.

To investigate this, we aimed to:

- Determine the effect of Dex treatment on T cell distribution and survival over time by analyzing key splenic T cell subsets and their viability at multiple time points following *in vitro* Dex exposure.
- Characterize the time-dependent dynamics of T cell activation, proliferation, and exhaustion in the presence of Dex by profiling markers such as CD69, CD25, Ki-67, and PD-1 across different time points.
- Identify concentration-dependent effects of Dex on T cell responses by distinguishing sensitive and resistant T cell populations and evaluating the potential of their recovery after initial suppression.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Animals

Young female BALB/c mice, aged 1-month-old, were used in this experiment. The animals were obtained from the Laboratory Animal Centre, Institute of Biomedicine and Translational Medicine, Tartu. The mice were housed in communal cages under standard laboratory conditions, including a 12-hour light/dark cycle, and were provided with food and water *ad libitum*.

3.1.2 Spleen collection

Mice were euthanized by isoflurane overdose. Spleens were collected in 6-well plates containing 2ml 1×HBSS on ice and mashed between the frosted ends of clean glass slides. The cell suspensions were filtered through a pre-wetted 100 µm cell strainer and collected into 50 ml Falcon tubes. Cells were washed by adding up to 30 ml of chilled FACS buffer (Fluorescent Activated Cell Sorting buffer – 5% BSA, 500 mM EDTA in PBS (phosphate buffered saline)) and centrifuged at 350g, 7 °C, for 7 minutes (same unless specified otherwise). To resuspend the cell pellet, 2 ml Ammonium-Chloride-Potassium (ACK) lysis buffer was added and incubated at room temperature (RT) for 3 min to lyse red blood cells. The lysis was stopped by adding chilled FACS buffer to a final volume of 25 ml and centrifuged. The resulting cell pellet was resuspended in 1 ml FACS buffer, and the suspension was filtered through a pre-wetted 100 µm cell strainer. The total volume was made up to 20 ml with chilled FACS buffer. Cells were counted using Acridine orange/ propidium iodide stain on LUNA-FL™ Dual Fluorescence Cell Counter (Logos Biosystems).

3.1.3 *In vitro* assay

The samples were adjusted to a concentration of 1 x 10^6 cells/ 100 µl in complete RPMI (RPMI 1640 Medium + 10% FBS (fetal bovine serum) + 1% Penicillin-Streptomycin). The wells were pre-wetted with 80-98 µl complete media RPMI, and 100 µl of cell suspension was added to each well. The plates were kept at 37 °C in a 5% CO2 incubator for an hour. Dexamethasone (Dex; D4902, Sigma-Aldrich) was dissolved in absolute ethanol to prepare a 10 mM stock solution. The specific concentrations of Dex (0.01 nM, 1 nM, 100 nM, 1 µM, 100 µM) were made using RPMI and added to each well respectively. Untreated and vehicle-treated (absolute

ethanol) cells were added as controls. Plates were incubated at 37°C in a humidified atmosphere containing a 5% CO_2 for 24, 48, and 72 hours.

3.1.4 Antibody staining and spectral flow cytometry

At the end of the specific incubation time, the plates were centrifuged. The supernatants were collected and stored at -20 °C. PBS was added, and the cells were counted using LUNA-FL™ Dual Fluorescence Cell Counter. The cells were pelleted by centrifugation.

The cells were resuspended in 50 µl of Zombie NIR viability dye (Biolegend, 1:400 dilution) and incubated for 15 min at RT in the dark for live/dead staining. 10 µl of FcX blocking antibody solution (Biolegend, 1:400 dilution) was added to each well to prevent the non-specific binding of antibodies, and cells were incubated for 10 min at RT. 10 µl of surface stain antibodies (Biolegend; listed in **Table 1**) were added, and cells were incubated for 30 min at 4°C in the dark. After incubation, the cells were washed thrice with 180 µl chilled FACS buffer and centrifuged.

For intracellular staining, cells were fixed with the fixative (1 part of eBio Fix concentrate + 3 parts of Fix Diluent (eBioscience™ Foxp3 / Transcription Factor Fixation/Permeabilization kit, Thermo)) and incubated for at least 50 minutes at RT in the dark. Cells were washed twice with 1× Permeabilization buffer (1 part of 10× concentrate and 9 parts of Milli-Q water) and centrifuged. 70 µl of intracellular stain antibodies (Biolegend, except Ifng – eBioscience and Rorgt – Invitrogen; listed in **Table 1**) were added, and cells were incubated for 30 min at RT in the dark. Additionally, Fluorescence Minus One (FMO) controls for specific intracellular markers were included to determine background fluorescence. Cells were washed twice with FACS buffer, resuspended in 150 µl of FACS buffer, and analyzed using the Sony ID7000 Spectral Cell Analyzer or stored at 4°C in the dark till acquisition.

Table 1. Antibodies used in spectral flow cytometry.

Antigen	Clone	Conjugate	Dilution
Surface staining			
CD45	MCA1031SBB810	SBB810	1:200
CD4	GK1.5	PE-F700	1:400
CD8	53-6.7	BV650	1:400
CD69	H1.2F3	PB	1:200
CD25	PCGI	BV785	1:200

IL7Rα	S18006K	PE-F640	1:200
CD44	IM7	BV711	1:400
CD62L	MEL-14	APC-Fire750	1:200
CD5	53-7.3	BV421	1:200
CD45R	RA3-6B2	APC/Fire810	1:200
MHCII	M5/114.15.2	PE/Cy5	1:2000
PD1	29F.1A12	BV510	1:100
Intracellular staining			
Ki67	16A8	AF700	1:400
CD3	145-2C11	PerCP/Cy5.5	1:50
Tbet	4B10	BV605	1:100
Rorgt	B2D	PE	1:200
Gata3	16E10A23	APC	1:50
Bcl6	7D1	PE/Dazzle594	1:100
Foxp3	156D	AF488	1:100
Ifng	XM61.2	PE-Cy7	1:100
Tnfa	MP6-XT22	AF647	1:100

3.1.5 Statistical analysis

Statistical significance for flow cytometry data was assessed using one-way ANOVA followed by Tukey's multiple comparisons test, performed with GraphPad Prism 8 software. P-values lower than 0.05 were considered significant and are mentioned in the figures accordingly. All data plots are expressed as mean ± standard error mean (SEM) of three technical replicates per group.

3.2 RESULTS & DISCUSSION

Dex is a synthetic GC that induces suppressive effects on the immune system. To investigate the impact of Dex on splenocytes *in vitro,* we collected murine splenocytes from young mice. After the splenocyte collection, we treated the cells with five different concentrations of Dex (0.01 nM, 1 nM, 100 nM, 1 μM, 100 μM). The cells were incubated for 24, 48, and 72 h before being analyzed and compared among the groups using spectral flow cytometry.

3.2.1 Effects of Dex on the viability of splenocytes

Spleens were isolated from mice and mechanically dissociated to obtain single-cell suspensions. Splenocyte viability and cell numbers were assessed using the automatic cell counter and plated with equal seeding density of 1 x 10^6 cells per well in a 96-well plate. Cells were cultured *in vitro* and treated with various concentrations of Dex (0.01 nM, 1 nM, 100 nM, 1 μM, 100 μM) or vehicle control. Splenocyte viability and absolute cell numbers were evaluated at 24, 48, and 72 h post-treatment.

The viability of splenocytes showed a decreasing trend over time (**Figure 3A**). The most significant decrease in cell viability was observed after 72 h of Dex treatment (**Figure 3B**). Higher concentrations of Dex (from 100 nM onwards) demonstrated a more potent suppressive effect compared to the vehicle. A similar decreasing pattern was observed for the absolute cell numbers of splenocytes collected after respective incubation periods (**Figure 3C**). After Dex treatment, the most pronounced trend was observed after 72 h in the number of surviving splenocytes, especially with higher concentrations from 1 μM (**Figure 3D**). No significant difference was found between untreated and vehicle-treated samples in cell viability or numbers (**Figure 3B and 3D**).

The observed time and dose dependent decline in splenocyte viability following Dex treatment aligns with the well-established immunosuppressive and pro-apoptotic effects of GCs. At 24 h, there was a reduction in the viability and cell numbers in vehicle and Dex-treated groups, indicating stronger solvent effects at this time point. However, at 72 h, increasing concentrations of Dex led to a progressive reduction in both cell viability and absolute splenocyte numbers compared to the vehicle. This suggests that Dex induces cumulative cytotoxic effects over prolonged culture periods, which is consistent with previous reports demonstrating GC-induced lymphocyte apoptosis and suppression of immune cell proliferation (Distelhorst, 2002; Smith & Cidlowski, 2010; Varga et al., 2014). The marked decrease in viability and cell counts at Dex concentrations ≥100 nM suggests a threshold beyond which GCs robustly induces apoptosis pathways in splenocytes. Interestingly, the effect of Dex treatment became more pronounced at 72 h, suggesting that its immunomodulatory action is not immediate but accumulates with sustained receptor activation and transcriptional reprogramming. Moreover, the lack of difference between untreated and vehicle controls confirms that the observed effects are specific to Dex and not due to solvent exposure.

These findings provide a valuable baseline for subsequent analyses of specific T cell subset dynamics under GC exposure. Understanding how Dex compromises total splenocyte viability

helps contextualize downstream phenotypic changes in T cell activation, proliferation, and survival, as explored in later sections of this study.

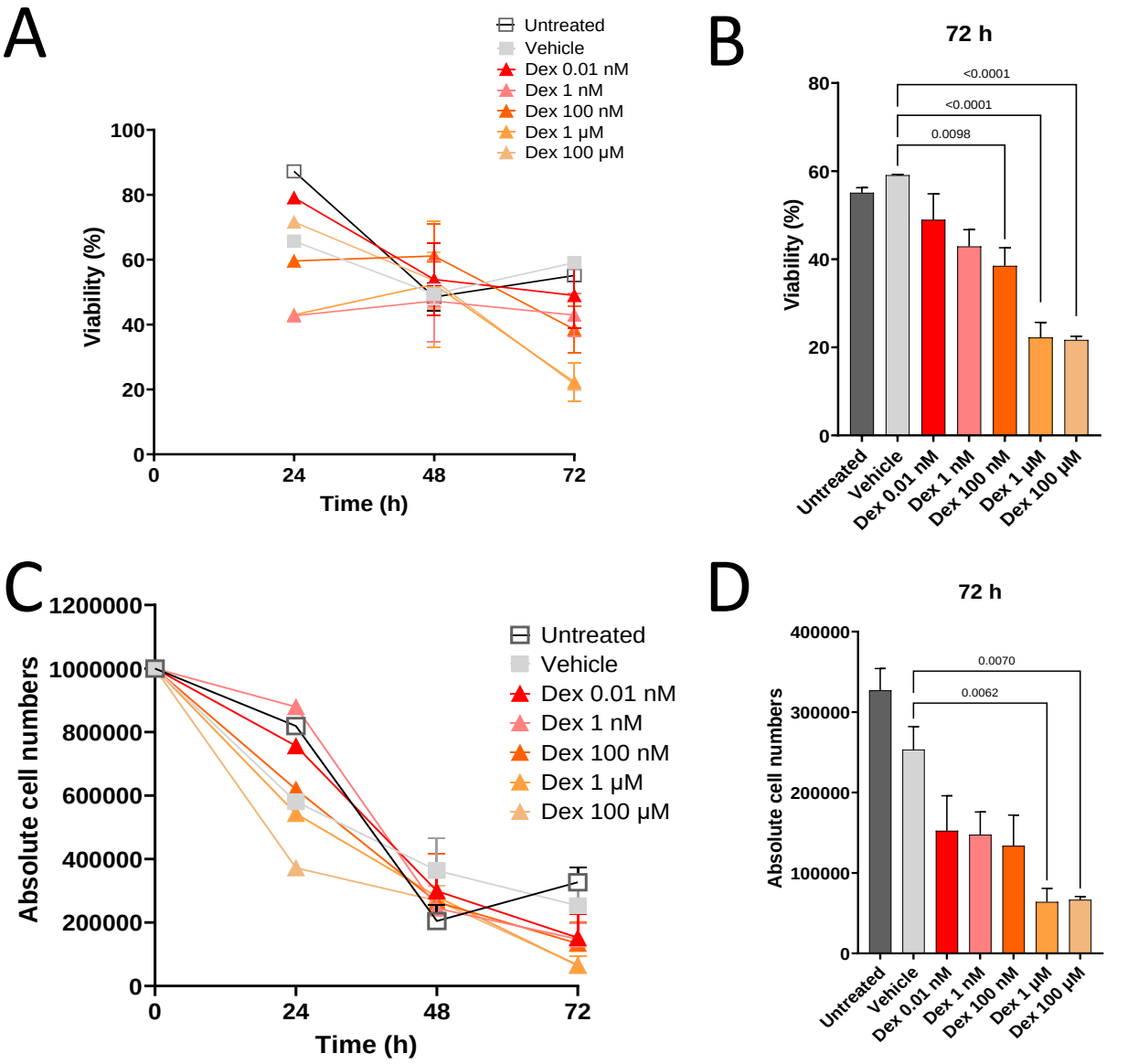


Figure 3. **The viability and absolute cell numbers of murine splenocytes after *in vitro* culture.** A) Viability of murine splenocytes at 24, 48, and 72 h following Dex treatment. B) Viability of murine splenocytes at 72 h after Dex treatment. C) Absolute cell number of murine splenocytes at 24, 48, and 72 h following Dex treatment. D) Absolute cell number of murine splenocytes at 72 h after Dex treatment. Data are expressed as mean ± SEM of three technical replicates per group for viability and absolute cell numbers. *Dex – dexamethasone. Statistical analyses were conducted using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.*

3.2.2 Effects of Dex on major splenic T cell populations

The spleen contains various immune cell types, including T cells at different activation and differentiation stages. We used a 22-color flow cytometry panel to distinguish the various T cell subsets and analyze their activation, proliferation, and function. The first step in flow cytometric analyses is to exclude debris, dead cells, and doublets. This is done by gating out the target cells using forward and side scatter channels and using the viability dye to negatively choose the live cells. After singlets and live cells selection, splenocytes were gated based on CD45, B220, MHCII, and CD3 expression. Leukocytes were identified as $CD45^+$, and further B220 expression was used to exclude the B cells ($CD45^+$ $B220^+$). Within the $CD45^+$ $B220^-$ population, antigen-presenting cells (APCs) were defined as $CD45^+$ $B220^-$ $MHCII^+$ $CD3^-$, and T cells were identified as $CD45^+$ $B220^-$ $MHCII^-$ $CD3^+$ cells (**Figure 4A**). Further, within the T cells, $CD4^+$ and $CD8^+$ T cells were identified based on the respective markers (**Figure 4A**).

The frequency of overall leukocyte population remained unchanged at both 24 and 48 h time-points (**Figure 4B**). At 72 h, there was an increase in the frequency of $CD45^+$ cells, indicating a drop in the $CD45^-$ cells, which represents the stromal populations. Regarding T cells, although no significant differences were detected at 24 h in the proportion of T cells after Dex treatment, significant increases were observed at higher Dex concentrations at 48 and 72 h (**Figure 4C**). This was in lieu of the decrease in $CD45^+$ $B220^-$ $MHCII^{-/+}$ populations representing non-T and non-B cells, such as NK cells and APCs, respectively (**Figure 4D**). Previously it has been shown that Dex inhibits the expression of MHCII and co-stimulatory molecules (CD80, CD86) on APCs affecting their ability to present antigen and activate T cells (Orlikowsky et al., 2005; Pan et al., 2001) as well as induces apoptosis of NK cells and APCs such as pDC cells (Abe & Thomson, 2006; L. Chen et al., 2018). Notably, the increase in proportions of T cells was not seen in terms of cell numbers wherein Dex treatment as low as 0.01 nM induced a reduction in T cell numbers, while concentrations of 1 μM and above resulted in more than a three-fold decrease compared to vehicle-treated controls (**Figure 4E**).

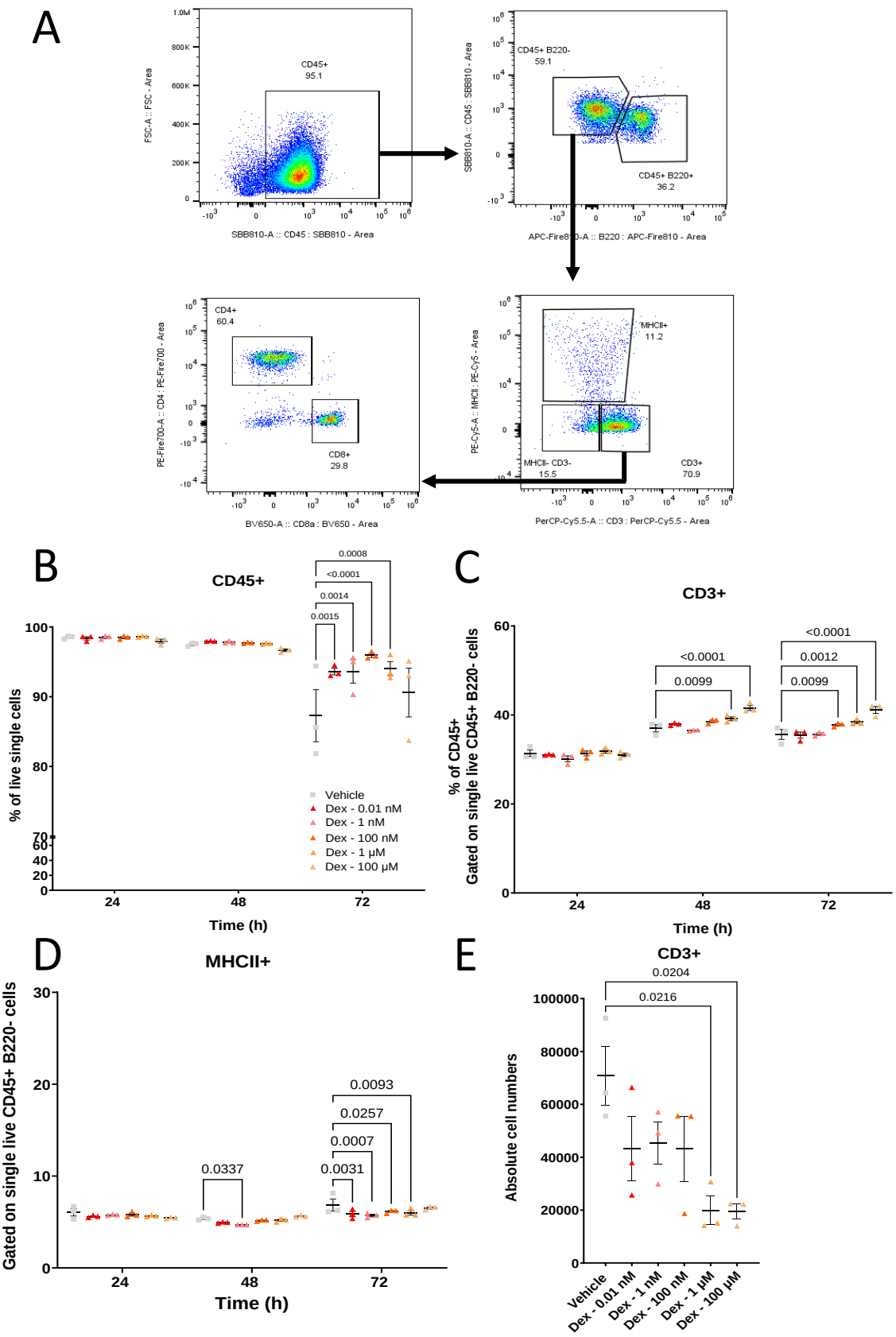


Figure 4. Flow cytometry analysis of T cells. A) Representative flow cytometry graph of lymphocytes and T cells. B) Frequency of leukocytes ($CD45^+$) population in mouse spleen at 24, 48, and 72 h following Dex treatment. C) Frequency of T cell ($CD45^+$ $B220^-$ $MHCII^-CD3^+$)

population in mouse spleen at 24, 48, and 72 h following Dex treatment. D) Frequency of antigen-presenting cells ($CD45^+B220^-MHCII^+$) population in mouse spleen at 24, 48, and 72 h following Dex treatment. E) Absolute cell number of T cells in mouse spleen at 72 h after Dex treatment. Data are expressed as mean ± SEM of three replicates per group for absolute cell numbers. *Dex – dexamethasone. Statistical analyses were conducted using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.*

Although the overall proportion of T cells remained unchanged at 24 h post-Dex treatment, transient changes in $CD4^+$ and $CD8^+$ T cell ratio were observed. Specifically, Dex treatment led to a decrease in the proportion of $CD4^+$ T cells, accompanied by a relative increase in the proportion of $CD8^+$ T cells (**Figure 5A, B**). This altered ratio may be attributed to the differential sensitivity of T cell subsets to GC-induced effects. $CD4^+$ T cells may be more vulnerable to GC-induced apoptosis than $CD8^+$ T cells, due to greater dependence on co-stimulatory and cytokine signaling for survival (Bischof & Melms, 1998; Franchimont et al., 2000). $CD8^+$ T cells, especially memory-like or activated subsets, may exhibit increased resistance to GC-mediated cell death, possibly due to the upregulation of anti-apoptotic factors such as Bcl-2 (Ploner et al., 2008). Interestingly, by 72 h, the proportions of $CD4^+$ and $CD8^+$ T cells returned to levels comparable to controls, suggesting an adaptive or homeostatic rebound in T cell distribution or survival following initial glucocorticoid exposure.

Similar to the change in T cell absolute cell numbers, both $CD4^+$ and $CD8^+$ T cells decreased across all three time points (24, 48, and 72 h), with significant reductions detected at 72 h (**Figure 5C, D**). The decline in absolute cell numbers was dose dependent, with higher concentrations of Dex (from 1 μM onwards) leading to around a three-fold decrease compared to vehicle-treated controls. The sensitivity of both $CD4^+$ and $CD8^+$ T cell subsets to Dex may reflect similar GR-mediated signaling pathways that suppress proliferation and promote apoptosis. These results highlight the vulnerability of adaptive immune cells to GC-induced modulation and underscore the importance of dosage and duration in shaping immune cell dynamics under stress or therapeutic regimens.

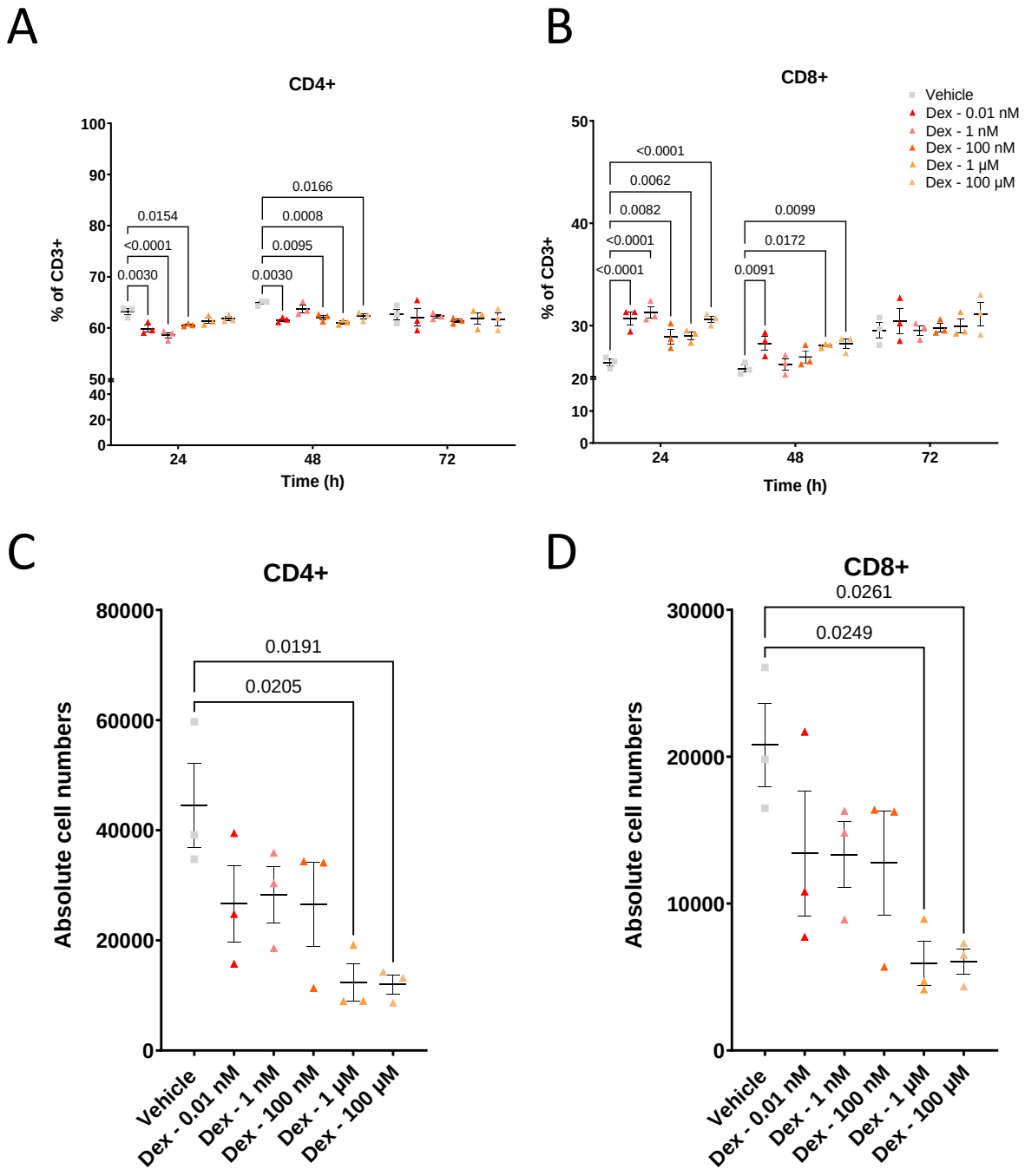


Figure 5. Flow cytometry analysis of CD4⁺ and CD8⁺ T cells. Frequency of A) CD4⁺ and B) CD8⁺ T cell populations in mouse spleen at 24, 48, and 72 h following Dex treatment. Absolute cell number of C) CD4⁺ and D) CD8⁺ T cells in mouse spleen at 72 h after Dex treatment. Data are expressed as mean ± SEM of three technical replicates per group for absolute cell numbers. *Dex – dexamethasone. Statistical analyses were conducted using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.*

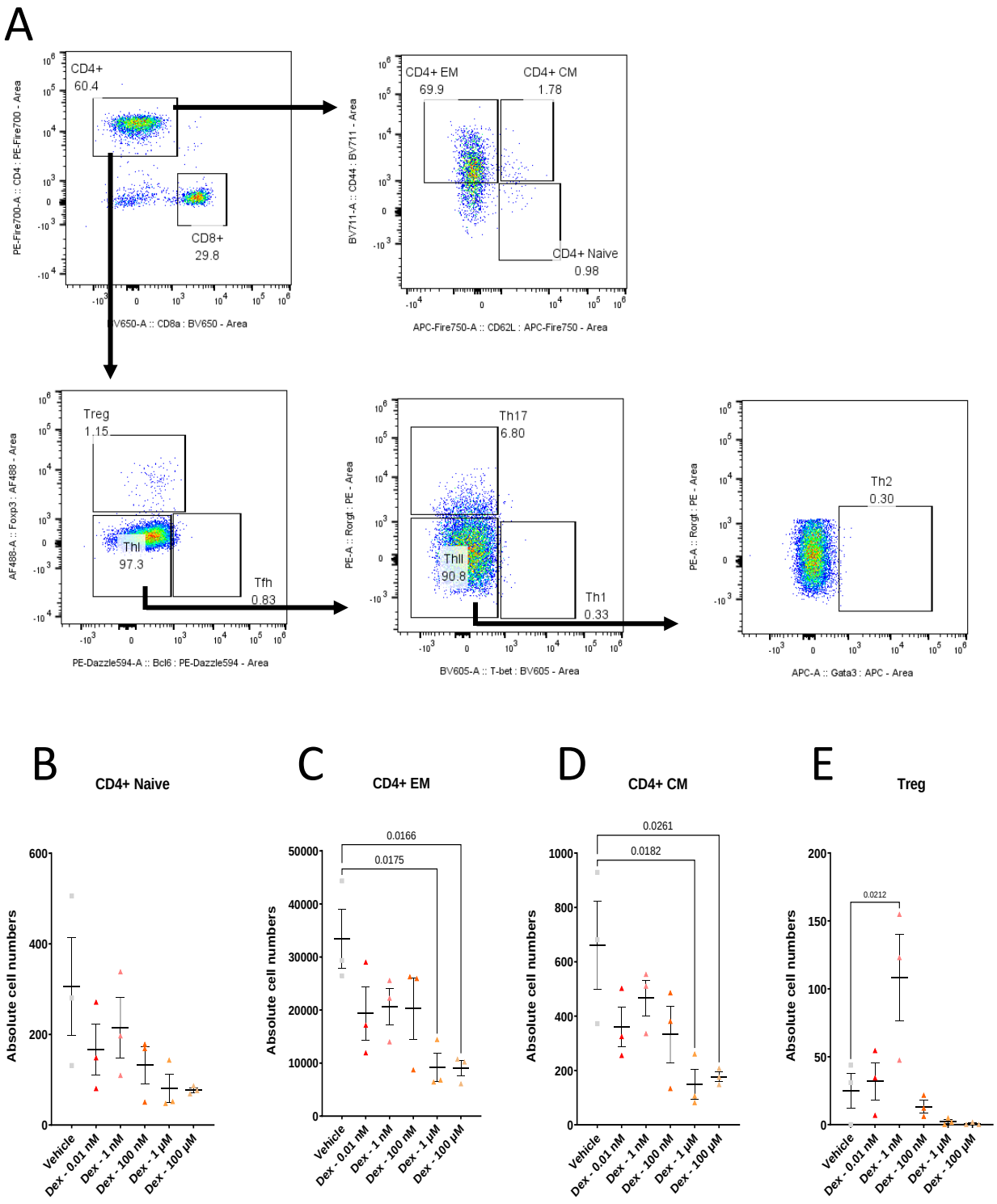
3.2.3 Effects of Dex on $CD4^+$ T cell subsets

To further dissect the effects of Dex on $CD4^+$ T cells, we analyzed their differentiation status using CD44 and CD62L to identify naïve ($CD44^-CD62L^+$), central memory (CM; $CD44^+CD62L^+$), and effector memory (EM; $CD44^+CD62L^-$) $CD4^+$ T subsets. Dex treatment led to a time and dose dependent reduction in the absolute numbers of all three subsets, with the most substantial decline observed at 72 h post-treatment (**Figure 6B-D**). At higher concentrations (1 μM and 100 μM), both CM and EM subsets demonstrated a significant reduction, with approximately a four-fold decrease compared to vehicle-treated controls (**Figure 6C, D**). These results indicate that Dex exerts a broad suppressive effect on $CD4^+$ T cell populations, irrespective of their phenotype. The pronounced reduction in both memory subsets (CM and EM) is contradictory to a previously published report that shows that antigen-primed effector T cells are more resistant to GC-induced apoptosis than naïve T cells *in vivo* (Bier et al., 2021).This discrepancy may be due to the absence of activation cues and survival cytokines in our *in vitro* system.

Further assessment of other $CD4^+$ T cell subsets revealed differential sensitivity to Dex. Regulatory T cells (Tregs; $Foxp3^+$ $CD4^+$) displayed a biphasic response: their numbers increased at a low Dex concentration (1 nM) but decreased markedly at higher doses (1 μM and 100 μM) (**Figure 6E**). This suggests that low-dose Dex may favor Treg survival or expansion, while higher doses exert an overall immunosuppressive effect, consistent with the depletion observed across $CD4^+$ subsets. The biphasic response observed in Tregs is consistent with prior studies showing dose dependent effects of GCs. *In vivo*, high doses of Dex reduce the frequency of splenic $CD4^+Foxp3^+$ Tregs, while *in vitro*, low doses (<100 nM) promote their expansion or survival (W. Wang et al., 2015). This suggests that physiological or low pharmacological doses may preferentially support the maintenance of regulatory pathways, whereas high doses suppress both effector and regulatory arms of the immune system.

Analysis of $CD4^+$ T helper (Th) subsets revealed that Th1 cells ($Tbet^+$ $CD4^+$) initially increased suggesting early resilience followed by suppression at pharmacologically relevant doses (**Figure 6F**). In contrast, Th17 ($Rorgt^+$ $CD4^+$) and Th2 ($Gata3^+$ $CD4^+$) subsets showed a general downward trend across all concentrations, although changes did not reach statistical significance (**Figure 6G, H**). T follicular helper (Tfh; $Bcl6^+$ $CD4^+$) cells were particularly sensitive to Dex, exhibiting significant reductions even at the lowest tested doses (**Figure 6I**). The marked reduction in Tfh cells across all concentrations is notable, given their role in providing help to B cells and coordinating germinal center responses. Their loss may contribute to impaired humoral immunity and antibody production following prolonged GC exposure, in

line with observed defects in vaccine responses in chronically stressed individuals (Pedersen et al., 2009). These findings highlight the differential sensitivity of CD4⁺ T cell subsets to GC exposure.



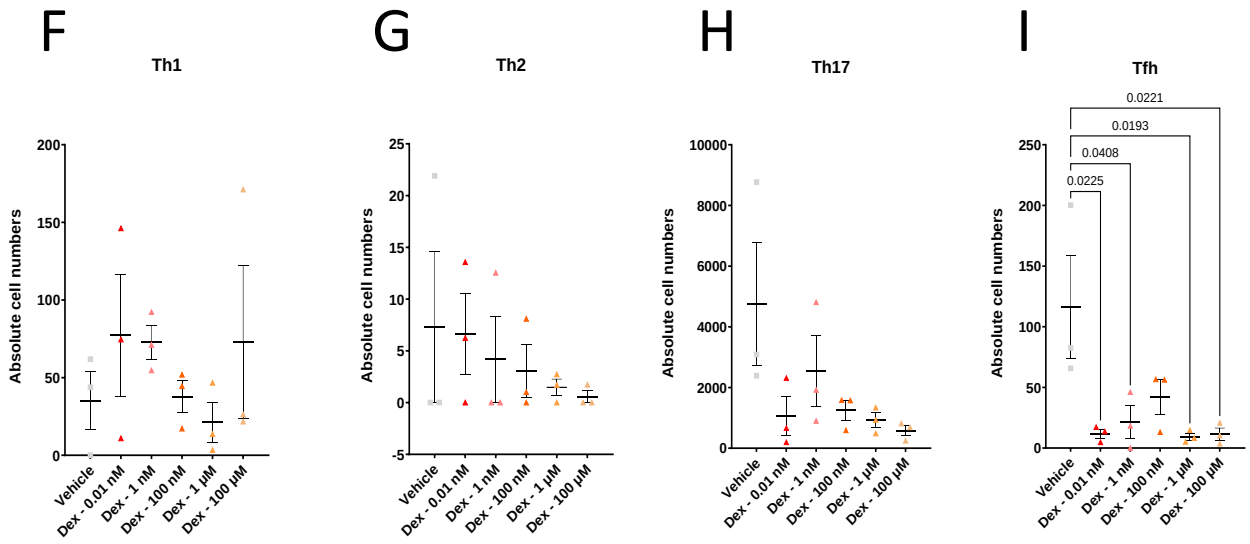


Figure 6. Flow cytometry analysis of CD4⁺ T cell subsets at 72 h after Dex treatment. A) Representative flow cytometry graph of CD4⁺ T cell subsets. Absolute cell number of CD4⁺ B) naïve C) effector memory D) central memory E) regulatory F) T helper 1 G) T helper 2 H) T helper 17 I) T follicular helper cells in murine splenocyte culture after 72 h in presence or absence of Dex treatment. Data are expressed as mean ± SEM of three technical replicates per group for absolute cell numbers. *Dex – dexamethasone; EM – effector memory; CM – central memory; Treg – regulatory T cell; Th – helper T cell; Tfh – T follicular helper cell. Statistical analyses were conducted using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.*

3.2.4 Effects of Dex on CD8+ T cell subsets

CD8⁺ T subsets showed a decrease in absolute numbers across all time points following Dex treatment, with the most significant reductions observed at 72 h (**Figure 7A-C**). The decline in the absolute numbers of naïve (CD44⁻CD62L⁺) CD8⁺ T cells was dose dependent, with higher Dex concentrations showing a more distinct decrease compared to vehicle-treated control. A similar effect was observed for CM (CD44⁺CD62L⁺) and EM (CD44⁺CD62L-) CD8⁺ T cells with a significant decrease in absolute cell numbers for higher concentrations (1 µM onwards).

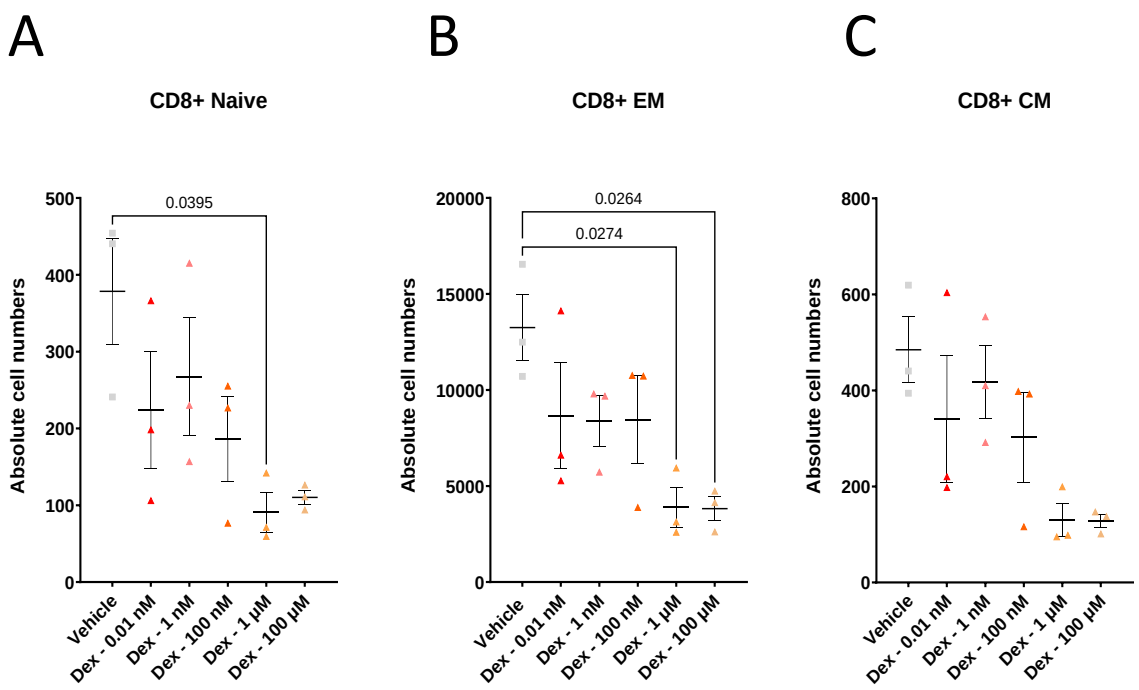


Figure 7. Flow cytometry analysis of CD8⁺ T cell subsets at 72 h after Dex treatment. Absolute cell number of CD8⁺ A) naïve B) effector memory C) central memory T cells in mouse spleen at 72 h after Dex treatment. Data are expressed as mean ± SEM of three technical replicates per group for absolute cell numbers. *Dex – dexamethasone; EM – effector memory; CM – central memory. Statistical analyses were conducted using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.*

These results indicate that Dex broadly suppresses CD8⁺ T cell subsets in a dose and time dependent manner. Notably, both naïve and CM CD8⁺ T cells were highly susceptible, showing significant depletion at high concentrations of Dex. The heightened sensitivity of naïve CD8⁺ T cells is consistent with previous studies demonstrating that GCs promote apoptosis in resting T cells by inducing pro-apoptotic molecules such as Bim and repressing survival genes like Bcl-2 (Brewer et al., 2003; Herold et al., 2006). EM CD8⁺ T cells also decreased with significant differences for higher Dex concentrations (1 µM onwards). Prior studies have reported that antigen-experienced effector and memory CD8⁺ T cells can be more resistant to GC-induced apoptosis due to their activation history and cytokine exposure (Tehseen et al., 2022, 2024). However, in our *in vitro* model, the absence of activating signals or homeostatic cytokines may have diminished this survival advantage, rendering memory subsets more vulnerable.

The observed dose dependent suppression highlights the dual nature of GCs: at physiological or low pharmacologic levels, they may support certain regulatory functions, but at higher levels, they induce widespread immunosuppression. This is especially relevant in clinical contexts

such as high-dose GC therapy or chronic stress, where $CD8^+$ T cell depletion may impair cytotoxic responses to infections and tumors (Aston et al., 2019; Hong et al., 2020; Iorgulescu et al., 2021).

3.2.5 Effects of Dex on IL7R

To evaluate the impact of Dex on T cell homeostasis and survival, the expression of IL7R or IL7Rα or CD127 marker on $CD4^+$ and $CD8^+$ T cells was further assessed. While the frequency of $CD4^+$ $IL7R^+$ and $CD8^+$ $IL7R^+$ T cells remained relatively unchanged across treatment groups (**Figure 8A, D**), a significant dose dependent reduction in absolute cell numbers was observed (**Figure 8B, E**). This decline was most pronounced at 72 h post-treatment, particularly at higher Dex concentrations (1 μM onwards), where both $CD4^+$ $IL7R^+$ and $CD8^+$ $IL7R^+$ T cells showed approximately a four-fold decrease compared to vehicle-treated controls (**Figure 8C, F**).

IL-7 and its receptor IL7Rα play a critical role in T cell survival, homeostasis, and memory formation by delivering anti-apoptotic signals, particularly through Bcl-2 induction (Kondrack et al., 2003; Maraskovsky et al., 1997; Schluns et al., 2000). Despite stable IL7R expression frequencies, the sharp reduction in absolute cell counts of $IL7R^+$ T cells following Dex treatment indicates that GCs impair IL-7-dependent survival pathways, potentially by downregulating downstream survival effectors or increasing susceptibility to apoptosis. GCs such as Dex are well known to induce apoptosis in T cells, particularly under prolonged or high-dose exposure. (Perandones et al., 1993). Although IL7R expression was preserved on a per-cell basis, overall T cell loss suggests that Dex overrides survival cues provided by IL-7, contributing to lymphopenia. This aligns with a previous study which shows that GCs reduce T cell numbers despite preserved IL7R expression, likely due to caspase activation which drives apoptosis (Amos et al., 1998). These findings suggest that $IL7R^+$ T cells are not intrinsically resistant to GC-induced apoptosis, and their apparent loss reflects global T cell depletion rather than selective downregulation of IL7R.

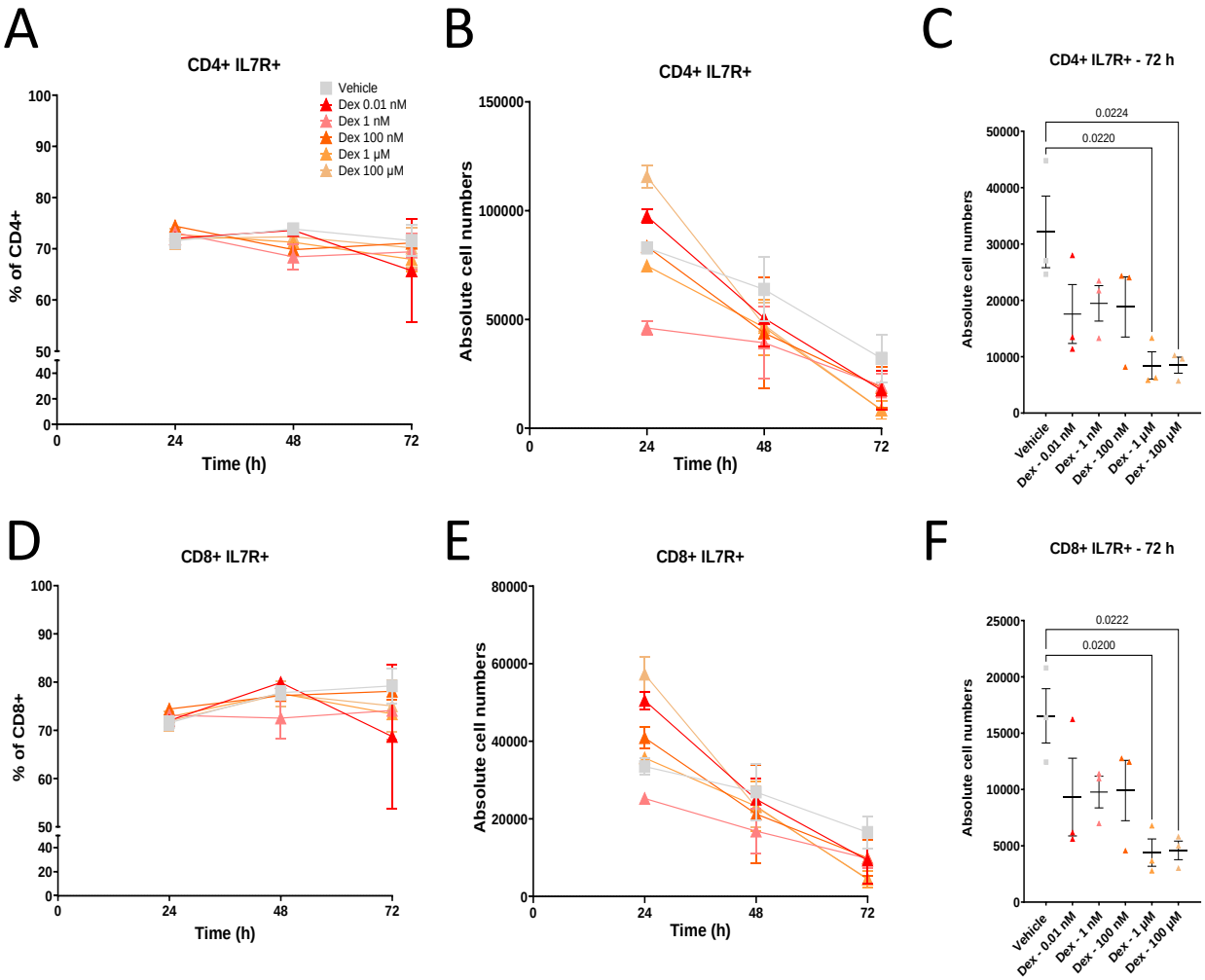


Figure 8. Flow cytometry analysis of CD4⁺IL7R⁺ and CD8⁺IL7R⁺ T cell subsets. A) Frequency and B) Absolute cell number of CD4⁺IL7R⁺ T cell populations in mouse spleen at 24, 48, and 72 h post Dex treatment. C) Absolute cell number of CD4⁺IL7R⁺ in mouse spleen at 72 h post Dex treatment. D) Frequency and E) Absolute cell number of CD8⁺Il7R⁺ T cell populations in mouse spleen at 24, 48, and 72 h post Dex treatment. F) Absolute cell number of CD8⁺IL7R⁺ in mouse spleen at 72 h post Dex treatment. Data are expressed as mean ± SEM of three technical replicates per group for absolute cell numbers. *Dex – dexamethasone. Statistical analyses were conducted using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.*

3.2.6 Effects of Dex on T cell proliferation

In order to study the effects of Dex on the T cell proliferation state, we analyzed the expression of Ki-67 on CD4⁺ and CD8⁺ T cells across the three time points. Ki-67 is a nuclear protein widely used to assess cellular proliferation due to its presence during the active phases of the cell cycle (G1, S, G2, and M) and its absence in the quiescence (G0) state (Scholzen & Gerdes,

2000). In $CD4^+$ T cells, Ki67 expression increased at 48 hours irrespective of the treatment group compared to 24 h but showed a decrease in both frequency and absolute cell number at 72 h (**Figure 9A, B**). At 72 h, there was a significant decrease in the Ki-67^+ $CD4^+$ T cells upon Dex treatment. A similar trend was observed in $CD8^+$ T cells, where Ki-67^+ cells modestly increased at 48 hours but showed a significant drop in absolute numbers at 72 hours in a dose-dependent manner (**Figure 9D–F**).

The increase in Ki-67 frequencies and numbers at 48 h after Dex treatment suggests that GCs initially promote the proliferation of $CD4^+$ and $CD8^+$ T cells. However, this increase was not sustained. By 72 h, it was followed by a reduction in both frequency and absolute cell numbers, indicating suppression of T cell proliferation. This biphasic pattern indicates that while GCs may permit or even stimulate T cell proliferation in the short term, prolonged or high-dose exposure ultimately leads to proliferative decline, most likely due to apoptosis of actively cycling cells. These findings are consistent with the known pro-apoptotic effects of GCs (Gruver-Yates et al., 2014; Perandones et al., 1993). An *in vivo* study further supports this, demonstrating that high doses of Dex inhibit Ki-67 expression correlating with increased apoptosis rates and reduced cell cycling (Xu et al., 2020).

Overall, our data indicates that while low or early exposure to Dex might not immediately suppress proliferation, prolonged or high-dose exposure leads to a strong anti-proliferative effect, reinforcing the immunosuppressive role of GCs in T cell biology.

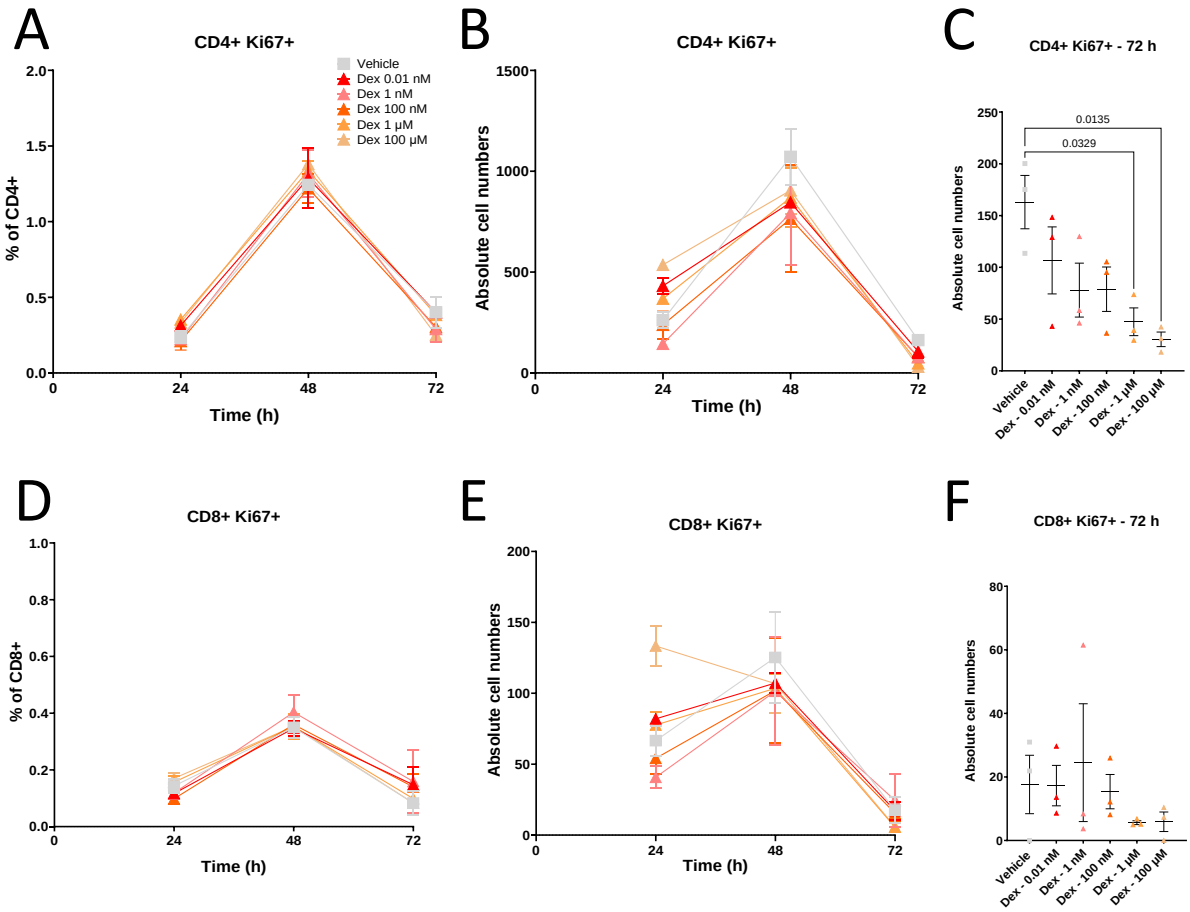


Figure 9. Flow cytometry analysis of CD4⁺Ki67⁺ and CD8⁺Ki67⁺ T cell subsets. A) Frequency and B) Absolute cell number of CD4⁺Ki67⁺ T cell populations in mouse spleen at 24, 48, and 72 h following Dex treatment. C) Absolute cell number of CD4⁺Ki67⁺ in mouse spleen at 72 h following Dex treatment. D) Frequency and E) Absolute cell number of CD8⁺Ki67⁺ T cell populations in mouse spleen at 24, 48, and 72 h following Dex treatment. F) Absolute cell number of CD8⁺Ki67⁺ in mouse spleen at 72 h following Dex treatment. Data are expressed as mean ± SEM of three technical replicates per group for absolute cell numbers. *Dex – dexamethasone. Statistical analyses were conducted using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.*

3.2.7 Effects of Dex on T cell activation and exhaustion

To evaluate how Dex modulated T cell activation and exhaustion, we analyzed the expression of CD69, CD25, and PD-1 markers on CD4⁺ and CD8⁺ T cell populations at 24, 48, and 72 h post-treatment. CD69, an early activation marker, oppositely showed a time dependent modulation as observed with Ki-67. In CD4⁺ T cells, CD69 expression decreased at 48 h from 24 h but showed a partial recovery at 72 hours in both frequency and absolute cell numbers (**Figure 10A, B**). In contrast, CD8⁺CD69⁺ showed a continuous decline over time in the

absolute cell number, but this change was not reflected in frequencies of the populations (**Figure 10D, E).** The most pronounced reduction in $CD69^+$ frequencies and cell numbers for both $CD4^+$ and $CD8^+$ subsets occurred at 72 h after the Dex treatment (**Figure 10A-F**). Higher concentrations of Dex (1µM and 100µM) exhibited a significantly stronger suppressive effect on both subpopulations compared to the vehicle-treated group (**Figure 10C, F**).

CD69 is rapidly upregulated upon engagement with a T cell receptor, signifying recent activation and immune response (Cibrián & Sánchez-Madrid, 2017). The decline in frequency and absolute cell numbers of $CD4^+CD69^+$ at 48 h post-treatment with Dex supports prior studies that showed Dex reduced the expression of CD69 on $CD4^+$ and $CD8^+$ T cells in activated populations, both *in vitro* and *in vivo* (Iorgulescu et al., 2021; Meyer-Heemsoth et al., 2023). Partial recovery of $CD69^+CD4^+$ T cells by 72 h may reflect compensatory reactivation or survival of resistant subsets.

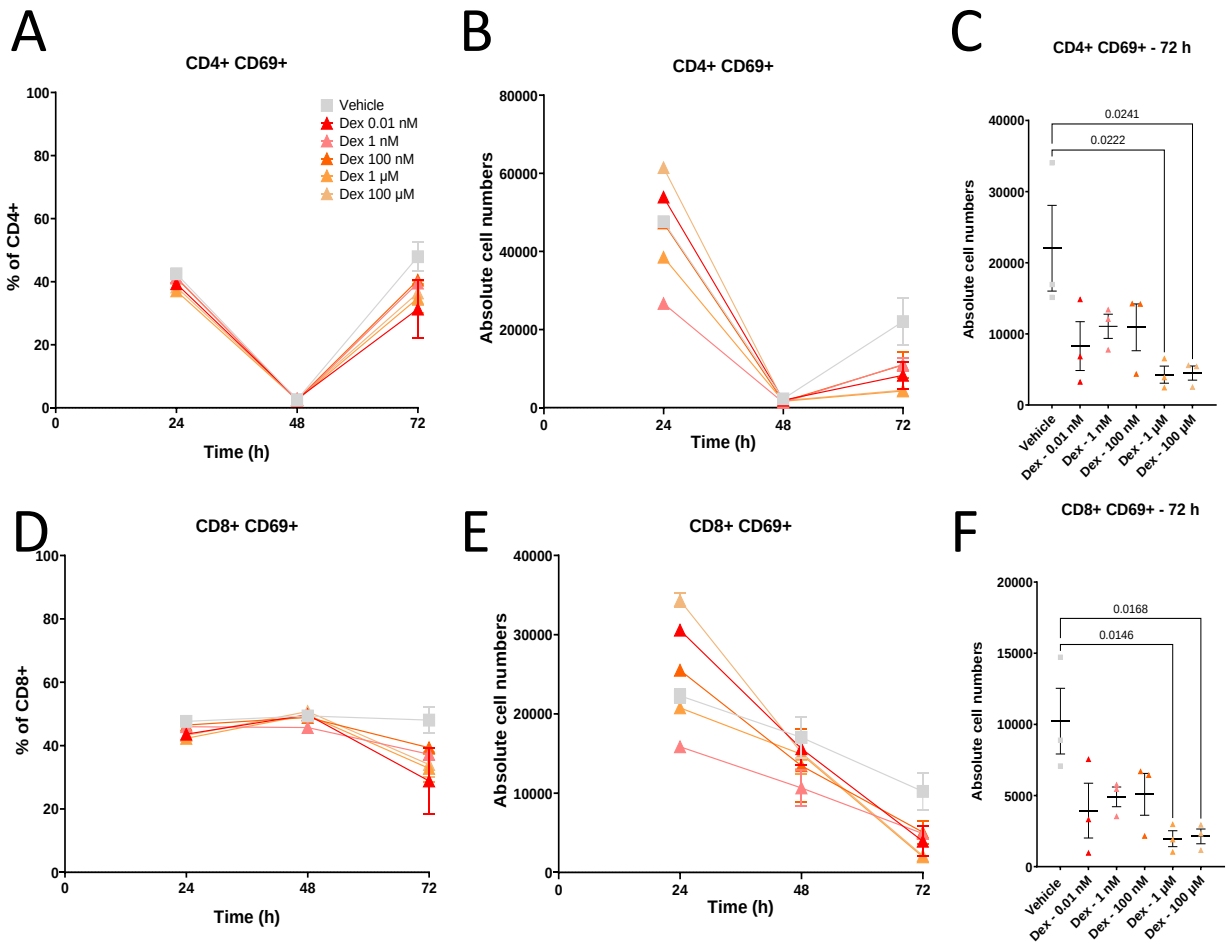


Figure 10. Flow cytometry analysis of $CD4^+CD69^+$ and $CD8^+CD69^+$ T cell subsets. A) Frequency and B) Absolute cell number of $CD4^+CD69^+$ T cell populations in mouse spleen at 24, 48, and 72 h following Dex treatment. C) Absolute cell number of $CD4^+CD69^+$ in mouse spleen at 72 h following Dex treatment. D) Frequency and E) Absolute cell number of

$CD8^+CD69^+$ T cell populations in mouse spleen at 24, 48, and 72 h following Dex treatment. F) Absolute cell number of $CD8^+CD69^+$ in mouse spleen at 72 h following Dex treatment. Data are expressed as mean ± SEM of three technical replicates per group for absolute cell numbers. *Dex – dexamethasone. Statistical analyses were conducted using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.*

On the other hand, CD25, a late activation marker, followed the opposite expression pattern. $CD4^+CD25^+$ T cells showed an increase in frequencies and absolute cell number at 48 h from 24 h but decreased at 72 h irrespective of Dex treatment (**Figure 11A, B**). On the contrary, there was a suppressive effect on the frequency and absolute number of $CD8^+CD25^+$ T cells over time (**Figure 11D, E**). The prominent effect of Dex on the absolute cell number of $CD4^+CD25^+$ and $CD8^+CD25^+$ T cells was observed at 72 h after the Dex treatment, with higher concentrations exhibiting a stronger decline compared to vehicle-treated controls (**Figure 11C, F**).

CD25, the IL-2 receptor α-chain, is expressed on T cell activation and is a hallmark marker for regulatory T cells (Létourneau et al., 2009). Dex facilitates the expression of $CD4^+CD25^+$, immobilizing late T cell activation. This result aligns with a previous study which shows that Dex treatment increases the proportion and ratio of $CD4^+CD25^+$ T cells relative to $CD4^+CD25^-$ (Chen et al. 2004) and synergizes with IL-2 to promote T cell survival (Chen et al. 2006). While $CD4^+$ T cells exhibit a transient increase in CD25 expression, peaking at 48 h, $CD8^+$ T cells demonstrate a continuous decline over time suggesting higher sensitivity GC-induced apoptosis. The subsequent decline at 72 h in $CD4^+CD25^+$ and $CD8^+CD25^+$ T cells, especially under high dose treatment, reflects the overarching suppressive and pro-apoptotic effects of GCs on activated T cells.

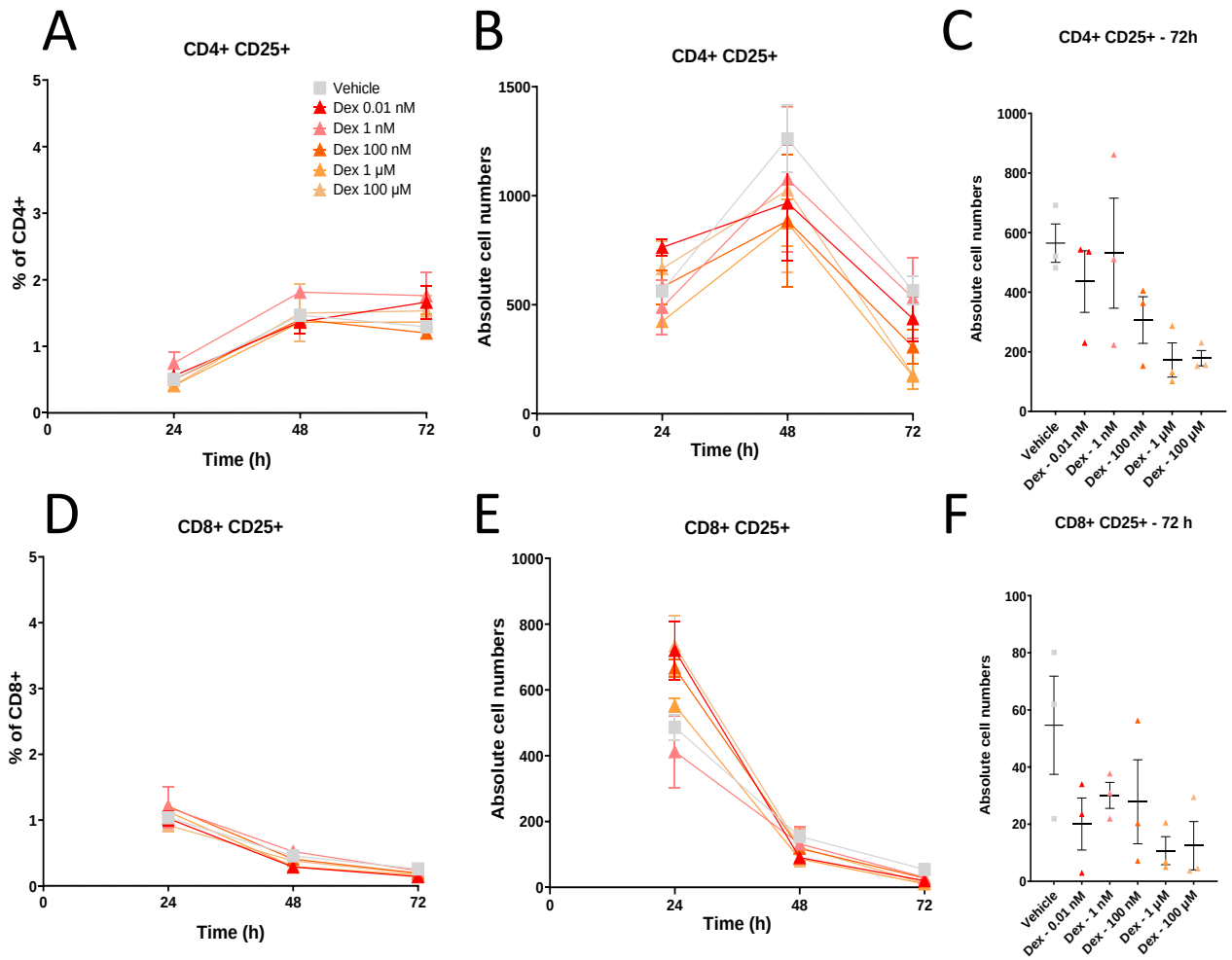


Figure 11. Flow cytometry analysis of CD4$^+$CD25$^+$ and CD8$^+$CD25$^+$ T cell subsets. A) Frequency and B) Absolute cell number of CD4$^+$CD25$^+$ T cell populations in mouse spleen at 24, 48, and 72 h following Dex treatment. C) Absolute cell number of CD4$^+$CD25$^+$ in mouse spleen at 72 h following Dex treatment. D) Frequency and E) Absolute cell number of CD8$^+$ CD25$^+$ T cell populations in mouse spleen at 24, 48, and 72 h following Dex treatment. F) Absolute cell number of CD8$^+$CD25$^+$ in mouse spleen at 72 h following Dex treatment. Data are expressed as mean ± SEM of three technical replicates per group for absolute cell numbers. *Dex – dexamethasone. Statistical analyses were conducted using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.*

PD-1, a key marker for T cell exhaustion, showed a similar trend for both CD4$^+$ and CD8$^+$ with an increase in frequencies and absolute cell numbers at 48 h after the Dex treatment (**Figure 12A, B, D, E**). However, by 72 h, the frequency and absolute cell number of CD4$^+$ PD-1$^+$ and CD8$^+$ PD-1$^+$ cells decreased in the presence of Dex as compared to vehicle treated group. High Dex doses exhibited a stronger decline in both subpopulations, showing a more drastic reduction in CD8$^+$PD-1$^+$ even at lower concentrations compared to vehicle-treated group (**Figure 12C, F**). PD-1 acts as an immune checkpoint for T cell exhaustion and effector

functions (Jubel et al., 2020; Terawaki et al., 2011). Our experiment showed an initial increase at 48 h post-treatment followed by a significant dose dependent decrease in both frequencies and absolute cell numbers of $CD4^+PD\text{-}1^+$ and $CD8^+PD\text{-}1^+$. This aligns with a previous study which showed that Dex facilitates the expression of PD-1 on mouse and human T cells *in vitro* with highest expression at 48 h (Xing et al., 2015).

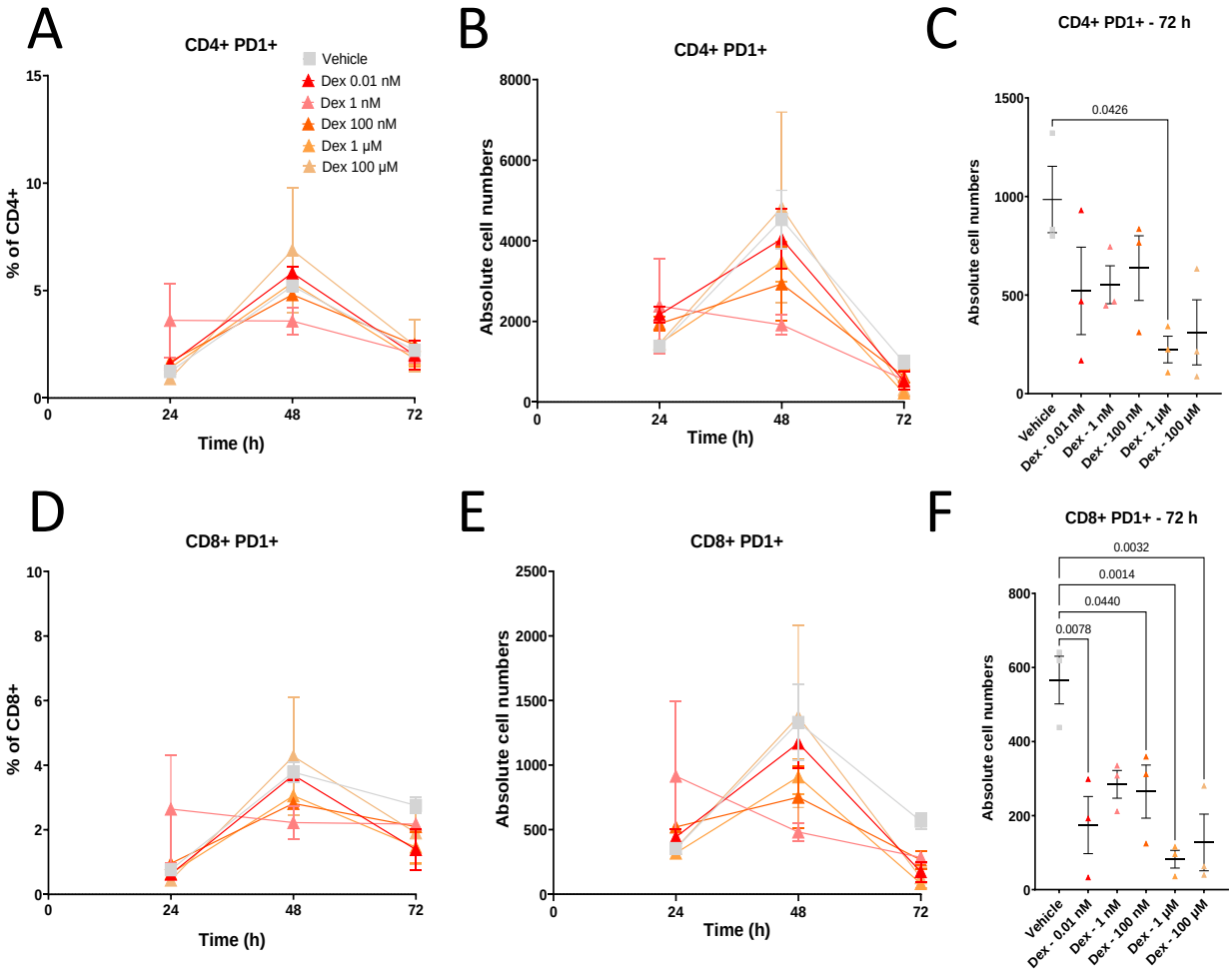


Figure 12. Flow cytometry analysis of $CD4^+PD\text{-}1^+$ and $CD8^+PD\text{-}1^+$ T cell subsets. A) Frequency and B) Absolute cell number of $CD4^+PD\text{-}1^+$ T cell populations in mouse spleen at 24, 48, and 72 h following Dex treatment. C) Absolute cell number of $CD4^+PD\text{-}1^+$ in mouse spleen at 72 h following Dex treatment. D) Frequency and E) Absolute cell number of $CD8^+PD\text{-}1^+$ T cell populations in mouse spleen at 24, 48, and 72 h following Dex treatment. F) Absolute cell number of $CD8^+PD\text{-}1^+$ in mouse spleen at 72 h following Dex treatment. Data are expressed as mean ± SEM of three technical replicates per group for absolute cell numbers. *Dex – dexamethasone. Statistical analyses were conducted using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.*

The combined analysis of CD69, CD25, and PD1 expression suggests Dex treatment exerts a complex, time and dose dependent effect on T cell activation and exhaustion. Dex modulates T cell activation in a stage-specific manner, repressing the early activation (CD69 expression), while maintaining or enhancing late activation (CD25 expression). The observed dynamics in PD-1 expression further indicate that Dex briefly promotes exhaustion-associated markers before inducing their downregulation, showcasing a broader immunosuppressive shift rather than selective checkpoint inhibition.

3.2.8 Effects of Dex on cytokine production

To examine how Dex influences T cell effector functions, we examined the production of intracellular cytokines, specifically IFN-γ and TNF-α, in $CD4^+$ and $CD8^+$ T cells.

$CD4^+IFN\text{-}\gamma^+$ T cells exhibited a marked decline in frequency and absolute cell number within 48 h of Dex treatment (**Figure 13A, B**). Interestingly, at lower Dex concentration at 72 h, there was an increase observed in the expression of IFN-γ on $CD4^+$ T cells, indicating that a low dosage of Dex stimulates while high doses inhibit cytokine production (**Figure 13C**). $CD8^+IFN\text{-}\gamma^+$ T cells showed a similar pattern with drastic declines in frequency, and absolute cell number were observed from 48 h after the administration of Dex, followed by a further decline at 72 h (**Figure 13D, E, F**).

IFN-γ is produced by activated T cells such as Th1-polarized $CD4^+$ and cytotoxic $CD8^+$ T cells (Keppel et al., 2015; J. Yu et al., 2006) and regulates the expansion of effector T cells (Sercan et al., 2006). In this study, Dex treatment led to rapid and progressive suppression of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells, observable as early as 48 h and exacerbated by 72 h in a dose dependent fashion. These findings are consistent with previous studies, which showed that Dex reduces Th-1 and IFN-γ production (Mazer et al., 2021; Skjolaas et al., 2002), indicating that Dex suppresses effector T cell function, particularly the cytokine-mediated responses.

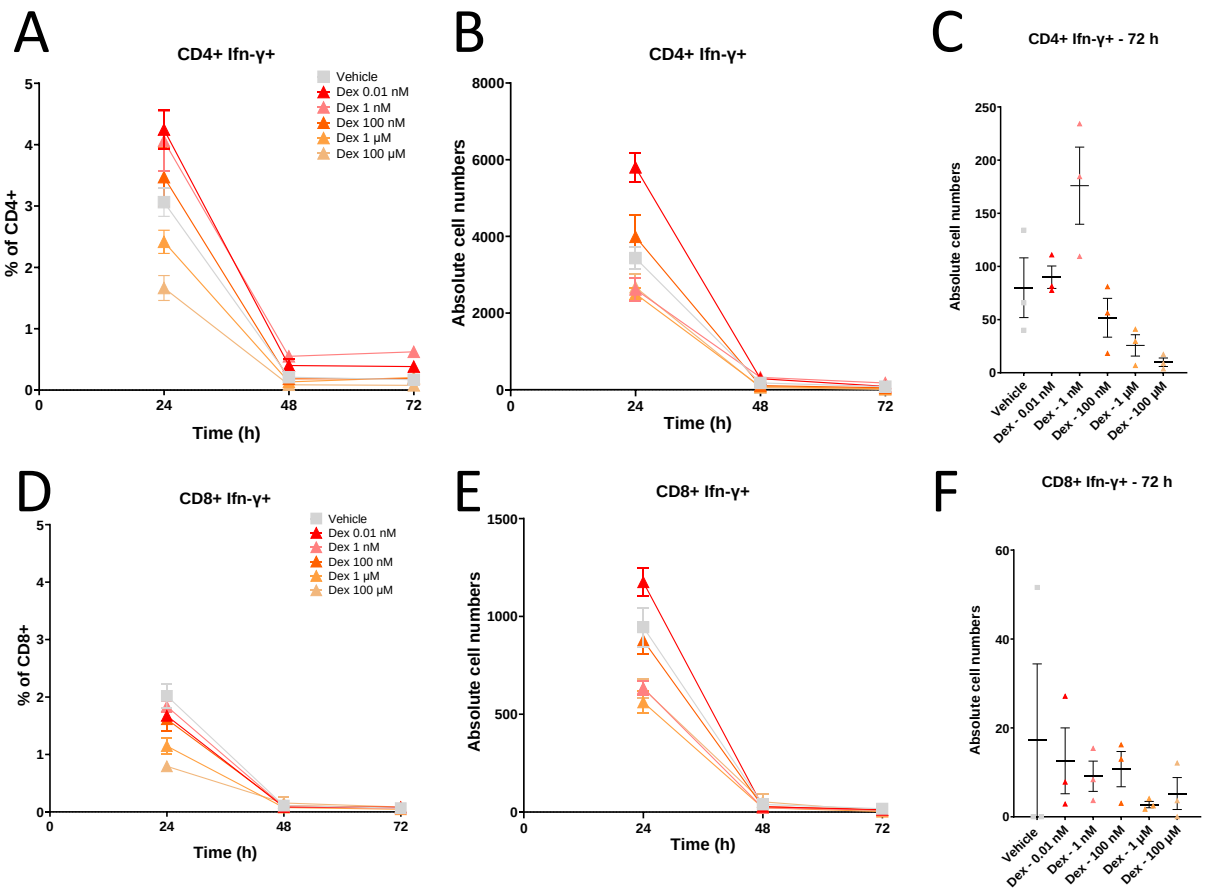


Figure 13. Flow cytometry analysis of CD4⁺IFN-γ⁺ and CD8⁺IFN-γ⁺ T cell subsets. A) Frequency and B) Absolute cell number of CD4⁺IFN-γ⁺ T cell populations in mouse spleen at 24, 48, and 72 h following Dex treatment. C) Absolute cell number of CD4⁺IFN-γ⁺ in mouse spleen at 72 h following Dex treatment. D) Frequency and E) Absolute cell number of CD8⁺IFN-γ⁺ T cell populations in mouse spleen at 24, 48, and 72 h following Dex treatment. F) Absolute cell number of CD8⁺IFN-γ⁺ in mouse spleen at 72 h following Dex treatment. Data are expressed as mean ± SEM of three technical replicates per group for absolute cell numbers. *Dex – dexamethasone; IFN-γ - interferon gamma. Statistical analyses were conducted using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.*

No significant change was observed in the frequencies of CD4⁺TNFα⁺ (**Figure 14A**). However, the absolute cell number of CD4⁺TNFα⁺ showed a decrease over time (**Figure 14B**). The distinct effects of Dex on TNF-α expression on CD4⁺T cells were seen at 72 h, although not significant (**Figure 14C**). While Dex-treated CD8⁺TNFα⁺ T cells decreased in both frequency and absolute cell numbers over time, an increase was observed in the vehicle-treated group at

72 h (**Figure 14D, E**). Higher concentrations of Dex showed a significant decrease in CD8$^+$TNFα$^+$ T cells compared to the vehicle-treated group (**Figure 14F**).

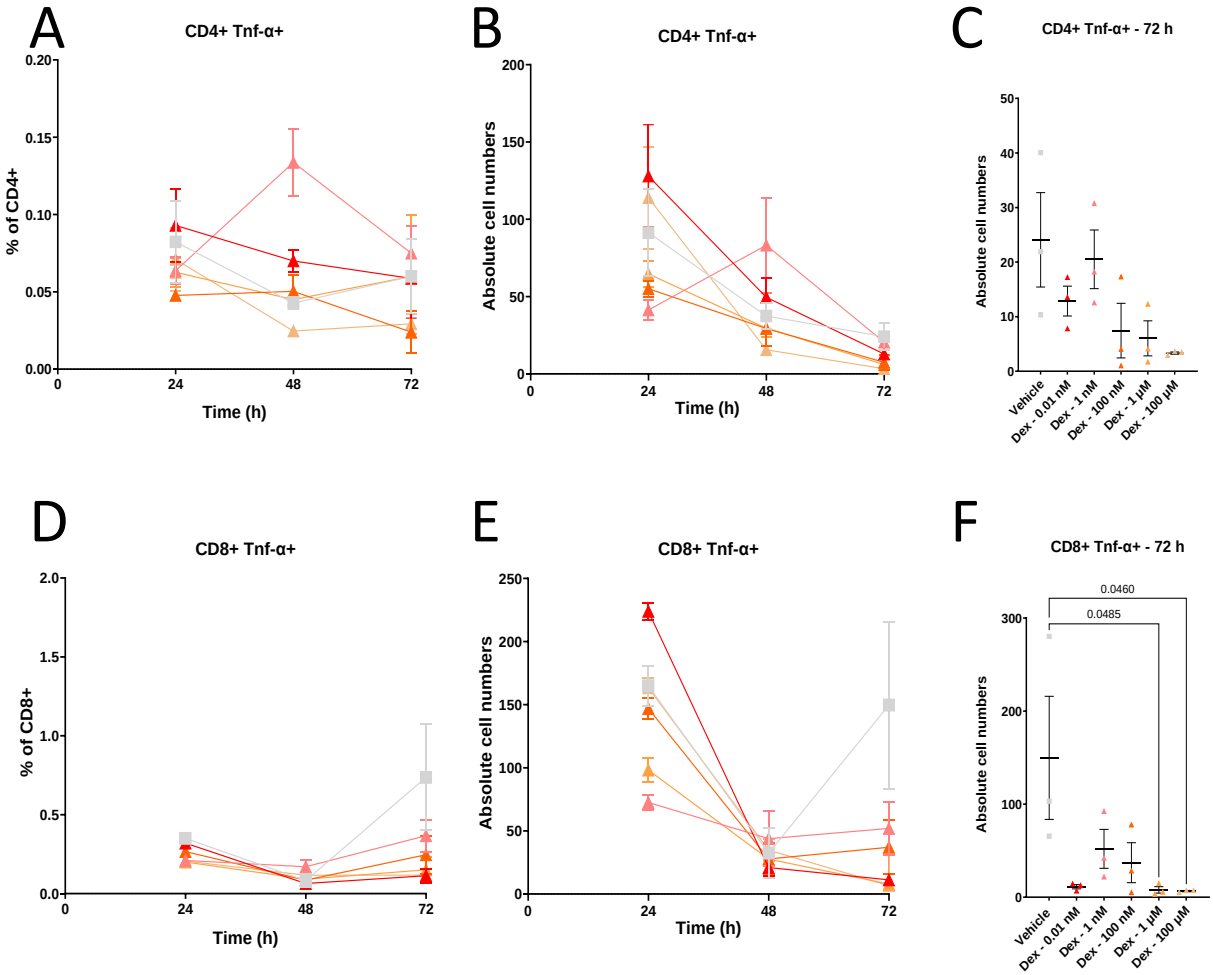


Figure 14. Flow cytometry analysis of CD4$^+$TNFα$^+$ and CD8$^+$TNFα$^+$ T cell subsets. A) Frequency and B) Absolute cell number of CD4$^+$TNFα$^+$ T cell populations in mouse spleen at 24, 48, and 72 h following Dex treatment. C) Absolute cell number of CD4$^+$TNFα$^+$ in mouse spleen at 72 h following Dex treatment. D) Frequency and E) Absolute cell number of CD8$^+$TNFα$^+$ T cell populations in mouse spleen at 24, 48, and 72 h following Dex treatment. F) Absolute cell number of CD8$^+$TNFα$^+$ in mouse spleen at 72 h following Dex treatment. Data are expressed as mean ± SEM of three technical replicates per group for absolute cell numbers. *Dex – dexamethasone; TNFα - tumor necrosis factor alpha. Statistical analyses were conducted using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.*

TNF-α is a key proinflammatory marker, expressed in several autoimmune and chronic inflammatory conditions (Bradley, 2008). It plays a role in activating of 11β-HSD1, an enzyme that activates GCs (Escher et al., 1997). In our data, Dex induced a gradual reduction in CD4$^+$TNFα$^+$ cell numbers and a significant decline in CD8$^+$TNFα$^+$ at higher Dex

concentrations, though frequency changes were not statistically significant. Our results align with a previous study in which Dex has been shown to suppress TNFα levels, contributing to its anti-inflammatory effects (Zhang et al. 2001).

These findings demonstrate the immunosuppressive capacity of Dex, highlighting its ability to dampen key proinflammatory cytokines such as IFN-γ and TNFα.

SUMMARY

In this study, we standardized an *in vitro* platform to model GC-induced immune responses, focusing on T cell responses to Dex. Splenocytes were isolated and stimulated under controlled culture conditions, followed by exposure to vehicle (control group) or various dexamethasone concentrations. Using a 22-color spectral flow cytometry panel, we tracked T cell viability, subset distribution, activation, proliferation, exhaustion, and cytokine production over 24, 48, and 72 hours.

Dex elicited a gradual time and dose dependent decline in splenocyte viability and total cell counts. Changes in specific T cell subsets were evaluated to examine how dexamethasone influences T cell survival and distribution. We observed that higher concentrations of dexamethasone had a more pronounced suppressive effect on naïve, CM, and EM $CD4^+$ and $CD8^+$ T cells, reflected by a dose-dependent reduction in their absolute numbers. In contrast, $CD4^+$ Tregs and Th1 cells showed a marked increase in absolute cell number under lower dexamethasone concentrations, suggesting a selective resistance of certain subsets and depletion of others under similar concentrations.

Dex treatment suppressed early and late activation in both $CD4^+$ and $CD8^+$ T cells while simultaneously promoting the state of exhaustion and suppression of cellular proliferation, indicating a shift toward a dysfunctional and immunosuppressed state under prolonged or high-dose GC exposure. This shift was further supported by the impairment of effector function wherein IFN-γ and TNF-α production in both $CD4^+$ and $CD8^+$ T cells was significantly reduced by 48 h and further suppressed by 72 h.

Since most studies investigating GC-induced immune alterations have been conducted *in vivo*, where systemic and environmental variables may confound direct cellular effects, we standardized an *in vitro* platform to dissect the time and dose dependent impact of Dex on splenic T cells in isolation. This work demonstrates that prolonged or high-dose GC exposure drives a shift from early activation to proliferative decline, exhaustion, and cytokine suppression. Additional experimentation needs to be done to validate the findings and further analyze the utility of the *in vitro* system in studying GC-related immunosenescence. Our platform would set a path for future research into GC-induced mechanisms and testing interventions aimed at preserving T cell functions following chronic stress or therapeutic steroid administration.

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