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Tesis Doctoral

INFLAMMATION INDUCED BY GRANZYME A IN ABDOMINAL POLYMICROBIAL AND RESPIRATORY VIRAL SEPSIS

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DOCTORAL DISSERTATION

INFLAMMATION INDUCED BY GRANZYME A IN ABDOMINAL POLYMICROBIAL AND RESPIRATORY VIRAL SEPSIS

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Biochemistry and Molecular Biology Program

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D. **Julián Pardo Jimeno**, Doctor en Ciencias e Investigador ARAID en el Centro de Investigaciones Biomédicas de Aragón (CIBA) de la Universidad de Zaragoza, y D. **Maykel Alberto Arias Cabrero**, Doctor en Ciencias e Investigador Post Doctoral en el Instituto de Investigación Sanitaria de Aragón (IIS).

CERTIFICAN:

Que la Tesis Doctoral titulada: **"Inflammation induced by Granzyme A in abdominal polymicrobial and respiratory viral sepsis"**, ha sido realizada por la Ingeniera en Procesos Biotecnológicos **Marcela Estefanía Garzón Tituaña** en el Departamento de Bioquímica y Biología Molecular y Celular de la Facultad de Ciencias de la Universidad de Zaragoza bajo nuestra dirección y que reúne, a nuestro juicio, las condiciones requeridas para que su autora pueda optar al Grado de Doctora en Ciencias por la Universidad de Zaragoza.

Zaragoza, 17 de mayo del 2023

Fdo. Julián Pardo Jimeno.

Fdo. Maykel Alberto Arias Cabrero

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"Cree en ti mismo y en lo que eres. Sé consciente de que hay algo en tu interior que es más grande que cualquier obstáculo"

Christian D. Larso

Que difícil se me hace ahora mirar hacia atrás y recordar todo lo que ha pasado desde que llegué a España. Es difícil pensar ahora mismo en todas aquellas personas a la que he conocido y que a su manera me han ayudado a llegar hasta aquí. Han sido muchas las personas con quienes he tenido la suerte de coincidir en estos años y sé que estos agradecimientos se quedarán cortos, sé que seguramente me dejaré a alguien fuera y es por eso que de antemano me disculpo con aquellas a las que no llegue a mencionar en estas líneas, sepan que siempre tendrán un lugar en mi corazón y las gracias son infinitas.

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ratones o quién salía más rápido del animalario. Como he dicho al inicio, no hay palabras que puedan expresar el inmenso cariño que te tengo, así que por ahora solo te diré mil gracias por todo Mike, no cambies nunca, la gente como tu escasea en el mundo y te deseo siempre lo mejor para ti y tu familia. **Llipsy**, gracias por siempre estar ahí para ayudarme, para darme un consejo, para reírnos un rato de todo o para bailar en el laboratorio y hacer los días más llevaderos. Por enseñarme que 10 minutos cubanos no son lo mismo que 10 minutos de Llipsy y que el AKTA es el mejor amigo que puede tener el equipo proteínas. Gracias por todo lo que me has enseñado, la purificación de granzima no sería la misma sin todos tus conocimientos. Eres y siempre serás la mamá de los patitos, la que siempre nos cuida y se preocupa por todos. Siempre es un placer acudir a ti en busca de consejos o una palabra de aliento que te ayude a ver las cosas desde otra perspectiva. Estás loca, pero te cuento un secreto, las mejores personas lo están y agradezco que tu locura haya llegado a alegrarme la vida. Mil gracias por estar ahí desde el principio y junto con Maykel ser unos amigos incondicionales. Os quiero muchísimo a los dos, nunca olviden que para lo que sea, siempre pueden contar conmigo y que no importa el lugar del mundo en el que me encuentre, siempre los llevaré presentes en mi corazón. Gracias por haber hecho de estos años una experiencia increíble y llevadera en los días más duros, por haberme tratado como familia y por quererme como quieren. Mil gracias para siempre.

Pilar, nunca me olvidaré de mi primer día en el CIBA contigo en el animalario. Muy pocas personas comienzan una amistad de la forma en la que tú y yo lo hicimos. Gracias por ser tan buena desde el día que nos conocimos y por ser una amiga extraordinaria. Eres grande amiga, sigue volando alto y lejos que te lo mereces. **Laurita**, mi rubia favorita, no sabes cuanto te quiero yo a ti, dentro y fuera del laboratorio contigo la vida es una aventura. Desde aprender a montar experimentos, usar el citómetro, jugar con los ratones en el animalario, los cafés interminables, las conversaciones a la hora de la comida, ir por los pasillos del CIBA en el carrito de los residuos, hasta ir a las fiestas de Casetas. Has estado ahí en mis mejores días, pero sobre todo en los peores, siempre paciente, siempre con una sonrisa, dispuesta a dar lo que sea necesario por verme feliz, siempre animándome y sosteniéndome para no caer. Lo mejor de todo ha sido que, aunque la vida ha continuado para las dos, siempre estamos la una para la otra a pesar de la distancia o de las ocupaciones y eso es lo más bonito que me llevo de toda esta experiencia, una amiga para toda la vida. Tenemos recuerdos para aburrir y lo único que puedo decirte ahora mismo es GRACIAS, por absolutamente todo. **Iratxe**, mi compañera de trabajo favorita, como aburrirme si te tengo a ti para siempre mantenerme alerta. Siempre aportando cositas con tu manera única de ver la vida y ese humor tuyo tan único que siempre me hace reír. Te he visto crecer y cambiar tanto en estos años que estoy segura de que conseguirás todo lo que te

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AKI:	Acute Kidney Injury
ALC:	Absolute Lymphocyte Count
ALI:	Acute Lung Injury
AP-1:	Adaptor Protein
APC:	Activated Protein C
APC:	Allophycocyanin
Ape1:	Apurinic Apyrimidinic Endonuclease 1
ARDS:	Acute Respiratory Distress Syndrome
Arg:	Arginine
Asp:	Aspartic Acid
AT:	Antithrombin
ATCC:	American Type Culture Collection
BAL:	Bronchoalveolar Fluid
BHI:	Brain Hearth Infusion
Bid:	BH3 Interacting-domain Death Antagonist
BMDMs:	Bone Marrow Derived Macrophages
CARS:	Compensatory Antiinflammatory Response Syndrome
CASP:	Stent-Induced Peritonitis in the Ascending Colon
CLP:	Ligation and Puncture of the Cecum
CLRs:	C-type lectin receptors
CMV:	Cytomegalovirus
COPD:	Chronic Obstructive Pulmonary Disease
COVID-19:	Corona Virus Disease 2019
CPE:	Cytopathic Effect
CRC:	Colorectal Cancer
CRP:	C-Reactive Protein
CSFs:	Colony-Stimulating Factor
cSMAC:	Central Supramolecular Activation Cluster
CTLA-4:	Cytotoxic T lymphocyte antigen-4
CTLs:	Cytotoxic T Lymphocytes
DAMPs:	Damage-associated molecular Patterns
DCs:	Dendritic Cells
DIC:	Disseminated Intravascular Coagulation

EBV:	Epstein-Barr virus
ECM:	Extracellular Matrix
EDHF:	Endothelium-Dependent Hyperpolarization Factor
Epi:	Epinephrine
ER:	Endoplasmic Reticulum
ERAD:	ER Associated Protein Degradation
ERK1/2:	Extracellular Signal-Regulated Kinase 1/2
FBS:	Fetal Bovine Serum
FIO2:	Fraction of Inspired Oxygen
FITC:	Fluorescein Isocyanate
G-CSF:	Granulocyte Colony-Stimulating Factor
GM-BMDM:	GM-CSF-Differentiated Macrophages
GM-CSF:	Granulocyte-Macrophage Colony-Stimulating Factor
GM-CSF:	Granulocyte-Monocyte Colony-Stimulating Factor
GzmA:	Granzyme A
GzmB:	Granzyme B
GzmK:	Granzyme K
GzmM:	Granzyme M
Gzms:	Granzymes
HIV:	Human Immunodeficiency Virus
HMGB2:	High Mobility Group Box 2
hnRNP:	Heterogenous Nuclear Ribonucleoprotein
HP:	Hypersensitivity Pneumonitis
HpEV:	Human Parechovirus
HSV:	Herpes Simplex Virus
HUVECs:	Human Umbilical Vein Endothelial Cells
I.p:	Intraperitoneal
ICAD:	Caspase-Activated DNase
ICON:	Intensive Care Over Nations
ICU:	Intensive Care Unit
IFN-γ:	Interferon-γ
IL-1:	Interleukins 1
IL-6:	Interleukins 6

IRF3: Interferon Regulatory Factor 3

IRF7: Interferon Regulatory Factor 7

KO: Knock Out

LPS: Lipopolysaccharide

Lys: Lysine

MAC: Membrane Attack Complex

MAMPs: Microbe-Associated Molecular Patterns

MAP: Mean Arterial Pressure

MAPK: Mitogen-Activated Protein Kinase

MARS: Mixed Antagonist Response Syndrome

Mc: Monoclonal

MCDK: Madin-Darby canine kidney

M-CSF: Macrophage Colony-Stimulating Factor

M-CSF: Macrophage Colony Stimulating Factor

MDSCs: Myeloid-Derived Suppressor Cells

Met: Methionine

MHC-I: Major Histocompatibility Complex class I

MHC-II: Major Histocompatibility Complex class II

MIF: Macrophage Migration Inhibitory Factor

MSS: Murine Sepsis Score

MTOC: MicroTubule-Organizing Center

MyD88: Myeloid Differentiation Protein 88

NF-kB: Nuclear Factor Kappa-light-chain-enhancer of activated B cells

NKs: Natural Killers

NKT: Natural Killer T Cells

NLRs: Nod-like receptors

NNT: Nicotinamide Nucleotide Transhydrogenase

NO: Nitric Oxide

Nor: Norepinephrine

NP: Nuclear Protein

OD: Optical Density

ON: Overnight

$PaCO_2$: Pressure of Carbon Dioxide

PAF:	Platelet-Activating Factor
PAI-1:	Plasminogen Activator Inhibitor 1
PaO2:	Partial Pressure of Oxygen
PAR:	Protease Activated Receptor
PARs:	Protease-Activated Receptors
PBLs:	Peripheral Blood Lymphocytes
PBMCs:	Peripheral Blood Mononuclear Cells
Pc:	Polyclonal
PCT:	Procalcitonin
PD1:	Programmed Cell Death Protein-1
PD-L1:	Programmed Cell Death Ligand-1
PE:	Phycoerythrin
Perf:	Perforin
PFA:	Paraformaldehyde
Phe:	Phenylalanine
PICS:	Persistent Inflammation, Immunosuppression and Catabolism Syndrome
PICU:	Pediatric Intensive Care Unit
PIM:	Pediatric Index of Mortality
PMS:	Polymicrobial Sepsis
PrC:	Protein C
PRRs:	Pattern Recognition Receptors
pSMAC:	Peripheral Supramolecular Activation Cluster
qSOFA:	quick SOFA
RA:	Rheumatoid Arthritis
RLRs:	RIG-receptors
ROS:	Reactive Oxygen Species
RSV:	Respiratory Syncytial Virus
SIRS:	Systemic Inflammatory Response Syndrome
SMAC:	Supramolecular Activation Cluster
SMCs:	Smooth Muscle Cells
SOAP:	Sepsis Occurrence in Acutely Ill Patients
SOFA:	Sequential Organ Failure Assessment
TcR:	T cell Receptor

TF:	Tissue Factor
TFPI:	Tissue Factor Pathway
TGF-β:	Transforming Growth Factor Beta
TGF-β:	Transforming Growth Factor-β
Th2:	T helper 2
TIGIT:	T cell Ig and ITIM domain
TIM-3:	T cell immunoglobulin domain and mucin domain 3
TIRAP:	TIR Domain-Containing Adaptor Protein
TLRs:	Toll-like receptors
TNFα:	Tumor Necrosis Factor Alpha
TRAM:	TRIF-Related Adaptor Molecule
Tregs:	T regulatory
TRIF:	TIR Receptor-Inducing Interferon-Beta
VCP:	Vasolin-Containing Protein
vWF:	von Willebrand Factor
VZV:	Varicella Zoster Virus
WHO:	World Health Organization

Sepsis is a serious global health problem. In addition to a high incidence, this syndrome has a high mortality and is responsible for huge health expenditure. The pathophysiology of sepsis is very complex and it is not well-understood yet. However, it is widely accepted that the initial phase of sepsis is characterized by a hyperinflammatory response while the late phase is characterized by immunosuppression and immune anergy, increasing the risk of secondary infections. Sepsis is defined as a life-threating organ dysfunction caused by a dysregulated host response to a bacterial, viral or fungal infection.

The granule exocytosis pathway is one of the most important defense mechanisms of the immune system against infected or transformed cells. It is specifically used by effector cytotoxic cells and consists in the liberation of cytotoxic granules that after the immunological synapse releases perforin allowing the entrance of granzymes in the cytosol of the target cell.

Perforin is a glycoprotein which function is to allow the entry of granzymes into the target cell causing cell death or inflammation. Granzymes are a family of serine proteases classified according to their cleavage specificity. Recent evidence suggests that GzmB is the one with the greatest cytotoxic capacity, while the cytotoxicity of others such as GzmA and GzmK is not clear. It has been suggested that GzmA could act as a proinflammatory mediator and could play an important role in the pathogenesis of sepsis. Furthermore, recent *in vitro* findings suggest that GzmK may act as a proinflammatory mediator. However, its role in sepsis is unknown.

We decided to analyze the role of GzmA in abdominal sepsis using a model of CLP induced sepsis and an *E. coli* induced sepsis model. In the CLP model, our results showed that GzmA deficient mice are protected from sepsis. In addition, WT mice treated with an extracellular GzmA inhibitor increased their survival, which was correlated with a reduction in the expression of proinflammatory cytokines. It was also demonstrated that the absence of GzmA did not influence the bacterial load in blood and spleen and GzmA did not affect bacterial replication in macrophages *in vitro,* suggesting that GzmA has no role in the bacterial control. Furthermore, the analysis of GzmA in lymphoid cells following CLP showed that it was mainly expressed in NK cells. We also analyzed the role of GzmK in an *E. coli* induced sepsis model due to the similarities of this protease with GzmA. Our results suggested that, although GzmA and GzmK are not involved in the control of the infection, mice deficient in GzmA or GzmK showed a lower sepsis score in comparison with WT mice suggesting the role of both proteases in sepsis pathophysiology. However, only GzmA deficient mice exhibit increased survival, which correlated with reduced expression of proinflammatory cytokines. Besides, the inhibition of GzmA during bacterial sepsis using serpinb6b improved survival and reduced the expression of IL-6 confirming that the inhibition of GzmA might be sufficient to improve survival irrespectively of the presence of other inflammatory granzymes, like GzmK. Mechanistically, we found that

active extracellular GzmA induces the production of IL-6 in macrophages by a TLR4 and MyD88 pathway. Finally, we analyzed the role of Gzms in a viral model of sepsis using Influenza A virus. Our results showed that the absence of GzmB and GzmK reduced survival suggesting the importance of both proteases in the control of the viral infection. While the absence of GzmA increased survival in influenza viral sepsis suggesting that this protease play an important role in influenza viral sepsis pathophysiology. Furthermore, we studied the role of Gzms after an influenza A infection to understand their importance in a secondary infection by *S. pneumoniae*. Our results showed that GzmA and GzmB deficient mice are protected against a secondary infection suggesting that both proteases play an important role in the immunosuppression state of sepsis.

La sepsis es un grave problema de salud mundial. Además de una alta incidencia, este síndrome tiene una elevada mortalidad y es responsable de un enorme gasto sanitario. La fisiopatología de la sepsis es muy compleja y aún no se comprende bien. Sin embargo, es ampliamente aceptado que la fase inicial de la sepsis se caracteriza por una respuesta hiperinflamatoria mientras que la fase tardía se caracteriza por una fase de inmunosupresión y anergia inmune, aumentando el riesgo de infecciones secundarias. La sepsis se define como una disfunción orgánica potencialmente mortal causada por una respuesta desregulada del huésped a una infección bacteriana, viral o fúngica.

La vía de exocitosis granular es uno de los mecanismos de defensa más importantes del sistema inmune frente a células infectadas o transformadas. Es utilizado específicamente por células citotóxicas efectoras y consiste en la liberación de gránulos citotóxicos que, luego de la sinapsis inmunológica, liberan perforina permitiendo la entrada de granzimas en el citosol de la célula diana.

La perforina es una glicoproteína cuya función es permitir la entrada de granzimas en la célula diana provocando la muerte celular o inflamación. Las granzimas son una familia de serin-proteasas clasificadas según su especificidad de corte. Se ha observado que la GzmB tiene mayor capacidad citotóxica, mientras que la citotoxicidad de otras como la GzmA y la GzmK no está clara. La GzmA podría actuar como mediador proinflamatorio y jugar un papel importante en la patogenia de la sepsis. Además, hallazgos *in vitro* recientes sugieren que la GzmK puede actuar como un mediador proinflamatorio. Sin embargo, se desconoce su papel en la sepsis.

En este estudio analizamos el papel de la GzmA en la sepsis abdominal utilizando un modelo de sepsis inducida por CLP y un modelo de sepsis inducida por *E. coli*. En el modelo de CLP, nuestros resultados mostraron que los ratones deficientes en GzmA están protegidos contra la sepsis. Además, los ratones WT tratados con un inhibidor extracelular de GzmA aumentaron su supervivencia, lo que se correlacionó con una reducción en la expresión de citoquinas proinflamatorias. También se demostró que la ausencia de GzmA no influyó en la carga bacteriana en sangre y bazo y la GzmA no afectó la replicación bacteriana en macrófagos *in vitro*, lo que sugiere que la GzmA no tiene ningún papel en el control bacteriano. Además, el análisis de GzmA en células linfoides después del CLP mostró que se expresaba principalmente en células NK. También analizamos el papel de GzmK en un modelo de sepsis inducida por E. coli debido a las similitudes de esta proteasa con la GzmA. Nuestros resultados sugirieron que, aunque la GzmA y la GzmK no están involucradas en el control de la infección, los ratones deficientes en GzmA o GzmK mostraron una puntuación de sepsis más baja en comparación con los ratones

WT, lo que sugiere el papel de ambas proteasas en la fisiopatología de la sepsis. Sin embargo, solo los ratones deficientes en GzmA exhiben una mayor supervivencia, lo que se correlaciona con una expresión reducida de citoquinas proinflamatorias. Además, la inhibición de GzmA durante la sepsis bacteriana utilizando serpinb6b mejoró la supervivencia y redujo la expresión de IL-6, lo que confirma que la inhibición de GzmA podría ser suficiente para mejorar la supervivencia independientemente de la presencia de otras granzimas inflamatorias, como GzmK. Respecto al mecanismo molecular, encontramos que la GzmA extracelular activa induce la producción de IL-6 en macrófagos por una vía TLR4 y MyD88.

Finalmente, analizamos el papel de las Gzms en un modelo viral de sepsis utilizando el virus de Influenza A. Nuestros resultados mostraron que la ausencia de GzmB y GzmK redujo la supervivencia, lo que sugiere la importancia de ambas proteasas en el control de la infección viral. Mientras que la ausencia de GzmA aumentó la supervivencia en la sepsis viral por influenza, lo que sugiere que esta proteasa juega un papel importante en la fisiopatología de la sepsis viral por influenza. Además, estudiamos el papel de las Gzms después de la infección por influenza para entender su importancia en una infección secundaria por la bacteria *S. pneumoniae*. Nuestros resultados mostraron que los ratones deficientes en GzmA y GzmB están protegidos contra una infección secundaria, lo que sugiere que ambas proteasas juegan un papel importante en el estado de inmunosupresión de la sepsis.

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INTRODUCTION

1 Introduction

1.1 Sepsis definition

Sepsis is one of the most important public health problems and the leading cause of deaths worldwide. A recent study reported 49 million cases and 11 million sepsis-related deaths in 2017, which represents approximately a 20% of all annual deaths globally (World Health, 2020). Furthermore, the average hospital-wide cost for sepsis treatment for a patient was estimated to be more than US$ 32 000, although these estimates were based on data from countries with high incomes (World Health, 2020). The latest studies of sepsis incidence and outcome in Europe date back to 2002 where the Sepsis Occurrence in Acutely Ill Patients (SOAP) study showed sepsis as the most prevalent disease in intensive care units (ICUs), which included higher mortality rates and higher economic costs than other pathologies of similar prevalence (Vincent et al., 2006). In another study, developed a decade later in 2012, comparing the SOAP study form 2002 with the Intensive Care Over Nations (ICON) audit from 2012, was affirmed that sepsis presence had slightly increased from 29.6% to 31.9%. Nevertheless, ICU stays, hospital mortality and 60-day mortality remained stable over time (Vincent et al., 2018). In Spain, the latest statistics show a sepsis incidence estimated in 367 cases per 100 000 inhabitants per year, with an associated hospital mortality of 12.8%. For severe sepsis, 104 cases per 100 000 inhabitants per year with a mortality of 20.7% have been established. For septic shock cases, an incidence of 31 cases per 100 000 inhabitant per year has been estimated, with an associated mortality of 45.7%. A total of 17 000 deaths per year due to sepsis, of which 70% of patients died during the first three days after diagnosis have been reported (Esteban et al., 2007). From 2008 to 2017, sepsis incidence increased 2.7 times, as well as the total costs in the Spanish public health system (Alvaro-Meca et al., 2018).

Despite its worldwide importance, public awareness of sepsis is poor. Even for experienced clinicians, diagnosing sepsis is difficult, as reflected in the evolution of its definition (Sabir, Wharton, & Goodacre, 2022). The concept of sepsis has evolved over time as medical knowledge of its physiopathology, aetiological factors, and clinical progression have increased and improved (World Health, 2020). A task force of experts in pathobiology, clinical trials, and epidemiology addressed the problem in the first international consensus definition for sepsis and septic shock (Sepsis 1). Since then, sepsis definition has been revised in 2001 (Sepsis 2) and in 2016 (Sepsis 3), being in the latter where the most recent definition of sepsis was established (Singer et al., 2016).

Sepsis is defined as a life-threating organ dysfunction caused by a dysregulated host response to infection. This definition emphasizes the non-homeostatic host response to infection, the potential lethality, and the need of urgent recognition. No current clinical measures reflect the concept of a dysregulated host response. However, many bedside examination findings and routine laboratory test results are indicative of inflammation and organ dysfunction (Singer et al., 2016).

Existing scores for inflammation or organ disfunction are used for sepsis diagnose being SOFA score (Sequential Organ Failure Assessment) the best known and used. SOFA is a simple and objective score that allows for calculation of the number and severity of organ dysfunction based on blood pressure, hypoxemia, creatinine, bilirubin, platelet count and the Glasgow Coma Scale. It has been validated as a predictor associated with in-hospital survival in diverse patient populations with various illnesses (Table 1) (Miller, Han, Peek, Charan Ashana, & Parker, 2021).

Following the SOFA score, organ dysfunction can be identified as an acute change in total SOFA score ≥2 points as a consequence of the infection. It has been determined that SOFA score baseline can be assumed to be zero in patients not known to have preexisting organ dysfunction. Patients with SOFA score of 2 or more have a mortality risk of 10% in general hospital population with presumed infection (Singer et al., 2016).

A quick diagnostic score has been defined to identify sepsis in patients with suspected infection who are at high risk for a poor outcome outside the ICU. This score is the qSOFA (quick SOFA) which uses three criteria, assigning one point for low blood pressure (SBP≤100mmHg), high respiratory rate (≥22 breaths per min), or altered mentation (Glasgow coma scale <15). The score ranges from 0 to 3 points. The presence of 2 or more in qSOFA points near the onset of infection is associated with greater risk of death or prolonged ICU stay (Singer et al., 2016).

The Third International Sepsis Consensus Definition Task Force (Sepsis-3) also redefined septic shock as a subset of sepsis in which underlying circulatory and cellular metabolism abnormalities are profound enough to substantially increase mortality. Patients with septic shock can be identified with a clinical assessment of sepsis with persisting hypotension requiring vasopressors to maintain MAP ≥65 mmHg and a serum lactate level >2 mmol/L (18mg/dL). With these criteria the hospital mortality is more than 40% (Singer et al., 2016).

1.1.1 Viral Sepsis

Though Sepsis-3 defines better the concept of sepsis and it helped to improve the clinical management of the disease, it fails to correctly define viral sepsis. The real incidence of viral sepsis, especially in the pediatric population, remains unknown. It is widely known that bacterial and fungal infections are the most common causes of sepsis and that it is unfrequently related to viral infections. This is because most of the large-scale epidemiological studies on sepsis have not considered cases of viral origin and did not specify the proportion of the viral sepsis. Microorganisms that contribute to sepsis can be identified in 59-69% of the cases, where bacteria usually are the responsible of 70% of sepsis cases and virus only contribute in approximately 1% of the documented cases. What is more, the number of negative bacteria cultures is up to 42% on septic patients, for whom the possible cause could be a virus (Gu, Zhou, Wang, Fan, & Cao, 2020; Gupta, Richter, Robert, & Kong, 2018; Lin, McGinley, Drysdale, & Pollard, 2018). The lack of information for viral sepsis is because viral diagnosis tests are not performed in septic patients, therefore viral sepsis is underdiagnosed (Lin et al., 2018). In an epidemiological study performed in children with severe sepsis, an infectious cause was only demonstrated in 65% of patients and from these group, one-third had a viral infection. The most frequent infected site was the respiratory tract (40%) and the bloodstream (20%) where rhinovirus, RSV (Respiratory Syncytial Virus), and adenovirus were most commonly isolated (Gupta et al., 2018; Weiss et al., 2015). In another similar study carried out in Australia and New Zealand a pathogen was identified in 50% of septic cases and one-fifth of the cases had a viral etiology where the most common virus detected where RSV, CMV (Cytomegalovirus), EBV (Epstein-Barr virus), HSV (Herpes Simplex Virus), VZV (Varicella Zoster Virus) and influenza (Amin & Amin, 2015). It has been reported that 16% of pediatric patients with sepsis and septic shock had first a viral disease (Gupta et al., 2018). It has also been reported that influenza virus is a leading cause of viral sepsis in PICU (Pediatric Intensive Care Unit) (Dorofaeff, Mohseni-Bod, & Cox, 2012; Gupta et al., 2018). RSV has been found to cause severe bronchiolitis and may present with sepsis, especially in children with history of premature birth, chronic lung disease or congenital heart disease of primary immunodeficiency (González-Granado et al., 2022; Perk & Özdil, 2018). In neonatal patients, sepsis has been observed with HSV, HpEV (Human Parechovirus) and enteroviral infections. Also, immunodeficient patients due to HIV (Human Immunodeficiency Virus) infection are also susceptible to viral sepsis where the most common viral infections detected are RSV, influenza, parainfluenza, adenovirus, CMV, EBV, and VZV (Gupta et al., 2018).

Finally, recent studies showed that respiratory viral infections have been underdiagnosed in patients with sepsis and septic shock. In these studies, viruses were detected in one-third of adult septic patients. Some of the viruses found, which can cause a severe disease, included influenza A, and B, respiratory syncytial virus, coronavirus, human metapneumovirus, parainfluenza virus types 1-3, adenovirus, enteroviruses and rhinovirus (Gu et al., 2020). In addition, the novel coronavirus outbreak has brought back coronavirus into the light. To date, there are seven types of coronaviruses which are known to cause human disease, four of them causing mild infections, while the other three (SARS-CoV, MERS-CoV and SARS-CoV-2) cause fatal cases (Dhama et al., 2020; Zhu et al., 2020).

It has been reported that patients suffering from sepsis in COVID-19 (Corona Virus Disease 2019) had higher rates of comorbidity compared with non-septic patients. In addition, the incidence of sepsis in COVID-19 is estimated at 11% and some predictors for developing sepsis, which are inflammatory markers, such as CRP (C-Reactive Protein), PCT (Procalcitonin) and ferritin are identified in COVID-19 (Abumayyaleh et al., 2021). In these patients, the immune response seems to be enhanced, which might be due to macrophage-activation syndrome, viral sepsis-induced immune paralysis and dysregulation of an intermediate functional state of the immune system. In another study, it was shown that 119 patients with COVID-19 presented and increase of inflammatory parameters. In patients infected with SARS-CoV-2 it was observed changes in the coagulation function such as an increase in the procoagulant activity due to inflammation which could contribute to thrombus formation (Abumayyaleh et al., 2021). On the other hand, similar to COVID-19, comorbidities like diabetes, chronic renal disease, obesity, hypertension, chronic cardiac diseases and lung diseases increased the risk of severe MERS-CoV infection. It has been reported that the virus receptor DPP4 is highly expressed on type I and II pneumocytes and alveoli as well as in epithelial cells of other organs such as thymus, liver, intestine and kidneys (Memish, Perlman, Van Kerkhove, & Zumla, 2020). MERS-CoV antigens have been detected by immunohistochemical staining on type I and II pneumocytes and *in vitro* studies showed that the virus has a significant replication in differentiated and undifferentiated primary cultures of human epithelial cells (Prescott et al., 2018). The lack of autopsies has limited the amount of information available for MERS-CoV infection, but it is safe to say that due to the fact that DPP4 receptor is widely expressed on epithelial cells it is possible that other organs are involved in the pathogenesis (Barth, Buja, Barth, Carpenter, & Parwani, 2021; Liang et al., 2020). Like SARS-CoV-2, MERS-CoV can overcome the immune response, produce high virus titers and induce a cytokine imbalance (Liang et al., 2020). With all available evidence it can be said that COVID-19, SARS-CoV-2 and MERS-CoV pathology can be considered a septic response.

Sepsis has been listed by the WHO (World Health Organization) and the World Health Assembly as one of the global health priorities for the next years, it is undeniable the importance of the understanding of sepsis epidemiological burden and to better comprehend it, it is urgent to understand viral sepsis epidemiology (Gu et al., 2020; Lin et al., 2018).

Table 1.1 SOFA score assessment

SOFA score	**0**	**1**	**2**	**3**	**4**
Respiration **PaO_2/FIO_2 mmHg (kPa)**	≥400 (53.3)	<400 (53.3)	<300 (40)	<200 (26.7) with respiratory support	<100 (13.3) with respiratory support
Coagulation **Platelets ($x10^3/mm^3$)**	≥150	<150	<100	<50	<20
Liver **Bilirubin (mg/dl)** **(µmol/l)**	< 1.2 (20)	1.2-1.9 (20-32)	2.0-5.9 (33-101)	6.0-11.9 (102-204)	>12.0 (204)
Cardiovascular **Hypotension**	No hypotension	MAP <70 mmHg	Dopamine ≤5 or dobutamine (any dose) *	Dopamine >5 Epi ≤0.1 Nor ≤0.1	Dopamine >15 Epi >0.1 Nor >0.1
Central nervous system **Glasgow coma score[1]**	15	13-14	10-12	6-9	< 6
Renal **Creatinine (mg/dl)** **(µmol/l) or urine output**	< 1.2 < (110)	1.2-1.9 (110-170)	2.0-3.4 (171-299)	3.5-4.9 (300-440) <500 ml/day	> 5.0 > (440) < 200 ml/day
Urine output, mL/d				<500	<200

Abbreviations: PaO2, partial pressure of oxygen; FIO2, fraction of inspired oxygen; MAP, mean arterial pressure; Epi, epinephrine; Nor, norepinephrine.

[1]Glasgow Coma Score range from 3 to 15; higher score indicates better neurological functions.

*Adrenergic agents administered for at least 1h (doses given in µg/kg/min).

1.2 Sepsis Pathophysiology

1.2.1 General overview

In contrast to a localized infection, sepsis is a multifaceted disruption of the strictly coordinated immunological balance between inflammatory and anti-inflammatory responses (Jacobi, 2002; Y. Y. Zhang & Ning, 2021). When a microorganism overcomes the natural defense

barriers, an inflammatory response that activates the host's immune system is produced. Bacteria, virus, fungi, or parasites may trigger sepsis where a systemic spillover of inflammatory mediators triggers a more generalized reaction. The systemic inflammatory response syndrome (SIRS) caused by an infection includes fever or hypothermia, leukocytosis or leukopenia, tachycardia and tachypnea. This inflammatory response is initiated by the recognition of the pathogen by cells of the innate immune system through different mechanisms, as it will be described in more detail in the next sections, which is subsequently enhanced due to a failure in the regulatory mechanisms, creating a detrimental amplification loop (Figure 1). Even though there is little evidence to determine if the pathophysiology of viral and bacterial sepsis differs from each other, it has been observed that the respiratory viral sepsis pathophysiology is limited to specific immune response to viral infections. For instance, for the influenza virus, haemagglutinins of different strains determine attachment to the epithelial of which the viral polymerase is associated for viral replication and cytokine production. Seasonal influenza (H3N2 and H1N1) targets epithelium in trachea, bronchi and bronchioles. Viral infections such as influenza virus can trigger the host immune system and recruitment of leukocytes through PRRs (Pattern Recognition Receptors) (Lin et al., 2018). Also, several studies have demonstrated that both toll-like receptors 2 and 4, the main receptors for Gram-positive and negative bacteria such as *S. pneumoniae, K. pneumoniae* (Branger et al., 2004), *S. aureus* (Kielian, Esen, & Bearden, 2005), *M. tuberculosis* (Pennini, Pai, Schultz, Boom, & Harding, 2006), *P. aeruginosa* (McIsaac, Stadnyk, & Lin, 2012), among others, are also related to influenza pathogenicity. Inflammatory response varies according to the viral strain, for instance, H5N1 virus produces a different response than H1N1pdm09 virus and H7N7 in blood macrophages, while H1N1pdm09 produces a most robust cytokine production than other strains (Kalil & Thomas, 2019). For example, in H5N1 infected patients, the production of IL-6, IL-1ra and MIP-1α varied greatly among blood donors and did not correlate with replicative ability of macrophages, while cytokines production in H1N1pdm09 infected patients is enhanced in all donors and induce higher levels of several cytokines compared with some H5N1 strains (Friesenhagen et al., 2012; Sakabe et al., 2011). The main mechanism involved in influenza pathophysiology is related with lung inflammation and damage as a consequence of viral infection of the respiratory epithelium, combined with the effects of lung inflammation due to exacerbated immune response aiming to control virus spreading. This inflammation can spread systemically, therefore, cardiac sequelae including the risk of myocardial disease have been observed during influenza infection (Kalil & Thomas, 2019). Similar to bacterial sepsis, endothelial damage and microvascular permeability changes have been observed in influenza infections, leading to edema and organ failure (Kalil & Thomas, 2019). For respiratory viral sepsis the organ dysfunction generated by sepsis includes, but is not

limited, to the lung. Extrapulmonary organ dysfunction includes acute kidney and heart injury among cases with influenza infection, acute kidney injury and thrombocytopenia associated to MERS-CoV infection, high viral load in gut and liver and moderate viral loads in the kidney among fatal cases with SARS-CoV infection and liver disfunction reported for syncytial virus infection (Gu et al., 2020). Finally, influenza virus infection increases by 6-fold the progression to secondary bacterial sepsis due to immunoparalysis. It is so that in adults with severe influenza-induced organ failure and in pediatric patients with high PIM (Pediatric Index of Mortality) scores and acute renal failure have greater risk of mortality (Kalil & Thomas, 2019).

There is not a complete understanding of the inflammatory dynamics that occurs during sepsis. At first, it was thought that the initial response in sepsis was a hyper-inflammatory state quickly followed by an antiinflammatory or immunosuppressive state (Hotchkiss & Nicholson, 2006; Nedeva, Menassa, & Puthalakath, 2019). It was postulated that early deaths during sepsis were due to excessive and uncontrolled release of proinflammatory cytokines such as CXCL-10, CXCL8/IL-8, TNFα (Tumor Necrosis Factor Alpha), IL-1 and IL-6 (Interleukins 1 and 6) (Iskander et al., 2013; Megha, Joseph, Akhil, & Mohanan, 2021). Broad antiinflammatory treatments have been investigated over the years such as the administration of high doses of glucocorticoids at the onset of sepsis, which has been proved unsuccessful. Nevertheless, glucocorticoids in lower doses have been found to have a beneficial effect in patients with septic shock. However, if the benefit of this treatment is through inhibition of inflammation or by counteracting a relative steroid refractoriness occurring during sepsis, remains unknown. Active agents against the endotoxin molecule were also studied which showed a lack of efficacy. Most recently, several clinical trials have focused on inhibiting specific host proinflammatory mediators (TNF, interleukins) where there were not convincing benefits observed, but when pooling the results of these trials it is suggested that this approach has a marginal effect, supporting a role for excessive inflammation in sepsis (Freeman & Natanson, 2000). The failure of antiinflammatory therapies to control sepsis in clinical trials has raised questions about whether mortality in sepsis is actually due to an uncontrolled inflammatory response (Angus & van der Poll, 2013; Nedeva et al., 2019; Remick, Bolgos, & Siddiqui, 2003). Due to all these drawbacks, the understanding of the traditional concept of the proinflammatory versus the antiinflammatory response has quickly evolved in the last years.

Subsequently, the multimodal hypothesis of sepsis was proposed. The SIRS (Systemic Inflammatory Response Syndrome), would be temporarily followed by CARS (Compensatory Antiinflammatory Response Syndrome), characterized by the production of antiinflammatory cytokines (McBride et al., 2020; Torres, Pickkers, & Poll, 2022; Venet & Monneret, 2018). Thus,

the risk of nosocomial infections would increase, as a prolonged state of immunosuppression would occur (D. Liu et al., 2022).

Experimental and clinical findings demonstrated that both pro and antiinflammatory cytokines are released in early stages of sepsis (Gogos, Drosou, Bassaris, & Skoutelis, 2000). In addition, immunosuppression signs are already observed in the acute stage of sepsis supporting the presence of MARS (Mixed Antagonist Response Syndrome) (Osuchowski, Craciun, Weixelbaumer, Duffy, & Remick, 2012). Therefore, sepsis does not progress with a previously determined disease pattern but needs to be perceived as a highly dynamic biological process where inflammatory and antiinflammatory responses are highly related and occur simultaneously (Torres et al., 2022).

For instance, together with acute phase protein release and complement activation, endothelial activation and TF (Tissue Factor) expression is promoted in monocytes, macrophages and endothelial cells and release to the circulation. In addition, microparticles of activated or apoptotic cells are also released to the circulatory system (Figure 1) (Iba & Levy, 2020; Joffre, Hellman, Ince, & Ait-Oufella, 2020).

The expression of the TF in the circulatory system will trigger the activation of the coagulation cascade, causing an imbalance in homeostasis, with a displacement towards a procoagulant state characterized by the formation of large amounts of thrombin and by the decrease of the anticoagulant systems and fibrinolytics (Nduka & Parrillo, 2009; Willers et al., 2021). This results in the intra and extravascular formation of fibrin, a process known as hypercoagulability. This accumulation of fibrin at the microvascular level is often associated with the development of multi-organ dysfunction and failure due to microcirculation disturbances (Abraham, 2000; Bray, Sartain, Gollamudi, & Rumbaut, 2020). Paradoxically, this sustained procoagulant state leads to coagulation factor consumption and platelet dysfunction, leading to the bleeding disorders that occur in this disease (Marcel Levi, Schouten, & van der Poll, 2008; Sang, Roest, de Laat, de Groot, & Huskens, 2021).

In addition to coagulation disorders, the endothelial cell is capable of responding to different stimuli from the pathogen or to signals produced by the host, such as cytokines and chemokines. This will lead to the activation of nuclear factor NF-kB and the expression on surface adhesion molecules such as P-selectin (CD62P), E-selectin (CD62E), intercellular adhesion molecule-1 (ICAM-1 or CD54) and vascular adhesion molecule-1 (VCAM-1 or CD106). All of them together will favor platelet adhesion and leukocyte infiltration from the bloodstream to the

underlying tissue, mainly in the precapillary territory and in the capillaries of organs such as the heart and lung (Norooznezhad & Mansouri, 2021).

In response to LPS or cytokines such as TNF-α, together with thrombin, an increase in endothelial permeability is also produced. This may favor the redistribution of fluids to the extravascular space, which constitutes one of the causes of hypotension. In addition, as a product of endothelial activation, vasodilator substances such as NO (Nitric Oxide), prostacyclin and EDHF (Endothelium-Dependent Hyperpolarization Factor) will be produced, which will favor the development of hypotension (Dolmatova, Wang, Mandavilli, & Griendling, 2020).

Taken together, coagulation disorders, interstitial edema, hypotension, and high levels of some proinflammatory cytokines will lead to reduced perfusion, tissue hypoxia, mitochondrial dysfunction, apoptotic, pyroptotic and necroptotic cell death, and autophagy. These disorders will be responsible for alterations in vital organs and functions. Virtually all organs can be damaged during the septic response, but the main injuries are renal, cardiovascular, pulmonary, hepatic and neurological (Wu et al., 2022).

Another of the most important complications in the septic response is immunosuppression that can lead to secondary infections. This complication is frequent in advanced stages of sepsis. Immunosuppression can be caused by increased apoptosis in lymphocytes and dendritic cells as well as by the expression of immune checkpoint inhibitors like PD1 (Programmed Cell Death Protein 1) (Darden et al., 2021).

1.2.2 Innate immunity and inflammatory mediators

The first step in the sepsis pathophysiology is the activation of the innate immune system. Once a pathogen enters the body it will eventually be spotted by the innate immune system which is the ensemble of cellular and humoral mechanisms that will immediately act against a threat trying to directly eliminate the pathogen and generating inflammation to enhance the immune response against the infection. The cells considered the key actors of the innate response are monocytes/macrophages, neutrophils, eosinophils, basophils, mast cells and NKs (Natural Killers) and its activation is made through the PRRs together with cytokine receptors (Chousterman, Swirski, & Weber, 2017; Jarczak, Kluge, & Nierhaus, 2021).

MAMPs (Microbe-Associated Molecular Patterns) or DAMPs (Damage-associated molecular patterns), from infecting microorganisms or necrotic/damaged cells respectively are the ligands for PRRs, responsible of generating a cascade of activation inducing an inflammatory response in host cells. Four types of PRRs have been identified in vertebrates: TLRs (Toll-like

receptors), NLRs (Nod-like receptors), RLRs (RIG-receptors) and the CLRs (C-type lectin receptors). The most studied PRR during sepsis has been TLR4 because it is the receptor for LPS (Lipopolysaccharide), which is the main component of gram-negative bacteria. The interaction of TLRs and its respective ligands induces a cascade of activation of various mediators such as MyD88 (Myeloid Differentiation Protein 88), TIRAP (TIR Domain-Containing Adaptor Protein), TRIF (TIR Receptor-Inducing Interferon-Beta), TRAM (TRIF-Related Adaptor Molecule) and several tyrosine kinases. As a result of these interactions, the activation of transcription factors such as NF-kB (Nuclear Factor Kappa-light-chain-enhancer of activated B cells), IRF3 (Interferon Regulatory Factor 3), IRF7 (Interferon Regulatory Factor 7), or AP-1 (Adaptor Protein), induce the expression of large number of inflammatory genes including those encoding TNF-α, IL-1β, IL-6, IL-12p40 and cyclooxygenase-2 for NF-kB transcription factor (T. Liu, Zhang, Joo, & Sun, 2017), systemic interferon response for IRF3 and IRF7 (Dalskov et al., 2020), and the activation of complement and coagulation pathways for AP-1 (Chousterman et al., 2017).

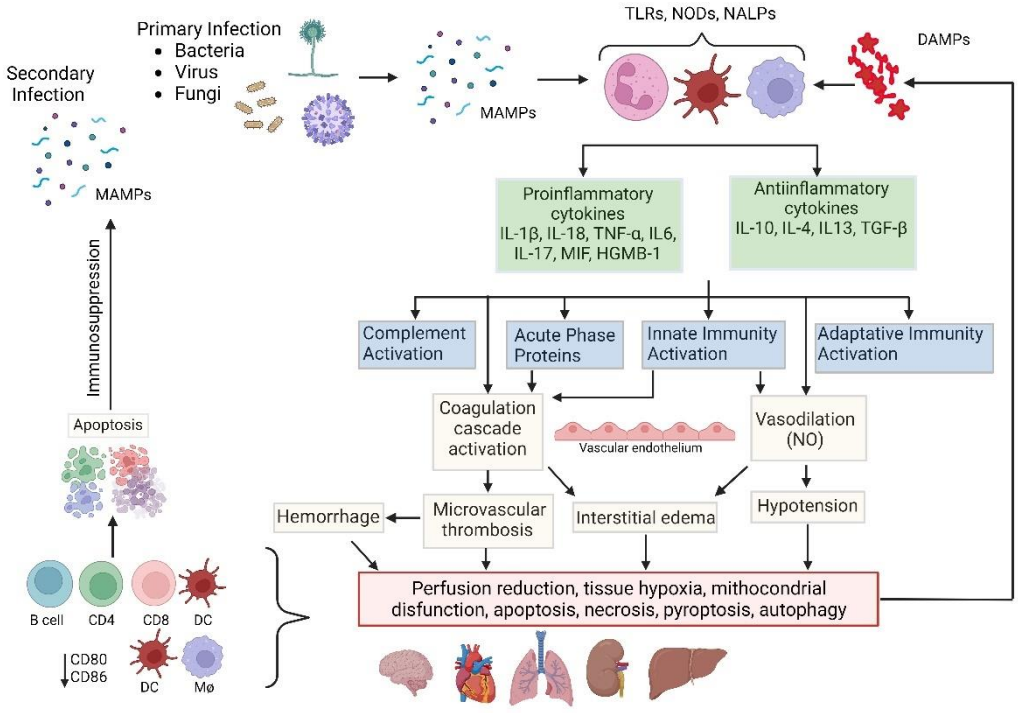


Figure 1.1 Sepsis physiopathology. Microbe Associated Molecular Patterns (MAMPs), generated by a primary infection due to bacteria, virus, or fungi, are recognized by TLR, NOD or NALP receptors of immune cells. This recognition triggers the expression of both, proinflammatory and anti-inflammatory cytokines. The expression of cytokines initiates several processes like complement activation, expression of acute phase proteins, activation of the innate and adaptative immunity. Together with the coagulation cascade activation and vasodilation, all these processes generate hypotension, interstitial edema, microvascular thrombosis, and hemorrhage, resulting in perfusion reduction, tissue hypoxia, mitochondrial dysfunction, apoptosis, necrosis, pyroptosis and autophagy. An important reduction in the number of immune cells due to uncontrolled cell death results in an immunosuppressive state that enhances susceptibility to secondary infections.

1.2.3 Pro-inflammatory and Anti-inflammatory Cytokines Release in Sepsis

The result of the activation of the first line of defense is an excessive release of cytokines and other inflammatory regulators. Cytokines regulate a great variety of inflammatory responses which includes the migration of immune cells to the locus of infection, an important step in containing a localized infection and preventing it from become systemic. A disrupted epithelial barrier allows pathogens and their products to invade the host organism, to disrupt regulatory mechanisms and finally, to cause organ disfunctions. Furthermore, immune, and inflammatory responses are highly related to physiological processes such as coagulation, metabolism, and neuroendocrine activation. For instance, coagulation processes disrupted by inflammation, significantly aggravates the deleterious effects of sepsis, and can result in a lethal DIC (Disseminated Intravascular Coagulation) (M. Huang, S. Cai, & J. Su, 2019; Schulte, Bernhagen, & Bucala, 2013).

Cytokines include autocrine, paracrine, endocrine activities and immunomodulating functions by binding to specific receptors on different cell types. They are divided in different categories: interleukins, chemokines, interferons, tumor necrosis factor and growth factors. Interleukins are released during infectious processes and encompass a large variety of proteins secreted by leukocytes and endothelial cells (among others). They contribute to cell signaling, proliferation, death and motility of immune cells and are divided in pro- and anti-inflammatory (Chousterman et al., 2017; Megha et al., 2021). Pro-inflammatory cytokines stimulate a systematic inflammation by acting as endogenous pyrogens that up-regulate the synthesis of secondary mediators and other cytokines in cells such as macrophages, fibroblasts, epithelial and endothelial cells, while at the same time they stimulate the production of acute phase proteins or attract inflammatory cells. The overproduction of these cytokinesis also able to cause an immunosuppressive state on the host due to the apoptosis generated in circulating and tissue lymphocytes such as B cells, $CD4^+$ T-cells and dendritic cells which may be responsible for late death on septic patients. On the other hand, anti-inflammatory cytokines are a group of immunoregulatory molecules involved in the prevention of harmful effects of persistent or excessive inflammation and may play an important role in sepsis (Chaudhry et al., 2013; Megha et al., 2021). The most important cytokines in sepsis are listed in table 2.

Chemokines, on the other hand, are not only specialized in recruitment but also in activation of immune cells. For instance, they induce leukocyte recruitment to the site of an infection and the release of immune cells from the bone marrow or spleen. Lack of chemokines lead to a quasi-immunosuppressed state that makes the body more susceptible to infections.

Moreover, growth factors are directly involved in the sepsis pathogenesis. The ones involved in the so called "cytokine storm" are the hematopoietic targeted CSFs (Colony-Stimulating Factor), GM-CSF (Granulocyte-Macrophage Colony-Stimulating Factor), M-CSF (Macrophage Colony-Stimulating Factor) and G-CSF (Granulocyte Colony-Stimulating Factor) (Chaudhry et al., 2013; A. Kumar, Taghi Khani, Sanchez Ortiz, & Swaminathan, 2022; Schulte et al., 2013).

The sequential release of cytokines is referred to a "cytokine cascade" which in the 1990s was associated to sepsis and to an exacerbated release of mainly proinflammatory cytokines such as TNF-α, IL-1, IL-6, IL-12, IFN-γ (Interferon-γ) and MIF (Macrophage Migration Inhibitory Factor). However, nowadays it is known that the profound proinflammatory response is counteracted by certain anti-inflammatory cytokines such as IL-10, TGF-β (Transforming Growth Factor-β) and IL-4, aiming to restore the immunological equilibrium (Megha et al., 2021; Schulte et al., 2013).

Table 1.2 Pro-inflammatory and anti-inflammatory cytokines found in sepsis

CYTOKINES	SECRETED BY	FUNCTION
INFLAMATORY CYTOKINES		
IL-1β	Activated macrophages, monocytes, neutrophils, erythrocytes, and platelets.	Adhesion molecules synthesis. Cell proliferation, differentiation, and apoptosis. Induces expression of iNOS and COX-2. Potent pyrogen Favors the synthesis of IL-6 and IL-8 (Chaudhry et al., 2013).
IL-1α	Keratinocytes, epithelial cells, endothelial cells, astrocytes, and myeloid cells.	Acts as an alarmin. Extracellular binding to IL-1R1 and nuclear function as a transcription factor (Cavalli et al., 2021)
IL-6	T-cells, macrophages, monocytes, DCs, endothelial cells and fibroblasts.	Important pyrogen. Induces hepatic synthesis of proteins in acute phase, regulates growth and development of hematopoietic cells. Differentiation of Th17 cells. Its level in serum is used as a sepsis marker.

		Stimulate immune response to trauma or to specific microbial molecules. Increases the release of corticosteroids by increasing the production of ACTH (Song et al., 2019)
TNF-α	Monocytes, macrophages, dendritic cells, NK cells, T-cells, neutrophils, B cells, endothelial cells, fibroblasts.	Induce apoptotic cell death, inhibits tumorigenesis, and viral replication. Induces expression of iNOS and COX-2 in endothelial cells. Stimulates the expression of adhesion molecules in endothelial cells. Induces expression of procoagulant proteins and tissue factor and reduces the expression of anticoagulants such as thrombomodulin (Idriss & Naismith, 2000).
IFN-I (α γ β)	Epithelial cells, fibroblasts, tissue resident macrophages and DCs, subcapsular sinus macrophages, parenchymal cells, marginal zone macrophages, monocytes, and stromal cells.	Activate antiviral state in infected and neighboring cells that limits spread of infection. Modulates innate immune responses like antigen presentation and NK cells functions while restraining proinflammatory pathways. Activates the adaptative immune system for the development of high-affinity antigen-specific T and B cell response (Schreiber, 2020).
IFN-γ	CD4, CD8 T-cells, and NK cells.	Critical for innate and adaptative immunity against viral and intracellular bacterial infections and tumor control (Schroder, Hertzog, Ravasi, & Hume, 2004).
GM-CSF (Granulocyte macrophage colony-stimulating factor)	Macrophages, T-cells, mast cells, endothelial cells, and fibroblasts.	Stimulates white blood cells. Induces stem cells to develop into neutrophils, eosinophils, basophils, and monocytes (Egea, Hirata, & Kagnoff, 2010).
IL-8/CXCL8	Macrophages, epithelial cells, and endothelial cells.	Induction of chemotaxis in its target cells. Neutrophil chemotactic factor (Bernhard et al., 2021)

IL-12	Dendritic cells, macrophages, and B-lymphoblastoid cells.	Differentiation of naïve T-cells into Th1 cells. Stimulates production of IFN-γ and TNF-α from T and NK cells. Reduces IL-4-mediated suppression of IFN-γ (Tugues et al., 2015).
IL-17	Helper T-cells, NKs and NKTs	Potent mediator in the regulation of hemopoiesis and inflammation by recruiting monocytes and neutrophils. Induces the expression of IL-6, TNF-α, IL-1β, G-CSF, GM-CSF and chemokines (Zenobia & Hajishengallis, 2015).
IL-18	Macrophage DCs, monocytes, endothelial cells, T-cells, B cells, osteoblasts, Kuffer cells, microglial cells	Stimulate NK cells and T-cells to release IFN-γ or type II interferon. Promotes the expression of adhesion molecules. Promotes the production of nitric oxide and chemokines (Dinarello, Novick, Kim, & Kaplanski, 2013).
IL-7	Fetal liver cells, stromal cells in the bone marrow, T cells, B cells, NK cells, ILCs, monocytes, macrophages, DCs, neutrophils, eosinophils, thymus, keratinocytes, enterocytes, and other epithelial cells.	Decrease in thymic cell account. Cell atrophy and impairment of T-cell functions. T-cell and B-cells apoptosis. Impairment of B differentiation potential. Decrease in $CD56^{bright}$NK cell count. Pronounced reduce of NK cell cytotoxicity. Impairment of ILC differentiation and generation. Inhibition of monocyte activity. Reduce of cytokine secretion. Decrease in DC, neutrophils, and eosinophils count. Recruitment delay of neutrophils. Inhibition of eosinophil survival. (Chen, Tang, Deng, Yang, & Tang, 2021)
HMGB-1	Macrophages, monocytes, NK cells, DCs, endothelial cells and platelets.	Maintenance of nucleosome structure and regulation of gene transcription. Important role in host response to infections.

		Active secretion of HMGB1 is regulated by NF-kB activation. Induces the expression of proinflammatory cytokines and ROS (Reactive Oxygen Species) in macrophages and neutrophils (Yang, Antoine, Andersson, & Tracey, 2013)
CXCL-10	Leukocytes, neutrophils, eosinophils, monocytes, epithelia, endothelial and stromal cells, and keratinocytes.	Promotes the chemotactic activity of CXCR3+ cells. Induces apoptosis. Regulates cell growth and proliferation as well as angiogenesis in infectious and inflammatory diseases (M. Liu et al., 2011).
CCL2	Endothelial cells, fibroblasts, epithelial cells, smooth muscle cells, mesangial cells, astrocytic cells, monocytic cells, microglial cells, monocytes, and macrophages.	Regulates the migration and infiltration of monocytes, memory T lymphocytes, and NK cells (Bose & Cho, 2013).
MIP-1α	Macrophages	Recruits inflammatory cells. Wound healing. Inhibition of stem cells. Maintenance of effector immune response (Bhavsar, Miller, & Al-Sabbagh, 2015).
ANTI-INFLAMMATORY CYTOKINES		
IL-1Ra	Monocytes, macrophages, neutrophils, keratinocytes, epithelial cells, and fibroblasts.	Specific inhibitor for IL-1α and IL-1β (Garlanda, Anders, & Mantovani, 2009).

IL-4	T-cells, mast cells, and basophils	Inhibits Th1 response. Stimulate proliferation of B-cells and T-cell. Induce the differentiation of $CD4^+$ T-cells into Th2 cells. Reduces the expression of IL-1β and nitric oxide in macrophages (Keegan, Leonard, & Zhu, 2021).
IL-10	$CD4^+$ Th2 cells, monocytes, macrophages, DCs, Tregs, NK, NKTs and B-cells	Inhibits the expression of Th1 cytokines, including IL-2 and IFN-γ. Inhibits IL-1β, TNFα and IL-6 release in macrophages and monocytes. Induces expression of IL-1 receptor (IRAP-1) and the soluble receptor of TNF-α (Ouyang & O'Garra, 2019).
IL-11	Bone marrow stromal cells, osteoblasts, brain cells, joints, and testes.	Hematopoietic, immunomodulatory, and epithelial cell-protective activities. Acts as Th2-type cytokine. Induces IL-4 expression and inhibits IFN-γ, IL-2, and Th1-type cytokines production (Allanki et al., 2021).
IL-13	Many cell types specially Th2 cells, NKTs and mast cells.	Controls the expression of different surface receptors in macrophages and monocytes. Reduces the expression of CD14. Reduces TNF-α, IL-1, IL-8, IFN-γ, IL-12 and MIP-1α expression. Improves the expression of mucus of endothelial cells (Tubau & Puig, 2021).
IL-35	Regulatory T -cells	Converts naïve T-cells into IL-35-producing induced regulatory T-cells. Reduces Th17 cell activity. May contribute to infectious tolerance (Choi & Egwuagu, 2021).
TGF-β	Multiple lineages of leukocytes and stromal cells	T- and B-cells proliferation and differentiation. Inhibits IL-2, IFN-γ and TNF-α production. Inhibits activation of lymphocytes and monocytes-derived phagocytes (Hao, Baker, & ten Dijke, 2019).

1.2.4 Dysregulation of hemostasis

DIC is a complication in septic patients found in a 25% to 50% of the cases. It is characterized by a generalized intravascular coagulation activation, microvascular damage, systemic thrombin generation and endothelial injury causing organ failure (Fujishima, 2016; Hoppensteadt et al., 2015; Rinaldi, Sudaryo, & Prihartono, 2022).

Inflammation is strongly linked to hemostasis, several inflammation mediators amplify thrombin generation, upregulate procoagulant pathways, downregulate physiological anticoagulants and suppress fibrinolysis. Also, coagulation proteases influence inflammation through their interaction with PARs (Protease-Activated Receptors). Inflammation also results in cell apoptosis or necrosis and the cell products released in the process are able to generate more inflammation, to induce coagulation, cell death and organ failure (Hoppensteadt et al., 2015; Posma et al., 2019).

As summarized in figure 2, inflammatory cytokines exert different effects on the coagulation pathway including direct platelet activation. For instance, proinflammatory cytokines alter the levels of coagulation inhibitors such as AT (Antithrombin) and can stimulate the production of PAI-1 (Plasminogen Activator Inhibitor 1), which results in the simultaneous suppression of fibrinolysis along with coagulation activation. In DIC, there is a decrease in the levels of PrC (Protein C), protein S and a downregulation of the expression of thrombomodulin at the endothelial surface, which is regulated by proinflammatory cytokines TNF-α and IL-1β (Hoppensteadt et al., 2015). Protein C is a natural anticoagulant that interrupts several of the pathophysiologic pathways in sepsis. Acquired protein C is present in the majority of septic patients and is associated with unfavorable outcomes (Catenacci, Sheikh, Patel, & Fox-Robichaud, 2022; Looney & Matthay, 2007). Additionally, protein S is a critical regulator of coagulation that functions as a cofactor for the APC (Activated Protein C) and to the TFPI (Tissue Factor Pathway) pathways. It has an important function in complement activation pathways and has direct anticoagulant functions by inhibiting the intrinsic tenase and prothrombinase complexes. Its deficiency causes life-threatening thrombotic conditions (Gierula & Ahnström, 2020; Majumder & Nguyen, 2021). Studies of sepsis in human patients are normally centered in circulating fibrinolytic markers where it has been reported a sustained increase in PAI-1 and also it turned out to be a prognostic marker. On the other hand, some evidence shows that thrombin causes resistance to fibrinolysis by forming more compact and less permeable clots and reducing plasmin formation, so enhanced thrombin generation is the hallmark of sepsis and might influence in the fibrin structure (Semeraro, Ammollo, Semeraro, & Colucci, 2012).

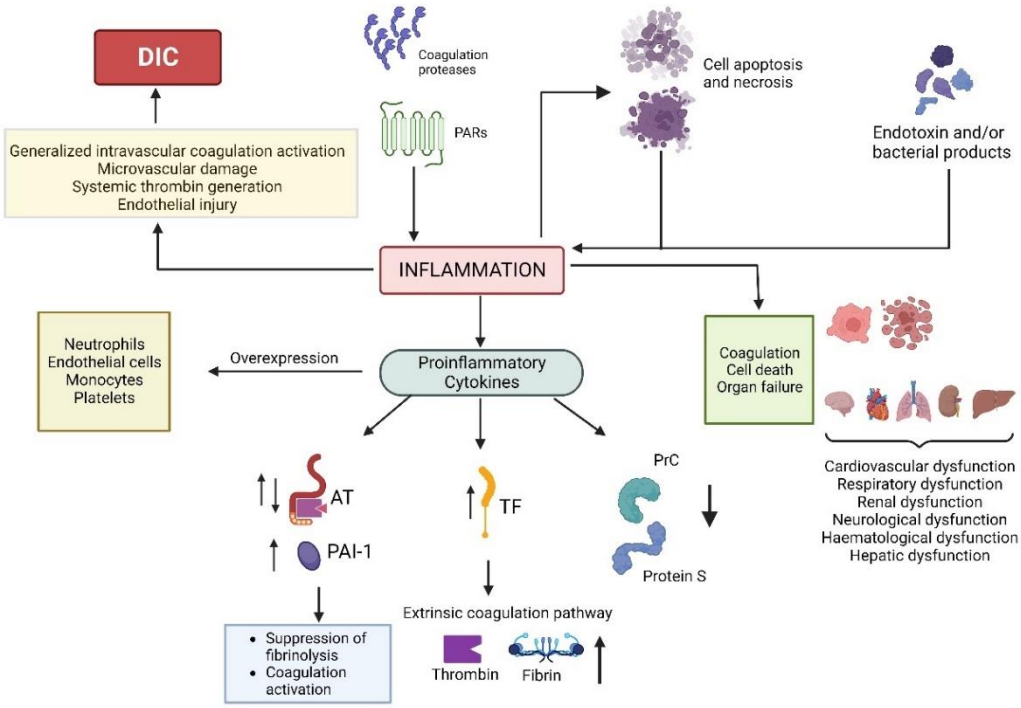


Figure 1.2 Hemostasis dysregulation. Acute inflammation, produced by apoptotic or necrotic cell residues or bacterial products, triggers the expression of proinflammatory cytokines which are responsible of several changes in the homeostasis such as: the dysregulation of antithrombin (AT) and the increase of plasminogen activator inhibitor 1(PAI-1) suppressing fibrinolysis and activating coagulation. An increase of tissue factor (TF) which interacts with the extrinsic coagulation factor and promotes the expression of thrombin and fibrin. And a decrease in the levels of protein C (PrC) and protein S. Protein C is a natural anticoagulant that interrupts several of the pathophysiologic pathways in sepsis. While protein S is a critical regulator of coagulation that functions as a cofactor for the activated protein C (APC) and to the tissue factor pathway (TFPI) pathways, it has an important function in complement activation pathways and has direct anticoagulant functions. Likewise, acute inflammation is responsible of generalized intravascular coagulation activation, microvascular damage, systemic thrombin generation and endothelial injury causing a disseminated intravascular coagulation (DIC). Coagulation proteases generated in this process act as inflammatory mediators through PAR receptors. Finally, acute inflammation disrupts coagulation, induces cell death and organ failure.

Although inflammation is the main target for sepsis treatments, in the last years the coagulation cascade has been considered as another target point which may be linked to the pro-inflammatory pathway as described above. The activation of the coagulation cascade in sepsis is manly driven by the TF. TF blockage in sepsis animal models has shown to attenuate the coagulant response and prevent death (Pawlinski & Mackman, 2010). TF interacts with factors of the extrinsic coagulation pathway leading via a cascade of protease activation, to thrombin and fibrin generation. The activity of TF has been detected in several cells that are related to sepsis pathogenesis such as neutrophils, eosinophils, activated platelets and more often, activated monocytes-macrophages. The presence of endotoxin and other bacterial products induce the expression of proinflammatory cytokines and this provokes the expression of TF in monocytes and endothelial cells which is thought to be the trigger for the development of DIC during sepsis (M. Levi, Schultz, & van der Poll, 2013; Semeraro et al., 2012; Wang, Bastarache, & Ware, 2008).

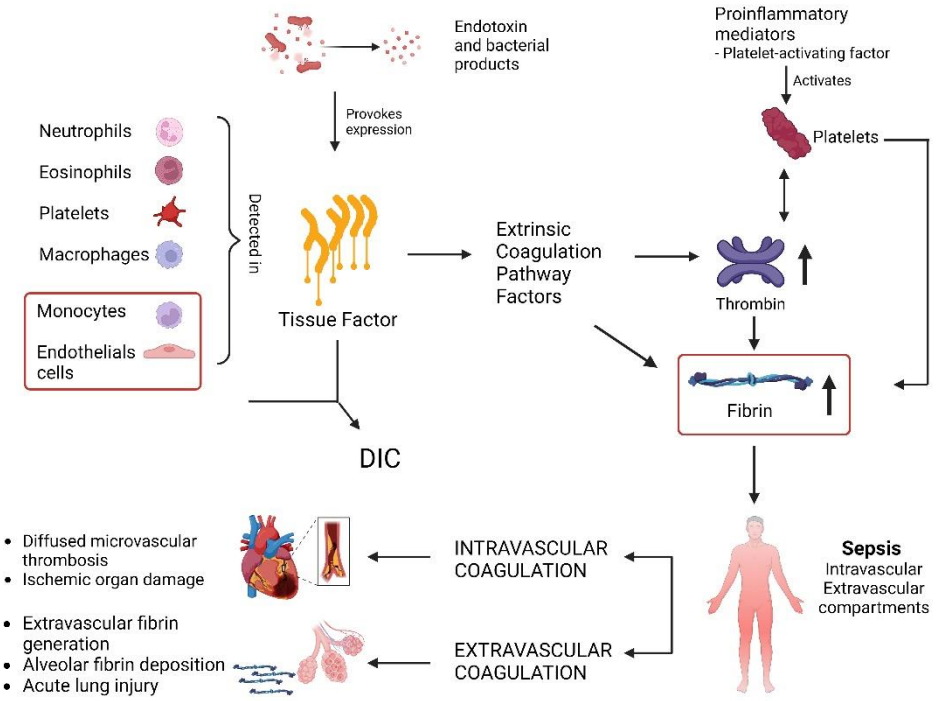


Figure 1.3 **Tissue factor and platelets in the coagulation pathway.** Endotoxin and bacterial products induce the expression of tissue factor (TF) mainly detected in neutrophils, eosinophils, platelets, active macrophages, monocytes and endothelial cells being in this two last the most abundant expression of TF in presence of endotoxin or cytokines which trigger the development of DIC during sepsis. TF interacts with factors of the extrinsic coagulation pathway that results in the expression of thrombin and fibrin. Proinflammatory mediators as platelet-activator factor activate platelets and once thrombin is formed, it will activate more platelets. Activated platelets may accelerate fibrin formation. The generation of fibrin is both a pathological and a physiological indication of sepsis that occurs in intravascular and extravascular compartments.

Platelets play an important role in the coagulation pathogenesis in sepsis. Platelets can be activated directly by proinflammatory mediators such as platelet-activating factor (M. Levi et al., 2013). Following the coagulation cascade, once thrombin is formed, it will also activate more platelets. Activation of platelets may accelerate the fibrin formation by another mechanism (Swieringa, Spronk, Heemskerk, & van der Meijden, 2018). Activated platelets not only alter fibrin structure, but also release inorganic polyphosphates that modify fibrin structure increasing its resistance to fibrinolysis. The generation of fibrin is both a pathological and a physiological indication of sepsis that occurs in intravascular and extravascular compartments. Intravascular coagulation is mainly characterized by diffused microvascular thrombosis which contributes to a widespread ischemic organ damage. On the other hand, the alveolar fibrin deposition is an important feature of the acute lung injury in sepsis which is an example of extravascular fibrin generation (M. Levi et al., 2013; Semeraro et al., 2012; Wang et al., 2008).

1.2.5 Cellular, tissue, and organ dysfunction

Organ dysfunction is the most important clinical event during sepsis and it is highly related to mortality and morbidity. It is the result of the global hyper-inflammatory response during sepsis and although several mechanisms have been proposed to explain and understand this event, the detailed molecular pathways leading to organ dysfunction are still not solved. While any organ system can be affected in sepsis, for practical reasons in the clinical evaluation, six organ systems are evaluated and have been more widely studied. Those organ systems are cardiovascular, respiratory, renal, neurological, haematological, and hepatic (Fujishima, 2016; Lelubre & Vincent, 2018).

- *Cardiovascular dysfunction*

Cardiovascular dysfunction is characterized by arterial hypotension which is mainly caused by hypovolaemia, a reduced vascular tone, and myocardial depression. The principal clinical presentation of cardiovascular dysfunction in septic patients is a poor tolerance to fluids administration, associated to a low central venous oxygen saturation (Lelubre & Vincent, 2018).

- *Respiratory dysfunction*

A decrease in the arterial partial pressure of oxygen known as hypoxaemia is the hallmark of pulmonary disfunction in septic patients. Clinically is recognized as hyperventilation (increased respiratory rate), which may lead to a low arterial $PaCO_2$ (Pressure of Carbon Dioxide). Hypoxaemia treatment requires oxygen administration and in the most severe cases, mechanical ventilation (Lelubre & Vincent, 2018).

ARDS (Acute Respiratory Distress Syndrome) or ALI (Acute Lung Injury) is a lung disfunction frequently associated with sepsis. Recent epidemiological studies show that ALI was complicated in 40.2% of patients with severe sepsis or septic shock and had a very poor outcome (Fujishima, 2016; Matuschak & Lechner, 2010). On the other side, ARDS was associated with an even poorer outcome than non-sepsis-related ARDS. The principal pathophysiology of ARDS is an increase in microvascular permeability that comes as a result of the dysregulation of cell-to-cell interaction or tissue destruction (Gonzales, Lucas, & Verin, 2015). Also, it has been shown that neutrophils play an important role in ARDS and in the dysfunction of other organs due to the release of granular enzymes, reactive oxygen metabolites, bioactive lipids and cytokines (Fujishima, 2016). Neutrophils can induce the formation of extracellular traps which can directly or indirectly injure tissues, leading to an increase in microvascular permeability and resulting in pulmonary edema. Some cytokines are related to ARDS pathophysiology such as IL-8, TNF-α and IL-1β. Along with neutrophils-mediated tissue injury, apoptosis and autophagy have been also involved in sepsis- induced tissue damage related to ARDS (Fujishima, 2016; Semeraro et al., 2012).

Influenza and SARS-CoV-2 viruses may be involved in the development of ARDS. The course of respiratory failure may differ between COVID-19 and influenza despite the several similarities they have (Maamar et al., 2023). Approximately 49-72% of pneumonia cases develop ARDS, which leads to higher morbidity and mortality rates. Influenza virus infection is a major and recurrent case of ARDS and it has been the focus of attention since the 2009 H1N1 pandemic (Gacouin et al., 2020). On the other hand, it has been reported that ARDS develops in 42% of COVID-19 patients, follows a predictable timeline, appearing at day 8 or 9 after symptoms onset (Gibson, Qin, & Puah, 2020).

- *Renal dysfunction*

In septic patients, renal dysfunction is clinically present as oliguria. The increase of urea and creatinine in serum are common and even the slightest creatinine concentration increase is associated with worse outcomes in critically ill patients (Lelubre & Vincent, 2018).

Kidneys are the main controllers of water and electrolytes metabolism under normal conditions. Therefore, under septic conditions, their role becomes even more critical for maintaining vital organs circulation, cellular electrolytes balance and for protecting the lungs from life-threatening pulmonary edema. AKI (Acute Kidney Injury) has been reported in 31-40% of patients with severe sepsis. The key features of AKI are a disturbed microcirculation and

hypoxia which leads to renal tubular epithelial cell injury and excessive inflammation leading to a downregulation of metabolism and cell cycle arrest (Fujishima, 2016).

- *Neurological dysfunction*

Cerebral dysfunction in sepsis is characterized by a mental state that includes disorientation, confusion and where also coma can be developed. It is important to notice that cerebral dysfunction can be present in absence of any other organ dysfunction, that it has a high mortality rate and that patients with this dysfunction often have prolonged cognitive and functional sequelae (Lelubre & Vincent, 2018).

- *Haematological dysfunction*

Most of sepsis patients have coagulopathy, which can range from subtle subclinical coagulations disorders to a prolongation of prothrombin time and activated partial thromboplastin time. Low platelet count and elevated D-dimer levels are some common features of haematological dysfunction, together with DIC which, as explained above, is characterized by widespread thrombosis in small and midsize vessels with simultaneous hemorrhage at various sites. DIC is indicative of severe sepsis and associated to a poor prognosis and higher mortality rates (Fujishima, 2016; Lelubre & Vincent, 2018).

- *Hepatic dysfunction*

During an infection, the liver plays an important role in modulating host defense, regulating inflammation and producing acute phase proteins such as C-reactive protein and serum amyloid A. Abnormalities in the liver caused by sepsis are primarily reflected by an increase in bilirubin levels. Sepsis-induced liver injury has a substantial effect in sepsis outcome. It is mainly caused by a reduced hepatocyte-mediated LPS clearance, increased release of proinflammatory cytokines that promote dysfunction of distal organs (lung injury) and to increased release of anti-inflammatory cytokines such as IL-10 (Fujishima, 2016; Lelubre & Vincent, 2018).

1.2.6 Immunosuppression

Sepsis survivors can suffer from multiple sequelae that can dramatically alter the life expectancy and the quality of life such as a prolonged state of immune suppression. It has been reported that patients who survive to the initial acute pro-inflammatory episode of sepsis frequently suffer from secondary infections or reactivation of latent viruses indicating that the immune system is unable to eradicate otherwise harmless or low-virulent microbial strains. Several causes for this condition have been proposed and studied in the last years. Nevertheless, the multiple suspected cause of immune suppression (Figure 3) makes difficult to choose

appropriate targets for treatment (Markwart et al., 2014). Long-term sepsis leads to immunosuppression which is characterized by different immune cell dysfunction and the activation of multiple signaling pathways.

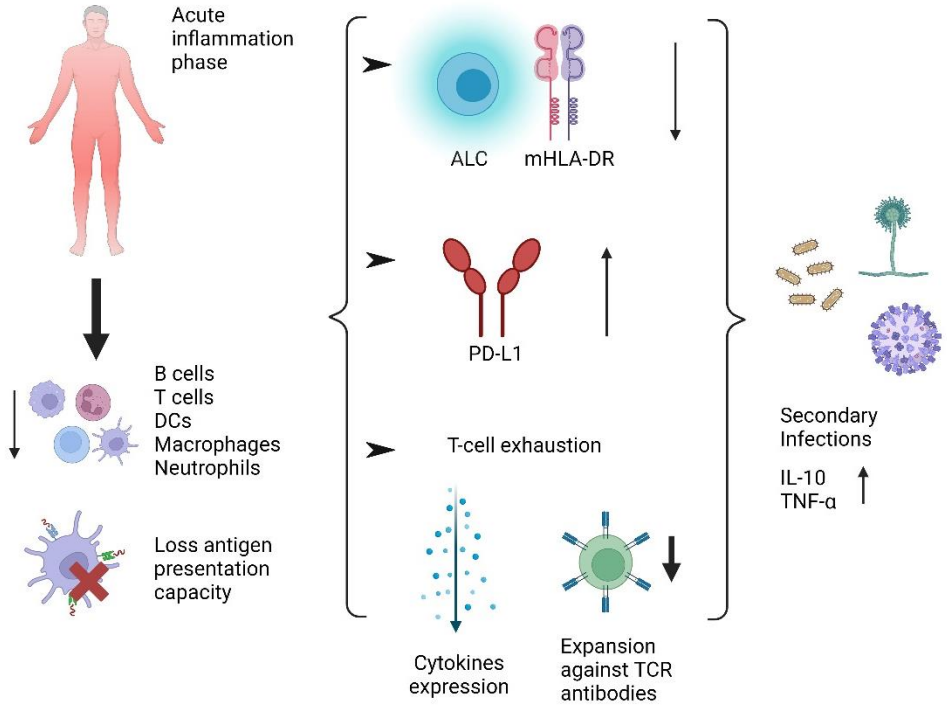


Figure 1.4 Immunosuppression state diagram. Acute inflammation phase generates a reduction in the number of immune cells like B cells, T cells, DCs, macrophages, neutrophils, among others, and also the loss of the antigen presentation capacity. The immunosuppressive state is characterized by a reduced absolute lymphocytes count (ALC) and monocyte membrane (m) HLA-DR expression beside an increase in soluble programmed cell death ligand-1 (PD-L1). Finally, there is a T-cell "exhaustion" characterized by a decreased ability to respond to antigen presentation and to express cytokines. All these immune dysfunctions, increase the vulnerability to secondary infections after sepsis.

- Increased release of anti-inflammatory cytokines

Anti-inflammatory cytokines related to sepsis include IL-4, IL-10 and IL-37. IL-4 is secreted by activated T cells and mast cells. Among other functions it induces the differentiation of $CD4^+$ T cells into Th2 (T helper 2), promoting autocrine signaling through a positive feedback that activates the expression of anti-inflammatory cytokines and inhibits the release of proinflammatory cytokines. IL-10 is a cytokine mainly expressed by monocytes macrophages and Th2 cells. IL-10 functions include inhibiting T-cell proliferation and function, inhibiting the release of proinflammatory cytokines and promoting the proliferation of immunosuppressive cells like Tregs (T regulatory) and MDSCs (Myeloid-Derived Suppressor Cells) (D. Liu et al., 2022).

It has been reported that there is a correlation between a high ratio of IL-10 and TNF-α in patients with community-acquired infections (Joshi et al., 2015). In another study where whole blood from septic and non-septic patients was stimulated with endotoxin, it was reported that the production of TNF-α, IL-1β and IL-6 was less than 10-20% compared with the production of these cytokines in non-septic patients . In addition, it has been determined that LPS-stimulated monocytes from septic patients have a considerable decrease in the production of IL-1β, TNF-α and IL-6 against healthy donors. Likewise, it has been demonstrated that fewer than 5% of monocytes from septic patients produce cytokines compared to the 15-20% of monocytes from controls (Santos et al., 2016). All these results suggest that some patients with sepsis rapidly produce both pro and anti-inflammatory cytokines whereas other patients have either predominance for anti-inflammatory cytokines or globally depressed cytokine production (Hotchkiss, Monneret, & Payen, 2013).

- Loss of immune effector cells

One of the principal causes of immunosuppression in sepsis is lymphodepletion due to death of $CD4^+$, $CD8^+$ T and B cells by apoptosis. Higher apoptosis rates have been found in all the lymphoid organs including spleen, thymus, lymph nodes and lymphoid tissue associated with the gastrointestinal tract (D. Liu et al., 2022).

In 1999, Hotchkiss and co-workers presented a report where it was shown that there is a widespread loss of lymphocytes in animal models for acute sepsis (Hotchkiss et al., 1999). B and T lymphocytes loss was later confirmed in septic patients (Markwart et al., 2014). Also, CD4+ T lymphocytes, an important population for survival in sepsis, was particularly susceptible to apoptotic cell death in polymicrobial sepsis models. Beyond lymphodepletion, a significant loss in antigen presentation capacity has been related to a compromised T-cell immunity in post-acute sepsis. Moreover, T-cells collected from deceased septic patients manifested signs of exhaustion showing a reduction in both cytokine production and cell expansion after activation with agonist TcR (T cell Receptor) antibodies (Jensen, Sjaastad, Griffith, & Badovinac, 2018). Another study showed that there was a striking apoptosis-induced loss of cells of the innate and adaptative immune system including CD4, CD8 T cells, B cells and dendritic cells in tissues and blood of patients dying from sepsis. Therefore, severe depletion of immune cells is a common finding in septic patients in all age groups with the notable exception of Tregs and MDSCs that show an increase in septic patients as they are less vulnerable to sepsis-induced apoptosis (Lai, Qin, & Shu, 2014).

- Expansion of regulatory cells

Overactivation of regulatory cells like Tregs and MDSCs plays a critical role in sepsis-induced immunosuppression. Tregs play an important role in maintaining the immunological homeostasis and self-tolerance. The main mechanisms of Tregs-induced immunosuppression include (Smigiel, Srivastava, Stolley, & Campbell, 2014): release of anti-inflammatory cytokines like TGF-β (Transforming Growth Factor Beta) and IL-10; upregulating the negative costimulatory receptors on immune effector cells like TIM-3 (T cell immunoglobulin domain and mucin domain 3), PD-1 and TIGIT (T cell Ig and ITIM domain), CTLA-4 (cytotoxic T lymphocyte antigen-4) and neutropilin-1; epigenetic modifications of the Foxp3 gene to enhance the stability of Tregs during lymphopenia; metabolic shift of Tregs from glycolysis to oxidative phosphorylation that enhances their suppressive capacity. MDSCs are a group of immature myeloid cells that include the progenitors of monocytes, neutrophils and dendritic cells. MDSCs suppress the innate and adaptative immune response (D. Liu et al., 2022).

- Decreased expression of HLA-DR

Immunosuppression after sepsis is clearly reflected in a reduction of ALC (Absolute Lymphocyte Count) and monocyte membrane (m) HLA-DR expression. In addition, there is an increase in soluble PD-L1 (Programmed Cell Death Ligand-1), which persists for weeks after sepsis (Darden et al., 2021). HLA-DR is a MHC-II (Major Histocompatibility Complex class II) molecule that is mainly albeit not exclusively expressed on monocytes, macrophages, dendritic and B cells. HLA-DR is critical for the activation of the adaptative immune system. High levels of HLA-DR correspond to a well activated immune function, which supports the idea that it is an indicator for assessing immune status in septic patients. HLA-DR correlates with reduced cytokine response to LPS and in septic patients the decreased expression of HLA-DR in bone marrow monocytes is closely related to the clinical prognosis (Skirecki, Mikaszewska-Sokolewicz, Hoser, & Zielińska-Borkowska, 2016). The expression of HLA-DR in patients with sepsis is 70% lower than in non-septic patients which indicates that its expression is inversely correlated with SOFA scores. Also, an expression of HLA-DR of less than 30% is an indicator of immunosuppression suggesting that this could be a good biomarker for immunosuppression and adverse clinical outcomes in septic patients (D. Liu et al., 2022).

- Increased expression of negative costimulatory molecules

Negative costimulatory molecules, also known as immune checkpoints, such as PD-1, TIM-3, CTLA-4, BTLA-4, LAG-3 and 2B4, and their respective ligands, are expressed in different immune and nonimmune cells. In preclinical models of sepsis, inhibitors and antibodies to block the engagement of negative costimulatory molecules have been shown to improve immune cell

functions and increase the host resistance to sepsis. An increased expression of PD-1 indicates a poor prognosis in septic patients. In addition, the expression of PD-1 and PD-L1 in neutrophils and monocytes in patients with septic shock is higher and it is positively correlated with sepsis severity and mortality (D. Liu et al., 2022).

Some findings suggest that T-lymphocytes from patients in the acute phase of sepsis accumulate inhibitory cell surface co-receptors such as CTLA-4 and PD-1, which provides an explanation for the impaired functional responses to antigens and the immune paralysis (Hotchkiss et al., 2013; D. Liu et al., 2022; Markwart et al., 2014).

- T lymphocyte anergy

T lymphocytes from patients who have suffered from sepsis have been shown to have a decreased ability to respond to mitogenic agents like concanavalin A and phytohemagglutinin. This immunoparalysis has been associated with an increase in T lymphocyte expression of inhibitory coreceptors (PD-1, CD47, CTLA-4) and a decrease in the expression of coactivator receptors such as CD28 (N. S. Ward, Casserly, & Ayala, 2008).Macrophages and T-lymphocyte dysfunction is an important contributor to PICS (Persistent Inflammation, Immunosuppression and Catabolism Syndrome)-associated immunosuppression. It is also known that in late sepsis, a state of immune “paralysis” develops as bacterial clearance, cytokine release and capacity for antigen presentation all decline. Additionally, there is a lymphocyte “exhaustion” characterized by dysfunctional T cell differentiation and decreased ability to respond to the antigenic presentation (Cabrera-Perez, Condotta, Badovinac, & Griffith, 2014). All these immune dysfunctions, increase the vulnerability to secondary infections after sepsis. Finally, a 25-32% of patients are readmitted to Hospital and from that percentage, 52-66% is a consequence of recurrent sepsis. From sepsis survivors, more than the half of them experience secondary and nosocomial infections. Mortality approaches the 40% of patients due to sepsis relapse(Darden et al., 2021; D. Liu et al., 2022).

1.3 Granule Exocytosis Pathway

The granule exocytosis pathway is one of the most important defense mechanisms of the immune system against infected or transformed cells. It is specifically used by effector cytotoxic cells such as CTLs (cytotoxic T lymphocytes) and NKs (Martínez-Lostao, Anel, & Pardo, 2015). CTL generation and activation is mediated by professional APCs (Antigen-Presenting Cells) together with MHC class I molecules and newly synthesized antigen peptides derived from tumor cells or

infected cells, together with cytokines like IL2 and IL12 provided by CD4+ Th1 cells. Meanwhile NK cells respond to the balance of stimulatory versus inhibitory signals that are expressed by target cells.

The process of releasing the content of the cytotoxic granules can be divided into four stages (de Saint Basile, Ménasché, & Fischer, 2010).

1. The formation of the immunological synapse and reorganization of the actin cytoskeleton.
2. Polarization of the MTOC (MicroTubule-Organizing Center) and the cytotoxic granules towards the immunological synapse.
3. Movement of cytotoxic granules towards areas close to the plasma membrane.
4. Fusion of the cytotoxic granules with the plasma membrane of the effector cells, and release of their contents towards the plasma membrane of the target cell.

The recognition of the target cell is the first step for granular exocytosis to occur. This is produced through the TCR in the case of CTL, or through activating receptors in NK cells. When this recognition takes place, the cytotoxic cell adheres to the target cell through the integrin LFA-1, which recognize the adhesion molecule ICAM-1 on the surface of the target cell (Grudzien & Rapak, 2018). This connection is known as the SMAC (Supramolecular Activation Cluster) or Immunological Synapse. Two parts can be distinguished in this region, a central one (cSMAC) and a peripheral one (pSMAC). An adhesion ring is formed around the initial point of contact inside which two domains are located, a signaling domain through which signal transduction begins inside the cell and a secretory domain through which it is secreted the content of the granules (Dustin & Long, 2010; Kabanova, Zurli, & Baldari, 2018; J. C. Stinchcombe, Bossi, Booth, & Griffiths, 2001). Actin polymerization and cytoskeletal reorganization at the lytic synapse are required for granule exocytosis. This reorganization of the cytoskeleton involves the accumulation of actin filaments (F-actin) in the pSMAC region of the synapse, while the cSMAC is largely free of actin, allowing cytotoxic granules to come in contact with the plasma membrane of this region (Dustin & Long, 2010; Kabanova et al., 2018; J. C. Stinchcombe, Bossi, et al., 2001).

Another important process that is necessary for the granule exocytosis is the reorientation of the MTOC towards the point of contact with the target cell (Jane C. Stinchcombe, Majorovits, Bossi, Fuller, & Griffiths, 2006). This depends on the signaling of proteins such as ZAP70, LAT, Fyn or SLP76, involved in signal transduction through the TCR in CTL or the activating receptors in NK cells. The MTOC produces a network of tubulin microtubules oriented towards the target cell, which allows the translocation of the granules towards the point of contact with it (Kurowska et al., 2012; Mentlik, Sanborn, Holzbaur, & Orange, 2010). The cytotoxic granules then move through the microtubules in a process mediated by the adapter protein AP-3 (Clark et al., 2003). The granules are then released from the microtubules at the synapse site via the Rab27a protein (J. C. Stinchcombe, Barral, et al., 2001) and subsequently fuse with the plasma membrane in a process controlled by the Munc 13-4 protein (Feldmann et al., 2003). Finally, acid sphingomyelinase provides elasticity to the membrane allowing the granule contents to be expelled directionally (Herz et al., 2009).

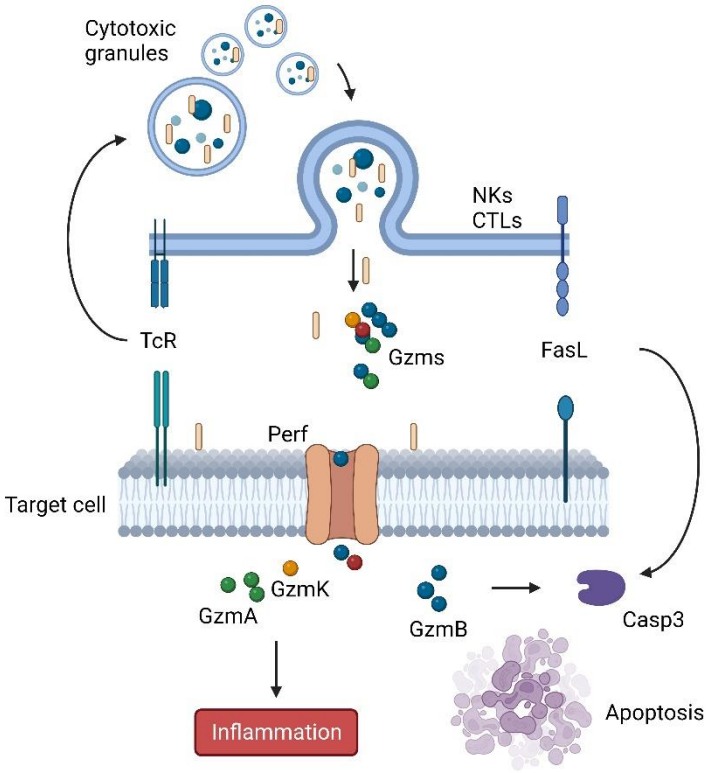


Figure 1.5 Granule Exocytosis Pathway. The cytotoxic granules of NK cells and CTLs are released after the immunological synapse mediated by TcR or FasL recognition takes place. Perf (Perforin) exerts its function and generates pores in the target cell membrane allowing the granzymes to enter the cytosol. Once in the cytosol of the target cell, Gzms (Granzymes) can exert their functions, both cell death and inflammation.

1.3.1 Cytotoxic granules

Cytotoxic granules are specialized lysosomes composed of both, proteins that are only found in this type of granules and others that are also found in lysosomes. Table 3 shows the content of the cytotoxic granules (de Saint Basile et al., 2010; Pardo et al., 2009; Smyth et al., 2001; Voskoboinik, Whisstock, & Trapani, 2015).

Table 1.3 Content of cytotoxic granules

Molecule	Function
Perforin	Pore formation
Granzymes	Serine proteases
Granulysin *	Antimicrobial agent
Calreticulin	Perforin inhibitor
Cathepsin C	Activation of granzymes
Serglicin	Binding to granzymes
Cathepsin B	Perforin degradation
Serpins	Granzyme inhibitors
Acid sphingomyelinase	Sphingomyelin degradation/granular exocytosis
Cathepsin D	Protease
Cathepsin L	Protease
Mannose 6-phosphate receptor	Protein trafficking
ATPase-H$^+$	Granule acidifier
Arylsulfatase	Degradation of polysaccharides
β- Hexosamidase	Degradation of polysaccharides
β- Glucoronidase	Degradation of polysaccharides
CD63	Lysosomal marker
Lamp 1	Lysosomal marker

*Expressed in ruminants and primates and absent in rodents

Adapted from "Unlocking the secrets of cytotoxic granule proteins". 2001. Journal of Leukocyte Biology.

1.3.1.1 Perforin

Perf is a glycoprotein with the ability of forming pores in the cell membrane. Perforin polymerization and pore formation require Ca^{2+} ions that promotes polymerization into de the cell membrane and pore formation. Both steps, polymerization and incorporation depend on various factor like temperature, phosphatidylserine content, PAF (Platelet-Activating Factor) and activation of membrane receptors (Voskoboinik et al., 2015). Perforin is active under the neutral pH conditions of the immunological synapse, but at acidic pH, such as within the granules, it is inactive. Perforin requires a concentration of at least 100 µM Ca^{2+} to efficiently bind to the target cell membrane. In the extracellular medium, the concentration of Ca^{2+} is in the millimolar order, while in the intracellular medium it is in the nanomolar range, regulating the activity of this protein under these conditions (Praper et al., 2010; Voskoboinik et al., 2015).

Calreticulin is an enzyme that works as a chaperon for perforin protecting it from activation by binding itself to Ca^{2+} ions and inhibiting perforin spontaneous polymerization inside

the granules (Andrin et al., 1998). Perforin presents structural homology with the C9 complement protein responsible for the formation of the MAC (Membrane Attack Complex). It has an N-terminal domain with lytic activity, a 150 amino acid fragment with unknown function, a core domain that forms an α-amphipathic helix structure through which it inserts into the membrane, an EGF-like domain, and a C2C domain. -terminal binding to Ca^{2+}, determinant for its activation (Voskoboinik et al., 2015).

In the granules, perforin undergoes a process of post-transductional modification, in which the last 20 residues of the C-terminus are cleaved, exposing the C2 domains. Once the content of the granules is released into the extracellular space, the calreticulin-perforin binding disappears due to the action of calcium, leaving perf free to exert its action(House et al., 2017; Sankar, Arora, Joshi, & Kumar, 2022)

Perforin under physiological conditions is not capable of causing death in target cells because they are capable of repairing the pore, either by endocytosis or by mobilizing internal membranes. Perforin causes cell death by necrosis only when added in vitro at high concentrations. The function of this protein is to allow the entry of the gzms into the target cells, which will be responsible for causing cell death or causing inflammation. Although different mechanisms have been proposed for this process including receptor mediated ones (Motyka et al., 2000), most scientific evidences suggest that the formation of membrane pores is the main mechanism involved in perf-mediated Gzm intracellular delivery (Baran et al., 2009; Law et al., 2010; S. S. Metkar et al., 2015).

1.3.1.2 Granzymes

Gzms are a family of serine proteases mainly classified by their cleavage specificity and known for their role in promoting death of infected or neoplastic cells. Structurally, granzymes are similar to chymotrypsin, with a triad of key residues (histidine, aspartic acid, and serine) conserved at the catalytic site. They represent about the 90% of the mass of the cytolytic granules and are responsible of inducing apoptotic cell death mediated by a CTLs or NK cells. Also, granzymes can be released into the extracellular milieu where they will exert functions as regulation of inflammation, pathogen inactivation, or extracellular matrix remodeling (Bouwman, van Daalen, Crnko, ten Broeke, & Bovenschen, 2021; Garzón-Tituaña et al., 2020). (Martínez-Lostao et al., 2015)

A total of five granzymes have been identified in humans (GzmA, B, H, K, and M) that are located on chromosome 5 (Gzms A and K), chromosome 14 (Gzms B and H), and chromosome 19 (GzmM) and ten in mice (A, B, C, D, E, F, G, K, M and N) being GzmA and B the

best characterized and most studied in both cases. Traditionally, it was assumed that all Gzms acted as cytotoxic proteases. However, nowadays it is known that intracellular GzmB is the one with the greatest cytotoxic capacity, while the cytotoxicity of the others is still in controversy. Furthermore, in addition to cytotoxicity, diverse roles of granzymes have been described in inflammation, ECM (Extracellular Matrix) degradation, impaired wound healing, scarring, basement membrane disruption, blistering, loss of epithelial barrier function, vascular permeability and autoimmunity. Functional extracellular characteristics of granzymes are summarized in Table 4 (Garzón-Tituaña et al., 2020; Richardson, Jung, Pardo, Turner, & Granville, 2022). It is known that Gzms circulates in the extracellular milieu and it has been suggested different mechanism for its release: i) escape from the immunological synapse, ii) granzyme release post degranulation, iii) degranulation induced by chemokines or iv) cytokines and granzyme release following integrin-ECM proteins interactions (M. Sharma et al., 2016).

Table 1.4 Extracellular activities of Gzms in different cell types

Enzyme substrate specificity	**Species**	**Cell type**	**Functions**	**Receptors Involved**
GzmA Tryptase Lys, Arg	H m	Monocytes Lung fibroblasts Intestinal fibroblasts Skin fibroblast Intestinal epithelial cells Neurons Macrophages Dendritic cells	Induce expression of IL-6, IL-8, TNF-α and MCP-1 Inactive GzmA potentiates the effect of LPS Neurite retraction Induce expression of IL-1β, TNF-α and cell maturation Neurotoxicity	Inflammasomes/caspase 1 TLR4 PAR-1 TLR9
GzmB Aspartase Asp	H m	Neurons Smooth muscle cells Endothelial cells	Neurotoxicity Cell death Disruption of endothelial cell layer integrity	PAR-1 –

GzmK Tryptase Lys, Arg	H m	Lung fibroblasts Endothelial cells Macrophages	Induce expression of IL-6, IL-8, and MCP-1 Induce expression of IL-6, IL-8, and MCP-1	PAR-2 PAR-2
GzmM Metase Met	H	Endothelial cells	Cleaves vWF and avoid plasma FVII activation.	–
GzmC-G Chymase Phe	m	–	–	–
GzmH Chymase Phe	H	–	–	–

H, human; m, mouse; Lys, lysine; Arg, arginine; Asp, aspartic acid; Met, methionine; Phe, phenylalanine; PAR, protease activated receptor; TLR, Toll like receptor; vWF, von Willebrand factor.
Adapted from "The Multifaceted Function of Granzymes in Sepsis: Some Facts and a lot to Discover". Garzón-Tituaña, M. et al. 2020. Frontiers in immunology.

1.3.1.2.1 Granzyme B

GzmB (Granzyme B) is one the most studied granzymes. It was first discovered in the mid-1980s when several groups reported the presence of a protease in the granules of cytotoxic lymphocytes. GzmB is a 32 kDa serine protease that has homologues expressed in many different species. The gene that encodes GzmB is approximately 3500bp long, it has five exons and four introns and is located in the chromosome 14 of the human genome. GzmB cleaves peptides adjacent to aspartate (Asp) residues. The specificity of cleavage is due to the structure of its active site, which contains an arginine (Arg) residue similar to caspases. The interaction between Asp and Arg in the active site is the key for enzyme-substrate interaction (Wendy Anne Boivin, Cooper, Hiebert, & Granville, 2009). At first, it was thought that GzmB vas exclusively expressed by natural killer (NK) cells and cytotoxic T cells (CTLs), but recently it has been reported that GzmB can be expressed by various additional cell types. Under certain pro-inflammatory conditions GzmB can be expressed by CD4+ T cells, mast cells, activated macrophages, neutrophils, basophils, DCs (Dendritic Cells), Tregs and nonimmune cell types such as SMCs (Smooth Muscle Cells), chondrocytes, keratinocytes, type II pneumocytes, Sertoli cells, primary spermatocytes, granulosa cells and syncytial trophoblasts as well as by tumour

cells, like leukemia cells and breast, urothelial, prostate, pancreatic and colorectal cancer cells (Maykel Arias et al., 2017; Wendy Anne Boivin et al., 2009).

Previous works have made it possible to determine that GzmB can induce apoptosis by two different mechanisms: directly activating caspases or through the mitochondrial apoptotic pathway (Sunil. S. Metkar et al., 2003; Pardo et al., 2009).

Due to its aspartase activity, GzmB can directly activate caspases. Initially, purified GzmB was shown in vitro to be capable of directly activating caspase 3 (Darmon, Nicholson, & Bleackley, 1995). Subsequently, the role of caspase-3 activation in GzmB-induced death was demonstrated using lymphocytes and caspase inhibitors or GzmB-deficient lymphocytes (Chinnaiyan et al., 1996; Pardo et al., 2008). It has also been shown in vitro that GzmB can activate caspases 7, 8 and 10 (I. S. Goping et al., 2003; Medema et al., 1997; Sunil. S. Metkar et al., 2003).

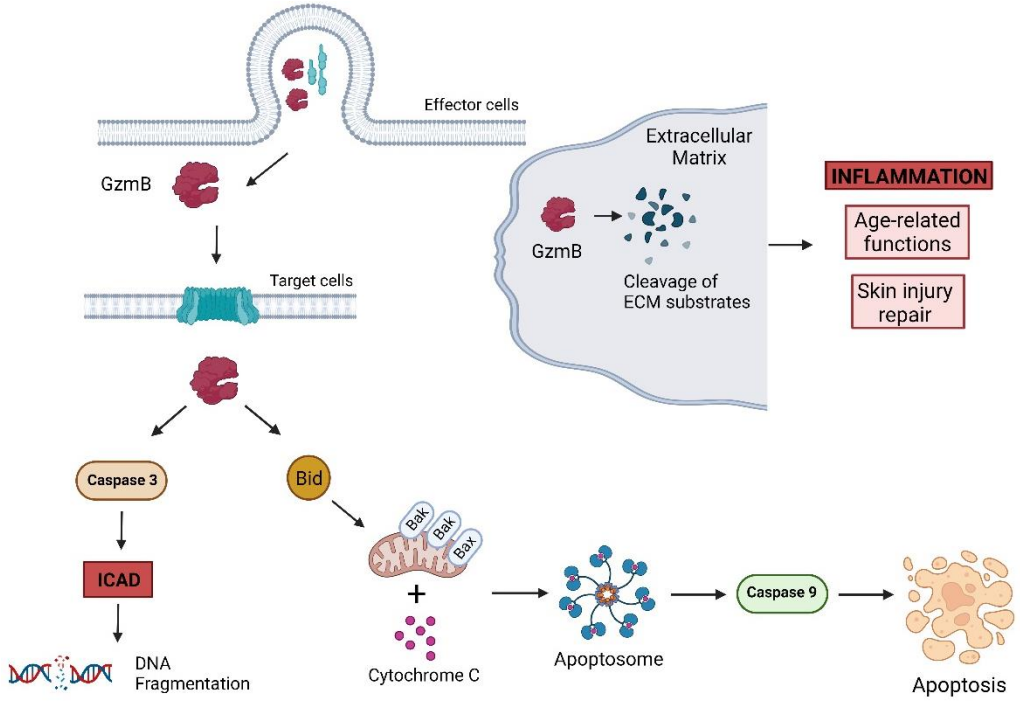


Figure 1.6 GzmB intracellular and extracellular substrates. GzmB induces cell death by two pathways. The first by activating caspase 3 and the second through mitochondrial apoptotic pathway by proteolysis of the protein Bid, the mitochondrial activation of Bax and Bak which promotes the expression of cytochrome C and subsequently the apoptosome formation and caspase 9 activation. GzmB extracellular substrates include ECM components, the cleavage of those substrates is responsible of inflammation, age related functions and skin injury and repair.

Active caspase-3 is capable of acting on various substrates, one of which is the inhibitor of ICAD (Caspase-Activated DNase). After ICAD degradation, CAD nuclease is released, which

causes DNA fragmentation. It has been reported that GzmB can directly process ICAD causing caspase-independent activation of CAD (Lopez & Bouchier-Hayes, 2022).

GzmB can also activate the mitochondrial apoptotic pathway, causing a fall in membrane potential. It has been described that GzmB can proteolyze the proapoptotic protein Bid, generating truncated Bid (tBid), which will move to the mitochondria and activate Bak and Bax, thus promoting the release of cytochrome c, which leads to the apoptosome formation and caspase-9 activation (Kiselevsky, 2020; Pardo et al., 2008). The loss of the membrane potential induced by GzmB can be induces through a pathway independent of Bid, Bak and Bax, and dependent on caspase 3 and 7 (Pardo et al., 2008). These specific affinity for substrates has been studied, showing that human GzmB shows more affinity for Bid (BH3 interacting-domain death antagonist) while mouse GzmB shows affinity for caspase 3 (Lavergne, Hernández-Castañeda, Mantel, Martinvalet, & Walch, 2021).On the other hand, it has been described that mouse GzmB could induce apoptosis by degrading the antiapoptotic protein Mcl1, which favors the release of the proapoptotic protein Bim, which in turn will collaborate in the activation of Bax and Bak (Catalan et al., 2015). At last, GzmB shares other substrates with the executing caspases. For instance, it has been described that this protease can induce cell death by causing the disorganization of the cytoskeleton by proteolyzing α-tubulin and filamin (Ing Swie Goping, Sawchuk, Underhill, & Bleackley, 2006).

GzmB not only exerts a perforin-dependent intracellular activity, but also an extracellular perforin-independent function, which consists in the cleavage of multiple extracellular substrates like ECM components, cytokines, cell receptors, angiogenic and clotting proteins. Aside from the previously mentioned functions of GzmB, some putative roles have been reported such as inflammatory, age-related functions, and the mediation of skin injury, inflammation, and repair (C. T. Turner, Lim, & Granville, 2019; Velotti, Barchetta, Cimini, & Cavallo, 2020).

Table 1.5 Extracellular GzmB substrates and receptors

Protein	**Implications**
Aggrecan	Disruption of structural integrity in cartilage
Cartilage proteoglycans	Disruption of structural integrity in cartilage
Von Willebrand factor	Prevention/delay of thrombosis
Plasminogen	Cleavage yields angiostatin, which is anti-angiogenic. Implications in angiogenesis
Plasmin	Reduction of angiogenesis

Neural glutamate receptor	Its cleavage forms an autoantigenic fragment
FGFR1	Cleavage activates pro-cell death functions as well as inactivates pro-growth signals
Notch1	Cleavage results in cell signaling affecting tumor survival and antiviral activities
Acetylcholine receptor	Cleavage results in a reduction of the receptor in neuromuscular junctions and yields an autoantigenic fragment
Vitronectin	Implications in cell adhesion, migration and anoikis
Fibronectin	Cell adhesion, migration and anoikis
Fibrinogen	Cleavage results in anti-thrombosis implications (C. T. Turner, Hiroyasu, & Granville, 2019).
Laminin	Cell adhesion, anoikis
Smooth muscle cell matrix	Cell adhesion, anoikis
Fibrillin-1	ECM cleavage
Decorin	Collagen disorganization and frailty in the skin of aging mice (Wendy A. Boivin et al., 2012).
Biglycan	Sequestration of active TGF-β and in collagen fiber spacing (Wendy A. Boivin et al., 2012).
Betaglycan	ECM cleavage
Vitronectin	ECM cleavage

Adapted from "Intracellular versus extracellular granzyme B in immunity and disease: challenging the dogma". Boivin, W. et al. 2009. Frontiers in immunology. Nature.

GzmB might play an important role in sepsis, for instance, a study carried out in 2012 by Napoli et al, demonstrated that there was a distinct increase in the expression of GzmB in the CTLs of patients with sepsis (Napoli, Fast, Gardiner, Nevola, & Machan, 2012). As GzmB is known to play important role in apoptosis, these results were the first direct evidence that the cytotoxic effect from activated CTLs might play a role in the inflammatory process and dysregulated apoptosis present in septic patients and GzmB may enhance the inflammatory activity of IL-1α. However, there are not studies yet analyzing if GzmB plays a direct role in sepsis, for example, by using inhibitor of GzmB deficient mouse models (Arias, Jimenez de Bagues, et al., 2014; Napoli et al., 2012).

1.3.1.2.2 Granzyme A

GzmA (Granzyme A) is the most abundant protease in the cytotoxic granules that has a unique quaternary structure that consists of a disulphide-linked homodimer of 60 kDa linked via Cys93 (van Daalen, Reijneveld, & Bovenschen, 2020). GzmA cleaves substrates after Arg or Lys similar to its closest homolog GzmK. Its dimerization creates a high degree of specificity due to an extended site for its substrates. In particular, because of the extended exosite, GzmA substrates do not share the same cleavage site peptide sequence. Therefore, when the cleavage site is mutated, alternate nearby residues may be cleaved instead. This selectivity has allowed the identification of different physiological relevant GzmA substrates (Lieberman, 2010; van Daalen et al., 2020).

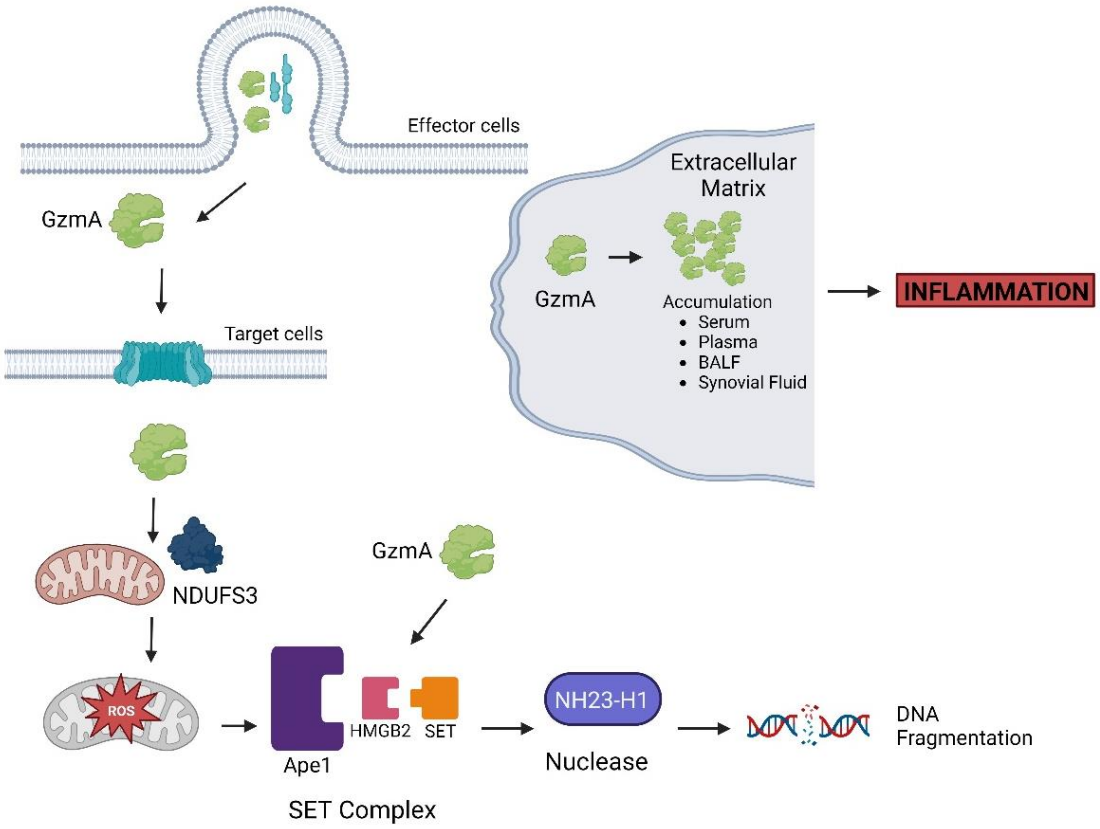


Figure 1.7 GzmA intracellular and extracellular substrates. GzmA cell death pathway consists in cleaving the mitochondrial protein NDUFS3 which result in the generation of ROS. The oxidative stress causes a response dependent on SET complex, translocating it to the nucleus and protecting the DNA. GzmA cleavages SET in Ape1, HMGB2 and SET and releases NH23-H1 nuclease responsible of DNA degradation.

The cytotoxic capacity of GzmA was demonstrated *in vitro* using heterologous models. Recombinant mouse or rat GzmA was used in these experiments in combination with rat perforin and mouse cells as target (van Daalen et al., 2020). With these experiments, it was

observed that while GzmB needed 4 h to induce cell death, GzmA-induced death occurred at 20 h (Tibbs & Cao, 2022). In contrast, when GzmA was tested in autologous models (perf, GzmA, and target cells of the same species), GzmA was not found to induce cell death, even at micromolar concentrations, whereas GzmB induced cell death at nanomolar concentrations in the same cell lines (Kaiserman et al., 2006; S. Metkar et al., 2008).

Although still controversial as indicated above, GzmA, that has a tryptase-like activity, has been proposed to activate caspase. Independent cell death pathways by cleaving the mitochondrial protein NDUFS3 which results in the generation of ROS (van Daalen et al., 2020). The oxidative stress generated causes a response dependent on SET complex, translocating it to the nucleus where it carries out its function of preventing DNA damage. There, the SET complex is degraded by GzmA, proteolyzing three components of this complex (Ape1 (apurinic apyrimidinic endonuclease 1), HMGB2 (High Mobility Group Box 2), and SET) and releasing the NM23-H1 nuclease, responsible for DNA degradation. Furthermore, GzmA can make DNA more accessible to nuclease action by proteolyzing histone H1. However, this mechanism has not been confirmed by independent labs (Gordy & He, 2012; Lieberman, 2010).

Other studies suggest alternative roles for GzmA, for instance, both human in vitro and mouse *in vivo studies* showed that GzmA is non-cytotoxic. Moreover, *in vitro* studies showed contrasting results with native human GzmA that showed lack of cytotoxicity *in vitro*, while recombinant mouse GzmA studies demonstrate cytotoxicity in several *in vitro* assays (van Daalen et al., 2020).

In addition to the intracellular functions, GzmA exhibits interesting extracellular functions due to its variated extracellular substrates (Table 5). For instance, accumulative clinical and biochemical evidence show high levels of extracellular GzmA in plasma, serum, synovial fluid and BAL (Bronchoalveolar Fluid) in patients with viral and bacterial infections or other pro-inflammatory conditions. The elevated levels of extracellular GzmA could reflect on spontaneous release of granzymes after elevated CTL/NK numbers in response to persistent inflammation. What is more, dendritic cells and macrophages can express GzmA but no perforin, suggesting a non-perforin dependent granzyme activity (van Daalen et al., 2020).

Active GzmA levels are locally increased in the BAL fluid, but not in blood, of patients with CD8+ T-cell-mediated HP (Hypersensitivity Pneumonitis) compared to control subjects and TNFα, IL-6, and IL-8 are increased in BAL of HP patients coinciding with GzmA levels. This is in contrast with increased plasmatic GzmA concentrations in several viral, bacterial, and parasitic infections(Tremblay, Wolbink, Cormier, & Hack, 2000; van Daalen et al., 2020).

GzmA positive cells and increased levels of GzmA in sputum are also found in smokers and nonsmoking patients with asthma. This supports a role for extracellular GzmA in the lung inflammatory response. In allergic asthma there was no increase of GzmA in BAL consistent with the absence of a lymphoid source (e.g., CTLs) (Annoni et al., 2015; Proklou et al., 2013; van Daalen et al., 2020).

In addition, immunohistology studies indicate GzmA expression by CTLs, NKs, alveolar macrophages, bronchiolar epithelium and type II pneumonocytes in both control subjects and COPD (Chronic Obstructive Pulmonary Disease) patients. GzmA expression is significantly increased in sputum and/or lung specimens of patients with COPD in comparison with controls, but not in blood (Hodge, Mukaro, Holmes, Reynolds, & Hodge, 2013; van Daalen et al., 2020; C.-z. Zhao, Fang, Wang, Tang, & Wang, 2010).

It has been reported that GzmA has the ability to contribute to the dysregulated innate host response observed in inflammatory and autoimmune disorders. GzmA affect notoriously to adherent mononuclear cells, suggesting that granzymes might stimulate these cells in the microenvironment, contributing to pathology. Different evidences support this asseveration, it has been found that GzmA positive cells are increased in synovial tissues and fluids like plasma and synovial fluid of patients with early RA (Rheumatoid Arthritis) (Tak et al., 1994). Furthermore, GzmA, has been reported to stimulate osteoclastogenesis within the joint, likely by stimulating TNF production, although the detailed mechanism is still unclear (Santiago et al., 2017). On an established mouse model of inflammatory arthritis, our group found that the absence of GzmA reduced substantially the inflammation and the pathologic changes in the synovium and contiguous bone, which correlated with a reduce number of osteoclasts in the joint of GzmA KO (Knock Out) mice and reduced levels of CTX-1 which is a marker for bone degradation and osteoclasts activity in RA. Furthermore, our *in vitro* data showed that GzmA was able to act on GFU-CM cells to produce TNF, which is known to have the capacity of stimulate osteoclastogenesis. In mice lacking GzmA, both the clinical and histologic scores were substantially reduced, and only 40% of GzmA KO mice developed polyarthritis versus an 80% and 60% of WT and Perf KO respectively (Santiago et al., 2017).

In other study developed by our group, we identified extracellular GzmA as a new mechanism by which the immune system facilitates CRC (Colorectal Cancer) development (Santiago et al., 2020). It was demonstrated that GzmA plays a critical role in promoting gut inflammation and tumor development in the DSS/AOM-induced CRC mouse model. This correlates with the fact that GzmA has the ability to promote NF-κB-dependent IL-6 expression

in macrophages which in turn results in IL-6-dependent pSTAT3 activation in CRC cells. This study also showed the potential of the inhibition of GzmA by serpinb6b, showing a proof of concept that GzmA is a possible therapeutic target in CRC and identifying it as the first Gzm to promote carcinogenesis (Santiago et al., 2020).

Related to bacterial infections, extracellular plasma GzmA levels increase in patients with tuberculosis compared with control patients suggesting that extracellular GzmA could be a potential therapeutic target in the inflammatory response to mycobacteria (Teo, Singh, Prem, Hsu, & Yi, 2019; van Daalen et al., 2020). Some studies in sepsis and experimental endotoxemia found that extracellular GzmA plasma levels significantly higher in severe sepsis, septic shock and endotoxemia. This has been demonstrated by inducing a systemic inflammation in volunteer patients by intravenous injection of endotoxin LPS where an increase of GzmA at 2h post administration and a decrease in the number of CTLs and NK cells was reported (Fanny N. Lauw et al., 2000; Van Amersfoort, Van Berkel, & Kuiper, 2003).

Patients with melioidoses caused by *Burkholderia pseudomallei* also show an increase in the levels of GzmA in comparison with control subjects. Additionally, patients infected with *Salmonella enteric* in typhoid fever and *Streptococcus pneumoniae* in community-acquired pneumonia have elevated levels of GzmA in acute phase plasma and BAL (Boogaard et al., 2016; Fanny N. Lauw et al., 2000; van Daalen et al., 2020; van Woensel, Biezeveld, Hack, Bos, & Kuijpers, 2005).

Other experimental results in mice suggest that GzmA-induced release of pro-inflammatory cytokines contribute to the development of sepsis during infection with *Brucella microti*¸ without being necessary for the pathogen clearance (Arias, M. et al. 2014). Similar studies reported that GzmA KO are more resistant to LPS induced toxicity compared to WT mice. These mice also showed higher survival rates and lower bacterial load in BAL during *S. Pneumoniae* infection and have reduced levels of proinflammatory cytokines (Boogaard et al., 2016; S. Metkar et al., 2008; van Daalen et al., 2020).With all these findings, it is likely to think that GzmA release is most likely a general immune response in the acute phase of a bacterial infection and non-specific for certain species (van Daalen et al., 2020).

Regarding viral infections, some studies showed high levels of GzmA in plasma samples of EBV-infected patients during the acute phase of the infection simultaneously with early markers of infection. Also, elevated levels of GzmA are found in patients with dengue fever and cytomegalovirus infection after renal transplantation, HIV and in patients with respiratory

syncytial virus infection (Bem et al., 2008; E. H. Spaeny-Dekking et al., 1998; E. H. A. Spaeny-Dekking et al., 1998; van Daalen et al., 2020).

Chikungunya virus infected patients show elevated circulating GzmA levels that coincide with the peak of IFN-γ levels, increased viral load and disease scores. These observations seem to suggest that GzmA contributes to the pathology of viral infections in humans by inducing a pro-inflammatory immune response with an upregulation of cytokines and extracellular GzmA in viral infections (Schanoski et al., 2020; van Daalen et al., 2020; Wilson et al., 2017).

Table 1.6 Extracellular substrates of GzmA and potential biological effects

Substrate	**Potential biological effect**
Basement membrane proteoglycans	Liberation basic fibroblast growth factor, protection against inhibition by natural high molecular weight inhibitors, lymphocyte migration.
Collagen IV	Influence on lymphocyte migration, anoikis, cell adhesion. Reduction of adhesion of epithelial cells with cell-basement membrane.
Fibronectin	Influence on lymphocyte migration (through fibrin clots), anoikis, cell adhesion.
Myelin basic protein (MBP)	Myelin destruction. Pathogenesis multiple sclerosis.
Pro-urokinase plasminogen activator	Convert single-chain human pro-urokinase into active two-chain enzyme. Role in plasmin generation.
Thrombin-like receptor on neurites	Neurite retraction, reversed stellation of astrocytes.
Platelet thrombin receptor	Desensitized response to thrombin-induced aggregation by platelets.
Unidentified (likely) cell surface receptor*	Pro-inflammatory cytokine production by fibroblasts, epithelial cells, monocytes, and macrophages
Proteinase-activated receptor 2 (PAR-2)	Protease-activated receptor-2 activating peptide (SLIGRL) is yielded. Roles in promoting inflammation.

* A 5-fold difference in potency between thrombin and GzmA has been reported suggesting that granule-associated proteases may signal through other membrane proteins than the thrombin receptor. However, no such receptor has been identified yet. Release of proinflammatory cytokines is suggested to be on their own potentiating LPS-induced responses (S. Metkar et al., 2008).

Adapted from “Modulation of Inflammation by Extracellular Granzyme A”. van Daalen, K. et al. 2020. Frontiers in immunology.

1.3.1.2.3 Granzyme K

GzmK (Granzyme K) was discovered in 1998 after purification from human PMBCs. GzmK is expressed by CTLs, NKT (natural killer T cells), γδ cells and $CD56^{bright+}$ cells. Similar to its closest homolog GzmA, GzmK has a tryptase-like activity cleaving Arg or Lys substrates. Since GzmA and K have similar activity, GzmK was proposed as a redundant enzyme for GzmA. Nevertheless, that concept has been changing in the last years due to the unique substrates and functionality of GzmK (Niels Bovenschen et al., 2009; Kim Plasman, Hans Demol, Philip I. Bird, Kris Gevaert, & Petra Van Damme, 2014). In its mature form, GzmK exists as a monomer with four disulfide bridges and no free cysteine residues. Various GzmK substrates have been reported, including nucleosome assembly protein SET, hnRNP (heterogenous nuclear ribonucleoprotein), β-tubulin, and α-tubulin. A physiological GzmK inhibitor is the inter-alpha inhibitor 1, which circulates in the plasma of healthy individuals. The traditional cytotoxic role of GzmK is still controversial, but new extracellular functions related with inflammation and infection are emerging (Bouwman et al., 2021).

GzmK has been observed in plasma of patients suffering from autoinflammatory diseases which suggests a potential role in this disease. Initially the pro-apoptotic potential of GzmK was performed using rat GzmK that showed the ability to induce oligonucleosomal DNA-fragmentation and chromatin condensation in YAC-1 cells (Bouwman et al., 2021). The fist *in vitro* study on the apoptotic capacity of human GzmK combined with perforin suggested that GzmK has the capacity to induce non-apoptotic cell death by provoking mitochondrial dysfunction and generating ROS (Bouwman et al., 2021). Also, several *in vitro* studies showed that the cleavage of GzmK substrates such as proteins of the SET complex, Bid, VCP (vasolin-containing protein) and P53, results in cytotoxicity (Bouwman et al., 2021; Joeckel et al., 2011a).GzmK targets other SET complex proteins such as DNA-binding protein HMG2 and redox Ape1, this last one responsible of facilitating the intracellular accumulation of ROS, which may be the first step in the GzmK-mediated cell death pathway (Bouwman et al., 2021; T. Zhao et al., 2007; Zorov, Juhaszova, & Sollott, 2006).

Different from human and rat GzmK, *in vitro* mice studies report no cytotoxicity of mouse GzmK after analyzing apoptotic markers like phosphatidylserine externalization, mitochondrial membrane integrity and ROS accumulation (Joeckel et al., 2011b). Also, in an *in vivo* study comparing GzmK KO mice with WT mice, no essential role in cytotoxicity for GzmK was found (L. T. Joeckel, C. C. Allison, M. Pellegrini, C. H. Bird, & P. I. Bird, 2017). Nevertheless, it has been reported that GzmK targets substrates like importin α1 or β *in vitro* and that the cleavage of these proteins raised the potential of a new cell death pathway that differs from

GzmA. Due to all the previously mentioned evidence, the potential biological functions of GzmK remain controversial (Joeckel & Bird, 2014).

Albeit GzmK has been found in the extracellular milieu, little evidence exists about the potential physiological role of GzmK. The accumulation of *in vitro* evidences suggest that GzmK-mediated extracellular cleavage of different substrates might play a role in the endothelial activation and the induction of proinflammatory cytokine response (Bouwman et al., 2021). Recently, it has been shown that GzmK is abundant in burn wounds and plays an important role in inflammation, epithelialization and remodeling. It has also been reported that in burn wounds GzmK is mostly localized in the $CD68^+$ monocyte/macrophage cell populations within the dermis. Classically activated M1 macrophages express and secrete GzmK, whereas M2a macrophages showed a negligible GzmK expression. Thus, GzmK may contribute to the proinflammatory response after burn injury (C. T. Turner, Zeglinski, et al., 2019). In other study, it has been reported that in human lesional atopic dermatitis samples, there was an increase in the number of $GzmK^+$ cells compared with healthy controls. In mice, GzmK KO exhibited a reduced overall severity characterized by the reduction in scaling, erosions and erythema. The presence of GzmK did not notably increase the overall proinflammatory response or epidermal barrier permeability in WT mice, whereas GzmK impaired angiogenesis, increased microvascular damage and microhemorrhage. Therefore, GzmK may provide a potential therapeutic target or skin conditions with persistent inflammation, vasculitis and pathological angiogenesis (Christopher T Turner et al., 2022).

Endothelium activation mediated by GzmK is performed through the cleavage and activation of the PAR-1 receptor and, in addition to expression of adhesion molecules, GzmK-mediated PAR-1 activation leads to production and secretion of cytokines, thereby promoting inflammation (M. Sharma et al., 2016). As mentioned before, it is reported that GzmK induces the expression of IL-1β, a response that has been observed in monocytes, human lung fibroblasts, HUVECs (Human Umbilical Vein Endothelial Cells), human keratinocytes and skin fibroblasts. The release of proinflammatory cytokines from these cells is dependent on PAR-1 activation and downstream ERK1/2 (extracellular signal-regulated kinase 1/2) and MAPK (Mitogen-Activated Protein Kinase) p38 phosphorylation and independent on NF-kB (Bouwman et al., 2021; Mehul Sharma et al., 2016; Annette C. Wensink et al., 2014).

Regarding bacterial infections it has been found elevated levels of extracellular GzmK in sepsis and experimental endotoxemia, which correlated with reduced expression of the GzmK inhibitor Iαlp. Elevated levels were also found during *P. aeruginosa* infection. *In vitro*

experiments found that GzmK has the potential to bind to LPS independent of its catalytic activity. LPS and CD14 form a complex that binds to TLR4 on the cell membrane leading to an inflammatory cytokine response, especially TNF-α release from primary monocytes (Bouwman et al., 2021; Annette C. Wensink et al., 2014). However, other experimental models have shown that the inflammatory activity of GzmK requires from its catalytic activity. For instance, it has been demonstrated that GzmK induces proinflammatory cytokines from human lung fibroblasts through the activation of PAR-1, this supports the idea that GzmK is not involved in immune-mediated cytotoxicity, but rather in the stimulation of inflammation (Cooper, Pechkovsky, Hackett, Knight, & Granville, 2011). In other study, it has been reported that human GzmK showed multiple pro-apoptotic pathways that could participate in viral immunity. Similar to human GzmA, GzmK has also been reported to cleave SET which occurs with a DNA fragmentation in target cells and to cleave p53 in three products which seem to have pro-apoptotic activities (Joeckel et al., 2011b).

Regarding viral disease, it has been reported that in Influenza A infection in vitro, intracellular GzmK cleaves importing 1α or β inhibiting the replication of the virus (Zhong et al., 2012). In other viral infections such as dengue or cytomegalovirus infections, GzmK might exert extracellular functions as in both, high levels of soluble GzmK levels have been detected (Bouwman et al., 2021; Lars T Joeckel, Cody C Allison, Marc Pellegrini, Catherina H Bird, & Phillip I Bird, 2017).

Table 1.7 Intracellular and extracellular GzmK substrates and suggested biological impact

Substrate	(Extra) cellular location	(Suggested) Biological Impact
Intracellular substrates		
SET complex	Nucleus	NM23H1-induced DNA nicks, chromatin condensation and apoptotic morphology.
Bid	Mitochondria	Disruption of the outer mitochondrial membrane and release of cytochrome c and endonuclease G.
Ape1	Nucleus	Inhibits its redox activity facilitating intracellular ROS accumulation and enhancing GzmK-induced cell death.
VCP	ER	Inhibition of ERAD components and initiation of ER stress leading to ROS accumulation and cytotoxicity.

p53	Nucleus, mitochondria	Cleavage products, p13, p35 and p40 induce transcription of p21, PG13, MDM and mitochondrial disruption, leading to ROS accumulation and cytotoxicity.
Importin 1α/β	Nucleus	Inhibition of viral replication by preventing NP/viral RNA complex formation.
B-tubulin	Cytoskeleton	Potential novel cell death pathway and terminating viral production in infected cells during NK cell attack.
hnRNP K	Nucleus	Potential novel cell death pathway and/or terminating viral production in infected cells during NK cell attack.
Extracellular substrates		
PAR-1	Cell membrane	Activation of PAR-1 mediating endothelial activation and release of proinflammatory cytokines.
PAR-2	Epithelial cells	Release of inflammatory cytokines IL-6 and IL-8 from cells (Kaiserman et al., 2022).
LPS	ECM	Removal of LPS molecules from micelles and transfer to CD14 and TLR4, promoting cytokine expression.

Bid, BH3 interacting-domain death agonist; Ape1, Apurine/apyrimidine endonuclease 1; VCP, Vasolin-containing protein; ER, Endoplasmic Reticulum; ROS, reactive oxygen species; ERAD, ER associated protein degradation; ECM, Extracellular matrix; NK cell, Natural Killer cell; NP, Nuclear protein; LPS, Lipopolysaccharide; PAR-1, Proteinase-activated receptor 1.

Adapted from "Intracellular and Extracellular Roles of Granzyme K". Bowman, A. et al. 2021. Frontiers in immunology.

1.4 Murine models for sepsis study

Various animal models have been developed to mimic human sepsis. A suitable animal model aims to investigate the biology of the normal and disease process, understand the disease mechanism, perform preclinical testing of potential therapeutic agents and test the ability of any intervention to interrupt or modify the disease process (Lewis, Seymour, & Rosengart, 2016; Stortz et al., 2017).

Mice are the most widely used preclinical animal model for sepsis research. The fact of being small in size, easy to transport and handle, as well as the availability of different strains of genetically modified mice, are some of the key characteristics for which mice are preferred to

larger animals such as dogs, cats, horses, and primates in sepsis models (Fink, 2014; Lewis et al., 2016).

Murine models for the study of sepsis can be divided into six types:

1. Models of endotoxemia.
2. Live pathogen inoculation models.
3. Bacteria and fibrin clot implantation.
4. Cecal slurry injection.
5. Models of stent-induced peritonitis in the ascending colon (CASP).
6. Models of peritonitis induced by ligation and puncture of the cecum (CLP).

1.4.1 Models of endotoxemia

This model consists of the administration of bacterial-derived endogenous toxins, generally LPS, although other toxins have also been used. Administration can be as a bolus or infusion via the i.p (intraperitoneal), intravenous or intrathecal route (Lewis et al., 2016).

The immune response in the bolus injection model is characterized by a potent innate immune response with very high production of inflammatory cytokines such as IL-1β, TNFα and IL-6. This model is not capable of faithfully reproducing the hemodynamic changes that occur in human sepsis. For example, bolus administration of LPS induces an almost immediate hypodynamic cardiovascular state, something that does not occur in the septic response in humans. Also with this model, it is not possible to mimic the cytokine profiles that occur in sepsis in humans. With the administration of LPS, large elevations of cytokines are produced, but transiently while human sepsis is characterized by a prolonged systemic elevation of cytokines, of variable magnitude but lower than the values reached with endotoxemia (Lewis et al., 2016; Nemzek, Hugunin, & Opp, 2008).

Nevertheless, in this model is not possible to analyze the contribution of the immune system in the pathogen control and therefore the impact of immunomodulators over it. Despite these drawbacks, endotoxemia models are popular because they are convenient and reproducible. LPS is a relatively pure compound that is reliably quantified and its use can be easily standardized in experimental studies. In addition, the injection of low doses of endotoxin in healthy human volunteers produces pathophysiological alterations similar to those described in patients with sepsis (Lewis et al., 2016).

1.4.2 Inoculation of live pathogens

These models vary widely with respect to the route of infection (endovenous, intraperitoneal, subcutaneous, intranasal, etc.), the frequency of administration, the bacterial strain and the size of the inoculum.

Although this model cannot recapitulate many important clinical features of sepsis, it does provide insight into host response mechanisms against pathogens. Different bacterial species have been used to perform this model such as *E. coli, Staphylococcus* and *Pseudomonas,* virus and fungi can also be administered in this model. One of the disadvantages is that the administration of high doses of bacteria can cause a large part not to colonize and replicate within the body, being rapidly lysed by the complement system. Therefore, this may lead to a possible model of endotoxin poisoning rather than a true septic model (Fink, 2014; Lewis et al., 2016; Nemzek et al., 2008).

1.4.3 Bacteria and fibrin clot implantation

This is a sophisticated version of the bacterial injection model that consists in the inclusion of standardized amounts of bacteria into fibrin clots, which are implanted into the peritoneal cavity. The fibrin clot acts as a reservoir for bacterial seeding of the bloodstream and elsewhere in the body, replicating in a better way the human abdominal sepsis. This is a highly reproducible model as bacterial quantities can be controlled and source control procedures can be employed during a second laparotomy for fibrin clot removal. This model has been used to show the importance of early antibiotic administration in sepsis (Lewis et al., 2016).

1.4.4 Cecal slurry injection

This model is based on an intraperitoneal injection of cecal contents that has been standardized in quantity and suspended in fluid from a donor rodent. Mortality rates from cecal slurry can be titrated to mimic that of a particular CLP model, the kinetics of mortality between these two models still vary. One clear difference between this model and CLP is the lack of surgical tissue trauma and ischemic tissue generated. A clear advantage is that the cecal slurry injection model can be performed in neonatal mice, helping in this way to cover the existing gap for sepsis in neonate studies (Lewis et al., 2016).

1.4.5 Stent-induced peritonitis in the ascending colon (CASP)

This model involves implantation of a stent in the ascending colon, which leads to persistent leakage of fecal contents into the peritoneal cavity and infection with intestinal microbial flora. The severity of sepsis can be manipulated by varying the diameter of the stent used. It represents a model of polymicrobial peritonitis in which SIRS and CARS responses are

appreciated. Furthermore, it is a very useful model to study multi-organ failure (Lewis et al., 2016; Nemzek et al., 2008).

1.4.6 Peritonitis induced by cecal ligation and puncture

The CLP model is one of the most stringent models of sepsis and is considered by many researchers as one of the most relevant models for the study of sepsis. Compared to other models, CLP provides a better representation of the complexity of human sepsis. CLP involves a combination of three stimuli: tissue trauma from laparotomy, necrosis from cecal ligation and infection from microbial leakage. This leakage results in peritonitis followed by the translocation of bacteria into the bloodstream, which activates the systemic inflammatory response. The advantage of CLP is that the pathogens are endogenous, thus mimicking the traumatic injury that leads to peritonitis in humans. This technique is widely used because it satisfies many of the essential criteria required in a powerful septic model: simple procedure, polymicrobial in nature, with localization of the infectious focus, etc. In addition, CLP sepsis shows a high degree of similarity to the progression of human sepsis, triggering the hyper- and hypoinflammatory responses characteristic of human sepsis (Korneev, 2019; Lewis et al., 2016).

BACKGROUND AND OBJECTIVES

2 Background and objectives

2.1 Background

Sepsis is life-threatening organ dysfunction caused by a dysregulated host response to an infection. If not diagnosed promptly it can lead to a septic shock, multiple organ dysfunction and death. Sepsis is the most serious complication of infection, particularly in low- and middle-income countries where it represents the major cause of mortality and morbidity in maternal and neonatal patients. Sepsis in the community can result from clinical deterioration of common and preventable infections, but also result from infections acquired in healthcare settings. Infections associated to the healthcare system are often more resistant to antibiotics and can lead to deteriorating clinical conditions and antimicrobial resistance is a major factor to determine clinical response to treatment and a rapid evolution against sepsis and septic shock (Rudd et al., 2020).

Most of sepsis cases have been thought to be caused by bacterial pathogens. Nevertheless, up to 42% of sepsis cases are culture negative, suggesting a non-bacterial cause. Despite this knowledge, viral sepsis remains rare and needs further investigation (Lin et al., 2018).

It is necessary to implement preventing measures against infections, such as good hygiene practices, ensure access to vaccination programs, and improve sanitation and water quality. Early diagnosis and appropriate clinical management of sepsis are crucial to increase survival. Thus, sepsis requires a multidisciplinary approach (Reinhart et al., 2017).

Sepsis is a global burden that kills approximately 11 million people annually. In December 2019 a novel condition named as coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged and was responsible of more than 1,535,982 deaths until December 2020. Both, sepsis, and COVID-19, share many pathophysiological and clinical features such as thrombocytopenia, hemolytic anemia, vascular microthrombosis, multi-organ dysfunction syndrome, coagulopathy, septic shock, respiratory failure, fever, leukopenia, hypotension, leukocytosis, high cytokine production and high predisposition to opportunist infections. Considering the parallelism between these two conditions it is likely that the existing well stablished health system for sepsis care, could help and inform on COVID-19 management(Gu et al., 2020).

During sepsis, the innate immune system activated by MAMPs and DAMPs, release various inflammatory cytokines generating a “cytokine storm” which result in a persistent and

severe inflammatory response (Ding, Meng, & Ma, 2018). It has been known for some time that sepsis in humans or animals exhibits evidence of a loss in the regulation of the inflammatory response, resulting in the expression of high levels of proinflammatory mediators (IL-1β, TNF-α, IL-6, IL-8) in plasma. This has been referred as the 'systemic inflammatory response syndrome' (SIRS). Over time during sepsis, there is often the presence of anti-inflammatory mediators (IL-10, IL-12, IL-13), originally considered to be a response to the earlier appearing pro-inflammatory mediators of SIRS. This situation has been described as a 'compensatory anti-inflammatory response syndrome' (CARS). In addition, there is many evidence showing that sepsis causes major changes in phagocytic cells and endothelial cells, leading to a proinflammatory state that stimulates accumulation of polymorphonuclear leucocytes (PMNs) in tissues which may perhaps be related to attendant tissue and organ damage during sepsis (P. A. Ward & Gao, 2009).

Even though there is an important amount of evidence suggesting that cytokines play an important role in the infection process, there are no effective specific inflammation-targeting therapies. This problem could be explained by many reasons, for instance, septic patients are heterogenous due to the varied manifestations of sepsis depending on the source of the infection, the different mechanism of development, and the different degrees of inflammation, all of them leading to a different response to treatment. Also, the fact that hundreds of factors are involved in the inflammation response in sepsis makes that the therapeutic strategies targeting specific cytokines to be highly ineffective. Finally, the fact that each cytokine generated during sepsis is double-edge sword, meaning that in one hand, proinflammatory cytokines cause damage, but on the other hand they are necessary to remove pathogens. Making it hard to control sepsis pathogenesis by regulating the expression of cytokines (Ding et al., 2018).

Taking these in consideration, it is important to study new targets that help to control the pathogenic inflammatory process without comprising the pathogens control of the body. Thus, the importance of the granular exocytosis pathway. The granular exocytosis mechanism consists of a transfer process by which lymphocytes release perforin and granzymes. The perforin exerts its action fulfilling the passage of the Gzms to the cell interior. It has traditionally been assumed that all gzms act as cytotoxic proteases. However, recent evidence suggests that GzmB is the one that mainly exerts cytotoxic functions, while other granzymes such as GzmA, GzmK and GzmM would act as proinflammatory mediators (Anthony et al., 2010; Catalan et al., 2015; Cooper et al., 2011; Pardo et al., 2009).

Although macrophages are important promoters or septic shock, cells like Tc and NKs have an important role in the pathological outcome too. Therefore, several studies have shown a positive correlation between the severity of sepsis and the expression of GzmA and GzmB in lymphocytes (Schaer, Schaer, Schoedon, Imhof, & Kurrer, 2006; Sherwood et al., 2003; Zeerleder, Hack, et al., 2005).

GzmA is capable of inducing the production of proinflammatory cytokines such as IL-1β, TNFα and IL-6 in human monocytes and mouse macrophages (S. Metkar et al., 2008). It has also been shown in models of LPS endotoxemia that the absence of GzmA or GzmM increases the survival of mice deficient in these Gzms compared to WT mice (Anthony et al., 2010; S. Metkar et al., 2008). In addition, LPS-stimulated GzmM-deficient mice were shown to have lower serum levels of proinflammatory cytokines(Anthony et al., 2010).

GzmK is another serine protease with proinflammatory capacity. Joeckel and collaborators found that GzmK induced IL-1β maturation in LPS-stimulated mouse macrophages(Joeckel et al., 2011a). It has also been shown that GzmK can activate PAR-1 receptors on fibroblasts, inducing the production of IL-6, IL-8, and MCP-1(Cooper et al., 2011).

It has been suggested that GzmK augments GzmA-induced proinflammatory processes by cleaving the same substrates differentially based on the specificity of GzmK. Mouse GzmK induces the expression of proinflammatory cytokines like IL-1β which can explain its proinflammatory role (Bouwman et al., 2021; Lars T Joeckel et al., 2017). Intra and extracellular GzmK target physiological substrates independent of its catalytic activity.

Elevated serum levels of GzmA, GzmB and GzmK have been found in patients with sepsis. In addition, various studies have shown the relationship between the expression of granzyme A and B in lymphocytes with the severity of sepsis (M Isabel García-Laorden et al., 2021; Napoli et al., 2012). However, it is not clear whether the contribution of perforin and Gzms to the pathophysiology of sepsis is due to the induction of cell death or pro-inflammatory effects, so further studies are needed to elucidate this (Min Huang, Shaoli Cai, & Jingqian Su, 2019; Jarczak et al., 2021).

Unfortunately, LPS endotoxemia models are not capable of reproducing all events that occur in sepsis, so other animal models need to be used in which clinical signs of sepsis develop after exposure to endogenous bacterial pathogens.

Animal models for sepsis not always recapitulate most of the facets of the septic shock response and that why there is a need for an animal model where clinical signs of sepsis develop

after exposure to endogenous bacterial pathogens. Our group employed a mouse gram-negative bacterial pathogen *B. microti* to analyze Tc and NKs cells capacity to control bacterial infection and their contribution sepsis where it was shown that GzmA and GzmB, expressed in Tc and NK cells contribute differentially to bacterial immunopathogenesis. Also, by blocking GzmA the bacteria-associates sepsis may be reduced without compromising the ability of the immune system to control de infection (Arias, Jimenez de Bagues, et al., 2014).

In a murine sepsis model with intra-abdominal infection of *E. coli* García-Laorden and collaborators showed that there was an association between granzymes deficiency and an enhanced bacterial growth, as well as a relation between differences in organ damage in advanced peritonitis and sepsis. In this study, it was shown for first time that there is a difference in the pattern of expression of intracellular granzymes in different lymphocytes populations before and after *E. coli* expression. Also, after analyzing the effect of the deficiency of GzmA and GzmB on the host during *E. coli* peritonitis and sepsis it was reported that there was an increase of bacterial load in distant organs of granzyme deficient mice. At a late point of the disease, it was observed that granzymes, mainly GzmB, have a role in controlling bacterial outgrowth (Garcia-Laorden et al., 2017).

In another study, using *K. pneumoniae* in WT mice and deficient GzmA and/or B mice, it was observed that Gzm-deficient mice displayed an unaltered pattern of bacterial dissemination, distant organ damage and mortality when compared to WT mice, suggesting that GzmA and B play a modest role in the pathogenesis of Gram-negative pneumonia-derived sepsis. Also, it was shown that NK cells represented the predominant cell type expressing Gzms and while GzmA levels decreased in NK cells during *Klebsiella* sepsis, GzmB expression increased, which suggests a different regulation of these mediators. Finally, the host response to *K. pneumonia*-derived sepsis was analyzed. For instance, bacterial loads did not differ between Gzm-deficient and WT mice at any site. Thus, Gzms played little, if any, role in the control of local infection and subsequent dissemination to distant organs. The mechanism by which Gzms contribute to the regulation of lug inflammation during *Klebsiella* pneumonia remains to be stablished (M. I. García-Laorden et al., 2016).

Taking in consideration the previously known background, the hypothesis we have stablished is that Gzms A and K are implicated in bacterial sepsis, viral sepsis of respiratory origin, and bacterial peritonitis while GzmB is important for the pathogens control by killing infected cells.

2.2 Objectives

The main objective of this work is to establish the relevance of granzymes during bacterial and viral sepsis. Therefore, the following secondary objectives have been set:

1. Analyze the levels of extracellular GzmA in human patients undergoing abdominal sepsis.
2. Determine the role of granzyme A in polymicrobial sepsis induced by CLP.
    a. Compare survival rates between WT and GzmA KO.
    b. Determine the levels of proinflammatory cytokines and bacterial load and species.
    c. Identify the main cellular groups that express GzmA during sepsis.
3. Determine the Biological relevance of granzyme A and K in sepsis induced by *E. coli.*
    a. Compare survival rates between WT and GzmA KO and GzmK KO.
    b. Determine the levels of proinflammatory cytokines and bacterial load.
    c. Identify the main cellular groups that express GzmA and K.
4. Determine the therapeutic effect of the inhibition of extracellular GzmA using the specific inhibitor Serpinb6b.
    a. Compare survival rates between WT and GzmA KO and GzmK KO after the administration of Serpinb6b during sepsis induced by both *E. coli* and CLP.
    b. Determine the levels of proinflammatory cytokines after the administration of Serpinb6b during sepsis induced by both *E. coli* and CLP.
5. Determine the mechanism by which extracellular GzmA induces the expression of proinflammatory cytokines in different cells groups.
6. Analyze the relevance of granzymes during viral sepsis induced by influenza virus.
    a. Compare survival rates between WT, GzmA KO, GzmK KO, GzmB KO, and Perf KO during sepsis induced by influenza virus.
    b. Determine the levels of proinflammatory cytokines and viral load in lungs from infected mice.
    c. Identify the main lung immune cellular groups that express GzmA, B and K.
    d. Determine anatomic damage produced in the lung tissue due to influenza infection.
7. Analyze the role of granzymes in a model of super infection with influenza and *S. pneumoniae*.
    e. Optimize an *in vivo* model of superinfection.

f. Compare survival rates between WT, GzmA KO, GzmK KO, GzmB KO, and Perf KO.

MATERIALS AND METHODS

3 Materials and Methods

3.1 Bacteria and virus strains culture and storage

All manipulations and experiments with *E. coli* and influenza virus were carried out in a BSL-2 laboratory following all protocols and biosafety recommendations.

3.2 *E. coli* strain isolation, culture, and storage

An *E. coli* strain was isolated from blood of wild type C57BL/6 mice after 24 h of sepsis induced by CLP procedure. This strain was the most frequent gram-negative bacteria isolated from blood of this septic mouse. This strain was identified by biochemical tests and MALDI-TOF mass spectrometry. The *E. coli* strain was stored at -80 °C in Luria-Bertani medium (LB; Sigma) containing 10% glycerol.

3.2.1 E. coli dose preparation

20 µL of *E. coli* were added to 150 mL of LB medium and incubated ON (overnight) at 37 ºC and 200 rpm. At the next day, 50 mL of the initial culture was added to 250 mL of LB medium and incubated at 37 °C and 200 rpm for 4 h. OD_{600} (Optical density) must be at 0.2. After 4 h, with a DO_{600} of 0.5, 100 mL of culture were collected in two 50 mL falcon tubes and centrifuged at 3500 rpm for 10 mins. The remaining pellets were resuspended in 10 mL of PBS and joined in one 50 mL falcon tube. The 50 mL falcon tube with the bacteria was centrifuged one last time at 3500 rpm for 10 mins, the pellet was resuspended in 20 mL of sterile PBS.

The bacteria DO_{600} was adjusted to 0.285 ± 0.005 which represents the theoretical CFU of $1x10^9$ CFU/mL. The dose was confirmed by doing serial dilutions of the stock, seeding them in petri dishes with LB agar, incubating at 37 °C for 24 h, and counting colonies in each dilution.

3.2.2 Influenza virus culture and storage

Influenza A/Puerto Rico/8/34 mouse adapted (H1N1) virus and MCDK (Madin-Darby canine kidney) cells were purchased from bei resources which has a contract with the National Institute of Allergies and Infectious Disease and is administered by ATCC (American Type Culture Collection).

To propagate the virus, MDCK cells were cultivated previously in 75 cm^2 flask (Thermo Scientific, Nunc™) until they were confluent. MDCK growth medium was removed, and the cell monolayer was washed twice with room temperature PBS and once with DMEM (Sigma) + 7.5% BSA (Roche). The flask was carefully inoculated with the virus and rotated to cover the flask surface. The flask was incubated for 30 minutes at 37 °C to allow the inoculum to adsorb. DMEM

+ 7.5% BSA was added to the flask, and it was incubated at 37 ºC until CPE (Cytopathic Effect) was at least 75%.

To harvest the virus, the medium was decantated into a 15 mL falcon tube (Thermo Scientific, Nunc™) and centrifuged for 15 min at 300 x g and 4 ºC to pellet cellular debris. Clarified supernatant was transferred in a 15 mL falcon tube. Finally, the supernatant was dispensed into 2 mL cryovials and store up to 1 year at -80 ºC.

3.3 Cell culture

Cells were cultured in a thermostated incubator (Sanyo CO_2 incubator) at 37 ºC in an atmosphere enriched with 5% CO_2. All manipulations were performed in vertical laminar flow cabinet (Telstar BioII advance class II cabinet) under sterile conditions. Non-sterile materials and solutions were sterilized in an MLS-3751L, Sanyo autoclave for 20 min at 1 atmosphere pressure or filtered through 0.22 µm sterilization membranes (Merck). The cells were observed in a Leica DMi1 inverted microscope and counted in a Neubauer chamber.

3.3.1 Cell lines

The cell lines used in this work have been the following:

- L929: is a mouse fibroblastoma line of C3H (H-2k) origin that secretes macrophage colony-stimulating factor, M-CSF (Macrophage Colony Stimulating Factor), responsible for progenitor proliferation and differentiation myeloid cells in cells of the macrophage and monocyte lineage (Health Protection Agency, cat 85011425).
- X-63Ag8653: is a murine myeloma cell line transfected with a plasmid that produces the mouse GM-CSF, granulocyte-monocyte colony-stimulating factor. This line was provided by Dr. G. Stockinger of the National Institute for Medical Research in London (United Kingdom).
- Madin-Darby canine kidney (MDCK): is a line of epithelial cells isolated from normal kidney tissue from a normal, adult, female dog. They are widely used for the isolation and propagation of human influenza viruses (ATCC, cat NR-2628).

L929 and MDCK cells were cultured in DMEM medium with 10% decomplemented Fetal Bovine Serum (FBS) (Sigma) and 2 mM Glutamax (Gibco). The X63Ag8653 cell line was grown in RPMI (PAN Biotech) supplemented with 10% decomplemented FBS, 50 mM 2β-mercaptoethanol (Sigma) and antibiotics (penicillin (Sigma), 100 U/mL; streptomycin (Sigma), 100 µg/mL and geneticin (Sigma) 1mg/mL).

3.3.2 Maintenance of cell cultures

All cell lines were routinely grown in 25 or 75 cm^2 culture flasks, with filter stopper (Falcon). In the 25 cm^2 flasks, suspension cell cultures were carried out in a volume of 10 to 15 mL and in the 75 cm^2 ones, from 20 to 40 mL. In the case of adherent cells, the final volume was 5 mL in 25 cm^2 flasks and 15 mL in 75 cm^2 flasks. The cells were seeded at an initial density of $5x10^4$ cells/mL and cultured in an incubator at 37 ºC, in a saturated humidity atmosphere with 5% CO_2. Once the saturation densities were reached under these conditions, subcultures derived from the initial ones were established. To do this, the cells were counted with a Neubauer chamber, transferred to sterile conical-bottom tubes, and centrifuged for 5 min at 330 xg and 20 ºC. The supernatant was removed, and the cells were reseeded in fresh medium or used for the different experiments.

In the case of adherent cell lines, cells were detached by incubation with trypsin/EDTA (Sigma) at 37 °C for 5 min. After this time, and after inactivating the trypsin with 4 mL of culture medium with SFB, the cells were collected in conical tubes and subjected to the process described above.

The cell lines were stored in liquid nitrogen according to the following procedure: the cells, grown to their saturation density, were counted, centrifuged at 330 xg for 5 min and, after removing the supernatant, resuspended in their culture medium, usually with 10% DMSO (PanReac) at a cell density of 5-$10x10^6$ cells/mL. They were quickly aliquoted into sterile cryotubes and frozen at -80 ºC for 24-72 hours. Finally, they were preserved in liquid nitrogen, until the moment of their use.

Cells were thawed by gradually adding 10 mL of the appropriate culture medium for their growth and subsequent centrifugation at 330 xg for 5 min, after which the supernatant was removed. Cells were resuspended in a known volume of complete culture medium for counting and viability determination before establishing cell cultures according to the procedure already described.

3.3.3 Counting and determination of cell viability

Cell viability was determined by staining with Trypan blue (Sigma). For the cell count, a 50 µL aliquot of the cell suspension was taken and mixed with the same volume of a 0.4% sterile solution of Trypan blue in 0.15 M NaCl. This mixture was deposited on the Neubauer camera and counted under a microscope (Eclipse E 100, Nikon).

Cell viability was evaluated considering the percentage of viable cells with respect to the total number of cells. The viability in routine cultures was always higher than 90%.

3.3.4 Fetal Bovine Serum (FBS) complement inactivation

In the experiments performed, the serum used was decomplemented to prevent cell lysis by the complement system. For this, the serum was treated for 30 min at 56 ºC and after this time it was centrifuged for 15 min at 2000 xg. The supernatant was collected in a conical bottom tube and frozen until use.

3.3.5 Supernatant production of L-929 cells

For the generation of BMDMs (Bone Marrow Derived Macrophages), L-929 cell supernatant was used as a source of M-CSF. For this, $4.7x10^5$ L-929 cells were seeded in a 75 cm^2 flask with 55 mL of DMEM medium supplemented with 10% decomplemented SFB and 2 mM Glutamax. These cells were cultured for 7 days at 37 °C with 5% CO_2. After this time, the supernatant was collected and filtered using a 22 µm sterile filter. Aliquots of 50 mL were made and frozen at -20 °C.

3.3.6 Supernatant production of X-63Ag8653 cells

For the generation of bone marrow-derived dendritic cells, BMDCs (Bone Marrow Derived Dendritic Cells), or GM-BMDM (GM-CSF-differentiated macrophages), the supernatant of X-63Ag8653 cells was used as GM-CSF source. To obtain them, these cells were cultured in RPMI medium with 5% decomplemented SFB, Glutamax and 1 mg/mL geneticin until $60x10^6$ cells were obtained. Cells were centrifuged at 330 xg for 5 min to remove the geneticin medium and resuspended in 200 mL of RPMI 5% SFB decomplemented medium and Glutamax. The cells were then grown for 48 h until confluent, centrifuged at 330 xg for 5 min, the supernatant collected and aliquoted into 50 mL flasks that were frozen at -20 °C.

3.3.7 Macrophages isolation and differentiation derived from bone marrow (BMDM)

To obtain BMDM, mice with C57BL/6 genetic backgrounds were used. After being euthanized in a CO_2 chamber, their hind limbs were removed. After carefully removing the skin and muscle, they were washed with 70 % ethanol and DMEM, the ends of the bones were cut aseptically, to expose the bone marrow (BM), and the cells were eluted by injecting, under sterile conditions, 5 mL of DMEM medium through the bone cavity. This suspension was then homogenized and filtered through a 100 µm sterile nylon filter (Falcon). The erythrocytes were lysed and the suspension centrifuged at 330 xg for 5 min. The obtained cells were resuspended in DMEM medium supplemented with 10 % SFB and 10 % supernatant of the L-929 line as a source of M-CSF (hereinafter BMDM medium). Cells were counted, the concentration was

adjusted to $1x10^6$ cells/mL and 10 mL were deposited in sterile 90 mm culture dishes (Falcon). Three days later, the plates were washed 2 times with PBS and fresh BMDM medium was added. On day 6, the medium was removed, washed 2 times with PBS, and 3 mL of trypsin was added per plate. After an incubation of 15 min at 37 °C with 5% CO_2, 5 mL of DMEM medium supplemented with 10% SFB (complete medium) were added, the cells were collected in sterile conical-bottom tubes and centrifuged for 5 min at 330 xg. After the supernatant was removed, the cells were resuspended in 10 mL of complete medium, counted, and diluted to a concentration of $5x10^5$ cells/mL. For use in subsequent experiments, cells were distributed at 100 µL per well in 96-well cell culture plates (Falcon) and incubated at 37 °C with 5% CO_2. After 7 days, the macrophages were fully differentiated, showing a CD11b+ CD11c- phenotype, analysed by flow cytometry.

3.3.8 Isolation of Mouse Peripheral Blood Lymphocytes (PBLs)

After 24 h of sepsis induction, WT and $GzmA^{-/-}$ mice were sacrificed. Total blood was collected aseptically by cardiac puncture in presence of anticoagulants Sodium Citrate 3.8% (Sigma). PBLs were isolated by gradient centrifugation. 1 mL of total blood was mixed with 1 mL PBS. To a conical 15 mL tube, 2 mL of Histopaque-1077 (Sigma) was added and then blood was carefully added onto Histopaque-1077. Next, the sample was centrifuged at 400 xg for 30 min at room temperature. Mononuclear cells were collected from the opaque interface.

3.3.9 *Infection of bone marrow macrophages with* E. coli

M2 macrophages were differentiated from mouse bone marrow. Cells were aseptically collected from BM and resuspended in RPMI medium supplemented with 10% of FCS serum, 100 U/mL of penicillin/streptomycin, 50 mM of 2-ME, and 10% supernatant of L-929 cell culture as source of M-CSF. Cells were seeded at a density of 1×10^6 cells/mL and allowed to differentiate for 6 days at 37 °C and 5% CO_2 atmosphere. For *in vitro E. coli* infection experiments, $5x10^5$ macrophages/mL were seeded a volume of 100 µL per well in 96 well plates, incubated with 300 nM of active GzmA and infected with *E. coli* at a MOI of 1:100 for 1 h at 37 °C and 5% CO_2. Subsequently, medium was removed, cells were washed with PBS and further incubated with complete RPMI medium containing 30 µg/mL of gentamycin for 1 h. Cells were washed and incubated with complete RPMI medium for 4, 24, and 48 h. After incubation periods, macrophages were lysed with Triton-X 0.1% (Sigma) and CFU number was determined.

3.3.10 Inflammation induced by GzmA in bone marrow derived macrophages (GM BMDM)

M1 Macrophages were differentiated from WT and TLR4$^{-/-}$ mouse bone marrow. Cells were aseptically collected from femurs and tibias and 1x10^6 cells were culture on 100 mm petri dishes with 10 mL of RPMI 1640 medium containing 10% of FCS serum, 100 U/mL of penicillin/streptomycin, 50 mM of 2-ME, and 10% of supernatant of X63Ag8653 cell cultures as source of GM-CSF (complete medium). On days 3 and 5, the supernatant was removed, and 10 mL of fresh complete medium was added. On day 7, macrophages showed a complete differentiated phenotype expressing CD11b and F4/80. WT and TLR4$^{-/-}$ macrophages were stimulated with active GzmA (300 nM), GzmA inactivated with the inhibitor serpinb6b (2,4 µM), *E. coli*-derived LPS (100 ng/mL) or heat killed *S. aureus* (*S. aureus* HK,1 x10^8 CFU/mL). After 24 h of incubation, the supernatant was collected to determine the cytokine levels by ELISA (Invitrogen).

3.3.11 Inflammation induced by GzmA in macrophages from WT, TLR4$^{-/-}$, and MyD88$^{-/-}$

M1 macrophages were differentiated from mouse BM of WT, TLR4$^{-/-}$, and MyD88$^{-/-}$ mice. Cells were aseptically collected from femurs and tibias and resuspended in RPMI medium supplemented with 10% of FCS serum, 100 U/mL of penicillin/streptomycin, 50 mM of 2-ME, and 10% supernatant of X-63 cell culture as source of GM-CSF. Cells were seeded at a density of 1×10^6 cells/mL and allowed to differentiate for 6 days at 37 °C and 5% CO_2 atmosphere. For in vitro experiments, 5x10^5 macrophages were seeded in 96 well plates and incubated with 300 nM of active GzmA overnight at 37 °C and 5% CO_2. After incubation, supernatants were collected, and IL-6 levels were measured using a commercial ELISA kit. In some cases, the TLR4 pathway inhibitors TAK242 (Merck) (1 µM) and OxPAPC (InvivoGen) (30 µg/mL) were used in WT macrophages. Cells were preincubated with the inhibitors for 30 min at 37 °C/5% CO_2 and, subsequently, treated with LPS (10 ng/mL) or active GzmA (300 nM) overnight at 37 °C/5% CO_2. After incubation, supernatants were collected, and IL-6 was measured using a commercial ELISA kit.

3.3.12 Inflammation induced by human GzmA in human monocytes

Monocytes were isolated from peripheral blood of healthy donors by a ficoll (Sigma) gradient centrifugation. Diluted blood was carefully layered on top of 15 mL of Histopaque 1077 (1.077 g/mL) solution. Centrifugation was performed for 10 min at 1455 xg without brake. After centrifugation, the white ring of PBMCs (Peripheral Blood Mononuclear Cells) were collected, resuspended in 15 mL of complete DMEM medium (10% of FCS serum, 100 U/mL of

penicillin/streptomycin, 50 mM of 2-ME), placed on a 75 cm2 flask and incubated for 2 h at 37 °C and 5% CO_2 to allow monocytes to adhere at the flask bottom. After 2 h of incubation, cells were washed with PBS to remove cells in suspension. Adherent cells were trypsinized and $5x10^5$ monocytes were seeded in 96 well plates and incubated for 2 h to allow them to adhere. Monocytes were stimulated with LPS (100 ng/mL), human GzmA (300 nM), antithrombin III (8.6 µM), and heparin (430 µM). After 24 h of incubation, supernatant was collected to determine IL-6 by ELISA.

3.4 Mouse Strains

Animal experimentation protocols were established, approved, and updated by the Animal Experimentation Ethics Committee of the University of Zaragoza, numbers PI43/13 (CLP protocol) and PI63/17 (*E. coli* sepsis protocol).

The animals used to perform the experiments were:

3.4.1 CONTROL (Wild type -WT)

- C57BL/6j (B6) strain was purchased from two different sources. A group of control mice was purchased from Charles River and maintained at the Biomedical Research Centre of Aragon (CIBA). And the second group of WT mice was purchased from ENVIGO.

3.4.2 KNOCK OUT ANIMALS (KO)

- $GzmA^{-/-}$, $GzmB^{-/-}$, $GzmK^{-/-}$, and $Perf^{-/-}$ mice were kindly provided by Dr. Markus Simon from Max-Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany and maintained at the Biomedical Research Centre of Aragon (CIBA). The genetic background of these mice is C57BL/6.
- $TLR4^{-/-}$ mice were kindly provided by Dr. Marina Freudenberg from Max-Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany and maintained at the Biomedical Research Centre of Aragon (CIBA). The genetic background of these mice is C57BL/10.

Animals were maintained in a free pathogen environment with food and water "*ad libitum"*. Mice of 8–12 weeks of age were used in all the experiments and their genotypes were periodically analysed by PCR.

3.5 Human samples analysis

3.5.1 Patients and study design

A total of 10 patients with a diagnose of peritonitis and with a SOFA score ≥ 2 were prospectively recruited over a 3-month period in 2019 after admission to Lozano Blesa Hospital in Zaragoza, Spain. Blood samples were collected at admission and during the first 72 hours. Blood samples were spun down at 2000 xg for 10 min and serum was collected and stored at -80 °C. GzmA concentration in serum was determined by ELISA.

3.5.2 Human GzmA ELISA

Human serum GzmA levels from healthy donors and patients with confirmed abdominal sepsis at diagnose and during the first 72h was monitored by a commercial ELISA kit (Human Granzyme A DuoSet ELISA, R&D Systems) following the manufacturer recommendations.

3.6 *In-vivo* experimentation

3.6.1 Cecal Ligation and Puncture

PMS (Polymicrobial sepsis) was induced by CLP performed according to general guidelines. WT and GzmA$^{-/-}$ mice were anesthetized using 2% isoflurane in oxygen. After disinfecting the abdomen, a 1 cm midline incision was performed to expose the cecum. From the tip, 1 cm of the cecum was ligated using a non-absorbable suture 3-0 (Silkam black 3/0 HS26, Braun) and subsequently perforated twice by a through-and-through puncture with a 20G needle. After gently squeezing the cecum, to extrude enough faeces from the perforation, the cecum was returned to the peritoneal cavity and the abdominal musculature was sutured with absorbable suture 3-0 (Novosyn violet 3/0 HR26, Braun). The skin was then sutured with non-absorbable suture 3.0 and a subcutaneous dose of 0.05 mg/kg of buprenorphine in 1 mL of normal saline was administered. After 6 h of the intervention a mixture of antibiotics, ceftriaxone (30 mg/kg) + Metronidazole (12.5 mg/kg), was administered i.p. once a day for 5 days. WT and GzmA$^{-/-}$ sham operated mice underwent the same procedure but without CLP.

Mouse survival was monitored for 14 days. Mice were observed three times a day and if there were signs of respiratory distress, pain when touching, vocalisations, hunched posture, or inability of a supine animal to stand, the human endpoint was applied.

3.6.2 E. coli sepsis induction

To prepare the inoculum for sepsis induction, 20 µL of *E. coli* stock was cultured in LB medium at 37 ºC to exponential growth phase and washed twice with cold phosphate-buffered saline (PBS). The absorbance at 600 nm was measured in a spectrophotometer to estimate the

number of bacteria in the culture. An OD_{600} of 0.285 ± 0.005 represents a theoretical CFU of $1x10^9$ CFU/mL. The bacterial density was adjusted to $1x10^9$ bacteria/mL. Sepsis was induced in WT, $GzmA^{-/-}$ and $GzmK^{-/-}$ mice by i.p of $2x10^8$ bacteria in 200 µL of PBS.

3.6.3 Influenza virus sepsis induction

To prepare the inoculum for sepsis induction a dilution was made from the virus stock ($2,8x10^9$ PFU). A viral concentration of $2x10^3$ PFU was administered intranasally, for which mice were put to sleep using isoflurane (Isoflutek®). Mice were anesthetized in a mouse anesthesia induction chamber where 4-5% of isoflurane was used for induction and 1-2% was used for maintenance. Once asleep, two drops of 20 µL each were administered within a couple of minutes between each other to avoid damage to the respiratory tract.

3.6.4 Coinfection model

The coinfection model consisted in a double infection within 5 days between each other. First, we induced an influenza infection as explained in section 1.6.3. The second infection was done with a serotype 3 of *S. pneumoniae* for which we incubated 10 µL of the bacteria stock in 5 mL of BHI (Brain Hearth Infusion) medium and incubated it for 4 h at 37 °C and 180 rpm. At a OD_{600} of 0.98 the concentration of the bacteria was of $1.3x10^8$ CFU. The bacterial density was adjusted to various doses to carry out different experiments. For the pneumonia induction mice were anesthetized in a mouse anesthesia induction chamber where 4-5% of isoflurane was used for induction and 1-2% was used for maintenance. Once asleep, two drops of 20 µL each were administered within a couple of minutes between each other to avoid damage to the respiratory tract.

3.6.5 Survival experiments and Sepsis Score

Mice were weighed daily and observed twice a day determining a MSS (Murine Sepsis Score) as described in (Bradly Shrum et al., 2014). Briefly, seven variables were analysed (Appearance, Level of consciousness, Activity, Response to stimulus, Eyes, Respiration rate and Respiration quality), each one received a score between 0 and 4. Mice were euthanized when MSS was superior to 21, or when either the score of Respiration rate or Respiration quality increased more than 3. Survival was monitored for 5 days in polymicrobial sepsis and for 15 days in viral sepsis.

3.6.6 Collection of blood, peritoneal lavage fluid and organs

For CLP experiments, samples were collected after 6 and 24 h of sepsis induction, a group of septic WT and $GzmA^{-/-}$ mice as well as sham operated mice were sacrificed, and blood samples were obtained aseptically by cardiac puncture. Anticoagulated blood was kept on ice

until further processing for bacteriologic analysis. The rest of blood samples were centrifuged at 2000 xg for 15 min, and plasma was recovered and stored at -80 °C. To collect peritoneal lavage fluid, 5 mL of sterile PBS was slowly injected into the peritoneal cavity with a 18G needle. The abdomen was gently massaged and then the peritoneal fluid was recovered. Part of it was used for bacteriologic analysis while the rest was centrifuged at 2000 xg for 10 min and the supernatant was stored at -80 °C. Finally, spleen, liver and lungs were collected aseptically, homogenized in 1 mL of PBS, and used for bacteriological analysis.

For *E. coli* experiments, samples were collected after 24 and 48 h of sepsis induction, and blood, liver and spleen were collected aseptically. Both organs were homogenized, serial dilutions were carried out in PBS, and used for bacteriological analysis. Finally, blood samples were centrifugated at 3700 xg for 10 mins to obtain plasma.

3.6.7 Reverse transcription and PCR for Nnt detection

RNA was isolated from hearts of WT, GzmA-/-, GzmB-/- and GzmK-/- mice using the RNAeasy Protect Mini Kit (Qiagen) and following the manufacturer instructions. For cDNA preparation, 2 µg of RNA was reverse transcribed using the Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), for which 10 µL of 2X RT master mix and 10 µL of RNA sample were added into a reaction tube and mixed gently. The thermal cycler program used for the reverse transcription was:

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature	25 °C	37 °C	85 °C	4 °C
Time	10 minutes	120 minutes	5 minutes	Hold

Forward and reverse templates sequences used for Nnt were:

F1: AACAGTGCAAGGAGGTGGAC

R1: GTGCCAAGGTAAGCCACAAT

PCR was performed using a Hot Start Taq 2X Master Mix and following the recommended protocol:

Component	**25 µL REACTION**	**FINAL CONCENTRATION**
10 µM Forward Primer	0.5 µL	0.2 µM
10 µM Reverse Primer	0.5 µL	0.2 µM
cDNA	Variable	< 1000 ng

Hot Start Taq 2X Master Mix	12.5 µL	1X
Nuclease-free water	to 25 µL	

And the thermocycling conditions for a routine PCR we used were:

STEP	TEMPERATURE	TIME
Initial Denaturation	95 ºC	30 seconds
30 Cycles	52 ºC	60 seconds
Final Extension	68 ºC	5 minutes
Hold	4 ºC	

PCR product was visualized in a 1% agarose gel stained with SYBR safe and revealed in a BioRad Gel Documentation System.

3.6.8 Determination of Bacterial load in blood, liver, and spleen

A group of mice were sacrificed after sepsis induction, and blood, liver and spleen were collected aseptically. Both organs were homogenised, and serial dilutions were carried out in PBS, including the blood, plated onto LB agar, and incubated for 24 h at 37 ºC to determine the number of viable *E. coli* organisms.

3.6.9 Determination of cytokine levels

After sepsis induction, a group of mice was sacrificed, and blood samples were collected by cardiac puncture using anticoagulant. Blood samples were centrifuged at 3700 xg to obtain plasma. Levels of mouse IL-1α, IL-1β, TNFα and IL-6 were measured in peritoneal exudate and plasma using commercial ELISA kits.

3.6.10 Blood and lung collection in influenza virus sepsis

After influenza infection a group of mice were sacrificed with a lethal dose of Pentothal 200 mg/kg (i.p). Blood was taken by cardiac puncture and placed in an eppendorf with 10 µL of EDTA (0.5 M) as an anticoagulant. After blood collection, mice lungs were collected aseptically and avoiding damaging the tissue with the surgical material. Lungs were cut in 4 different parts. Half left lung used for anatomical pathology was placed in a 15 mL falcon tube for fixation using 10% of buffered formalin. Remaining left lung half and right lung inferior lobe were collected in a MACS C tube with 2 mL of RPMI medium. Right lung superior lobe for virus titration was placed in a MACS M tube with 500 µL of DMEM medium. Finally, right lung middle and post-caval lobes

for RNA analysis were pieced in small portions and placed in an Eppendorf tube with 500 µL of RNA later (RNA protect, Qiagen).

3.6.11 Therapeutic inhibition of extracellular GzmA with Serpinb6b6

For CLP experiments, after sepsis induction a group of WT, GzmA$^{-/-}$ mice were treated with 40 µg of serpinb6b, a specific mouse GzmA inhibitor, in 100 µL of PBS i.p. The inhibitor was administrated after 6 h and once a day for 5 days combined with ceftriaxone (30 mg/kg) + Metronidazole (12.5 mg/kg). Survival was monitored for 14 days.

For *E. coli* experiments, after sepsis induction a group of WT, GzmA and GzmK deficient mice were treated twice a day with 40 µg of serpinb6b in 100 µL of PBS i.p for 5 days. A control group of these mice were treated with 100 µL of PBS i.p twice a day. Mice were observed twice a day and survival was monitored for five days.

3.6.12 Anatomic Pathology Analysis

Lungs were collected aseptically as explained previously (1.5.7). The left lung was placed in the cassette with the costal side upwards then fixated in 10% pH 7.4 buffered formalin (PanReac). After 24 h the fixed organs were embedded in paraffin. Paraffin blocks were cut and prepared for Haematoxylin-Eosin staining.

3.7 Proteins Analysis

Analysis of protein expression in cells was performed on a Gallios flow cytometer (Beckman Coulter) and the results were analysed using the Kaluza Analysis 2.1 software. The antibodies used in flow cytometry are shown in tables 7 and 8.

3.7.1 Flow cytometry analysis for membrane proteins

Analysis of the presence of certain cell membrane proteins by flow cytometry was performed with antibodies linked to fluorochromes (Tables 7 & 8).

For this, 10^5 cells were centrifuged for 5 min at 330 x g and washed once with PBS supplemented with 5% FBS. Next, the cells were incubated in 100 µL of PBS with 5% FBS for 15 min at 4 °C, at the antibody concentration indicated in tables 7 & 8 respectively. After this time, they were washed 3 times with PBS with 5% SFB and fixed with 100 µL 4% PFA (Paraformaldehyde) for 15 min at 4 °C. Subsequently, the paraformaldehyde was diluted to 1% by adding 300 µL of PBS and the cells were analysed by flow cytometry.

3.7.2 Analysis of the intracellular expression of GzmA, B and K by flow cytometry

After 18 and 24 h of *E.coli* sepsis induction and CLP respectively, mice were sacrificed. Blood, spleen, and lungs were collected aseptically. PBLs were isolated from blood as described above and spleen was homogenized in 5 mL of RPMI medium. $2x10^5$ PBLs or $1x10^6$ splenocytes were stained with the antibodies of table 7. Subsequently, cells were fixed with PFA 1%, permeabilised with saponin 1% in PBS and incubated with anti GzmA and GzmK antibodies. Finally, intracellular expression of GzmA and GzmK was analysed by FACS.

For influenza infection, after 5 and 7 days of infection, WT, $GzmA^{-/-}$, $GzmB^{-/-}$, $GzmK^{-/-}$ and $Perf^{-/-}$ mice were sacrificed. Lungs were collected aseptically, washed with sterile PBS and collected in a MACS C tube (Miltenyi Biotec) with 5 mL RPMI (PAN Biotech), 20 µL liberase TM (Roche) and 20 µL DNAseI (Roche). Later, lungs in the MACS C tubes were shredded using the GentleMACS protocol Lung 01 and incubated for 30 mins in water at 37 ºC, moving each 5 min. After incubation, GentleMACS protocol Lung 02 was used to finish homogenising the lungs. Lungs were later centrifuged 5 min at 1500 rpm, supernatant was discarded, and cells were resuspended in 1 mL RBC lysis buffer (Alfa Aesar) for 3 min. Lysis was stopped by adding 10 mL of complete RPMI medium. The homogenisation was filtered in a 70 µm cell strainer, washed using RPMI medium in a 50 mL falcon tube, and centrifuged for 5 min at 1500 rpm. Finally, the supernatant was discarded, the cells resuspended 400 µL of RPMI and 100 µL were seeded in 3 different round bottom 96 well plates (ThermoScientific; Nunclon™). Cells were stained using the antibodies showed in table 8. Subsequently, cells were fixed and permeabilised using the fixation/permeabilization buffers from Miltenyi Biotec and incubated with anti GzmA and GzmB antibodies. Finally, Gzms expression was analysed by FACS.

Table 3.1 Antibodies used in E. coli sepsis and CLP experiments

ANTIBODY	CHARACTERISTICS	CONCENTRATION	SUPPLIER
CD3-FITC	Mc, rat	1/50	Miltenyi Biotec
CD8-APC	Mc, rat	1/50	Miltenyi Biotec
NK1.1-APC-VIO770	Mc, mouse	1/50	Miltenyi Biotec
CD4-VIOBLUE	Mc, rat	1/10	Miltenyi Biotec
CD4-VIOGREEN	Mc, rat	1/50	Miltenyi Biotec
CD45-BV421	Mc, rat	1/50	Biolegend
LY6G- FITC	Mc, human	1/50	Miltenyi Biotec
LY6C-VIOGREEN	Mc, rat	1/50	Miltenyi Biotec
CD11B-APC-VIO770	Mc, mouse	1/50	Miltenyi Biotec

CD11C-APC	Mc, mouse	1/50	Miltenyi Biotec
GZMA-PE	Mc, mouse	1/50	Invitrogen
GZMK-PE	Pc, rabbit	1/25	MyBioSource

Mc: monoclonal, Pc: polyclonal, PE: Phycoerythrin, APC: Allophycocyanin, FITC: Fluorescein Isocyanate.

Table 3.2 Antibodies used in influenza infection experiments

ANTIBODY	CHARACTERISTICS	CONCENTRATION	SUPPLIER
VIABILITY DYE GREEN-488	-	-	Miltenyi Biotec
SIGLECF-PE	Mc, rat	1/50	BD Bioscience
CD64- PE-DAZZLE (CF594)	Mc, mouse	1/50	Biolegend
LY6G- BB700	Mc, mouse	1/50	BD Bioscience
LY6C- PE-CY7	Mc, rat	1/50	Biolegend
CD11B- APC	Mc, rat	1/50	Biolegend
CD11C- ALEXA FLUOR 700	Mc, hamster	1/20	Biolegend
MHC-II- APC-FIRE750	Mc, rat	1/50	Biolegend
GZMB- BV421	Mc, rat	1/100	Biolegend
CD45- VIOGREEN	Mc, rat	1/50	Miltenyi Biotec
GZMA-PE	Mc, mouse	1/50	Invitrogen
CD3- PE-DAZZLE (CF594)	Mc, rat	1/50	Biolegend
CD4-BB700	Mc, rat	1/50	BD Bioscience
CD8A-PE-CY7	Mc, rat	1/50	Biolegend
NKP46-APC	Mc, rat	1/50	Biolegend
CD69-ALEXA FLUOR 700	Mc, hamster	1/20	Biolegend
CD62L-APC-FIRE750	Mc, rat	1/50	Biolegend
CD140A-PE	Mc, rat	1/50	Biolegend
PODOPLANIN-PE-DAZZLE (CF594)	Mc, hamster	1/50	Biolegend
CD31-BB700	Mc, rat	1/50	BD Bioscience
CD95-PE-CY7	Mc, hamster	1/50	BD Bioscience
CD44-APC	Mc, rat	1/50	Biolegend
LY6C-ALEXA FLUOR 700	Mc, rat	1/20	Biolegend
CD326 EP-CAM-APC-FIRE750	Mc, rat	1/50	Biolegend
A-SMOOTH MUSCLE-BIOTIN-STREPTAVIDIN	Mc, mouse	1/25	Invitrogen

Mc: monoclonal, PE: Phycoerythrin, APC: Allophycocyanin, FITC: Fluorescein Isocyanate.

3.7.3 Cytokines quantification by ELISA

To quantify the following cytokines in cell culture supernatants or serum: IL-1α, IL-1β, IL-6, and TNF-α, different mouse uncoated ELISA Kits from (Invitrogen) were used.

Generally, 100 µL of the capture antibody at the recommended concentration, in coating buffer, was added to the 96-well ELISA plate (NUNC Maxisorp) and incubated overnight at 4 °C. The plate was washed 3 times with 200 µL of washing solution (1X PBS with 0.05% TWEEN 20) and then blocked with 200 µL of assay buffer (¨Buffer Assay¨; ELISA/ELISPOT; Invitrogen) for 1 h at room temperature. The standard and blank were added as recommended by the manufacturer, in addition to the samples diluted in assay buffer, incubating for 2 h at room temperature. After washing the plate 3 times with washing solution, 100 µL of biotin-conjugated detection antibody at the recommended concentration was added and incubated for 1 h at room temperature. The plate was washed 3 times with wash solution, 100 µL of HRP-linked avidin enzyme ("horseradish peroxidase"; 1:4000 in assay buffer) was added and incubated for 30 min. Next, the plate was washed 5 times and 100 µL of TMB (3,3',5,5'-Tetramethylbenzidine) were added. After 15 min, the reaction was stopped by adding 50 µL of H2SO4 (2N). The absorbance was determined in a plate reader (Synergy ™ HT, BioTek) at 450 nm and 570 nm.

3.8 Recombinant proteins production

3.8.1 *Recombinant GzmA expression from* E. coli

Mouse proGzmA was cloned into pET21a and transformed into *E. coli* BL21. For protein production, *E. coli* expressing proGzmA were grown at 37 °C until the culture reached an optical density at 600 nm between 0.6 and 0.8. The protein expression was induced adding IPTG 1 mM and then was cultured 3 hours at 37 °C. The culture was centrifugated at 8300 xg for 15 min. The supernatant was discarded, and the pellet was resuspended in lysis buffer (Tris-HCl 20 mM, NaCl 100 mM, DTT 2 mM, lysozyme 2 mg/mL, DNAase 1 mg/mL and protein inhibitor). Cells were lysed by mechanical disruption by sonication, centrifugated at 48400 xg for 15 min at 4 °C and the supernatant was discarded. Previously obtained inclusion bodies were resuspended in buffer denaturation (100 mM Tris-HCl, 6 M GuCl2, 20 mM EDTA and 10 mM oxidized DTT) and then centrifugated at 48400 xg for 15 min at 4 °C. Proteins were refolding in 100 volumes of refolding buffer (100 mM Tris-HCl, 500 mM arginine, 10 % glycerol and 10 mM oxidized DTT) for 3 days at 4 °C and then were dialyzed five times in MT-PBS. Recombinant proGzmA was purified by cation exchange chromatography. Active GzmA was obtained using cathepsin C which hydrolyse the N-terminal dipeptide present in proGzmA.

3.8.2 Recombinant GzmA expression from P. pastoris

Pichia pastoris expressing mouse proGzmA was kindly provided by Phillip I. Bird from Monash University, Australia. *P. pastoris* expressing mouse proGzmA were grown at 30 °C for 36 h, and then allowed to settle for 12 h at room temperature; growth medium was replaced with induction medium containing 3 % methanol and 0.5 M arginine. Cells were induced at 23 °C for 60 h. Culture was centrifugated, the supernatant was collected and then filtered. Recombinant proGzmA was purified by cation exchange chromatography. Active GzmA was obtained using cathepsin C which hydrolyse the N-terminal dipeptide present in proGzmA.

3.8.3 *Recombinant mouse Serpinb6b expression from* P. pastoris

Pichia pastoris expressing serpinb6b was kindly provided by Phil Bird from Monash University, Australia. *P. pastoris* expressing serpinb6b was grown at 30 °C for 36 h and then allowed to settle for 12 h at room temperature; growth medium was replaced with induction medium containing 3% methanol and 0.5 M Arginine. Induction was performed at 23 °C for 60 h. Culture was centrifuged and a chemical and physical lysis was performed. After supernatant clarification, recombinant serpinb6b was purified by immobilized metal (Nickel) affinity chromatography.

3.8.4 LPS quantification in mouse and human GzmA preparation

Mouse and human proGzmA LPS quantification was carried out using the Toxin Sensor™ Endotoxin Detection System (GenScript). Kit reagents and standard were prepared following manufacturer instructions. Once the reagents and the samples were ready, 100 µL of the samples and the standard were carefully dispensed into different endotoxin-free vials with 100 µL of LAL-water and mixed thoroughly for 30 seconds with a vortex avoiding foaming. Later, 100 µL of reconstituted LAL (Limulus Amebocyte Lysate) was added to each tube, mixed gently and incubated at 37 ºC using a heating block for 30 mins. After proper incubation, 100 µL of chromogenic substrate was added in each vial and incubated for 6 mins at 37 ºC using a heating block. Finally, 500 µL of stop solution (color-stabilizer #1) was added to each vial and mixed gently. Then 500 µL of color-stabilizer #2 was added to each vial and mixed gently. At last, 500 µL of color-stabilizer #3 was added to each vial and mixed gently. The absorbance was read at 545 nm in a plate reader (Synergy ™ HT, BioTek). The amount of LPS in all protein preparations was always lower than 0.5 EU/µg protein.

3.9 Statistical Analysis

To analyse the differences in MSS and weight between mouse groups we performed a Mixed Linear Regression with the IBM SPSS Statistics 25 software. MSS was chosen as the

dependent variable, mouse group (WT, GzmA-/- or GzmK-/-) as subject (grouping element), time as an actor and weight as a covariable. Differences between single groups were performed using ANOVA and Bonferroni´s post-test for the variable that showed statistical significances in Mixed Linear Regression. Logrank and Gehan-Wilcoxon test for survival and GzmA therapeutic inhibition analyses, one-way ANOVA test with Bonferroni's post-test for bacterial load in blood and spleen analysis and extracellular expression of GzmA and unpaired t test for inflammation induced by GzmA and TLR4 pathway inhibition experiments were performed with the GraphPad Prism software.

RESULTS

4 Results

4.1 Role of Granzyme A in abdominal sepsis

4.1.1 Extracellular GzmA is increased in patients undergoing abdominal sepsis

We analyzed the levels of GzmA in serum from patients with abdominal sepsis diagnosed with peritonitis with a SOFA score >2 and compared them with the levels of GzmA from healthy donors. Patients' characteristics are included in table 4.1. There were 6 males and 4 females with a medium age of 76 years from which 3 patients deceased after 30 days. At the time of diagnosis (time = 0) and after 24, 48, and 72 h, septic patients showed elevated levels of GzmA compared to healthy donors GzmA levels (Figure 1). These results show that there are elevated levels of GzmA during abdominal sepsis and suggests that extracellular GzmA could be involved in its pathogenesis.

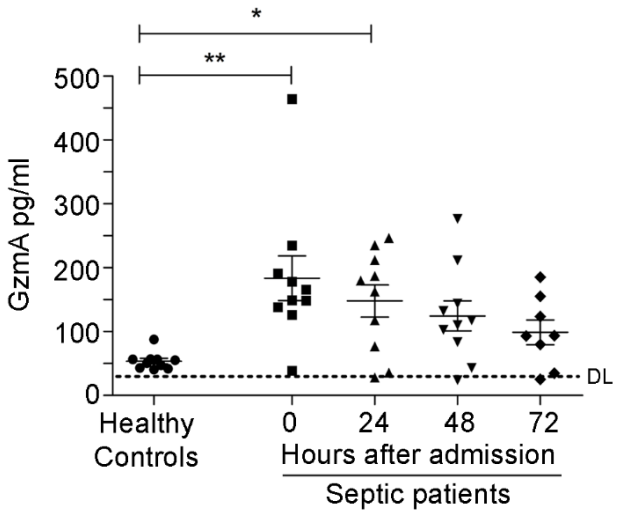


Figure 4.1 Increased levels of extracellular GzmA are observed in patients with abdominal sepsis. Serum levels of GzmA were analyzed by ELISA in healthy donors (n=10) and compared with GzmA levels from patients with a diagnosis of abdominal sepsis (n=10) at diagnosis (time 0 = disease onset) and during the first 72 h. Statistical analyses were performed by one-way ANOVA test with Bonferroni's post test *P < 0.05; **P <0.01- DL (ELISA Detection Limit).

Table 4.1 Clinical data of abdominal sepsis patients

Patient	SOFA score	Gender	Age	Status at day 30	Co-morbidities
1	2	Male	62	Alive	Gastric tumor + chemotherapy
2	2	Male	68	Alive	Coronary disease, peripheral vascular disease, peptic ulcer.
3	5	Female	75	Dead	Dementia, cerebrovascular disease, renal disease, diabetes with damage to target organs.
4	7	Female	72	Alive	Congestive heart failure, connective tissue disease (immunosuppression), renal disease.
5	7	Male	75	Alive	Diabetes, bile-duct tumor.
6	8	Male	67	Alive	Coronary disease, congestive heart failure, diabetes with damage to target organs.
7	6	Female	78	Dead	None
8	6	Male	90	Alive	None
9	7	Female	82	Dead	Cerebrovascular disease, peptic ulcer, renal disease, renal tumor.
10	9	Male	94	Alive	Chronic pulmonary disease

4.1.2 GzmA deficient mice are protected from CLP

With these results, observing that there is an increase in the levels of GzmA in patients with abdominal sepsis, we decided to study the relevance of GzmA in sepsis by testing its role in the CLP mouse model, one of the best murine models that mimics the complex septic response in human abdominal sepsis (Korneev, 2019). We applied a severe sepsis protocol to WT and GzmA$^{-/-}$ as described in materials and methods (3.6.1). To mimic the clinical management of septic patients, a broad-spectrum of antimicrobial treatment was administered 6 h after CLP, and survival was monitored for 14 days.

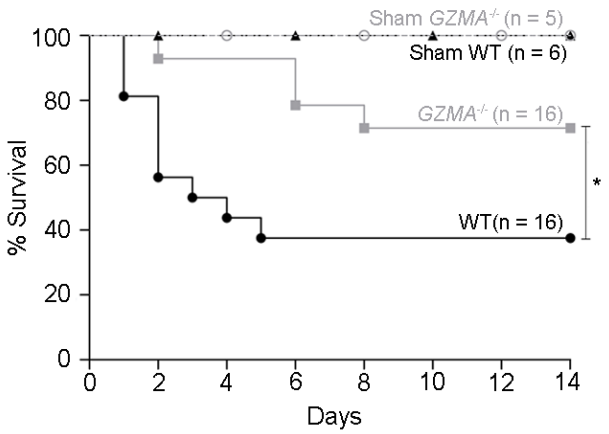


Figure 4.2 The absence of GzmA increases survival during CLP induced sepsis. Sepsis was induced by CLP in WT and GzmA$^{-/-}$ mice as described in material and methods. After 6 h, a mixture of antibiotics, ceftriaxone (30 mg/kg) + metronidazole (12.5 mg/kg), was administered i.p. every 24 h for 5 days. WT and GzmA$^{-/-}$ sham operated mice underwent the same procedure without the ligation and puncture of the cecum. Survival was monitored for 14 days. The shown data correspond to the indicated number of biological replicates (individual mice) from three independent experiments. Statistical analysis was performed using logrank and Gehan-Wilcox test, *p <0.05.

A shown in figure 2, survival of GzmA$^{-/-}$ mice was significantly higher than WT controls. Showing that a 70% of GzmA$^{-/-}$ mice survived after 2 weeks compared to a survival rate less than 40% of WT mice. All sham controls survived, confirming that the procedure was performed correctly.

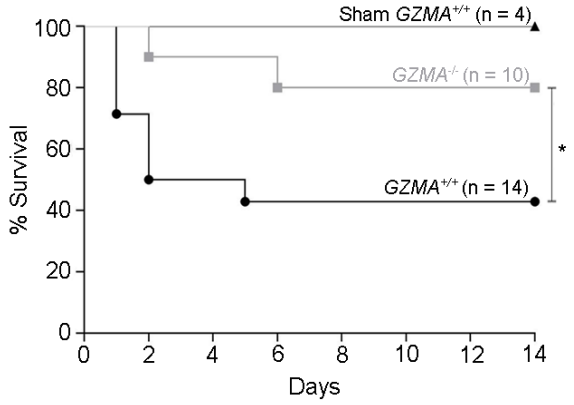


Figure 4.3 Littermates of GzmA KO mice have higher survival during CLP sepsis. Sepsis was induced by CLP in GzmA$^{-/-}$ and GzmA$^{+/+}$ littermates as described in materials and methods. After 6 h, a mixture of antibiotics, ceftriaxone (30 mg/kg) + metronidazole (12.5 mg/kg), was administered i.p. every 24 h for 5 days. GzmA$^{+/+}$ and GzmA$^{+/+}$ sham operated mice underwent the same procedure without the ligation and puncture of the cecum. Survival was monitored for 14 days. The shown data correspond to the indicated number of biological replicates (individual mice) from three independent experiments. Statistical analysis was performed using logrank and Gehan-Wilcox test, *p <0.05**.**

Backcrossing a targeted gene on a control mouse to obtain a knock-out strain, even with ten generations of backcrossing, has the risk of not necessarily cleaning up the genetic background (Holmdahl & Malissen, 2012). With this in mind, we performed our experiments using GzmA littermates mice to confirm that the genetic background had not interference in our results. As shown in figure 3, CLP was performed in $GzmA^{-/-}$ and $GzmA^{+/+}$ littermates showing that an 80% of $GzmA^{-/-}$ mice survived after 2 weeks compared to a survival rate of 40% in $GzmA^{+/+}$. All sham controls survived, confirming that the procedure was performed correctly. The use of littermates helped ensure that the genetic background and the environment are comparable, discarding the fact that minimal genetic difference could influence in the results.

4.1.3 GzmA deficient mice show reduced levels of proinflammatory cytokines during abdominal sepsis

The next step was to confirm that the protection observed in GzmA deficient mice during sepsis induced by CLP correlated with a reduction in the levels of proinflammatory cytokines, for which we analyzed the levels of IL-6, IL-1α, IL-1β and TNF-α in plasma and peritoneal lavage fluid samples.

As shown in figure 4, $GzmA^{-/-}$ mice showed significantly lower levels of all cytokines in both plasma and peritoneal lavage fluid after 6 and 24 h of sepsis induction. Nevertheless, it is important to note that, even though all cytokines were reduced in the biological fluids of GzmA KO mice after CLP, only IL-6 and IL-1α were significantly lower than controls at 6 h in both plasma and peritoneal fluid. At last, after 24 h, all analyzed cytokines were significantly reduced un $GzmA^{-/-}$ mice. These results helped us confirm that GzmA regulates the expression of proinflammatory cytokines *in vivo* during abdominal sepsis.

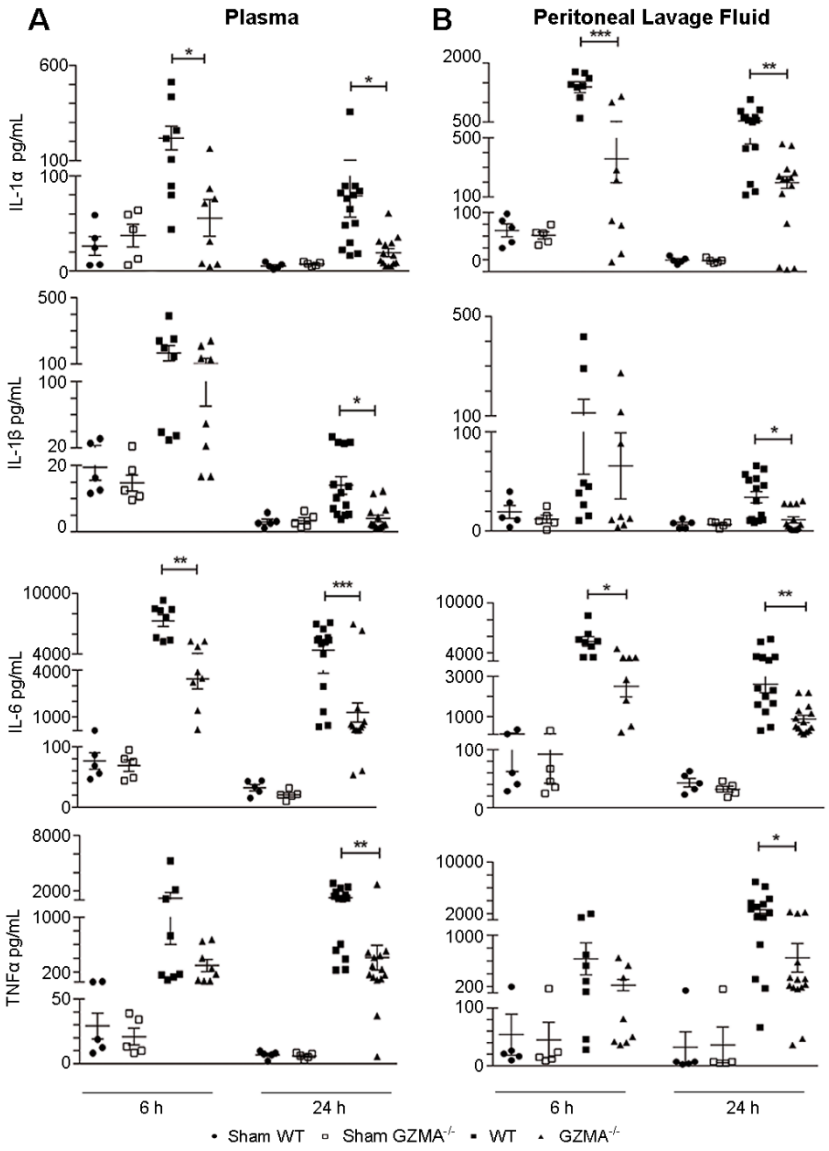


Figure 4.4 The absence of GzmA reduces the level of proinflammatory cytokines during sepsis induced by CLP. Sepsis was induced by CLP as described in materials and methods. After 6 and 24h of sepsis induction, mice were sacrificed, plasma and peritoneal lavage fluid samples were taken and the levels of IL-6, IL-1α, IL-1β and TNF-α were determined by ELISA. Data are presented as mean ± SEM of 5 (sham) and at least 8 (CLP) different biological replicates (individual mice) from 3 independent experiments. Statistical analysis was performed by a one-way ANOVA test with a Bonferroni's post-test *P <0.05; P** <0.01; P*** <0.001.

4.1.4 GzmA absence is not important to the pathogen control during CLP

As mentioned previously, GzmA absence reduces the production of inflammatory cytokines *in vivo.* Therefore, it is possible that the reduced inflammatory response affects the efficiency in the clearance of local and/or systemic bacterial infection after CLP. To demonstrate

this, we analyzed the total aerobic bacterial load in blood, spleen, liver, lungs and peritoneal fluid during CLP-induced sepsis.

WT and GzmA$^{-/-}$ mice exhibited similar bacterial loads in all the taken samples (peritoneal fluid, blood, spleen, liver and lung) after 6 and 24 h of sepsis induction as shown in figure 5A. Although total bacterial count was similar in both mice strains, we thought it could be possible that the absence of GzmA compromises the control of specific bacterial species. Hence, we characterized the different bacterial species presented in different organs and fluids and quantified them by MALDI-TOF mass spectrometry. As shown in figure 5B, the most frequent species found were *Lactobacillus murinus, Enterococcus faecalis, Staphylococcus sciuri, Streptococcus sp.* and *Escherichia coli.* The bacterial load of these pathogens in WT and GzmA$^{-/-}$ was similar in peritoneal fluid, blood, spleen, liver and lung after 6 and 24 h of sepsis induction. These results show that the absence of GzmA and its inflammatory effect does not compromise the control of aerobic bacteria during polymicrobial abdominal sepsis.

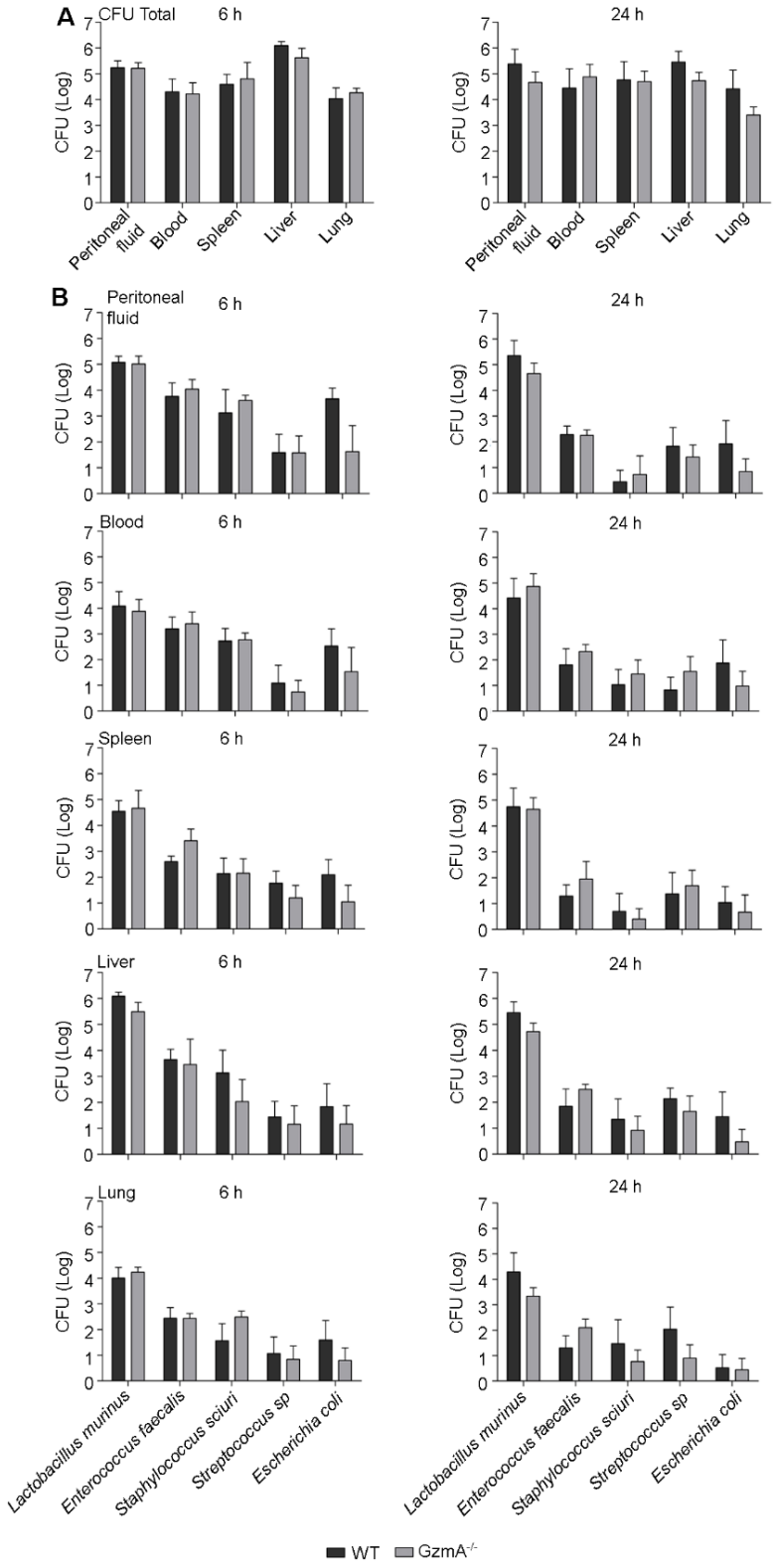


Figure 4.5 GzmA is not involved in the pathogen control during CLP-induced sepsis. Sepsis was induced by CLP in WT and GzmA$^{-/-}$ mice as described in materials and methods. (A) After sepsis induction, a group of mice were sacrificed and the total number of CFU from aerobic bacteria was determine in peritoneal fluid, blood, spleen, liver and lung. (B) The most frequent bacterial strains found were identified by MALDI-TOF mass spectrometry and the number of CFU of these strains was determine in peritoneal fluid, blood, spleen, liver and lung. Data is presented as mean ± SEM from 5 biological replicates (individual mice) in each group.

4.1.5 NK cells increase the expression of GzmA during abdominal sepsis

After confirming that the absence of GzmA increases mice survival during abdominal sepsis, correlated with a reduced inflammatory response that did not compromise the pathogen control, we decided to establish the cellular source of GzmA. We focused on the major cell populations known to express GzmA in blood and spleen (Maykel Arias et al., 2017), which included NK and T cells, including NKT cells. The followed gating strategy is shown in figure 6. We stained cells with NK1.1, CD3, CD4 and CD8 antibodies to differentiate between NK cells ($NK1.1^+CD3^-$), NKT cells ($NK1.1^+CD3^+$), $CD4^+$ T cells ($NK1.1^-CD3^+CD4^+$) and $CD8^+$ T cells ($NK1.1^-CD3^+CD8^+$).

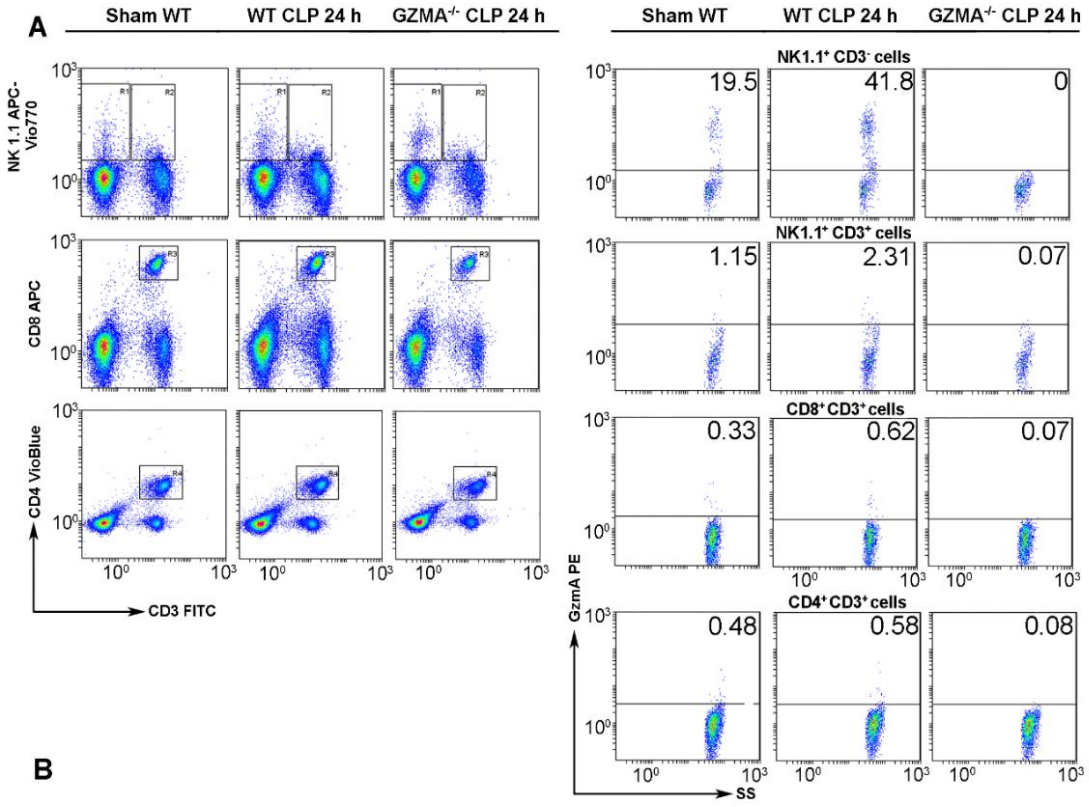


Figure 4.6 Gating strategy followed to analyze the expression of GzmA. The percentage of NK cells (NK1.1+CD3-), NKT cells (NK1.1+ CD3+), CD8+ T cells (CD8+ CD3+) and CD4+ T cells (CD4+, CD3+) and the intracellular expression of GzmA on each cells subset was analyzed in splenocytes and PBLs by flow cytometry. A representative experiment of GzmA expression in spleen is shown via dot plot. Numbers show the cell percentage in each quadrant.

As it is shown in figure 6, the presence of NK, NKT, CD4 and CD8 cells populations was not different between WT and $GzmA^{-/-}$ mice confirming that the absence of GzmA does not

influence the immune cell response. Therefore, as shown un figure 6, GzmA expression was significantly enhanced in NK cells from septic mice in both spleen and peripheral blood. On the other hand, the expression of GzmA in the main T cell subsets (NKTs, $CD8^+$ or $CD4^+$ T cells) was very low and did not increase during sepsis.

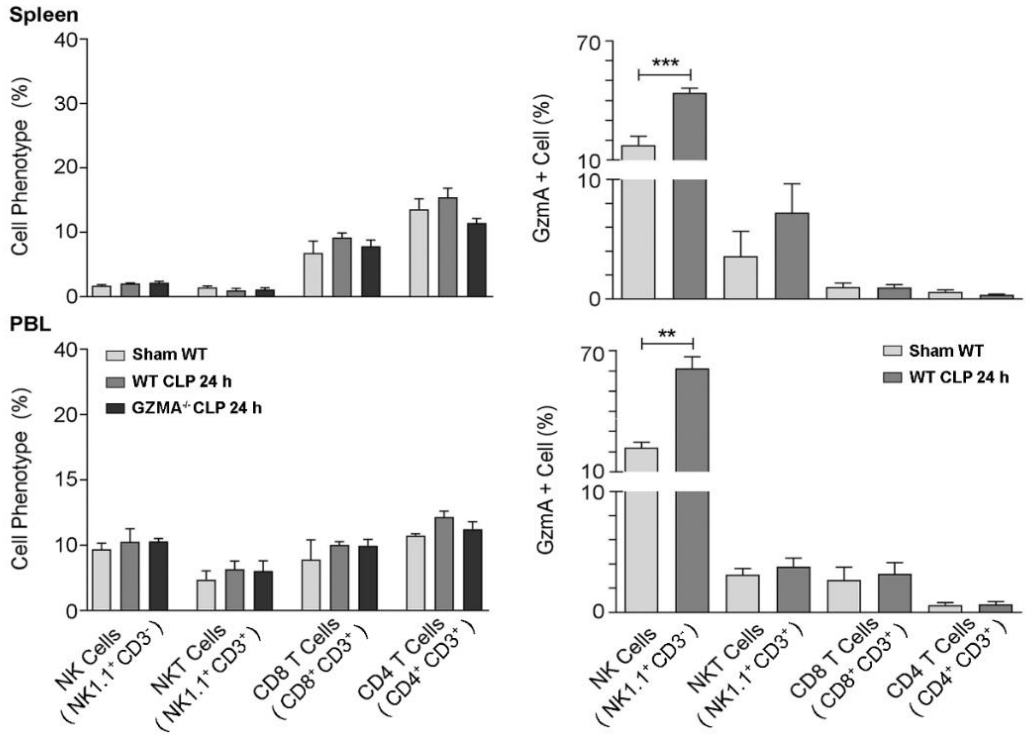


Figure 4.7 GzmA expression is increased in NK cells from septic mice. Sepsis was induced as described in materials and methods. After 24 h mice were sacrificed and the percentage of NK cells (NK1.1+CD3-), NKT cells (NK1.1+ CD3+), CD8+ T cells (CD8+ CD3+) and CD4+ T cells (CD4+, CD3+) and the intracellular expression of GzmA on NK cells, NKT cells, CD8+ T cells and CD4+ T cells were analyzed in splenocytes and PBLs by flow cytometry. Data in graphs represent the mean ± SEM of the percentage of GzmA positive cells of each phenotype (right) and the percentage of each subtype (left) from 2 independent experiments. Statistical analysis was performed by unpaired student's t test. **p < 0.01, ***p < 0.001.

4.1.6 Therapeutic inhibition of GzmA with Serpinb6b increases survival and reduces inflammation during CLP

Once the effect of GzmA absence was studied in a genetically deficient model, we thought it would be necessary to analyze the potential of using GzmA as a therapeutic target for abdominal sepsis treatment. To achieve this goal, we analyzed the effect of a GzmA inhibitor on WT mice undergoing abdominal sepsis. Serpinb6b, a natural specific inhibitor of mouse GzmA, was used in a group of WT and $GzmA^{-/-}$ mice after sepsis induction. Mice were treated with the inhibitor and survival was monitored for 14 days. As showed in figure 8, WT mice treated with antibiotics and serpinb6b showed a significant improvement in survival compared with WT mice

treated only with antibiotics. Survival of septic WT mice treated with serpinb6b was similar to the survival of GzmA$^{-/-}$ deficient mice. Contrasted to these results, the inhibitor did not have any effect on GzmA$^{-/-}$ deficient mice which suggests that the effect observed in WT mice was specifically a consequence of GzmA inhibition.

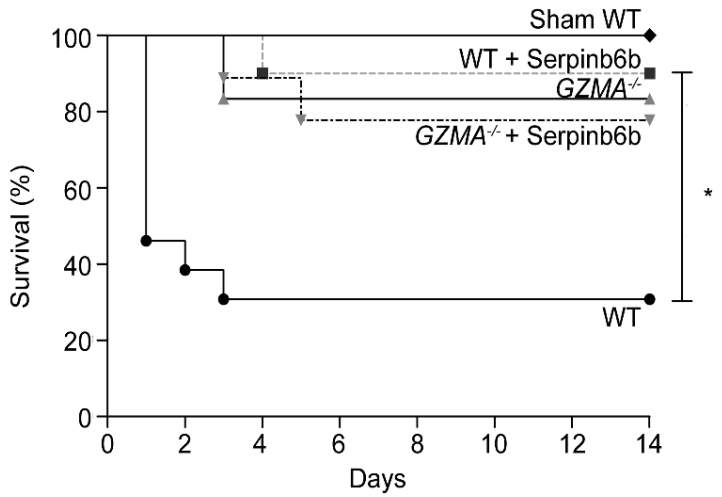


Figure 4.8 Inhibition of GzmA improves sepsis outcome. Sepsis was induced by CLP as described in materials and methods. Immediately after surgery, mice were treated with 40 µg of Serpinb6b in 100 µL of PBS (10 WT and 9 GzmA$^{-/-}$ mice). The treatment was repeated 6 h later and once a day for 5 days. Control mice only received 100 µL of PBS i.p. (13 WT and 6 GzmA$^{-/-}$ mice). Survival was monitored for 14 days and the shown data corresponds to the indicated number of mice combined from two independent experiments. Statistical analysis was performed using logrank and Gehan-Wilcox test. *$p < 0.05$.

Parallel to these, a group of mice non-treated and treated with serpinb6b were sacrificed and samples of plasma and peritoneal fluid were collected to determine the levels of IL-6. Since all cytokines were notably reduced in GzmA$^{-/-}$ mice after 24 h, we decided to focus only on IL-6 cytokine in WT mice treated with the inhibitor in order to confirm the previously obtained results with GzmA$^{-/-}$ mice. It was observed that WT mice treated with serpinb6b had a significant reduction of IL-6 levels in plasma and in peritoneal fluid. In contrast, the therapeutic inhibition of GzmA in GzmA$^{-/-}$ mice did not reduced the levels of IL-6, confirming once again the specificity of seprinb6b (Figure 9). Also, the ability of serpinb6b to inhibit the enzymatic activity of GzmA was confirmed (Figure 10). These results confirms that GzmA inhibition in septic WT mice mimics the effect observed in GzmA$^{-/-}$ mice and provided a proof of principle that the inhibition of extracellular GzmA could be useful for the treatment of abdominal sepsis.

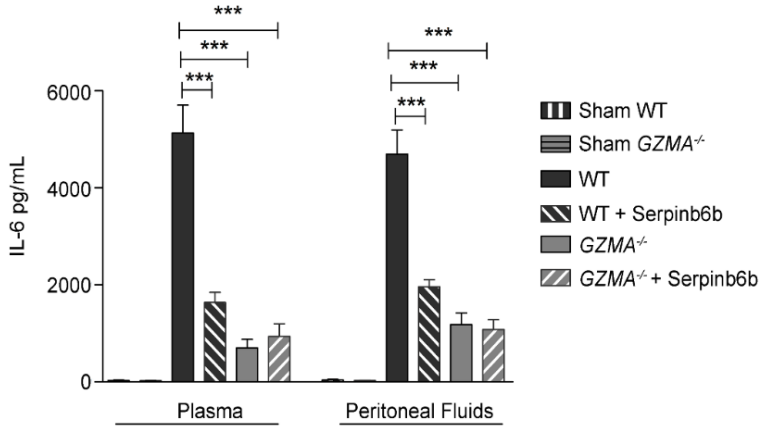


Figure 4.9 Inhibition of extracellular GzmA reduces inflammation in CLP induced sepsis. After 24 h of sepsis induction mice were sacrificed and the levels of IL-6 in plasma and peritoneal fluid was determined by ELISA. Data are presented as mean ± SEM of 4 (Sham) or 6 (CLP) biological replicates from 2 independent experiments. Statistical analysis was performed by one-way ANOVA test with Bonferroni's post-test *P < 0.05; **P < 0.01; ***P < 0.001.

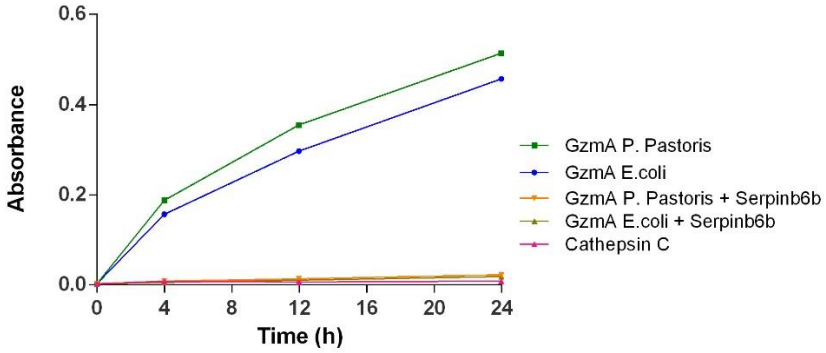


Figure 4.10 Serpinb6b inhibits de activity of GzmA. GzmA activity was confirmed by an activity essay incubating active and inactive GzmA produced in *E. coli*, GzmA inactivated with sepinb6b and cathepsin C with a specific mouse GzmA substrate. Optical density at 405 nm was measured after 4, 12 and 24 h.

4.2 Biological Relevance of Granzyme A and K during *E. coli* sepsis

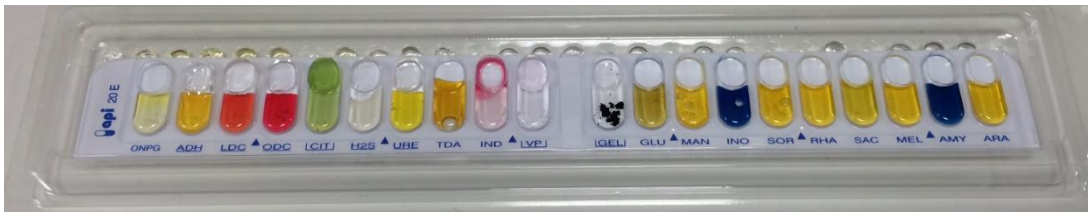
After studying the role of GzmA in the CLP-induced sepsis, we wanted to analyze the role of GzmK that has been reported to have a different but overlapping substrate specificities. GzmK is the closest homologue of GzmA mapping at the same chromosome locus in both human and mice (Hay & Slansky, 2022). GzmK and A belong to the same family of serine proteases and share similar structural features (Bouwman et al., 2021). GzmK cleaves preferentially after arginine residue, whereas GzmA cleaves after aspartic acid. Nevertheless, both enzymes can also cleave after lysine and glutamic acid residues (Plasman et al., 2013). By comparing both granzymes we wanted to determine GzmK biological relevance. Furthermore, it has been reported that, in

sepsis, GzmK has been implicated in both the pathogenesis and resolution of the disease (Cooper et al., 2011; Joeckel et al., 2011b). However, GzmK relevance has not been studied in an *in vivo* model of sepsis thus further investigation is needed to understand how GzmK contributes to sepsis and to identify potential therapeutic targets. Here, we aimed to analyze GzmK relevance in an *E. coli*-induced sepsis model, for which, using biochemical tests and MALDI-TOF mass spectrometry, we isolated the most frequent *Enterobacteriaceae* strain isolated from blood of a WT mice after 24h of CLP induced sepsis.

4.2.1 Characterization of E. coli isolated from CLP

Biochemical tests, growth curves and cytokines expression experiments were performed to correctly characterize the enterobacteria most frequently isolated from blood of WT mice 24h after CLP. Regarding the biochemical tests, bacterial identification was carried out using API 20E (20 characters for *Enterobacteriaceae*). API test strip is a miniaturized and standardized gallery of biochemical tests that helps with the complete identification of different types of bacteria. The API 20E contains 20 test mini chambers which help detect enzymatic activity, mainly related to fermentation of carbohydrates or the catabolism of proteins or amino acids (Tri Yahya et al., 2021). The result of the biochemical test API 20E showed that it was a strain of *E. coli* (Figure 11).

A



B

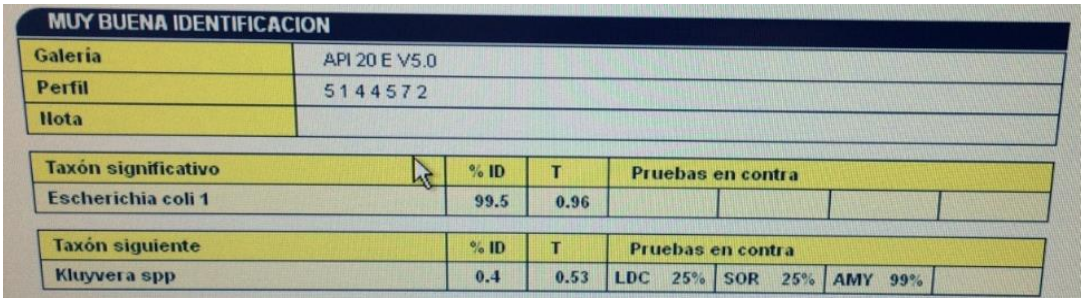


Figure 4.11 *E. coli* was the most common bacteria isolated from CLP-induced sepsis in mice. API 20 E test was performed to identify the most common bacteria found after CLP in mice. **(A)** Biochemical test of *E. coli* using API 20 E and **(B)** result confirmation *E. coli*.

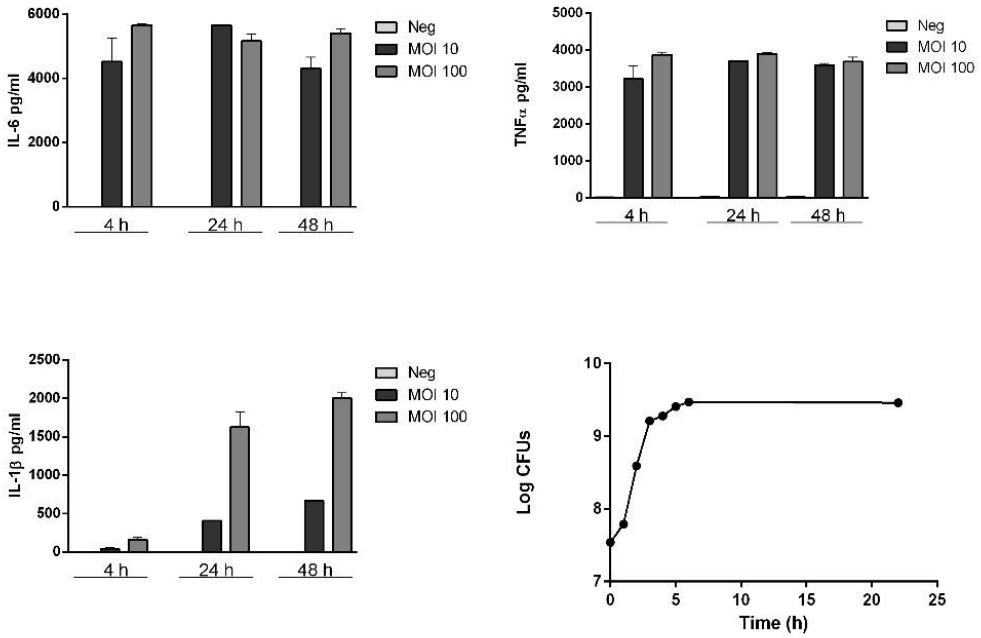


Figure 4.12 Isolated E. coli induces the expression of proinflammatory cytokines such as IL-6, TNFα and IL-1β. Macrophages M1 were stimulated with *E. coli* and incubated for 4, 24 and 48 h. Supernatants were collected and cytokines were analyzed by ELISA. *E. coli* growth curve was determined to confirm the exponential growth time of the bacteria.

E. coli is commonly used in mouse models of sepsis because it can cause a robust and reproducible systemic inflammatory response (Korneev, 2019). As it is shown in figure 12, isolated *E. coli* induces the expression of proinflammatory cytokines such as IL-6, TNF-α and IL-1β. The expression of these proinflammatory cytokines by immune cells in response to *E. coli* infection leads to the recruitment of other immune cells to the site of infection and the activation of endothelial cells. This generates the characteristic symptoms of inflammation, including redness, swelling, heat, and pain (Kany, Vollrath, & Relja, 2019). These results helped us determine that this bacteria was a good candidate to *study E. coli*-induced sepsis.

4.2.2 In vivo study of the pathogenicity of the isolated bacterial strain

As we have observed *in vitro* in our results and in previous bibliography, *E. coli* can induce the expression of several proinflammatory cytokines, including TNFα, IL-1β, IL-6, IL-8 among others (Agelaki, Tsatsanis, Gravanis, & Margioris, 2002; Marcinkiewicz et al., 2007; Tesh, Ramegowda, & Samuel, 1994). Due to the existing evidence in several *in vitro* assays, we have decided to use *E. coli* in our *in vivo* sepsis models.

Severity of sepsis can be regulated by the number of bacteria injected into the peritoneal cavity of mice (He, Ouyang, & Xu, 2021). Therefore, the importance of determining an optimal dose for our model. Based on previous literature, we stablished a bacterial dose to induce sepsis in our mouse model (He et al., 2021; Shrestha, Duwadi, Jukosky, & Fiering, 2019). Live *E. coli* bacteria were freshly prepared and mice were inoculated i.p. with $2x10^8$ CFU and $2x10^6$ CFU (Deitch, 2005; Poli-de-Figueiredo, Garrido, Nakagawa, & Sannomiya, 2008). Weight and score were analyzed as it is observed in figure 13A and 13B. Furthermore, as it is shown in figure 13C mice infected with $2x10^8$ CFU had a 50% survival rate against a 100% survival in the group infected with $2x10^6$ UFC. These results showed that the optimal bacterial concentration to perform our experiments was $2x10^8$ UFC of *E. coli* as it produced a severe pathology thus corroborating that this is a good model.

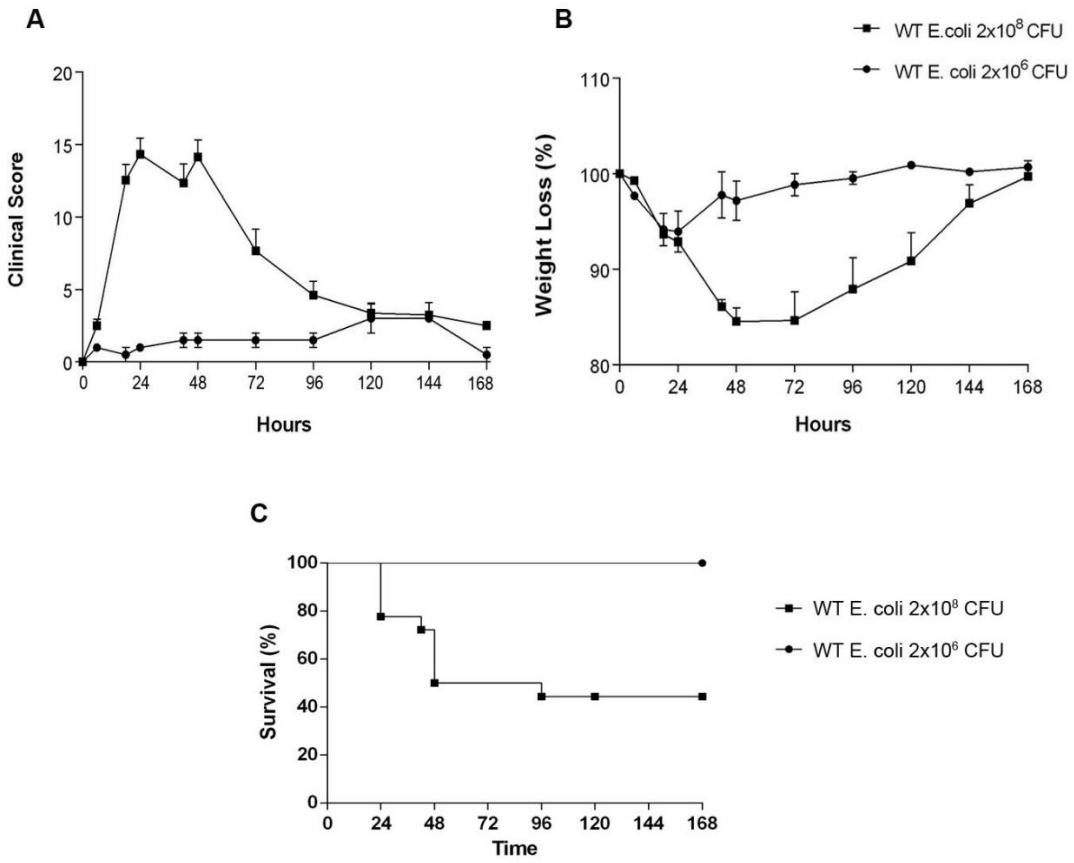


Figure 4.13 Sepsis score, weight loss and percentage of survival during study of *E. coli* pathogenicity. WT mice were infected with $2x10^8$ CFU and $2x10^6$ CFU i.p. of *E. coli*. Animals were weighted daily and the sepsis score was determined as described in material and methods. (A) Sepsis score. (B) Changes in weight and (C) Survival was monitored for seven days. Data are represented as mean ± SEM of sepsis score or the mean ± SEM of the percent of weight loss.

4.2.3 Clinical score and survival are improved in the absence of GzmA

Sepsis was induced in WT, GzmA and GzmK knock out mice by i.p. injection of $2x10^8$ CFU/mL of an *E. coli* strain isolated from CLP-induced septic mice. We used this model in order to analyze sepsis induced by a mice specific *E. coli* strain in order to avoid differences due to the use of non-species-specific strains.

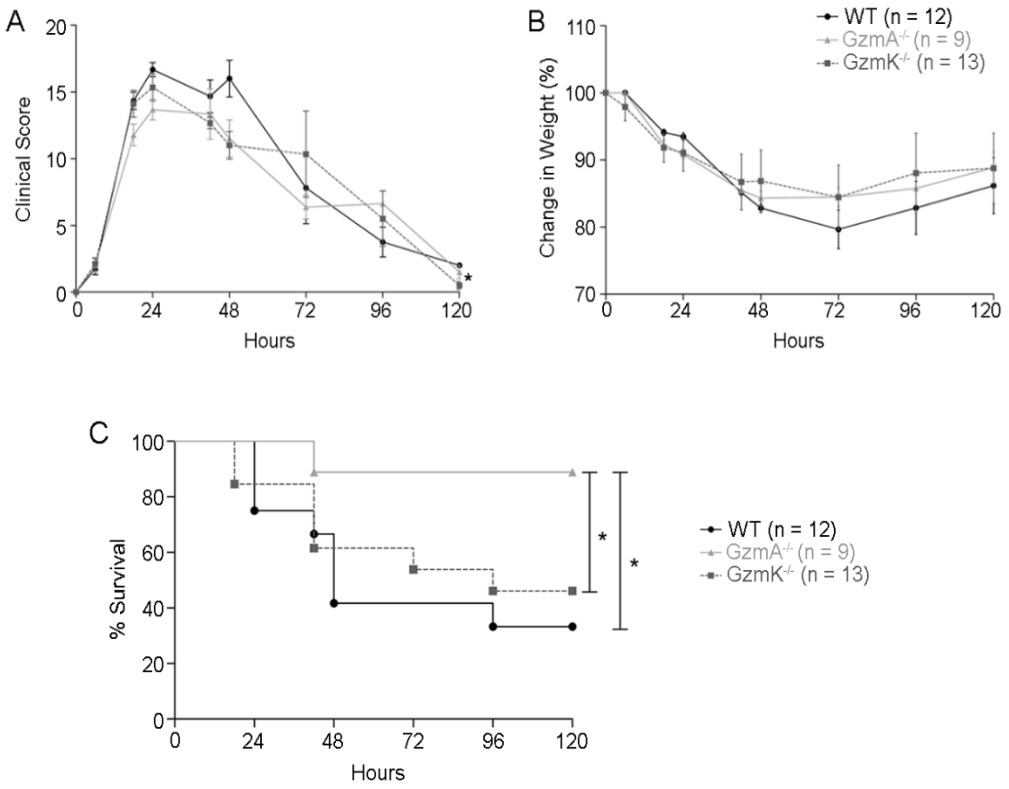


Figure 4.14. Sepsis score, weight loss and percentage of survival during E. coli-induced sepsis. WT, GzmA and GzmK mice were infected with $2x10^8$ CFU i.p. of E. coli. Animals were weighted daily and the sepsis score was determined as described in material and methods. (A) Sepsis score. (B) Changes in weight. (C) Survival was monitored for five days. Data are represented as mean ± SEM of sepsis score (A) or the mean ± SEM of the percent of weight loss (B). Statistical analyses were performed using a mixed linear regression as described in materials and methods.

As shown in figure 14B, all mice had a significant, but similar, loss of weight characteristic of sepsis. In addition, applying a murine sepsis score (MSS) described in materials and methods, mice showed other signs of sepsis such as piloerection, decreased movement, decreased respiratory rate, labored breathing and decreased response to auditory and tactile stimuli. 24 h later of sepsis induction, all mice strains reached their highest sepsis score, but there were not

significant differences between WT and KO mice. Nevertheless, after 48 h after sepsis induction, GzmA$^{-/-}$ and GzmK$^{-/-}$mice showed a slight but significant lower sepsis score than WT mice (Figure 14A).

A mixed linear regression analysis of these data was performed and showed significant differences between groups. These indicated that the absence of either GzmA or GzmK improved MSS ($p = 0.035$). In contrast to MSS, the covariance analysis showed that these differences were not due to a relation between MSS and weight ($p = 0.366$). The analysis of survival showed that, despite the fact that GzmA$^{-/-}$ and GzmK$^{-/-}$ mice showed a similar loss of weigh and sepsis score, only GzmA deficient mice showed a significant increase in survival compared with WT mice. In contrast, GzmK deficient animals behaved as WT controls, beginning to die 48 h after sepsis induction. At last, only 33% of WT and 46% of GzmK$^{-/-}$ mice remained alive after 5 days in contrast to a 90% of GzmA$^{-/-}$ animals (Figure 14C).

4.2.4 In vivo bacterial replication is not affected by GzmA or GzmK deficiency

We wanted to determine if GzmA and GzmK were necessary in the control of the infection for which we induced *E. coli* sepsis in WT, GzmA$^{-/-}$ and GzmK$^{-/-}$ mice, sacrificed them at 18 and 42 h and determined the bacterial load in blood and spleen. The results showed a similar bacterial load in both blood and spleen at 18 and 42 h, indicating that GzmA and GzmK were not involved in the control of *E. coli* infection, hence, the increased survival of GzmA KO mice is not due to differences in bacterial replication *in vivo* (Figure 15).

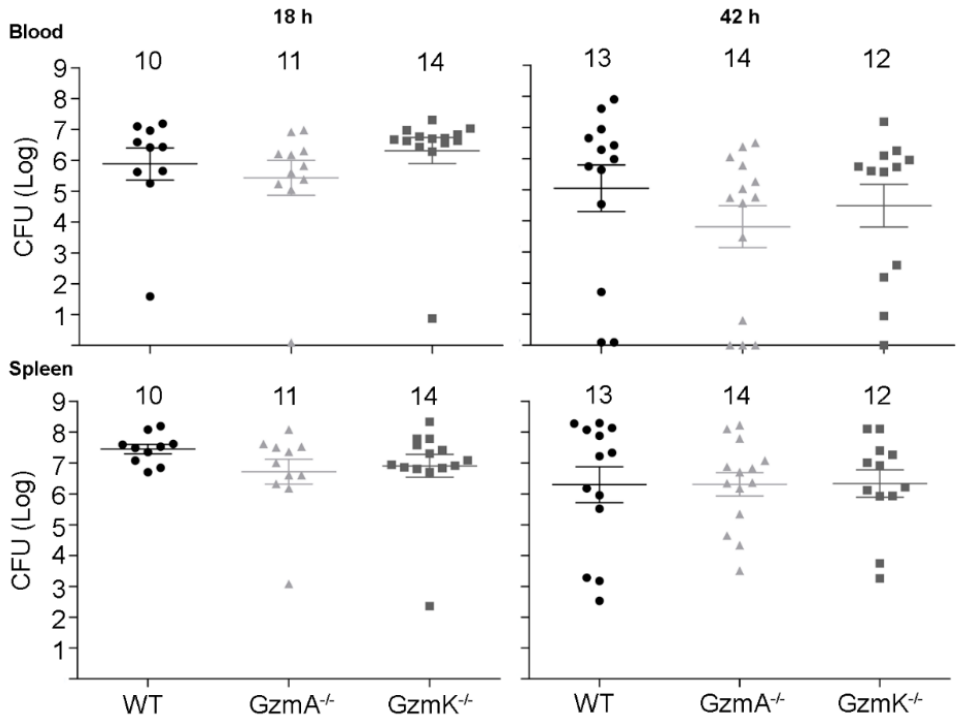


Figure 4.15 Bacterial load in blood and spleen during *E. coli* sepsis. WT, GzmA$^{-/-}$ and GzmK$^{-/-}$ mice were infected with 2x10^{8} CFU i.p. of *E. coli*. After 18 and 42 h of sepsis induction a group of animals was sacrificed and the bacterial load determined in blood and spleen. Data are represented as mean ± SEM CFU counts. Numbers at the top of each graph indicate the number of biological replicates from each group of two independent experiments. Statistical analysis was performed by one-way ANOVA test with Bonferroni's post-test.

4.2.5 Extracellular GzmA increases in WT and GzmK mice during E. coli sepsis

Once we confirmed that the protection observed in GzmA$^{-/-}$ mice was not related with the control of the infection, we analyzed if the levels of extracellular GzmA could be detected in serum from septic mice. And, as shown in figure 16, extracellular GzmA in serum was significantly increased in WT and GzmK$^{-/-}$ mice compared to healthy controls.

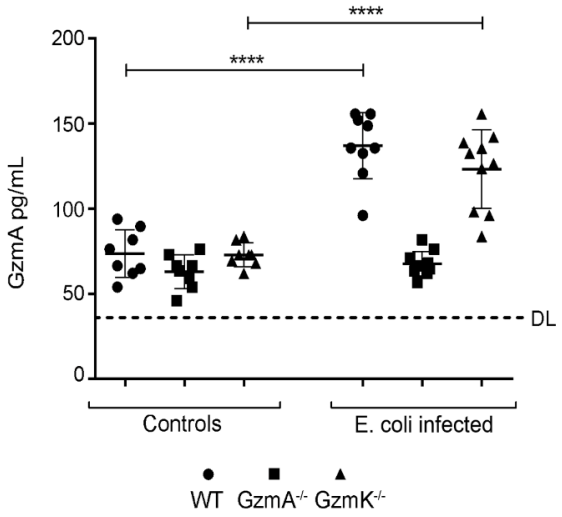


Figure 4.16 Extracellular GzmA levels in plasma during E. coli sepsis. WT, GzmA-/- and GzmK-/- mice were infected with $2x10^8$ CFU i.p. of *E. coli*. After 18 and 42 h of sepsis induction a group of animals was sacrificed and the levels of GzmA in plasma were determined by ELISA.

4.2.6 Proinflammatory cytokines are reduced in GzmA and GzmK deficient mice during E. coli sepsis

The next step was to analyze if the absence of GzmA and GzmK attenuated the inflammatory response in mice. To achieve that, we analyzed the presence of proinflammatory cytokines such as IL-1α, IL-1β, IL-6 and TNF-α in mice serum. As it is shown in figure 17, GzmA deficient mice had lower levels of all tested cytokines in comparison with WT mice, although only IL-1α, IL-1β and IL-6 were significantly different. On the other hand, GzmK deficient mice showed lower levels of IL-1β compared with WT mice which is supported by previous studies where it has been reported that GzmK has a proinflammatory potential (Joeckel et al., 2011b). Furthermore, the level of inflammatory cytokines was also reduced in GzmA deficient mice compared with $GzmK^{-/-}$ mice, although only IL-6 was significantly different. Finally, after 42 h, most animals had very low cytokines level in blood without differences between WT and KO mice.

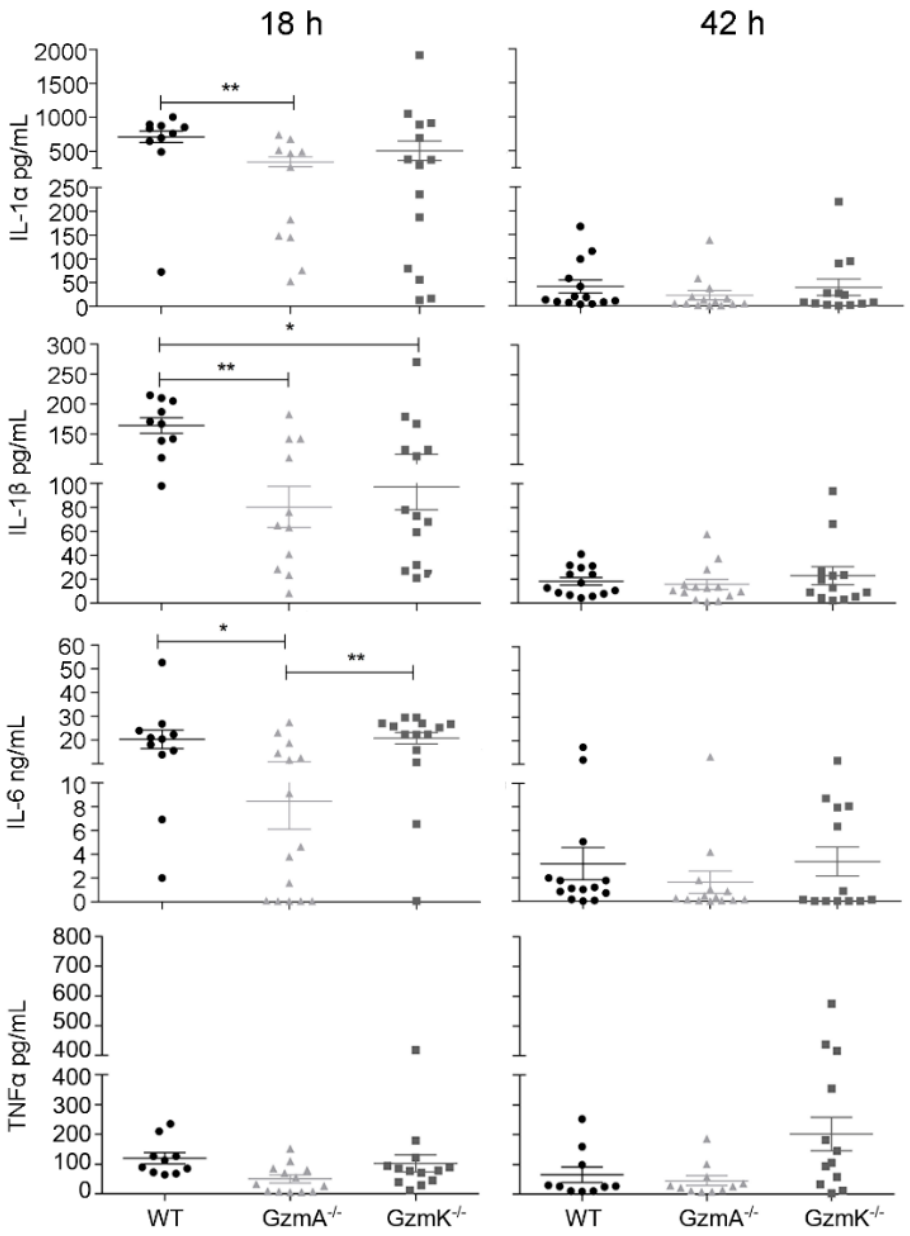


Figure 4.17 ***Proinflammatory cytokines levels in plasma during*** **E. coli** ***sepsis.*** WT, GzmA$^{-/-}$ and GzmK$^{-/-}$ mice were infected with 2x10^{8} CFU i.p. of E. coli. After 18 and 42 h of sepsis induction a group of animals was sacrificed and the levels of IL-1α, IL-1β, IL-6 and TNF-α in plasma were determined by ELISA. Data are represented as mean ± SEM of the values of each cytokine in 3 independent experiments. Statistical analysis was performed by one-way ANOVA test with Bonferroni's post-test *p < 0.05; **p < 0.01.

4.2.7 GzmA and GzmK are mainly expressed in NK and NKT cells during E. coli induced sepsis

Afterwards, we analyzed the cell source of GzmA and GzmK during *E. coli*-induced sepsis. Since our results showed that the reduction of inflammation in GzmA$^{-/-}$ and GzmK$^{-/-}$ was observed withing the first 24 h, we expected the main cell source of GzmA and K in this model to be a component of the innate immune system. Intracellular expression of GzmA and GzmK was analyzed in different immune spleen cell subsets 18 h after sepsis induction following the gating strategy showed in figure 18.

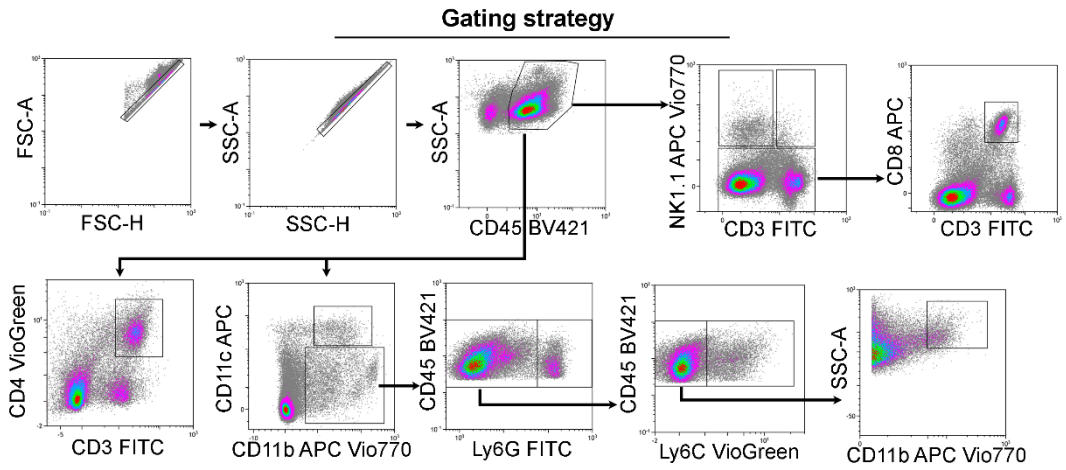


Figure 4.18 Gating strategy for GzmA and GzmK expression analysis. After 18 h of sepsis induction, WT, GzmA$^{-/-}$ and GzmK$^{-/-}$ mice were sacrificed. Spleens were collected aseptically, homogenized in 5 mL of RPMI medium and then erythrocytes were lysed. For analysis of GzmA and GzmK expression, 1x10^6 splenocytes were stained with extracellular fluorescent labelled antibodies. Doublets (FSC-H vs FSC-A and SSC-H vs. SSC-A) were excluded. SSC-A vs. CD45$^+$ gating was done to identify CD45$^+$ cells population. For the first cocktail 3 subpopulations were identified: NK cell (NK1.1+CD3-), NKT cell (NK1.1+CD3+) and CD8 lymphocyte (CD8+CD3+) which were gated from NK1.1 negative cells. For the second cocktail CD4 T lymphocyte (CD4+CD3+) subpopulation was identified. Finally, for the third cocktail 4 subpopulations were identified: dendritic cell (CD11b+CD11c+), from CD11b+CD11c- cells a Ly6G+ population was identified as neutrophils (CD11b+Ly6G+). Next, from Ly6G- population a Ly6C+ population was identified as monocytes (CD11b+Ly6C+Ly6G-). Finally, from Ly6C- population a CD11b++ population was identified as macrophages (CD11b++Ly6C-Ly6G-).

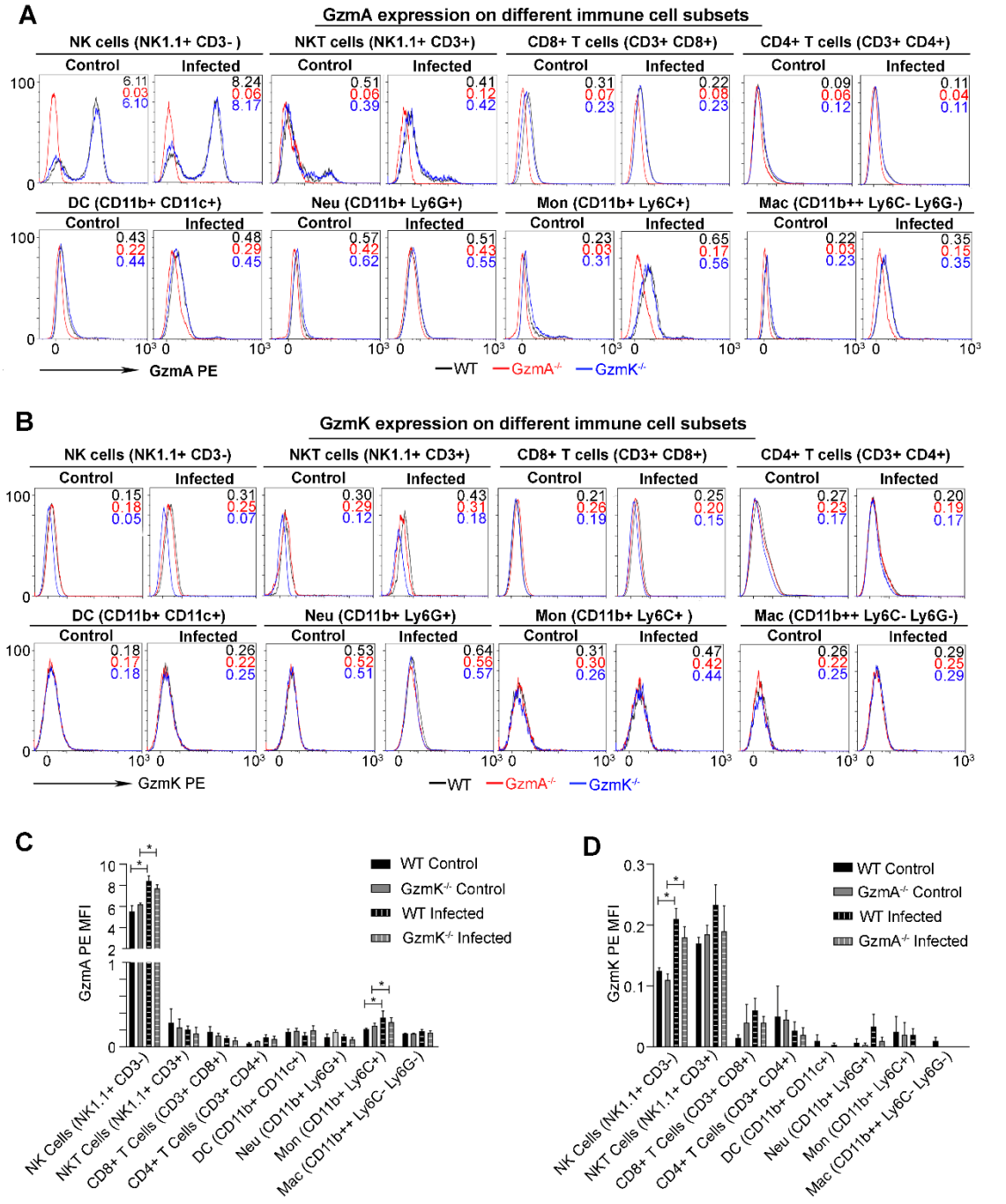


Figure 4.19 Intracellular expression of GzmA and GzmK during *E. coli* sepsis. WT, GzmA$^{-/-}$ and GzmK$^{-/-}$ mice were infected with 2x10^{8} CFU i.p. of *E. coli*. After 18 h of sepsis induction a group of animals were sacrificed and spleens were collected. The intracellular expression of GzmA and GzmK was analyzed in splenocytes by flow cytometry as indicated in materials and methods. (**A**) A representative histogram analysis is shown for GzmA (**B**) and GzmK. Numbers in the histograms show MFI (Mean Fluorescence Intensity) in each strain (WT in black, GzmA$^{-/-}$ in red and GzmK$^{-/-}$ in blue). Data in graphs represent the mean ± SEM of MFI for (**C**) GzmA-PE (**D**) and GzmK-PE minus the florescence of each granzyme deficient control mice in the immune cells subsets analyzed from three biological replicates. Statistical analysis was performed using one-way ANOVA test with Bonferroni's post-test. * $p < 0.05$.

As shown in figure 19, NK and NKT cells had an increase in the expression of GzmA and GzmK during *E. coli* sepsis if compared with Gzm KO mice which were used as controls. Additionally, we observed a slight increase in the expression of GzmA in monocytes (Ly6C+CD11b+Ly6G-CD11c-). Also, as expected, GzmA and GzmK had a similar expression profile in GzmK and GzmA deficient mice respectively, and they were not expressed in the corresponding deficient mice, confirming specific Gzm detection.

4.2.8 Therapeutic inhibition of GzmA with serpinb6b improves survival and reduces inflammation in E. coli sepsis

Finally, we studied the effect of therapeutic inhibition of GzmA in *E. coli* sepsis. After sepsis induction, a group of WT, GzmA and GzmK deficient mice were treated with the specific GzmA inhibitor serpinb6b. As it is shown in figure 20, there is a significant survival in WT and GzmK$^{-/-}$ mice treated with Serpinb6b compared with not treated mice. Likewise, as it is observed in figure 21, IL-6 expression is low in plasma of infected mice with *E. coli* and treated with Serpinb6b compared with the ones that have not been treated with serpinb6b. GzmA$^{-/-}$ mice did not show a significant difference on either groups.

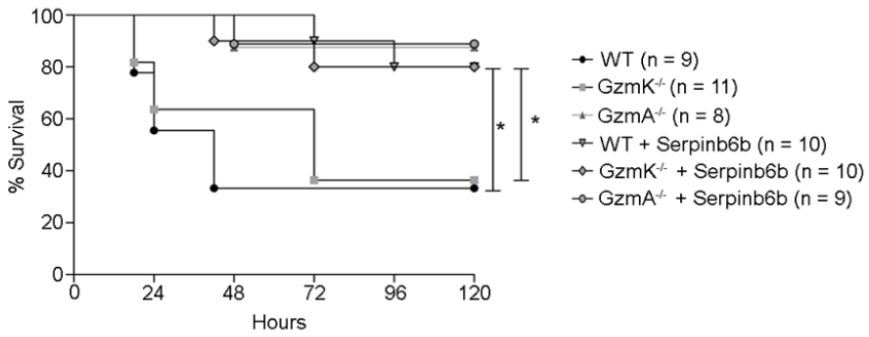


Figure 4.20 Therapeutic inhibition of GzmA with serpinb6b during E. coli sepsis increase survival in WT and GzmK deficient mice. WT, GzmA$^{-/-}$ and GzmK$^{-/-}$ mice were infected with 2x10^{8} CFU i.p. of E. coli. After E. coli sepsis induction, a group of WT, GzmA and GzmK deficient mice were treated twice a day with 40 µg of serpinb6b in 100 µl of PBS i.p. for five days. Mice were observed twice a day and survival was monitored for five days. Statistical analysis was performed using logrank and Gehan-Wilcoxon test. In the figure the number of biological replicates from each group (n) of two independent experiments are indicated. *p< 0.05; ***p < 0.001.

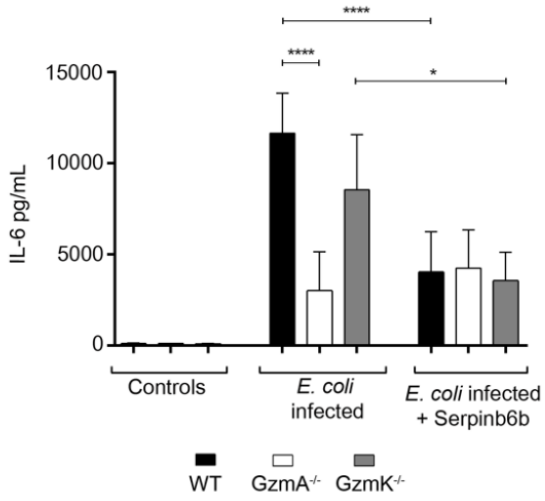


Figure 4.21. **Therapeutic inhibition of GzmA with serpinb6b during E. coli sepsis reduces serum IL-6 in WT and GzmK deficient mice.** WT, GzmA$^{-/-}$ and GzmK$^{-/-}$ mice were infected with 2x10^{8} CFU i.p. of *E. coli.* After *E. coli* sepsis induction, a group of WT, GzmA and GzmK deficient mice were treated twice a day with 40 µg of serpinb6b in 100 µl of PBS i.p. for five days. As control, a group of mice were treated with 100 µl of PBS i.p. twice a day. After 18 h a group of animals was sacrificed and IL-6 concentration in plasma was determined by ELISA. Data are represented as mean ± SEM of the values 2 independent experiments. Statistical analysis was performed by one-way ANOVA test with Bonferroni's post-test ****p < 0.0001.

4.3 Study of the mechanism of inflammation induced by GzmA in macrophages

As we have seen so far, extracellular GzmA plays an important role in the pathophysiology of sepsis induced by both CLP and *E. coli*, influencing inflammation and therefore the expression of proinflammatory cytokines such as IL-1α, IL-1β, IL-6, and TNFα. In order to find out how GzmA contributes to abdominal sepsis we decided to study the molecular mechanism by which GzmA exerts its effect. To achieve this, we differentiated macrophages M1 as described in materials and methods to analyze the generation of inflammatory cytokines. As shown in figure 22, mouse active GzmA significantly induced IL-6 expression in mouse macrophages. Inactive proGzmA or GzmA inhibited by serpinb6b did not induce IL-6 and as an additional control we used cathepsin C, the protease used to activate GzmA in the purification process. As expected, since cathepsin C is not active at a pH higher than 6, it did not induce IL-6 production. It is also important to mention that the amount of LPS in the used recombinant protein preparation was lower than 0.5 EU/µg and that the GzmA inhibitor serpinb6b completely blocked the activity of mouse GzmA (Figure 10) and prevented the expression of IL-6 in macrophages incubated with GzmA (Figure 23), confirming that potential contaminants in GzmA preparations were not responsible of the inflammatory effect of extracellular GzmA.

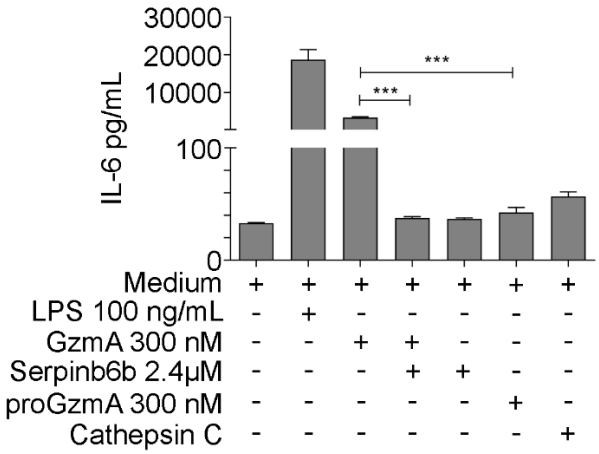


Figure 4.22 Active extracellular GzmA induces the expression of IL-6 in macrophages. WT bone marrow differentiated macrophages were stimulated with LPS 100 ng/mL, active and inactive GzmA produced in *E. coli*, and GzmA inactivated with sepinb6b and cathepsin C. After 24 h of incubation, supernatant was collected to determine the levels of IL-6 by ELISA.

As it has been reported in previous studies, extracellular GzmA is not typically considered a cytotoxic enzyme because it does not have access to the intracellular environment where it can induce apoptosis in target cells, suggesting alternative roles for GzmA (S. Metkar et al., 2008; van Daalen et al., 2020). Here, performing a viability assay, we observed that, indeed, GzmA at different concentrations did not exert a cytotoxic activity in macrophages (Figure 23).

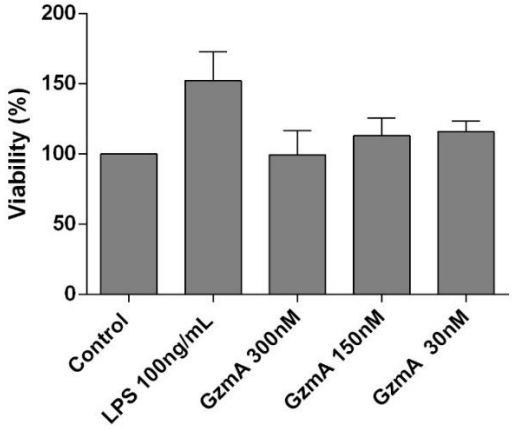


Figure 4.23 Granzyme A does not affect the viability of macrophages M1 in vitro. Macrophages M1 were isolated from WT mice bone marrow as described in materials and methods. Cells were stimulated with LPS (100 ng/mL) and GzmA (300 nM, 150 nM and 30 nM). Cells with no stimuli were used as control. After 24h of incubation, 1/10 of viability reagent was added and incubated for at least 3h. Fluorescence was measured using a fluorescence excitation wavelength of 560 nm and an emission of 590 nm.

4.3.1 Active GzmA induces the expression of IL-6 by a TLR4 and MyD88 dependent pathway

We analyzed the potential involvement of TLR4 and MyD88 in the molecular mechanism of GzmA to induce inflammation. TLR4 is a key receptor that is activated by microbial PAMPs like LPS and different endogenous ligands or DAMPs (Ciesielska, Matyjek, & Kwiatkowska, 2021) and it has been previously reported to be involved in abdominal sepsis. In order to confirm our findings and further get more insights into the potential mechanism activated by GzmA, we analyzed the expression of IL-6 GzmA-stimulated WT, TLR4$^{-/-}$ and MyD88$^{-/-}$ mice. First, we analyzed the effect of extracellular GzmA on the expression of IL-6 and TNF-α by M1 bone marrow-derived macrophages from WT and TLR4 deficient mice. We found that the recombinant mGzmA produced in *E. coli* or in *P. pastoris* induced the expression of IL-6 and TNFα on M1 WT macrophages and was reduced in absence of TLR4 as is shown in figure 24, indicating that GzmA induces the expression of IL-6 by a mechanism dependent on TLR4. Next, as we already demonstrated the importance of the TLR4 pathway in IL-6 expression, we decided to compare it with MyD88 deficient macrophages too. As showed in figure 25, the expression of IL-6 was significantly reduced in TLR4 and MyD88 deficient macrophages compared to WT.

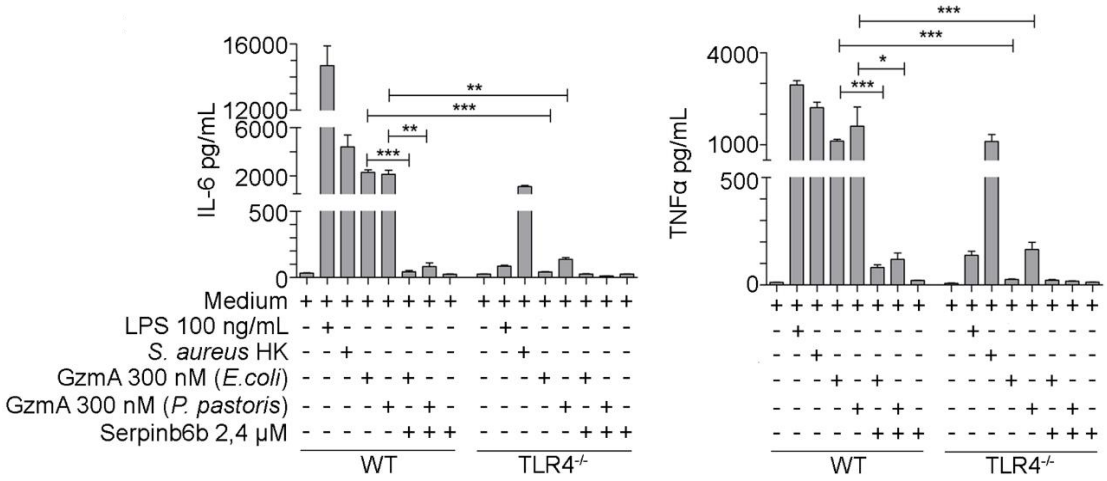


Figure 4.24 Active extracellular GzmA induces the expression of IL-6 and TNFα in WT but not in TLR4 deficient macrophages. Macrophages differentiated from WT or TLR4-/- mouse bone marrow were stimulated with active GzmA (300 nM) produced in *E. coli* or in *P. pastoris*, GzmA inactivated with serpinb6b, LPS 100 ng/mL or *S. aureus* HK (1 x10^6 CFU/mL). After 24 h of incubation, the supernatant was collected to determine the levels of IL-6 and TNFα by ELISA. Data are represented as the mean ± SEM of two independent experiments performed by duplicate. Statistical analyses were performed by one-way ANOVA test with Bonferroni's post-test, *P < 0.05, **P < 0.01; ***P < 0.001.

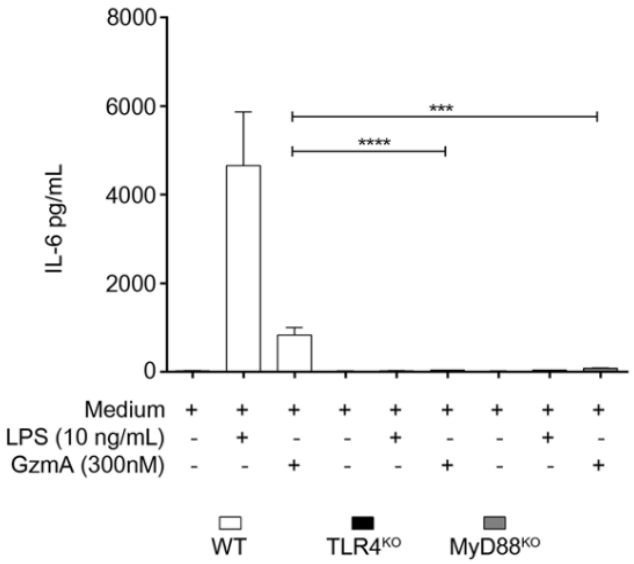


Figure 4.25 Active GzmA induces the expression of IL-6 by a TLR-4 and MyD88 dependent pathway. WT, TLR4$^{-/-}$ and MyD88$^{-/-}$ bone marrow differentiated macrophages were stimulated with LPS (1 ng/mL) and active GzmA (300 nM). After 24 h of incubation supernatants were collected to determine levels of IL-6 by ELISA.

Furthermore, to better understand if GzmA activates specific TLR4 signaling pathways we decided to use chemical inhibitors which targeted different molecules involved in the TLR4-mediatd signaling. We used TAK-242, a small molecule inhibitor that blocks the intracellular TLR4 domain that recruits TIRAP (MyD88 dependent signaling) and TRAM (MyD88 independent signaling) and OxPAPC (Oxidized 1-palmitol-2-arachidonyl-sn-glycero-3-phosphorylcholine), and oxidized phospholipid that inhibits LPS-mediated signaling by blocking its binding to LBP, CD14 and MD2, molecules required for TLR4 activation by LPS. As shown in figure 26, the expression of IL-6 induced by active GzmA was significantly reduced by both inhibitors. These results, together with the results using TLR4 and MyD88 deficient macrophages, suggest that GzmA activates the conventional MyD88 and TIRAP dependent TLR4 signaling.

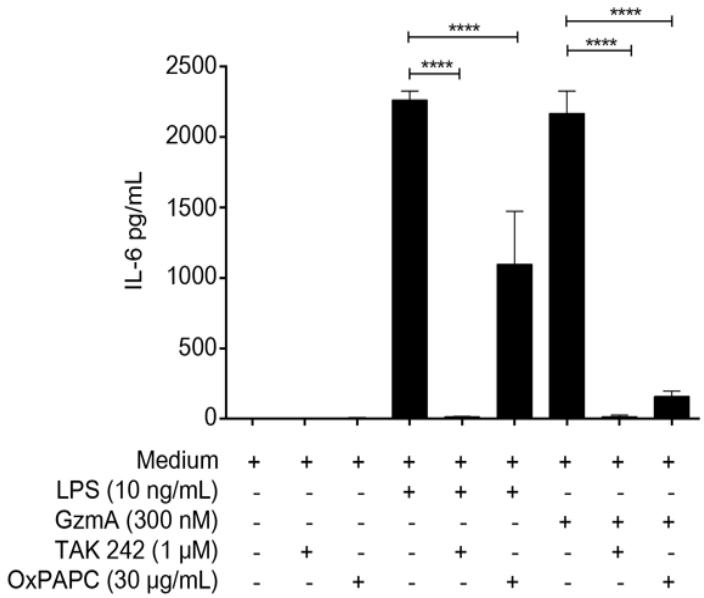


Figure 4.26 Chemical inhibitors for TLR4-mediated signaling reduces the expression of IL-6 in WT mice macrophages. WT bone marrow differentiated macrophages were first stimulated with TAK 242 (1 µM), OxPAPC (30 µg/mL) for 30 min at 37 °C and then stimulated with LPS 10 ng/mL and active GzmA (300 nM). After 24 h incubation, supernatants were collected to determine the levels of IL-6 by ELISA. Data are represented as the mean ± SEM of three independent experiments performed by triplicate. Statistical analyses were performed by unpaired t test ****p < 0.0001.

4.3.2 NNT expression in different strains of C57BL/6J mice and its impact on the studied sepsis models

In the last years, it has been published that the nicotinamide nucleotide transhydrogenase (NNT) protein functions as a redox-driven proton pump that it is located in the inner mitochondrial membrane thereby sustains mitochondrial antioxidant capacity through generation of NADPH which generates reactive oxygen species (ROS). ROS plays an important role in the immune responses including bacterial killing and the increased susceptibility to an infection when ROS is not present (Vera M. Ripoll et al., 2012). GzmA$^{-/-}$ mice have a background that retains the full-length NNT gene. Therefore, due to differences in the genetic background of our WT mice and our GzmA deficient mice, and the fact that redox regulation is involved in many cellular processes and genetic backgrounds are known to affect phenotypes (D. J. Rawle et al., 2022), we decided to analyze the difference between both genetic backgrounds and determine if there were significant differences that would affect our results. To achieve this, first, we performed a RT-PCR, as is described in materials and methods, to detect the presence of the NNT gene in our different mice strains. As we can see in figure 27, WT and GzmK mice have truncated the NNT (572 bp) whereas GzmA$^{-/-}$ and GzmB$^{-/-}$ express NNT (1320 bp).

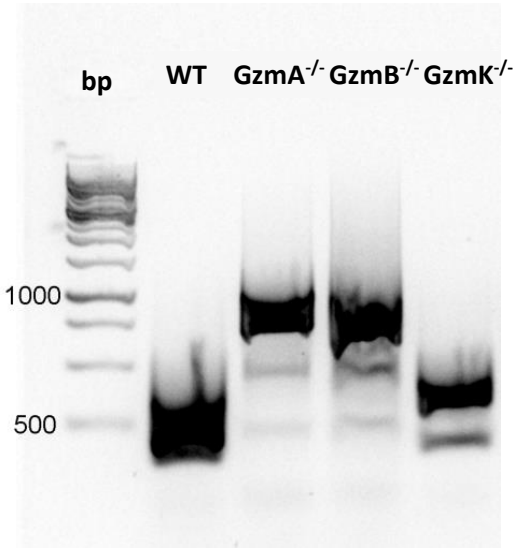


Figure 4.27 NNT detection in WT and GzmA, B and K deficient mice was performed by RT-PCR. Mice hearts were collected and used for RNA extraction. RT-PCR was performed using primers spanning exons 7-11 which produces a product of 1320 bp in hearts of C57BL/6 mice that express NNT (GzmA$^{-/-}$ and GzmB$^{-/-}$), while a product of 572 bp is the product of a truncated NNT gene (WT and GzmK$^{-/-}$).

Second, we decided to analyze the expression of NNT in the littermates of our GzmA strain in order to define the distribution of the gene and confirm whereas or not the NNT gene was closely related to the GzmA KO. As we can see in figure 28, GzmA mice with -/- genotype express NNT while mice with +/+ and +/- genotype express a truncated NNT.

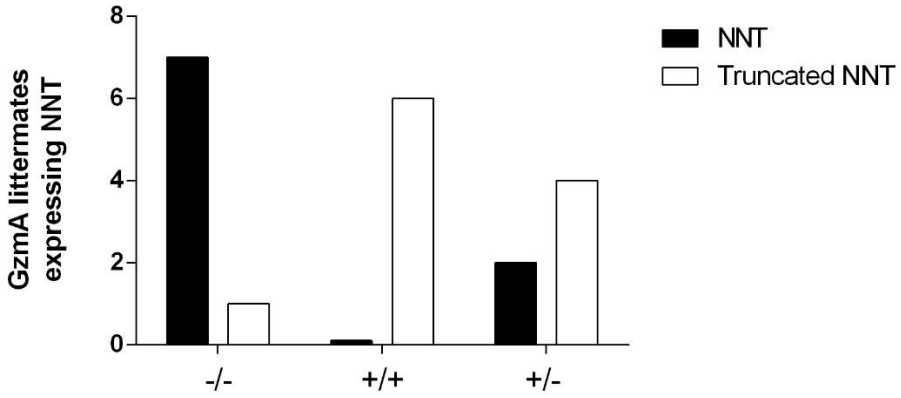


Figure 4.28 GzmA littermates genotyping showed the expression of NNT in -/- mice in comparison with +/+ and +/- which showed a truncated expression of NNT. A RT-PCR was performed in 20 GzmA littermates mice to detect NNT expression. 7 out of 8 mice with -/- genotype expressed NNT, 6 out of 6 mice with +/+ genotype showed a truncated expression of NNT and 4 out of 6 mice with +/- genotype showed a truncated expression of NNT while 2 out of 6 mice from this same group expressed NNT.

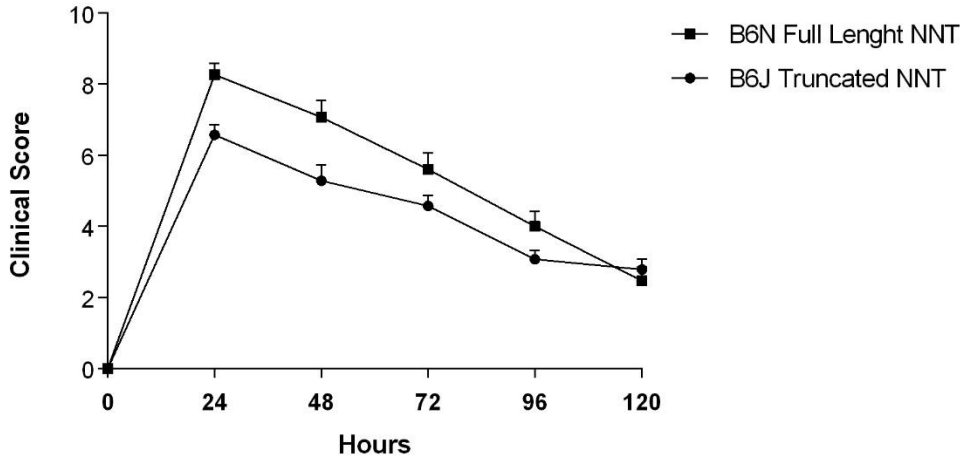


Figure 4.29 Clinical sepsis score showed no significant differences between WT mice which express NNT and those who express truncated NNT. WT mice from different genetic background were infected with $2x10^8$ UFC i.p of *E. coli.* Sepsis score was determined as described in material and methods.

As it has been reported, our results confirm that GzmA KO mice retained the expression of the full length NNT gen as it shown in figure 27, been its presence responsible of the amelioration of CHIKV arthritic foot swelling in GzmA KO mice in a study of inflammatory arthritis induced by CHIKV infection (Daniel J. Rawle et al., 2021). However, in our experimental model for sepsis we do not register significant differences in the results between the strain which expresses NNT and the one that expresses the truncated gen (figure 29).

4.4 Role of GzmA in viral sepsis due to Influenza virus

4.4.1 Influenza virus titration

As mentioned before, influenza virus is an important pathogen that causes respiratory diseases in humans and animals. Seasonal influenza virus infection causes high morbidity and mortality in populations of young people, the elderly, and immunocompromised patients (Thangavel & Bouvier, 2014). In previous studies, it has been shown that CD4 and CD8 T-cell-mediated immunity could provide heterologous protection against different influenza virus. Previous data has demonstrated that conserved CD4 and CD8 T-cell epitopes against the 2009 pandemic H1N1 virus exist in the general population, but *in vivo* support for whether T cells contribute to the control of H1N1 virus is lacking (Guo, Santiago, Lambert, Takimoto, & Topham, 2011). Thus, considering influenza virus pathogenesis is closely related to a septic condition, we decided to analyze the role of granzymes in an influenza-induced sepsis mouse model. To achieve this, the first step was to determine the *in vivo* pathogenesis of the virus, therefore the

optimal viral dose for experimentation. First, a group of mice were infected with different doses of virus and weight loss and survival were monitored. As shown in figure 30, three different doses were tested in WT mice. Mice inoculated with $2x10^5$ PFU and $2x10^4$ PFU showed a rapid weight loss and between days 6 and 8 all mice from the groups died. On the other hand, the mice groups inoculated with $2x10^3$ PFU showed a progressive change in weight. With a 20% of weight loss, we observed a 75% of survival. On the other hand, if we consider a weight loss of 25% the survival rates observed were of 50%. The mice surviving at day 14 regain body weight and finally recovered from the illness. Considering these results, we decided that the experimental conditions would be a virus concentration of $2x10^3$ PFU and a weight loss of 25% as the end point.

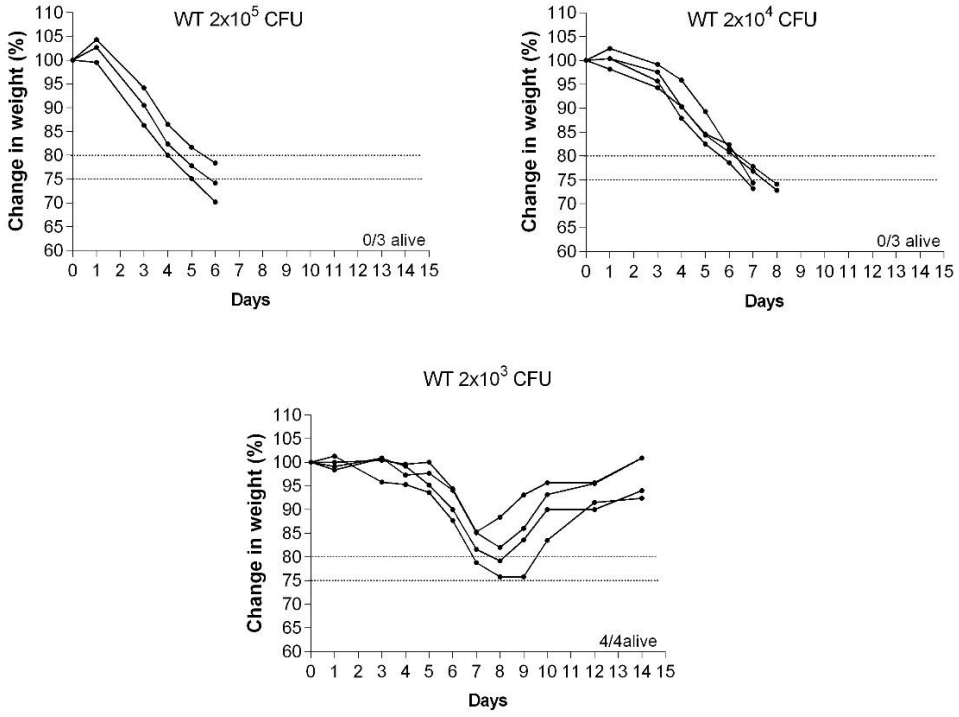


Figure 4.30 Influenza A virus titration for infective dose determination. WT mice were inoculated intranasally with three different virus doses ($2x10^5$ PFU, $2x10^4$ PFU, and $2x10^3$ PFU). Mice were anesthetized in a mouse anesthesia induction chamber where 4-5% of isoflurane was used for induction and 1-2% was used for maintenance. Once asleep, two drops of 20 µL each were administered within a couple of minutes between each other to avoid damage to the respiratory tract. Weight was monitored daily for 14 days.

Once we confirmed that NNT gene was indeed expressed in our GzmA and GzmB deficient mice, but not in our WT mice it was necessary to find a WT control with the same genetic characteristics as our knock-out mice. Thus, ENVIGO C57BL/6 mice were purchased. We infected Envigo WT mice with influenza virus and then compared them with our own WT strain.

As it is shown in figure 31, Envigo WT mice lost more than 25% of weight compared to our WT mice strain. Therefore, Envigo mice had a lower survival compared to our WT mice.

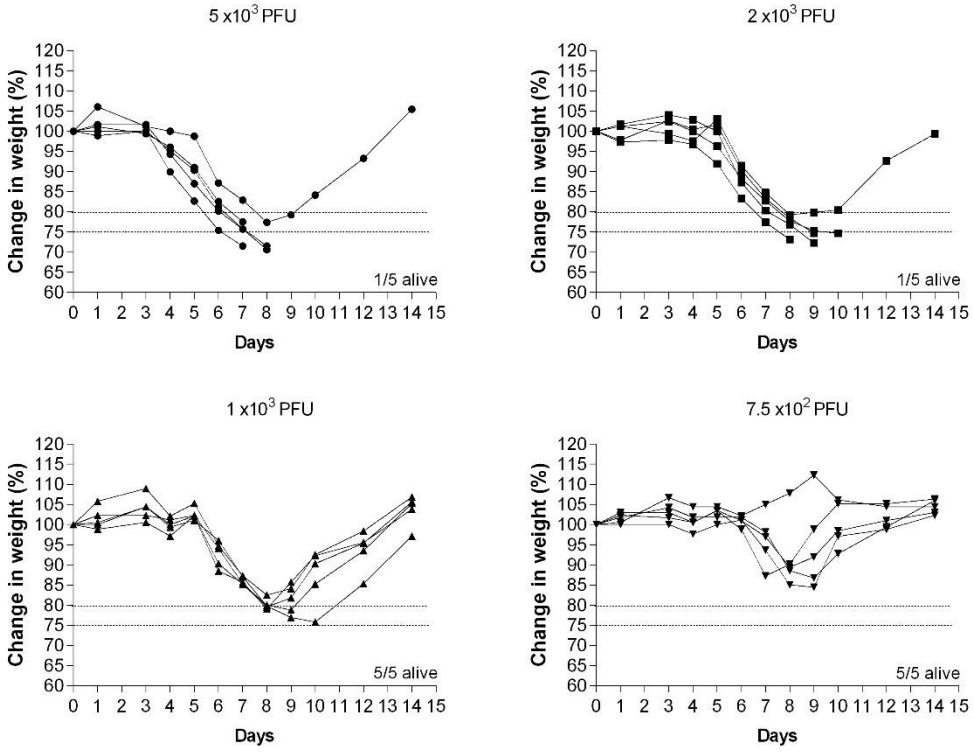


Figure 4.31 Influenza A virus titration in C57BL/6 ENVIGO strain. Mice were inoculated intranasally with 4 different virus doses ($5x10^3$, $2x10^3$, $1x10^3$ and $7.5x10^2$ PFU). Mice were anesthetized in a mouse anesthesia induction chamber. Once asleep, two drops of 20 µL each were administered within a couple of minutes between each other to avoid damage to the respiratory tract. Weight was monitored daily for 14 days.

4.4.2 GzmA deficient mice are protected from influenza virus infection while GzmB deficient mice are vulnerable to infection

After determining the optimal virus dose and the right experimental parameters, we decided to analyze the role of granzymes during an influenza-induced-sepsis. We infected different mouse strains with influenza virus as indicated in materials and methods. As shown in figure 32, mice began to lose weight between days 8 and 9. None of the GzmA deficient mice lost more than 25% of weight therefore had a survival of 100%. On the other hand, some GzmB deficient mice lost more than 25% of weight which meant a survival rate of 31%. Control group showed a survival rate of 77%. These results suggest that GzmA is involved in the disease pathogenesis and that the presence GzmB may be necessary for the pathogen control.

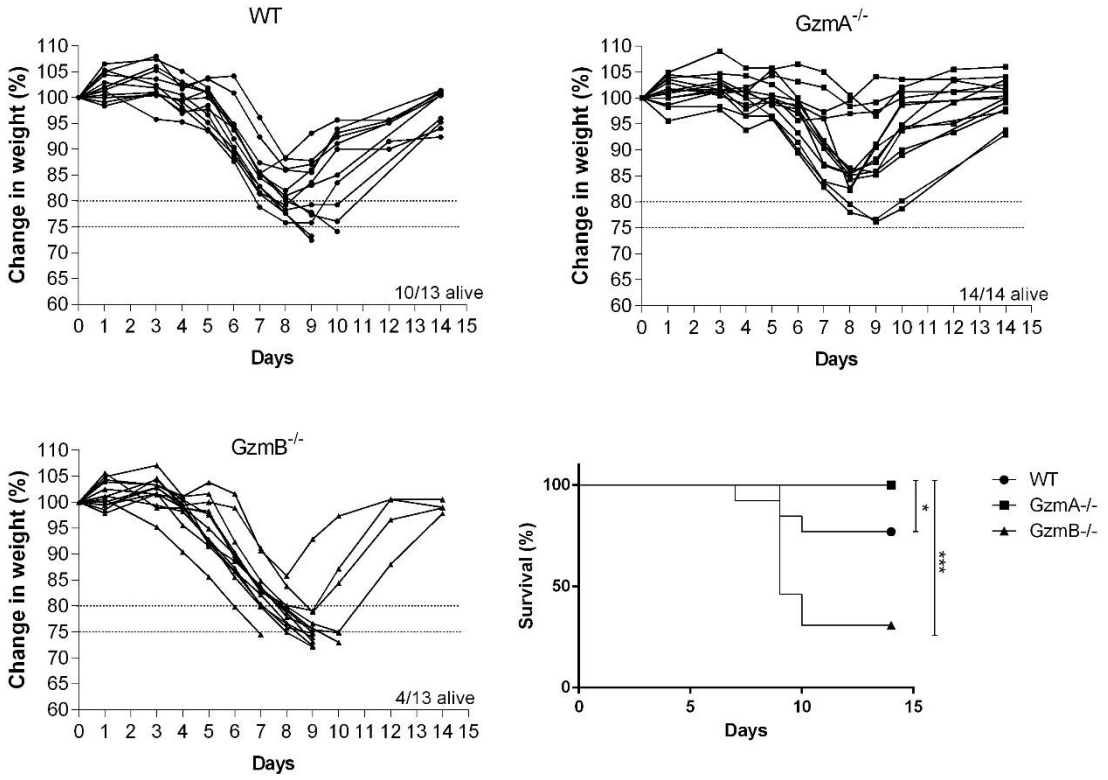


Figure 4.32 Weight loss and survival of WT, GzmA$^{-/-}$ and GzmB$^{-/-}$ mice during an influenza-induced-sepsis. WT, GzmA$^{-/-}$, and GzmB$^{-/-}$ mice were infected intranasally with a viral concentration of 2x10^3 PFU. Animals were weighted daily and monitored for 14 days. Sepsis score was determined as described in materials and methods. Statistical analysis was performed using logrank and Gehan-Wilcoxon test. In the figure the number of biological replicates from each group (n) of three independent experiments are indicated.

4.4.3 The absence of GzmK reduces survival in influenza infected mice

As we did in the bacterial sepsis, we decided to compare the role of GzmA and GzmK in sepsis induced by influenza virus. As it is shown in figure 33, weight loss began between days 7 and 8. Despite the fact that GzmA deficient mice lose almost 25% of their weight, they recover and no deaths are reported. Meanwhile GzmK deficient mice lose more than 25% of their weight therefore their survival is lower. Finally, as shown in figure 36, GzmK deficient mice had a lower survival rate compared to GzmA deficient mice (33% against 100%). If compared with each respective control, GzmK deficient mice had a lower survival than WT mice (C57BL/6N) with the truncated NNT expression and GzmA had a higher survival than WT Envigo mice (C57BL/6J) with the full length NNT expression.

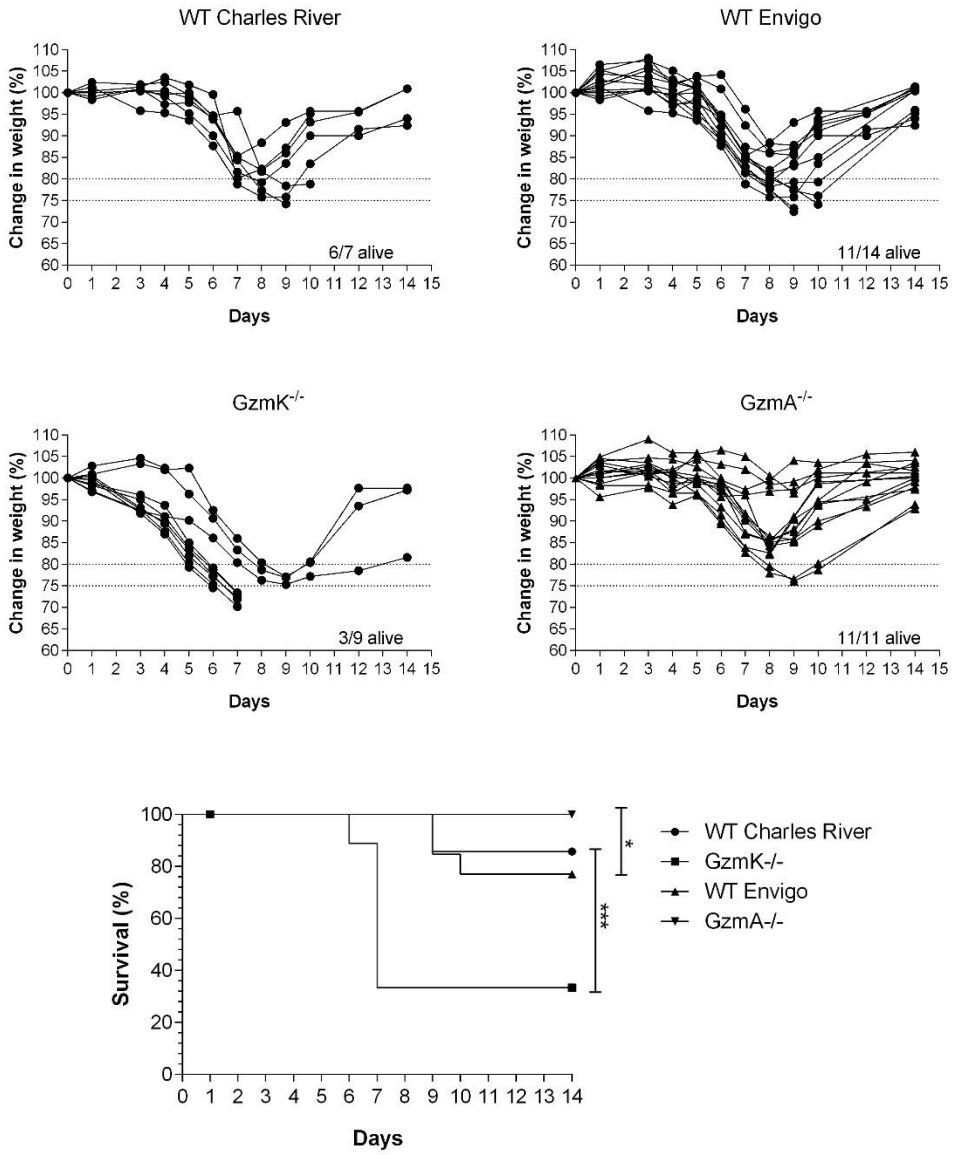


Figure 4.33. Weight loss and survival of WT, GzmA$^{-/-}$ and GzmK$^{-/-}$ mice during an influenza-induced-sepsis. WT$^{-/-}$, GzmA$^{-/-}$ and GzmB$^{-/-}$ mice were infected intranasally with a viral concentration of 2x10^{3} PFU. Animals were weighted daily, and the sepsis score was determined as described in material and methods. Statistical analysis was performed using logrank and Gehan-Wilcoxon test. In the figure the number of biological replicates from each group (n) of two independent experiments are indicated.

4.5 Biological relevance of granzymes on a model of coinfection after influenza sepsis

Secondary infections after influenza refers to the development of additional infections following a person`s recovery from the flu. Influenza virus damages the respiratory tract which creates an environment that is more conducive to bacterial growth and colonization (Kalil & Thomas, 2019; Morris, Cleary, & Clarke, 2017). The immune system is weakened by the viral

infection, making it difficult to the body to fight off the invading bacteria. The most common bacterial infections that follow influenza include pneumonia, sinusitis and ear infections. And it is now recognized that a high proportion of community-acquired pneumonia is caused by coinfections (Smith & McCullers, 2014). Secondary infections are common complications on influenza, particularly in people with weakened immune systems, the elderly and young children (Morris et al., 2017).

4.5.1 S. pneumoniae and influenza virus titration

With all our previous knowledge, we wanted to study the importance and the role of granzymes during secondary infections for which we needed to stablish the adequate experimental conditions to perform our experiments. Therefore, the first step was to determine the dose of both virus and bacteria. From our influenza study we determined the optimal influenza dose that will not kill the mice, but we needed to find the optimal dose of bacteria and the correct dose combination of virus and bacteria. As it is shown in figure 34, a bacterial concentration of $5x10^1$ CFU and $1x10^2$ CFU produced a loss in weight not strong enough kill the mice. Nevertheless, when virus and bacteria are combined the effect of both pathogens together is stronger. A combination of $5x10^4$ PFU of influenza virus and $5x10^1$ CFU of *S. pneumoniae* showed a survival of 0% at day 8 while a combination of $8x10^1$ PFU of influenza virus and the same bacterial concentration as before showed a survival rate of 60%. On the other hand, a combination of $4x10^1$ PFU of influenza virus and $1x10^2$ CFU of *S. pneumoniae* showed a survival of 80% while a combination of $2x10^2$ PFU of influenza virus and the same bacterial concentration as before showed a survival rate of 20%. Been this last combination the one we decided to use to perform the following experiments.

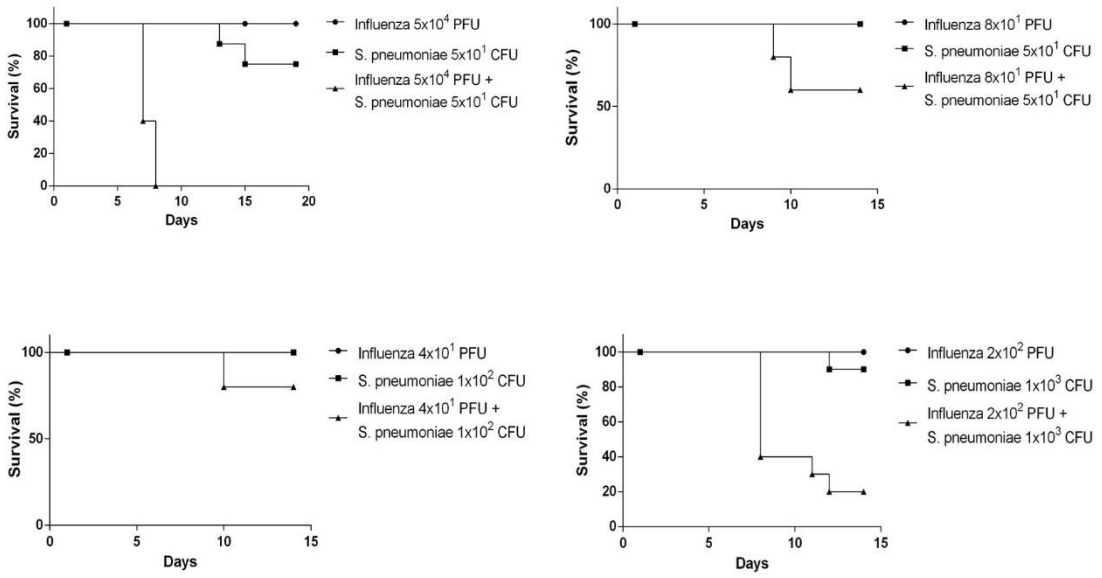


Figure 4.34. Influenza A virus and S. pneumoniae titration in C57BL/6 ENVIGO strain. Mice were inoculated intranasally with 4 different virus doses ($5x10^4$, $8x10^1$, $4x10^1$ and $2x10^2$ PFU). Mice were anesthetized in a mouse anesthesia induction chamber. Once asleep, two drops of 20 µL each were administered within a couple of minutes between each other to avoid damage to the respiratory tract. Five days after influenza infection mice were infected with two different doses of bacteria ($5x10^1$ and $1x10^3$). Weight was monitored daily for 14 days.

4.5.2 GzmA and GzmB deficient mice are protected against a secondary infection of S. pneumoniae

After stablishing the experimental conditions, we analyze the role of granzymes in our model of coinfection for which WT, $GzmA^{-/-}$ and $GzmB^{-/-}$ mice were, first infected with influenza virus ($2x10^2$ PFU) and after five days with *S. pneumoniae* ($1x10^3$ CFU). As it is shown in figure 35, GzmA deficient mice indicate a clear differentiation between those mice who lost weight and the ones who did not. This clear differentiation seems to be determinant to define survival. On the other hand, although GzmB mice also lose weight, they do so more slowly and do not lose more than 20% of their weight, so they manage to recover and survive. The survival rate of GzmA deficient mice was of 50% against an 88.8% survival of GzmB deficient mice. Our control group had a survival of 20%.

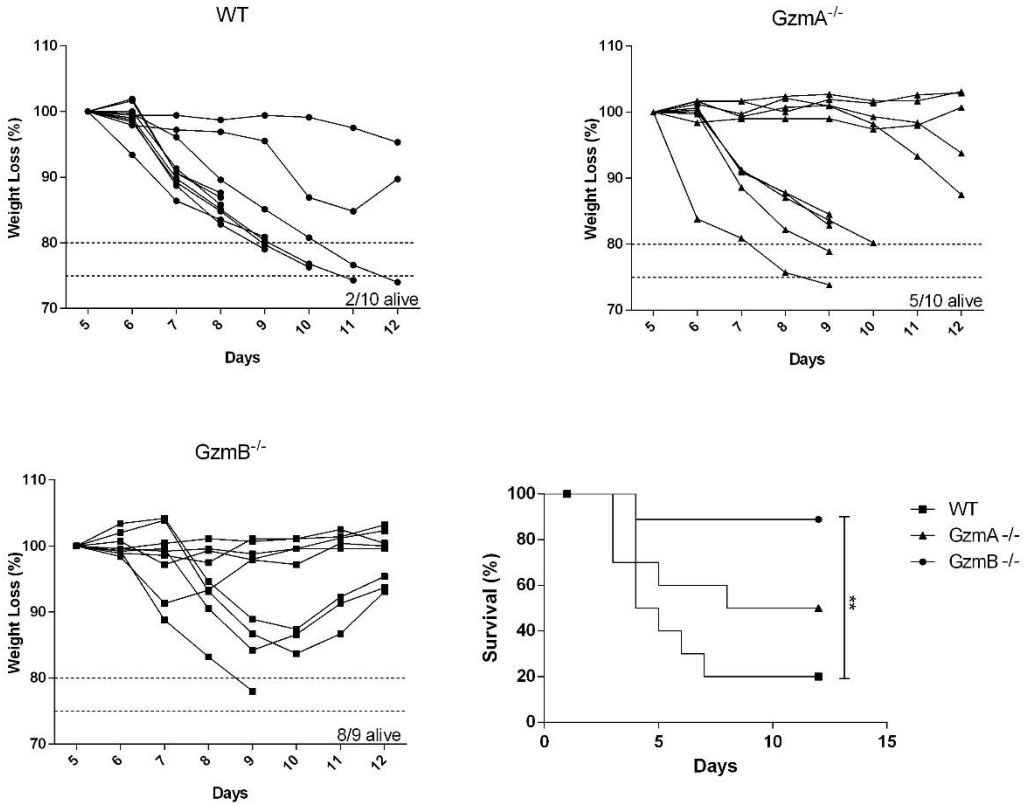


Figure 4.35 Weight loss and survival rate of WT, GzmA$^{-/-}$ and GzmB$^{-/-}$ mice during a secondary infection with *S. pneumoniae.* WT, GzmA$^{-/-}$ and GzmB$^{-/-}$ mice were infected intranasally with a viral concentration of 2x10^3 PFU. Five days after influenza infection mice were infected with 1x10^3 CFU. Animals were weighted daily, and the sepsis score was determined as described in material and methods. Statistical analysis was performed using logrank and Gehan-Wilcoxon test. In the figure the number of biological replicates from each group (n) of two independent experiments are indicated.

DISCUSSION

5 Discussion

In this work we have analyzed the importance and the contribution of GzmA in abdominal sepsis by looking into the presence of GzmA in the serum of patients suffering from peritoneal sepsis and using two mouse models, a mouse model of sepsis induced by cecal ligation and puncture model and a *E. coli* peritonitis sepsis model. Furthermore, in the *E. coli* sepsis model, we have analyzed the biological relevance of GzmA and compared it to its closest homolog GzmK. Additionally, we have analyzed the role of this proteases in a mouse model of viral sepsis induced by Influenza A virus as well as its role in the immunosuppression state caused by an influenza A virus infection in a coinfection model of influenza and *S. pneumoniae* infection.

As shown in our results, GzmA was found to be elevated in human serum from patients suffering from sepsis. The relevance of our findings was supported by the observation that GzmA deficient mice showed a higher survival and better outcome in CLP and *E. coli*-induced sepsis. GzmA deficiency reduced inflammation without changing bacterial load in both sepsis models. And it was observed that, the GzmA inhibitor serpinb6b, enhanced survival and reduced inflammation in septic WT mice to a similar level as the one observed in those with the GzmA genetic deficiency, suggesting a therapeutic potential in targeting GzmA in abdominal sepsis.

To perform our experiments in optimal conditions, it is important to understand the necessity of an adequate animal model to study polymicrobial abdominal sepsis physiopathology. Many animal models have been developed for the study of sepsis physiopathology, been the CLP mice model the most frequently used and continues to be considered the gold standard to inform novel pathways of sepsis physiology and its therapeutic direction (Alverdy, Keskey, & Thewissen, 2020). The reasons why we used the CLP model in our study is because the pathogens involved in sepsis development are endogenous, mimicking the traumatic injury that leads to peritonitis in humans. Also, CLP sepsis shows similarities with the progression of human sepsis, displaying both the hyper- and hypo-inflammatory responses characteristic of human sepsis (Korneev, 2019). On the other hand, we also used the model of inoculation of live pathogens which cannot evaluate important clinical features of sepsis, but allowed us to have an insight of the host response mechanisms against pathogens (Lewis et al., 2016).

Many studies have shown the possible contributions of GzmA in septic shock and sepsis. For instance, GzmA seems to play an important role in septic shock induced by LPS (S. Metkar et al., 2008) and augmenting the LPS-induced cytokine response in human monocytes (Annette C.

Wensink et al., 2016). Also, GzmA might contribute to sepsis induced by single bacterial agents like mice pathogen *B. microti* (Arias, Jimenez de Bagues, et al., 2014)*, M. tuberculosis* (Garcia-Laorden et al., 2015)*, S. pneumoniae* (van den Boogaard et al., 2016) or *E. coli* (Garcia-Laorden et al., 2017). However, the role of GzmA in abdominal polymicrobial sepsis had so far not been explored. Furthermore, the effect of GzmA inhibition in septic mice had not been analyzed in any of these models. Our study, together with previous literature, confirms that GzmA is a key mediator of sepsis associated with different bacterial pathogens.

Our data suggests that inflammation induced by GzmA plays a critical role in the development of sepsis during peritonitis. Our results show a lower production of IL-1α, IL-1β, IL-6 and TNFα in serum and peritoneal fluids in GzmA deficient mice. All these cytokines play an important role in sepsis (Matsumoto et al., 2018; Wensink, Hack, & Bovenschen, 2015). In addition, our results using an extracellular GzmA inhibitor, serpinb6b, *in vitro* and *in vivo,* strongly suggests that GzmA enhances the inflammatory pathological response in sepsis in the extracellular space. Thus, since serpinb6b should not affect intracellularly perforin-delivered GzmA, we suggest that GzmA does not contribute to sepsis by promoting pyroptosis (Zhou et al., 2020) or other types of cell death (Chowdhury & Lieberman, 2008). This suggestion is further supported by our results showing increased levels of extracellular GzmA in serum from patients with abdominal sepsis. Similar findings have been reported in other septic patients with human bacteremia, tuberculosis and typhoid fever where high serum levels of GzmA have been detected (de Jong et al., 2017; Garcia-Laorden et al., 2015; F. N. Lauw et al., 2000) suggesting this protease may have extracellular effects during other infectious diseases. But to try to understand how this protease regulate inflammation from the extracellular milieu, it is important to determine which is the cell source of GzmA during sepsis. In our study we have found that GzmA expression is increased in NK cells during CLP, supporting previous results in other models of sepsis (Arias, Jimenez de Bagues, et al., 2014; Garcia-Laorden et al., 2017). However, this finding does not exclude that GzmA expressed by other cell sources like platelets (Campbell et al., 2018) or other cells (Maykel Arias et al., 2017) could contribute to polymicrobial abdominal sepsis.

Furthermore, since GzmA and GzmK have been shown to activate inflammatory responses in macrophages and other cell types (M. Arias et al., 2017) and increased levels of GzmA and GzmK have been detected in serum of septic patients it has been thought that these proteases could have a role in bacterial sepsis (F. N. Lauw et al., 2000; Rucevic et al., 2007; Zeerleder, Voves, & Wuillemin, 2005). Using a mouse model of *E. coli* induced sepsis, the role of

GzmK in an *in vivo* mouse model of bacterial sepsis has been analysed for the first time in this study. In addition, the biological relevance of GzmA and GzmK in sepsis has been compared.

It has been found that, despite both proteases are playing a role in bacterial sepsis including disease progression and generation of inflammatory cytokines *in vivo*, only the absence of GzmA has a relevant impact in sepsis survival. In addition, it has been observed that neither GzmA nor GzmK are involved in the control of *E. coli* infection.

Both, GzmA and GzmK, were found to be expressed in NK cells during *E. coli* sepsis. This result is expected since NK cells play an important role in bacterial sepsis (de Pablo et al., 2012; Etogo, Nunez, Lin, Toliver-Kinsky, & Sherwood, 2008; Kerr et al., 2005) and this is one of the main cell populations in the innate immune system expressing Gzms (Martínez-Lostao et al., 2015). Moreover, this finding confirms previous results in other bacterial sepsis models, showing the critical role of NK cells and GzmA employing the mouse pathogen *B. microti* (Arias, Jimenez de Bagues, et al., 2014) or LPS (Anthony et al., 2010). Furthermore, GzmA and GzmK have been found to be expressed by NKT cells, a sub lineage of T cells that share characteristics between T and NK cells, which have also been shown to play a prominent role in bacterial sepsis (Szabo, Anantha, Shaler, McCormick, & Haeryfar, 2015).

During sepsis induced by a commensal strain of *E. coli* isolated from blood of a mice suffering from sepsis induced by CLP, a lower sign of sepsis was observed in GzmA or GzmK deficient mice when a murine sepsis score was applied, compared to WT mice. This murine sepsis score has been validated by Shrum et al and analyses several parameters such as respiratory function, animal behaviour and response to stimuli, allowing the evaluation of sepsis progression in mice (B. Shrum et al., 2014). However, only GzmA deficient mice showed a significant increment in survival compared with WT mice. In addition, GzmA deficient mice survived significantly more than GzmK deficient mice, suggesting that GzmA has a higher biological relevance than GzmK during *E. coli* sepsis. It is worth to mention again that we have employed a *E. coli* strain isolated from mice undergoing polymicrobial peritoneal sepsis (CLP) and, thus, our results are biologically relevant since a species-specific bacterial pathogen is employed.

These results, employing a mouse relevant sepsis model since *E. coli* is one of the most common pathogens causing sepsis in human, confirm previous studies on the inflammatory function of GzmA *in vitro* and *in vivo* during host-pathogen interaction. It has been previously found that GzmA deficient mice are resistant to endotoxemia induced by LPS (Anthony et al., 2010; S. S. Metkar et al., 2008). In addition, using a mouse model of sepsis induced by the mouse

pathogen *B. microti*, GzmA deficient mice showed an increment in survival compared with WT mice. The increment in survival was correlated with lower levels of proinflammatory cytokines IL-1α, IL-1β, TNFα and IL-6 (Arias, Jimenez de Bagues, et al., 2014).

GzmA was also found to be not involved in bacterial control in this model either (Arias, Jimenez de Bagues, et al., 2014). It has recently been shown that GzmA plays an important role in the damage associated with *S. pneumoniae* infection, the most common causative agent in community-acquired pneumonia (van der Poll & Opal, 2009). In addition, GzmA deficient mice also showed higher survival in a model of pneumonia due to *S. pneumoniae* (van den Boogaard et al., 2016). In our study, GzmA deficient mice express lower levels of IL-1α, IL-β and IL-6 in serum compared with WT mice, which correlates with increased mouse survival. These cytokines have been involved in the physiopathology of bacterial sepsis (Chong & Sriskandan, 2011; Mirzarahimi, Barak, Eslami, & Enteshari-Moghaddam, 2017; Vanden Berghe et al., 2014) and the lower levels of these cytokines in GzmA deficient mice could explain their higher survival during *E. coli* sepsis. However, it has also been observed that in murine models of *Klebsiella pneumoniae* pneumonia, a common pathogen in intrahospital pneumonias, GzmA does not play an important role during sepsis associated with this pathogen (Garcia-Laorden et al., 2016). A recent study has not been able to confirm a clear role for GzmA or GzmB during *E. coli* sepsis (Garcia-Laorden et al., 2017). However, the results obtained were not consistent and a clear conclusion on the role of GzmA and/or GzmB in bacterial control and sepsis could not be reached. Notably, a main difference among both studies is the use of different *E. coli* strains. Although an *E. coli* strain isolated from humans was used by Garcia-Laorden et al, we have employed a bacterial strain isolated from the blood of septic mice. Therefore, the biological relevance of this protease in sepsis seems to be dependent on the causal agent of the infection.

We found that similarly to GzmA, GzmK did not play an important role in the control of *E. coli* infection. This is the first time that the role of GzmK in the control of bacterial infection is analysed. Our finding is in line with those of Joeckel et al, showing that GzmK deficient mice controlled viral infections as efficiently as WT mice (L. T. Joeckel et al., 2017). However, further research is needed to confirm the role of inflammation induced by GzmK in the control of other bacterial infections.

Our results have confirmed that in contrast to GzmA, GzmK has a minor role during *E. coli* sepsis. Apparently, this result might be unexpected since both GzmA and GzmK are proteases with tryptase activity and share preferences to cleave substrates after basic residues (Lys or Arg). However, it has been shown that despite the similar protease activity, the substrate

specificity of GzmK differs from GzmA (N. Bovenschen et al., 2009; K. Plasman, H. Demol, P. I. Bird, K. Gevaert, & P. Van Damme, 2014) , which would explain the different contribution of these proteases to sepsis.

Despite we have not found a major role of GzmK in sepsis, our findings are important since they suggest that the detrimental role of GzmA during *E. coli* sepsis cannot be significantly compensated by its closest homologue GzmK and, thus, inhibition of GzmA might be sufficient to reduce damage and increase survival during sepsis.

Regarding the mechanism by which GzmA exerts its function, we describe that active GzmA induces the expression of TNFα and IL-6 by a mechanism dependent of both, its catalytic activity and TLR4. Supporting our findings in mouse macrophages, a recent study has found that human platelets acquire GzmA expression during aging and induce inflammation in human monocytes, a process inhibited by a TLR4 antagonist (Campbell et al., 2018). Confirming this finding, we have also shown that GzmA induces IL-6 generation in human monocytes, which similarly to mouse GzmA, was inhibited by SerpinC1/ATIII. Thus, it seems that both mouse and human extracellular GzmA regulates inflammation in monocytes and macrophages by a TLR4 dependent mechanism. However, it is not known yet if GzmA directly activates this receptor or could act on some other substrates that then act as ligands for TLR4. For example, GzmA can cleave fibronectin (Simon, Prester, Nerz, Kramer, & Fruth, 1988), and fibronectin fragments can activate TLR4 and induce inflammation (Kelsh, You, Horzempa, Zheng, & McKeown-Longo, 2014). Since extracellular GzmA induces the generation of IL-6 and TNFα in macrophages in absence of cell death (Santiago et al., 2020), it is unlikely that TLR4 is activated by the release of inflammatory mediators as a consequence of GzmA-mediated cell death. In addition, several lines of experimental evidence indicate that TLR4 is not activated by contaminants present in the protease preparations. Inactivated GzmA was not able to induce inflammatory cytokines in mouse macrophages or human monocytes, and inflammation was similarly triggered by GzmA generated in *E. coli* or in *P. pastoris* both of which were not contaminated with LPS. In addition, it has also been shown by others that GzmA generated in human cells induce inflammation via a TLR4-dependent mechanism (Campbell et al., 2018).

Under normal conditions, protease activity in blood is tightly regulated by extracellular inhibitors. Two circulating extracellular inhibitors are known for GzmA, antithrombin III (serpinC1) and α2-macroglobulin (Spaeny-Dekking, Kamp, Froelich, & Hack, 2000), suggesting that the protease activity can be regulated at the extracellular level. Interestingly, antithrombin III levels are markedly reduced in sepsis due to the reduction in liver synthesis, the consumption

of this protein by the formation of thrombin-antithrombin complexes and by its degradation mediated by neutrophil-released elastase (M. Levi, van der Poll, & Schultz, 2012). In addition, it has also been observed that in patients with sepsis the levels of α2-macroglobulin are decreased (Abbink et al., 1991). Therefore, supporting our findings, it is possible that in sepsis, as natural GzmA inhibitors decrease, the active fractions of this protease in the bloodstream increase, explaining why extracellular GzmA remains active during sepsis. It has been reported that human GzmA potentiates LPS induced cytokine responses in human monocytes, an effect independent of the catalytic activity of GzmA (Annette C. Wensink et al., 2016). In our study, employing mouse GzmA in mouse macrophages and human GzmA in human monocytes, we have found that enzyme activity is required for the induction of IL6 and TNFα. In addition, in our system, active or inactive GzmA did not potentiate the effect of LPS. Other studies employing inhibitors or enzymatically inactive mutants have also found that enzyme activity is required for the inflammatory action of mouse and human GzmA (S. Metkar et al., 2008; Schanoski et al., 2020). While the reasons for these apparently contradictory results are not known, it cannot be ruled out that both active and inactive forms of GzmA act as proinflammatory mediators in the plasma of septic patients.

On the other hand, respecting GzmK possible mechanism we have observed the following. Even though the impact of GzmK absence in *E. coli* sepsis seems to be less pronounced than that of GzmA absence, GzmK deficient mice have shown significantly lower levels of IL-1β in serum compared with WT mice. This result could explain the lower sepsis score observed in GzmK deficient mice during *E. coli* induced sepsis. This is the first time that the role of GzmK in bacterial sepsis is analysed *in vivo* and our findings are supported by previous *in vitro* findings indicating that GzmK may have a prominent role in regulating IL-1β function. Using recombinant GzmK, it has been demonstrated that GzmK induces the expression of IL-1β in peritoneal macrophages pre-stimulated with LPS (Joeckel et al., 2011b). Recently, it has also been observed that human GzmK is able to potentiate LPS-induced release of pro-inflammatory cytokines in human monocytes. The mechanism proposed for this synergy is that GzmK would promote the formation of the complex between LPS and CD14 by releasing individual molecules of LPS from the micelles (A. C. Wensink et al., 2014). In this same work it was observed, *in vivo*, that the combination of GzmK and LPS increased the production of TNFα, IL-1β and IL-6 in comparison with the administration of LPS alone (A. C. Wensink et al., 2014). In a recent work, Wensink et al, reported that similarly to GzmK, human GzmA also potentiates cytokine responses in human monocytes pre-stimulated with LPS or Gram-negative bacteria. They found that contrary to GzmK, GzmA does not bind to LPS or increase the LPS-CD14 complex formation and has a little

effect on LPS micelle disaggregation, concluding that GzmA and GzmK differentially modulate LPS-Toll-like receptor signalling in monocytes (A. C. Wensink et al., 2016). This result suggests that GzmA and GzmK have not a redundant function in antibacterial immune response.

Lately, some concerns have been raised regarding the presence and the influence of the NNT gene in inflammatory processes. It has been reported that NNT is one of the primary regulators of cellular redox balance due to its ability to supply mitochondria with NADPH. Furthermore, Ripoll et al. showed a mechanistic link between NNT and macrophage immune response where it was demonstrated that immune cells express high levels of NNT and showed that the overexpression of NNT resulted in the secretion of cytokines such as IL-6, TNFα and IL-1β (V. M. Ripoll et al., 2012). Also, it has been demonstrated that NNT has a significant effect in the immune response against pathogens. For instance, NNT overexpressing cells' inability to produce robust ROS had negative consequences in pathogen clearing as the cells were inefficient in clearing intracellular *E. coli*. Corroborating this data, mice that lacked NNT were more resistant to pulmonary infection by *S. pneumoniae*, whereas the NNT gene rescued mice were susceptible to the infection. Thus, the inability of NNT overexpressing cells to mount a robust immune response reveals a new role of NNT in regulating macrophages functions (Regan, Conway, & Bharath, 2022; V. M. Ripoll et al., 2012).

Although it has been reported by Rawle et al. that GzmA deficient mice have a mixed 6J/6N genetic background, express the full-length NNT gene and this expression is responsible for reducing the CHIKV arthritis phenotype (D. J. Rawle et al., 2022), our results showed that in our models of abdominal sepsis there was not significant differences between the mice that expressed full-length NNT against those that did not expressed it. We did not observe an equal distribution of the NNT gene in our $GzmA^{-/-}$ and $^{+/+}$ littermates showing that in fact $GzmA^{-/-}$ mice expressed NNT and that WT mice did not express it. Nevertheless, our *in vitro* assays showed that when using active GzmA there was an increased expression of proinflammatory cytokines while when using inactive GzmA those inflammatory cytokines levels were significantly reduced. Additionally, to confirm our results, when inducing sepsis to those mice who expressed NNT and those who did not, there were not significant differences in the sepsis score between the two groups. Furthermore, as it has been identified in previous studies, Serpinb6b is a specific intracellular inhibitor of mouse GzmA (Kaiserman et al., 2014) which is corroborated in our results where we observed how inflammation is reduced and survival increases when Serpinb6b was used as a therapeutic treatment in our septic mice, confirming in this way that in fact GzmA it is the responsible of regulate inflammation in abdominal sepsis induced by CLP and i.p. *E. coli* and that the presence of NNT has nothing to do with the phenotype of our septic models.

Regarding viral infections, it is known that they can cause a wide range of complications, including sepsis. Some viral infections that can lead to sepsis are dengue fever, Ebola, influenza virus and COVID-19 (Lin et al., 2018). The risk of developing sepsis from a viral infection depends on and can be increased by a variety of factors which include a weakened immune system, underlying medical conditions such as diabetes or heart disease and advanced age (Mayr, Yende, & Angus, 2014). Influenza, commonly known as the flu, is a highly contagious viral infection that affects the respiratory system. Complications on influenza infection include pneumonia, bronchitis, sinus infections, ear infections, myositis and rhabdomyolysis, encephalitis and sepsis, been the latter the main subject of our study. Influenza infections can trigger the deregulation of the innate immune system with excessive cytokines release and potential harmful consequences which includes endothelial damage, deregulation of coagulation and alteration of the microvascular permeability, tissue edema, shock, acute lung injury and acute encephalopathy (Florescu & Kalil, 2014).

Our results suggest that GzmA deficient mice have protection against influenza infection showing high survival rates compared to WT mice. On the other hand, GzmB deficient mice show a lower survival rate compared to WT mice which may indicate the importance of GzmB in the control of the pathogen during an influenza infection. Furthermore, when comparing the role of GzmA and GzmK we observe that GzmK deficient mice were more vulnerable to the infection compared to WT and GzmA KO mice. Unlike what was observed in the abdominal sepsis model, where GzmK did not play a representative role in inflammation when compared to GzmA, in our influenza sepsis model we have been able to observe that while the absence of GzmA protects the mice, the absence of GzmK increases mortality. With these results, we can think that, like GzmB, GzmK is involved in virus control.

Influenza infection causes significant induction of perforin and both GzmB and GzmA in activated $CD8^+$ T cells *in vivo.* $CD8^+$ cytolytic T lymphocytes (CTLs) are important in the viral clearance and recovery from influenza A infection in mice and in human. Its primary effector function is believed to be the antigen-specific lysis of infected cells in the respiratory epithelium mediated mainly by exocytosis of granules containing perforin and granzymes (Johnson et al., 2003). GzmB has been reported to be essential for the rapid apoptotic cell death detected in CTLs activity assays in some antiviral and antitumor responses (Johnson et al., 2003; Salti et al., 2011) which confirms our results that GzmB is responsible of the virus control. Furthermore, despite activating different and slower cell death pathways (Johnson et al., 2003), in our influenza sepsis model GzmA does not seem to be necessary for the control of the pathogen, which means it could be considered a good therapeutic target.

Regarding the role of GzmK, previous studies showed that the level of circulating GzmK in virus-infected patients is notably elevated, implying by this that GzmK has an important role in viral clearance (Bade et al., 2005). Also, GzmK has been observed to be elevated in the bronchoalveolar lavage (BAL) fluid of patients with allergic asthma and viral pneumonia (Cooper et al., 2011). It has also been reported that *in vivo* inhibition of GzmK by its specific inhibitor aggravated influenza virus infection which seems to demonstrate that Gzms may possess their own specificity depending on the pathogen they are fighting (Zhong et al., 2012). Even though GzmK shares some substrates specificity with GzmA it has its own substrates, for instance, lately it has been observed that GzmK cleaves importin α1 after Arg in the N-terminal IBB domain which is crucial to association with importin β. Moreover, GzmK also degrades importin β at its interaction regions in the C terminus. Thus, GzmK was found to degrade the importin α/β dimer, which means that GzmK can inhibit the classical importin α/β-mediated nuclear import pathway, which is responsible of the blockade of viral protein import to the host nucleus leading to efficient viral clearance (Zhong et al., 2012). In addition to confirming our results, explaining the importance of GzmK in the control of influenza virus, previous bibliography makes us fall in the need to perform further analysis such as cytokines expression and cell populations studies to broaden the knowledge, not only for the role of GzmK in influenza, but for the role of GzmA and B too, considering that cytokine secretion and direct killing are two major effector responses of NK cells (Cooper et al., 2011; Zhong et al., 2012).

Finally, interactions between different pathogens can lead to more severe outcomes, increased virulence and altered immune responses. Influenza is a highly contagious illness that may develop complications such as secondary infections that occur as the result of a weakened immune system that is susceptible to other infections (Metzger & Sun, 2013). Secondary infections challenge the immune system in ways that single infections do not, leading to complex and dynamic immune responses that can reveal important information about the immune system's mechanism for pathogen recognition, response and regulation (Chaplin, 2010). The understanding of the immunological mechanisms underlying these interactions can help identify strategies to better treat coinfections (N. Kumar, Sharma, Barua, Tripathi, & Rouse, 2018). Bacterial pneumonia is the most common and serious complication of influenza produced by *S. pneumoniae, H, influenzae, S. aureus* and a group of *Streptococcus* (MacIntyre et al., 2018).

When comparing our mice strains in the model of coinfection with influenza and *S. pneumonia*, our results showed a higher survival of GzmB deficient mice compared to WT mice, suggesting that the absence of GzmB would be beneficial in a coinfection. This result could be explained due to the fact that Treg express GzmB and it could be a mechanism to control

immune response after an infection. Thus, in the absence of GzmB the immune system would be less immunosuppressed after an infection and could react more efficiently to a secondary infection.

It has been demonstrated that Tregs are vital to the maintenance of naturally occurring and induced peripheral immune tolerance (Sula Karreci et al., 2017). Tregs have a crucial role in controlling immune responses and its deficiency causes dysregulated immunity with autoimmune disease affecting multiple organs. Treg cells use, among other mechanisms, perforin and GzmB to eliminate NK cells and activated T cells (X. Cao et al., 2007). In addition, it has been described that GzmB is capable of degrade the ζ chain of the TCR (Wieckowski, Wang, Gastman, Goldstein, & Rabinowich, 2002). Although the role of GzmB expressed in Treg cells in sepsis has not been studied, it has been shown that an increase in Treg cells occurs in sepsis (Taylor & Llewelyn, 2010; Venet et al., 2009). Treg cells play a significant role in the immunosuppression phase in sepsis by inhibiting the activation and proliferation of CD4+ T and CD8+ T lymphocytes. In addition, they can inhibit neutrophils, B lymphocytes, monocytes, macrophages, and DCs (C. Cao, Ma, Chai, & Shou, 2015; Y. Zhang et al., 2011).

Finally, our results also showed a higher survival of GzmA deficient mice compared to WT mice. This result could be explain by the fact that in the absence of GzmA the inflammatory response is more controlled (Arias, Jiménez de Bagües, et al., 2014) and therefore the compensatory anti-inflammatory mechanism would be more intense during a primary infection and the immune system will be strong enough to fight a second infection showing a better outcome of the disease. However, more analysis like a cytokine profile study and immune check points analysis such as PD-1 or CTLA-4 are needed in order to have a better understanding of the mechanisms by which GzmA participate in the immunosuppressive state in sepsis.

CONCLUSIONS

6 Conclusions

The results obtained in this work allow us to highlight the following conclusions:

1. The absence of granzyme A increases survival in mice subjected to CLP or *E. coli* induced sepsis.
2. Increased survival in GzmA deficient mice subjected to CLP or *E. coli* is associated with decreased production of IL-1β, TNFα and IL-6.
3. During CLP or *E. coli*-induced sepsis, the absence of GzmA does not affect pathogen control.
4. NK cells are the main source of GzmA during CLP or *E. coli*-induced sepsis.
5. The therapeutic inhibition of extracellular GzmA with serpinb6b increase survival in WT mice during CLP or *E. coli*-induced sepsis and could be associated with a reduction of a proinflammatory action of GzmA. These results suggest that extracellular GzmA is a promising target to treat peritoneal sepsis.
6. During *E. coli*-induced sepsis, the absence of GzmK reduce the severity of the disease and the levels of IL-β in plasma.
7. During *E. coli*-induced sepsis, the absence of GzmK does not affect pathogen control.
8. GzmA seems to play a more relevant role in the pathology of sepsis compared with GzmK.
9. Despite both GzmA and K, have been found to be involved in the physiopathology of bacterial sepsis. Inhibition of GzmA is sufficient to reduce inflammation and improve survival irrespectively of the presence of other inflammatory granzymes, like GzmK.
10. Extracellular GzmA induces the expression of IL-6 in macrophages and does not affect the viability of the cells.
11. Active GzmA seems to be inducing the expression of IL-6 in mouse macrophage and human monocytes by a TLR4 and MyD88 dependent pathway.
12. NNT expression does not change the phenotype of the pathogenesis in our models of abdominal sepsis.
13. GzmA deficient mice are protected from influenza virus suggesting that this protease play an important role in the pathophysiology of influenza viral sepsis.
14. During Influenza viral sepsis the absence of GzmB and GzmK reduced survival suggesting that both proteases are involved in the control of influenza A infection.
15. After an influenza A infection, GzmA and GzmB deficient mice are protected against a secondary infection of *S. pneumoniae* suggesting that both proteases are involved in the immunosuppression state of sepsis.

7 Conclusiones

Los resultados obtenidos en este trabajo nos permiten destacar las siguientes conclusiones:

1. La ausencia de GzmA aumenta la supervivencia en los ratones con sepsis inducida por CLP o *E. coli.*
2. El aumento de la supervivencia en ratones deficientes de GzmA con sepsis por CLP o *E. coli* se asocia a una menor producción de IL-1β, TNFα e IL-6.
3. La GzmA no está implicada en el control del patógeno en la sepsis inducida por CLP o *E. coli.*
4. Las células NK son la principal fuente de GzmA durante la sepsis inducida por CLP o *E. coli*.
5. La inhibición terapéutica de la GzmA extracelular utilizando Serpinb6b aumenta la supervivencia en ratones WT durante la sepsis por CLP o *E. coli* y podría estar asociada con una disminución en la acción proinflamatoria de GzmA. Estos resultados sugieren que la GzmA extracelular puede ser una diana terapéutica para el tratamiento de la sepsis peritoneal.
6. En la sepsis inducida por *E. coli*, la ausencia de GzmK disminuye la gravedad de la enfermedad y los niveles de IL-1β en plasma.
7. La ausencia de GzmK no afecta al control del patógeno en la sepsis inducida por *E. coli.*
8. La GzmA tiene un papel más relevante en la patología de la sepsis en comparación con GzmK.
9. A pesar de que tanto la GzmA como la K se encuentran involucradas en la fisiopatología de la sepsis bacteriana, la inhibición de GzmA es suficiente para reducir la inflamación y mejorar la supervivencia independientemente de la presencia de otras granzimas inflamatorias como GzmK.
10. La GzmA extracelular induce la expresión de IL-6 en macrófagos sin afectar la viabilidad de las células.
11. La GzmA activa induce la expresión de IL-6 en macrófagos de ratón y monocitos humanos mediante una vía dependiente de TLR4 y MyD88.
12. La expresión de NNT no cambia el fenotipo de la patología en nuestros modelos de sepsis abdominal.

13. Los ratones deficientes de GzmA están protegidos contra e virus de la influenza lo cual sugiere que esta proteasa juega un papel importante en la fisiopatología de la sepsis producida por el virus de la influenza.
14. Durante la sepsis producida por influenza, la ausencia de GzmB y GzmK reduce la supervivencia, lo cual sugiere las dos proteasas están involucradas en el control de la infección de influenza A.
15. Después de una infección por influenza A, los ratones deficientes de GzmA y B están protegidos contra una infección secundaria de *S. Pneumoniae*, lo que sugiere que las dos proteasas están involucradas en el estado de inmunosupresión de la sepsis.

BIBLIOGRAPHY

1. Abbink, J. J., Nuijens, J. H., Eerenberg, A. J., Huijbregts, C. C., Strack van Schijndel, R. J., Thijs, L. G., & Hack, C. E. (1991). Quantification of functional and inactivated alpha 2-macroglobulin in sepsis. *Thromb Haemost, 65*(1), 32-39.
2. Abraham, E. (2000). Coagulation Abnormalities in Acute Lung Injury and Sepsis. *American Journal of Respiratory Cell and Molecular Biology, 22*(4), 401-404. doi:10.1165/ajrcmb.22.4.f184
3. Abumayyaleh, M., Nuñez-Gil, I. J., El-Battrawy, I., Estrada, V., Becerra-Muñoz, V. M., Uribarri, A., . . . Akin, I. (2021). Sepsis of Patients Infected by SARS-CoV-2: Real-World Experience From the International HOPE-COVID-19-Registry and Validation of HOPE Sepsis Score. *Front Med (Lausanne), 8*, 728102. doi:10.3389/fmed.2021.728102
4. Agelaki, S., Tsatsanis, C., Gravanis, A., & Margioris, A. N. (2002). Corticotropin-Releasing Hormone Augments Proinflammatory Cytokine Production from Macrophages In Vitro and in Lipopolysaccharide-Induced Endotoxin Shock in Mice. *Infect Immun, 70*(11), 6068-6074. doi:doi:10.1128/IAI.70.11.6068-6074.2002
5. Allanki, S., Strilic, B., Scheinberger, L., Onderwater, Y. L., Marks, A., Günther, S., . . . Reischauer, S. (2021). Interleukin-11 signaling promotes cellular reprogramming and limits fibrotic scarring during tissue regeneration. *Science Advances, 7*(37), eabg6497. doi:doi:10.1126/sciadv.abg6497
6. Alvaro-Meca, A., Jimenez-Sousa, M. A., Micheloud, D., Sanchez-Lopez, A., Heredia-Rodriguez, M., Tamayo, E., . . . Group of Biomedical Research in Critical Care, M. (2018). Epidemiological trends of sepsis in the twenty-first century (2000-2013): an analysis of incidence, mortality, and associated costs in Spain. *Popul Health Metr, 16*(1), 4. doi:10.1186/s12963-018-0160-x
7. Alverdy, J. C., Keskey, R., & Thewissen, R. (2020). Can the Cecal Ligation and Puncture Model Be Repurposed To Better Inform Therapy in Human Sepsis? *Infect Immun, 88*(9). doi:10.1128/iai.00942-19
8. Amin, P., & Amin, V. (2015). Viral Sepsis. *Annual Update in Intensive Care and Emergency Medicine 2015, 2015*, 37-59. doi:10.1007/978-3-319-13761-2_4
9. Andrin, C., Pinkoski, M. J., Burns, K., Atkinson, E. A., Krahenbuhl, O., Hudig, D., . . . Michalak, M. (1998). Interaction between a Ca2+-binding protein calreticulin and perforin, a component of the cytotoxic T-cell granules. *Biochemistry, 37*(29), 10386-10394. doi:10.1021/bi980595z
10. Angus, D. C., & van der Poll, T. (2013). Severe sepsis and septic shock. *N Engl J Med, 369*(9), 840-851. doi:10.1056/NEJMra1208623
11. Annoni, R., Silva, L. F. F., Nussbaumer-Ochsner, Y., van Schadewijk, A., Mauad, T., Hiemstra, P. S., & Rabe, K. F. (2015). Increased expression of granzymes A and B in fatal asthma. *European Respiratory Journal, 45*(5), 1485-1488. doi:10.1183/09031936.00213814
12. Anthony, D. A., Andrews, D. M., Chow, M., Watt, S. V., House, C., Akira, S., . . . Smyth, M. J. (2010). A role for granzyme M in TLR4-driven inflammation and endotoxicosis. *J Immunol, 185*(3), 1794-1803. doi:10.4049/jimmunol.1000430
13. Arias, M., Martinez-Lostao, L., Santiago, L., Ferrandez, A., Granville, D. J., & Pardo, J. (2017). The Untold Story of Granzymes in Oncoimmunology: Novel Opportunities with Old Acquaintances. *Trends Cancer, 3*(6), 407-422. doi:10.1016/j.trecan.2017.04.001
14. Arias, M., Martínez-Lostao, L., Santiago, L., Ferrandez, A., Granville, D. J., & Pardo, J. (2017). The Untold Story of Granzymes in Oncoimmunology: Novel Opportunities with Old Acquaintances. *Trends in Cancer, 3*(6), 407-422. doi:https://doi.org/10.1016/j.trecan.2017.04.001
15. Arias, M. A., Jimenez de Bagues, M. P., Aguilo, N., Menao, S., Hervas-Stubbs, S., de Martino, A., . . . Pardo, J. (2014). Elucidating sources and roles of granzymes A and B during bacterial infection and sepsis. *Cell Rep, 8*(2), 420-429. doi:10.1016/j.celrep.2014.06.012

16. Arias, M. A., Jiménez de Bagües, M. P., Aguiló, N., Menao, S., Hervás-Stubbs, S., de Martino, A., . . . Pardo, J. (2014). Elucidating sources and roles of granzymes A and B during bacterial infection and sepsis. *Cell Rep, 8*(2), 420-429. doi:10.1016/j.celrep.2014.06.012
17. Bade, B., Lohrmann, J., ten Brinke, A., Wolbink, A. M., Wolbink, G. J., ten Berge, I. J., . . . Hack, C. E. (2005). Detection of soluble human granzyme K in vitro and in vivo. *Eur J Immunol, 35*(10), 2940-2948. doi:10.1002/eji.200526249
18. Baran, K., Dunstone, M., Chia, J., Ciccone, A., Browne, K. A., Clarke, C. J., . . . Trapani, J. A. (2009). The molecular basis for perforin oligomerization and transmembrane pore assembly. *Immunity, 30*(5), 684-695. doi:10.1016/j.immuni.2009.03.016
19. Barth, R. F., Buja, L. M., Barth, A. L., Carpenter, D. E., & Parwani, A. V. (2021). A Comparison of the Clinical, Viral, Pathologic, and Immunologic Features of Severe Acute Respiratory Syndrome (SARS), Middle East Respiratory Syndrome (MERS), and Coronavirus 2019 (COVID-19) Diseases. *Archives of Pathology & Laboratory Medicine, 145*(10), 1194-1211. doi:10.5858/arpa.2020-0820-SA
20. Bem, R. A., Bos, A. P., Bots, M., Wolbink, A. M., van Ham, S. M., Medema, J. P., . . . van Woensel, J. B. M. (2008). Activation of the Granzyme Pathway in Children With Severe Respiratory Syncytial Virus Infection. *Pediatric Research, 63*(6), 650-655. doi:10.1203/PDR.0b013e31816fdc32
21. Bernhard, S., Hug, S., Stratmann, A. E. P., Erber, M., Vidoni, L., Knapp, C. L., . . . Messerer, D. A. C. (2021). Interleukin 8 Elicits Rapid Physiological Changes in Neutrophils That Are Altered by Inflammatory Conditions. *Journal of Innate Immunity, 13*(4), 225-241. doi:10.1159/000514885
22. Bhavsar, I., Miller, C. S., & Al-Sabbagh, M. (2015). Macrophage Inflammatory Protein-1 Alpha (MIP-1 alpha)/CCL3: As a Biomarker. *General Methods in Biomarker Research and their Applications*, 223-249. doi:10.1007/978-94-007-7696-8_27
23. Boivin, W. A., Cooper, D. M., Hiebert, P. R., & Granville, D. J. (2009). Intracellular versus extracellular granzyme B in immunity and disease: challenging the dogma. *Laboratory Investigation, 89*(11), 1195-1220. doi:10.1038/labinvest.2009.91
24. Boivin, W. A., Shackleford, M., Vanden Hoek, A., Zhao, H., Hackett, T. L., Knight, D. A., & Granville, D. J. (2012). Granzyme B Cleaves Decorin, Biglycan and Soluble Betaglycan, Releasing Active Transforming Growth Factor-β1. *PLoS One, 7*(3), e33163. doi:10.1371/journal.pone.0033163
25. Boogaard, F. E. v. d., Gisbergen, K. P. J. M. v., Vernooy, J. H., Medema, J. P., Roelofs, J. J. T. H., Zoelen, M. A. D. v., . . . Poll, T. v. d. (2016). Granzyme A impairs host defense during Streptococcus pneumoniae pneumonia. *American Journal of Physiology-Lung Cellular and Molecular Physiology, 311*(2), L507-L516. doi:10.1152/ajplung.00116.2016
26. Bose, S., & Cho, J. (2013). Role of chemokine CCL2 and its receptor CCR2 in neurodegenerative diseases. *Arch Pharm Res, 36*(9), 1039-1050. doi:10.1007/s12272-013-0161-z
27. Bouwman, A. C., van Daalen, K. R., Crnko, S., ten Broeke, T., & Bovenschen, N. (2021). Intracellular and Extracellular Roles of Granzyme K. *Frontiers in Immunology, 12*. doi:10.3389/fimmu.2021.677707
28. Bovenschen, N., Quadir, R., van den Berg, A. L., Brenkman, A. B., Vandenberghe, I., Devreese, B., . . . Kummer, J. A. (2009). Granzyme K displays highly restricted substrate specificity that only partially overlaps with granzyme A. *J Biol Chem, 284*(6), 3504-3512. doi:10.1074/jbc.M806716200
29. Bovenschen, N., Quadir, R., van den Berg, A. L., Brenkman, A. B., Vandenberghe, I., Devreese, B., . . . Kummer, J. A. (2009). Granzyme K Displays Highly Restricted Substrate Specificity That Only Partially Overlaps with Granzyme A*. *Journal of Biological Chemistry, 284*(6), 3504-3512. doi:https://doi.org/10.1074/jbc.M806716200

30. Branger, J., Knapp, S., Weijer, S., Leemans, J. C., Pater, J. M., Speelman, P., . . . van der Poll, T. (2004). Role of Toll-like receptor 4 in gram-positive and gram-negative pneumonia in mice. *Infect Immun, 72*(2), 788-794. doi:10.1128/iai.72.2.788-794.2004
31. Bray, M. A., Sartain, S. E., Gollamudi, J., & Rumbaut, R. E. (2020). Microvascular thrombosis: experimental and clinical implications. *Transl Res, 225*, 105-130. doi:10.1016/j.trsl.2020.05.006
32. Cabrera-Perez, J., Condotta, S. A., Badovinac, V. P., & Griffith, T. S. (2014). Impact of sepsis on CD4 T cell immunity. *J Leukoc Biol, 96*(5), 767-777. doi:10.1189/jlb.5MR0114-067R
33. Campbell, R. A., Franks, Z., Bhatnagar, A., Rowley, J. W., Manne, B. K., Supiano, M. A., . . . Rondina, M. T. (2018). Granzyme A in Human Platelets Regulates the Synthesis of Proinflammatory Cytokines by Monocytes in Aging. *J Immunol, 200*(1), 295-304. doi:10.4049/jimmunol.1700885
34. Cao, C., Ma, T., Chai, Y. F., & Shou, S. T. (2015). The role of regulatory T cells in immune dysfunction during sepsis. *World J Emerg Med, 6*(1), 5-9. doi:10.5847/wjem.j.1920-8642.2015.01.001
35. Cao, X., Cai, S. F., Fehniger, T. A., Song, J., Collins, L. I., Piwnica-Worms, D. R., & Ley, T. J. (2007). Granzyme B and Perforin Are Important for Regulatory T Cell-Mediated Suppression of Tumor Clearance. *Immunity, 27*(4), 635-646. doi:https://doi.org/10.1016/j.immuni.2007.08.014
36. Catalan, E., Jaime-Sanchez, P., Aguilo, N., Simon, M. M., Froelich, C. J., & Pardo, J. (2015). Mouse cytotoxic T cell-derived granzyme B activates the mitochondrial cell death pathway in a Bim-dependent fashion. *J Biol Chem, 290*(11), 6868-6877. doi:10.1074/jbc.M114.631564
37. Catenacci, V., Sheikh, F., Patel, K., & Fox-Robichaud, A. E. (2022). The prognostic utility of protein C as a biomarker for adult sepsis: a systematic review and meta-analysis. *Critical Care, 26*(1), 21. doi:10.1186/s13054-022-03889-2
38. Cavalli, G., Colafrancesco, S., Emmi, G., Imazio, M., Lopalco, G., Maggio, M. C., . . . Dinarello, C. A. (2021). Interleukin 1α: a comprehensive review on the role of IL-1α in the pathogenesis and treatment of autoimmune and inflammatory diseases. *Autoimmunity Reviews, 20*(3), 102763. doi:https://doi.org/10.1016/j.autrev.2021.102763
39. Chaplin, D. D. (2010). Overview of the immune response. *J Allergy Clin Immunol, 125*(2 Suppl 2), S3-23. doi:10.1016/j.jaci.2009.12.980
40. Chaudhry, H., Zhou, J., Zhong, Y., Ali, M. M., McGuire, F., Nagarkatti, P. S., & Nagarkatti, M. (2013). Role of cytokines as a double-edged sword in sepsis. *In Vivo, 27*(6), 669-684.
41. Chen, D., Tang, T.-X., Deng, H., Yang, X.-P., & Tang, Z.-H. (2021). Interleukin-7 Biology and Its Effects on Immune Cells: Mediator of Generation, Differentiation, Survival, and Homeostasis. *Frontiers in Immunology, 12*. doi:10.3389/fimmu.2021.747324
42. Chinnaiyan, A. M., Hanna, W. L., Orth, K., Duan, H., Poirier, G. G., Froelich, C. J., & Dixit, V. M. (1996). Cytotoxic T-cell-derived granzyme B activates the apoptotic protease ICE-LAP3. *Curr Biol, 6*(7), 897-899. doi:10.1016/s0960-9822(02)00614-0
43. Choi, J. K., & Egwuagu, C. E. (2021). Interleukin 35 Regulatory B Cells. *Journal of Molecular Biology, 433*(1), 166607. doi:https://doi.org/10.1016/j.jmb.2020.07.019
44. Chong, D. L., & Sriskandan, S. (2011). Pro-inflammatory mechanisms in sepsis. *Contrib Microbiol, 17*, 86-107. doi:10.1159/000324022
45. Chousterman, B. G., Swirski, F. K., & Weber, G. F. (2017). Cytokine storm and sepsis disease pathogenesis. *Semin Immunopathol, 39*(5), 517-528. doi:10.1007/s00281-017-0639-8
46. Chowdhury, D., & Lieberman, J. (2008). Death by a thousand cuts: granzyme pathways of programmed cell death. *Annu Rev Immunol, 26*, 389-420. doi:10.1146/annurev.immunol.26.021607.090404

47. Ciesielska, A., Matyjek, M., & Kwiatkowska, K. (2021). TLR4 and CD14 trafficking and its influence on LPS-induced pro-inflammatory signaling. *Cell Mol Life Sci, 78*(4), 1233-1261. doi:10.1007/s00018-020-03656-y
48. Clark, R. H., Stinchcombe, J. C., Day, A., Blott, E., Booth, S., Bossi, G., . . . Griffiths, G. M. (2003). Adaptor protein 3-dependent microtubule-mediated movement of lytic granules to the immunological synapse. *Nat Immunol, 4*(11), 1111-1120. doi:10.1038/ni1000
49. Cooper, D. M., Pechkovsky, D. V., Hackett, T. L., Knight, D. A., & Granville, D. J. (2011). Granzyme K activates protease-activated receptor-1. *PLoS One, 6*(6), e21484. doi:10.1371/journal.pone.0021484
50. Dalskov, L., Narita, R., Andersen, L. L., Jensen, N., Assil, S., Kristensen, K. H., . . . Hartmann, R. (2020). Characterization of distinct molecular interactions responsible for IRF3 and IRF7 phosphorylation and subsequent dimerization. *Nucleic Acids Res, 48*(20), 11421-11433. doi:10.1093/nar/gkaa873
51. Darden, D. B., Kelly, L. S., Fenner, B. P., Moldawer, L. L., Mohr, A. M., & Efron, P. A. (2021). Dysregulated Immunity and Immunotherapy after Sepsis. *J Clin Med, 10*(8). doi:10.3390/jcm10081742
52. Darmon, A. J., Nicholson, D. W., & Bleackley, R. C. (1995). Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. *Nature, 377*(6548), 446-448. doi:10.1038/377446a0
53. de Jong, H. K., Garcia-Laorden, M. I., Hoogendijk, A. J., Parry, C. M., Maude, R. R., Dondorp, A. M., . . . Wiersinga, W. J. (2017). Expression of intra- and extracellular granzymes in patients with typhoid fever. *PLoS Negl Trop Dis, 11*(7), e0005823. doi:10.1371/journal.pntd.0005823
54. de Pablo, R., Monserrat, J., Torrijos, C., Martin, M., Prieto, A., & Alvarez-Mon, M. (2012). The predictive role of early activation of natural killer cells in septic shock. *Crit Care, 16*(2), 413. doi:10.1186/cc11204
55. de Saint Basile, G., Ménasché, G., & Fischer, A. (2010). Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules. *Nat Rev Immunol, 10*(8), 568-579. doi:10.1038/nri2803
56. Deitch, E. A. (2005). RODENT MODELS OF INTRA-ABDOMINAL INFECTION. *Shock, 24*, 19-23. doi:10.1097/01.shk.0000191386.18818.0a
57. Dhama, K., Khan, S., Tiwari, R., Sircar, S., Bhat, S., Malik, Y. S., . . . Rodriguez-Morales, A. J. (2020). Coronavirus Disease 2019-COVID-19. *Clin Microbiol Rev, 33*(4). doi:10.1128/cmr.00028-20
58. Dinarello, C., Novick, D., Kim, S., & Kaplanski, G. (2013). Interleukin-18 and IL-18 Binding Protein. *Frontiers in Immunology, 4*. doi:10.3389/fimmu.2013.00289
59. Ding, R., Meng, Y., & Ma, X. (2018). The Central Role of the Inflammatory Response in Understanding the Heterogeneity of Sepsis-3. *BioMed Research International, 2018*, 5086516. doi:10.1155/2018/5086516
60. Dolmatova, E. V., Wang, K., Mandavilli, R., & Griendling, K. K. (2020). The effects of sepsis on endothelium and clinical implications. *Cardiovascular Research, 117*(1), 60-73. doi:10.1093/cvr/cvaa070
61. Dorofaeff, T., Mohseni-Bod, H., & Cox, P. N. (2012). Infections in the PICU. *Textbook of Clinical Pediatrics*, 2537-2563. doi:10.1007/978-3-642-02202-9_268
62. Dustin, M. L., & Long, E. O. (2010). Cytotoxic immunological synapses. *Immunol Rev, 235*(1), 24-34. doi:10.1111/j.0105-2896.2010.00904.x
63. Egea, L., Hirata, Y., & Kagnoff, M. F. (2010). GM-CSF: a role in immune and inflammatory reactions in the intestine. *Expert Rev Gastroenterol Hepatol, 4*(6), 723-731. doi:10.1586/egh.10.73
64. Esteban, A., Frutos-Vivar, F., Ferguson, N. D., Penuelas, O., Lorente, J. A., Gordo, F., . . . de Luna, R. R. (2007). Sepsis incidence and outcome: contrasting the intensive care unit

with the hospital ward. *Crit Care Med, 35*(5), 1284-1289. doi:10.1097/01.CCM.0000260960.94300.DE

65. Etogo, A. O., Nunez, J., Lin, C. Y., Toliver-Kinsky, T. E., & Sherwood, E. R. (2008). NK but not CD1-restricted NKT cells facilitate systemic inflammation during polymicrobial intra-abdominal sepsis. *J Immunol, 180*(9), 6334-6345.
66. Feldmann, J., Callebaut, I., Raposo, G., Certain, S., Bacq, D., Dumont, C., . . . de Saint Basile, G. (2003). Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). *Cell, 115*(4), 461-473. doi:10.1016/s0092-8674(03)00855-9
67. Fink, M. P. (2014). Animal models of sepsis. *Virulence, 5*(1), 143-153. doi:10.4161/viru.26083
68. Florescu, D. F., & Kalil, A. C. (2014). The complex link between influenza and severe sepsis. *Virulence, 5*(1), 137-142. doi:10.4161/viru.27103
69. Freeman, B. D., & Natanson, C. (2000). Anti-inflammatory therapies in sepsis and septic shock. *Expert Opin Investig Drugs, 9*(7), 1651-1663. doi:10.1517/13543784.9.7.1651
70. Friesenhagen, J., Boergeling, Y., Hrincius, E., Ludwig, S., Roth, J., & Viemann, D. (2012). Highly pathogenic avian influenza viruses inhibit effective immune responses of human blood-derived macrophages. *J Leukoc Biol, 92*(1), 11-20. doi:10.1189/jlb.0911479
71. Fujishima, S. (2016). Organ dysfunction as a new standard for defining sepsis. *Inflamm Regen, 36*, 24. doi:10.1186/s41232-016-0029-y
72. Gacouin, A., Lesouhaitier, M., Reizine, F., Pronier, C., Grégoire, M., Painvin, B., . . . Tadié, J. M. (2020). Short-term survival of acute respiratory distress syndrome patients due to influenza virus infection alone: a cohort study. *ERJ Open Research, 6*(4), 00587-02020. doi:10.1183/23120541.00587-2020
73. Garcia-Laorden, M. I., Blok, D. C., Kager, L. M., Hoogendijk, A. J., van Mierlo, G. J., Lede, I. O., . . . van der Poll, T. (2015). Increased intra- and extracellular granzyme expression in patients with tuberculosis. *Tuberculosis (Edinb), 95*(5), 575-580. doi:10.1016/j.tube.2015.05.016
74. García-Laorden, M. I., Hoogendijk, A. J., Wiewel, M. A., van Vught, L. A., Schultz, M. J., Bovenschen, N., . . . van der Poll, T. (2021). Intracellular expression of granzymes A, B, K and M in blood lymphocyte subsets of critically ill patients with or without sepsis. *Clinical and Experimental Immunology, 205*(2), 222-231. doi:10.1111/cei.13601
75. Garcia-Laorden, M. I., Stroo, I., Blok, D. C., Florquin, S., Medema, J. P., de Vos, A. F., & van der Poll, T. (2016). Granzymes A and B Regulate the Local Inflammatory Response during Klebsiella pneumoniae Pneumonia. *J Innate Immun, 8*(3), 258-268. doi:10.1159/000443401
76. García-Laorden, M. I., Stroo, I., Blok, D. C., Florquin, S., Medema, J. P., de Vos, A. F., & van der Poll, T. (2016). Granzymes A and B Regulate the Local Inflammatory Response during Klebsiella pneumoniae Pneumonia. *Journal of Innate Immunity, 8*(3), 258-268. doi:10.1159/000443401
77. Garcia-Laorden, M. I., Stroo, I., Terpstra, S., Florquin, S., Medema, J. P., van, T. V. C., . . . van der Poll, T. (2017). Expression and Function of Granzymes A and B in Escherichia coli Peritonitis and Sepsis. *Mediators Inflamm, 2017*, 4137563. doi:10.1155/2017/4137563
78. Garlanda, C., Anders, H.-J., & Mantovani, A. (2009). TIR8/SIGIRR: an IL-1R/TLR family member with regulatory functions in inflammation and T cell polarization. *Trends in Immunology, 30*(9), 439-446. doi:https://doi.org/10.1016/j.it.2009.06.001
79. Garzón-Tituaña, M., Arias, M. A., Sierra-Monzón, J. L., Morte-Romea, E., Santiago, L., Ramirez-Labrada, A., . . . Pardo, J. (2020). The Multifaceted Function of Granzymes in Sepsis: Some Facts and a Lot to Discover. *Front Immunol, 11*, 1054. doi:10.3389/fimmu.2020.01054

80. Gibson, P. G., Qin, L., & Puah, S. H. (2020). COVID-19 acute respiratory distress syndrome (ARDS): clinical features and differences from typical pre-COVID-19 ARDS. *Med J Aust, 213*(2), 54-56.e51. doi:10.5694/mja2.50674
81. Gierula, M., & Ahnström, J. (2020). Anticoagulant protein S—New insights on interactions and functions. *Journal of Thrombosis and Haemostasis, 18*(11), 2801-2811. doi:https://doi.org/10.1111/jth.15025
82. Gogos, C. A., Drosou, E., Bassaris, H. P., & Skoutelis, A. (2000). Pro- versus Anti-inflammatory Cytokine Profile in Patients with Severe Sepsis: A Marker for Prognosis and Future Therapeutic Options. *The Journal of Infectious Diseases, 181*(1), 176-180. doi:10.1086/315214
83. Gonzales, J. N., Lucas, R., & Verin, A. D. (2015). The Acute Respiratory Distress Syndrome: Mechanisms and Perspective Therapeutic Approaches. *Austin J Vasc Med, 2*(1).
84. González-Granado, L. I., Martín-Nalda, A., Alsina, L., Neth, O., Santamaría, M., & Soler-Palacín, P. (2022). Respiratory syncytial virus infections requiring hospitalization in patients with primary immunodeficiency. *Anales de Pediatría (English Edition), 96*(6), 492-500. doi:10.1016/j.anpede.2022.03.002
85. Goping, I. S., Barry, M., Liston, P., Sawchuk, T., Constantinescu, G., Michalak, K. M., . . . Bleackley, R. C. (2003). Granzyme B-induced apoptosis requires both direct caspase activation and relief of caspase inhibition. *Immunity, 18*(3), 355-365. doi:10.1016/s1074-7613(03)00032-3
86. Goping, I. S., Sawchuk, T., Underhill, D. A., & Bleackley, R. C. (2006). Identification of α-tubulin as a granzyme B substrate during CTL-mediated apoptosis. *Journal of Cell Science, 119*(5), 858-865. doi:10.1242/jcs.02791
87. Gordy, C., & He, Y. W. (2012). Endocytosis by target cells: an essential means for perforin- and granzyme-mediated killing. *Cell Mol Immunol, 9*(1), 5-6. doi:10.1038/cmi.2011.45
88. Grudzien, M., & Rapak, A. (2018). Effect of Natural Compounds on NK Cell Activation. *Journal of Immunology Research, 2018*, 4868417. doi:10.1155/2018/4868417
89. Gu, X., Zhou, F., Wang, Y., Fan, G., & Cao, B. (2020). Respiratory viral sepsis: epidemiology, pathophysiology, diagnosis and treatment. *Eur Respir Rev, 29*(157). doi:10.1183/16000617.0038-2020
90. Guo, H., Santiago, F., Lambert, K., Takimoto, T., & Topham, D. J. (2011). T Cell-Mediated Protection against Lethal 2009 Pandemic H1N1 Influenza Virus Infection in a Mouse Model. *Journal of Virology, 85*(1), 448-455. doi:doi:10.1128/JVI.01812-10
91. Gupta, N., Richter, R., Robert, S., & Kong, M. (2018). Viral Sepsis in Children. *Front Pediatr, 6*, 252. doi:10.3389/fped.2018.00252
92. Hao, Y., Baker, D., & ten Dijke, P. (2019). TGF-β-Mediated Epithelial-Mesenchymal Transition and Cancer Metastasis. *International Journal of Molecular Sciences, 20*(11), 2767.
93. Hay, Z. L. Z., & Slansky, J. E. (2022). Granzymes: The Molecular Executors of Immune-Mediated Cytotoxicity. *Int J Mol Sci, 23*(3). doi:10.3390/ijms23031833
94. He, X. H., Ouyang, D. Y., & Xu, L. H. (2021). Injection of Escherichia coli to Induce Sepsis. *Methods Mol Biol, 2321*, 43-51. doi:10.1007/978-1-0716-1488-4_5
95. Herz, J., Pardo, J., Kashkar, H., Schramm, M., Kuzmenkina, E., Bos, E., . . . Utermöhlen, O. (2009). Acid sphingomyelinase is a key regulator of cytotoxic granule secretion by primary T lymphocytes. *Nat Immunol, 10*(7), 761-768. doi:10.1038/ni.1757
96. Hodge, G., Mukaro, V., Holmes, M., Reynolds, P. N., & Hodge, S. (2013). Enhanced cytotoxic function of natural killer and natural killer T-like cells associated with decreased CD94 (Kp43) in the chronic obstructive pulmonary disease airway. *Respirology, 18*(2), 369-376. doi:https://doi.org/10.1111/j.1440-1843.2012.02287.x

97. Holmdahl, R., & Malissen, B. (2012). The need for littermate controls. *European Journal of Immunology, 42*(1), 45-47. doi:https://doi.org/10.1002/eji.201142048
98. Hoppensteadt, D., Tsuruta, K., Hirman, J., Kaul, I., Osawa, Y., & Fareed, J. (2015). Dysregulation of inflammatory and hemostatic markers in sepsis and suspected disseminated intravascular coagulation. *Clin Appl Thromb Hemost, 21*(2), 120-127. doi:10.1177/1076029613509476
99. Hotchkiss, R. S., Monneret, G., & Payen, D. (2013). Immunosuppression in sepsis: a novel understanding of the disorder and a new therapeutic approach. *Lancet Infect Dis, 13*(3), 260-268. doi:10.1016/s1473-3099(13)70001-x
100. Hotchkiss, R. S., & Nicholson, D. W. (2006). Apoptosis and caspases regulate death and inflammation in sepsis. *Nat Rev Immunol, 6*(11), 813-822. doi:10.1038/nri1943
101. Hotchkiss, R. S., Tinsley, K. W., Swanson, P. E., Chang, K. C., Cobb, J. P., Buchman, T. G., . . . Karl, I. E. (1999). Prevention of lymphocyte cell death in sepsis improves survival in mice. *Proc Natl Acad Sci U S A, 96*(25), 14541-14546. doi:10.1073/pnas.96.25.14541
102. House, I. G., House, C. M., Brennan, A. J., Gilan, O., Dawson, M. A., Whisstock, J. C., . . . Voskoboinik, I. (2017). Regulation of perforin activation and pre-synaptic toxicity through C-terminal glycosylation. *EMBO reports, 18*(10), 1775-1785. doi:https://doi.org/10.15252/embr.201744351
103. Huang, M., Cai, S., & Su, J. (2019). The Pathogenesis of Sepsis and Potential Therapeutic Targets. *Int J Mol Sci, 20*(21). doi:10.3390/ijms20215376
104. Huang, M., Cai, S., & Su, J. (2019). The Pathogenesis of Sepsis and Potential Therapeutic Targets. *International Journal of Molecular Sciences, 20*(21), 5376.
105. Iba, T., & Levy, J. H. (2020). Sepsis-induced Coagulopathy and Disseminated Intravascular Coagulation. *Anesthesiology, 132*(5), 1238-1245. doi:10.1097/aln.0000000000003122
106. Idriss, H. T., & Naismith, J. H. (2000). TNF alpha and the TNF receptor superfamily: structure-function relationship(s). *Microsc Res Tech, 50*(3), 184-195. doi:10.1002/1097-0029(20000801)50:3<184::aid-jemt2>3.0.co;2-h
107. Iskander, K. N., Osuchowski, M. F., Stearns-Kurosawa, D. J., Kurosawa, S., Stepien, D., Valentine, C., & Remick, D. G. (2013). Sepsis: Multiple Abnormalities, Heterogeneous Responses, and Evolving Understanding. *Physiological Reviews, 93*(3), 1247-1288. doi:10.1152/physrev.00037.2012
108. Jacobi, J. (2002). Pathophysiology of sepsis. *Am J Health Syst Pharm, 59 Suppl 1*, S3-8. doi:10.1093/ajhp/59.suppl_1.S3
109. Jarczak, D., Kluge, S., & Nierhaus, A. (2021). Sepsis-Pathophysiology and Therapeutic Concepts. *Front Med (Lausanne), 8*, 628302. doi:10.3389/fmed.2021.628302
110. Jensen, I. J., Sjaastad, F. V., Griffith, T. S., & Badovinac, V. P. (2018). Sepsis-Induced T Cell Immunoparalysis: The Ins and Outs of Impaired T Cell Immunity. *J Immunol, 200*(5), 1543-1553. doi:10.4049/jimmunol.1701618
111. Joeckel, L. T., Allison, C. C., Pellegrini, M., Bird, C. H., & Bird, P. I. (2017). Granzyme K-deficient mice show no evidence of impaired antiviral immunity. *Immunol Cell Biol, 95*(8), 676-683. doi:10.1038/icb.2017.35
112. Joeckel, L. T., Allison, C. C., Pellegrini, M., Bird, C. H., & Bird, P. I. (2017). Granzyme K-deficient mice show no evidence of impaired antiviral immunity. *Immunology & Cell Biology, 95*(8), 676-683. doi:https://doi.org/10.1038/icb.2017.35
113. Joeckel, L. T., & Bird, P. I. (2014). Are all granzymes cytotoxic in vivo? *Biol Chem, 395*(2), 181-202. doi:10.1515/hsz-2013-0238
114. Joeckel, L. T., Wallich, R., Martin, P., Sanchez-Martinez, D., Weber, F. C., Martin, S. F., . . . Simon, M. M. (2011a). Mouse granzyme K has pro-inflammatory potential. *Cell Death & Differentiation, 18*(7), 1112-1119. doi:10.1038/cdd.2011.5

115. Joeckel, L. T., Wallich, R., Martin, P., Sanchez-Martinez, D., Weber, F. C., Martin, S. F., . . . Simon, M. M. (2011b). Mouse granzyme K has pro-inflammatory potential. *Cell Death Differ, 18*(7), 1112-1119. doi:10.1038/cdd.2011.5
116. Joffre, J., Hellman, J., Ince, C., & Ait-Oufella, H. (2020). Endothelial Responses in Sepsis. *Am J Respir Crit Care Med, 202*(3), 361-370. doi:10.1164/rccm.201910-1911TR
117. Johnson, B. J., Costelloe, E. O., Fitzpatrick, D. R., Haanen, J. B., Schumacher, T. N., Brown, L. E., & Kelso, A. (2003). Single-cell perforin and granzyme expression reveals the anatomical localization of effector CD8+ T cells in influenza virus-infected mice. *Proc Natl Acad Sci U S A, 100*(5), 2657-2662. doi:10.1073/pnas.0538056100
118. Joshi, L., Ponnana, M., Sivangala, R., Chelluri, L. K., Nallari, P., Penmetsa, S., . . . Gaddam, S. (2015). Evaluation of TNF-α, IL-10 and IL-6 Cytokine Production and Their Correlation with Genotype Variants amongst Tuberculosis Patients and Their Household Contacts. *PLoS One, 10*(9), e0137727. doi:10.1371/journal.pone.0137727
119. Kabanova, A., Zurli, V., & Baldari, C. T. (2018). Signals Controlling Lytic Granule Polarization at the Cytotoxic Immune Synapse. *Frontiers in Immunology, 9*. doi:10.3389/fimmu.2018.00307
120. Kaiserman, D., Bird, C. H., Sun, J., Matthews, A., Ung, K., Whisstock, J. C., . . . Bird, P. I. (2006). The major human and mouse granzymes are structurally and functionally divergent. *J Cell Biol, 175*(4), 619-630. doi:10.1083/jcb.200606073
121. Kaiserman, D., Stewart, S. E., Plasman, K., Gevaert, K., Van Damme, P., & Bird, P. I. (2014). Identification of Serpinb6b as a species-specific mouse granzyme A inhibitor suggests functional divergence between human and mouse granzyme A. *J Biol Chem, 289*(13), 9408-9417. doi:10.1074/jbc.M113.525808
122. Kaiserman, D., Zhao, P., Rowe, C. L., Leong, A., Barlow, N., Joeckel, L. T., . . . Bird, P. I. (2022). Granzyme K initiates IL-6 and IL-8 release from epithelial cells by activating protease-activated receptor 2. *PLoS One, 17*(7), e0270584. doi:10.1371/journal.pone.0270584
123. Kalil, A. C., & Thomas, P. G. (2019). Influenza virus-related critical illness: pathophysiology and epidemiology. *Crit Care, 23*(1), 258. doi:10.1186/s13054-019-2539-x
124. Kany, S., Vollrath, J. T., & Relja, B. (2019). Cytokines in Inflammatory Disease. *Int J Mol Sci, 20*(23). doi:10.3390/ijms20236008
125. Keegan, A. D., Leonard, W. J., & Zhu, J. (2021). Recent advances in understanding the role of IL-4 signaling. *Fac Rev, 10*, 71. doi:10.12703/r/10-71
126. Kelsh, R., You, R., Horzempa, C., Zheng, M., & McKeown-Longo, P. J. (2014). Regulation of the innate immune response by fibronectin: synergism between the III-1 and EDA domains. *PLoS One, 9*(7), e102974. doi:10.1371/journal.pone.0102974
127. Kerr, A. R., Kirkham, L. A., Kadioglu, A., Andrew, P. W., Garside, P., Thompson, H., & Mitchell, T. J. (2005). Identification of a detrimental role for NK cells in pneumococcal pneumonia and sepsis in immunocompromised hosts. *Microbes Infect, 7*(5-6), 845-852. doi:10.1016/j.micinf.2005.02.011
128. Kielian, T., Esen, N., & Bearden, E. D. (2005). Toll-like receptor 2 (TLR2) is pivotal for recognition of S. aureus peptidoglycan but not intact bacteria by microglia. *Glia, 49*(4), 567-576. doi:https://doi.org/10.1002/glia.20144
129. Kiselevsky, D. B. (2020). Granzymes and Mitochondria. *Biochemistry (Mosc), 85*(2), 131-139. doi:10.1134/s0006297920020017
130. Korneev, K. V. (2019). Mouse Models of Sepsis and Septic Shock. *Molecular Biology, 53*(5), 704-717. doi:10.1134/S0026893319050108
131. Kumar, A., Taghi Khani, A., Sanchez Ortiz, A., & Swaminathan, S. (2022). GM-CSF: A Double-Edged Sword in Cancer Immunotherapy. *Frontiers in Immunology, 13*. doi:10.3389/fimmu.2022.901277

132. Kumar, N., Sharma, S., Barua, S., Tripathi, B. N., & Rouse, B. T. (2018). Virological and Immunological Outcomes of Coinfections. *Clin Microbiol Rev, 31*(4). doi:10.1128/cmr.00111-17
133. Kurowska, M., Goudin, N., Nehme, N. T., Court, M., Garin, J., Fischer, A., . . . Ménasché, G. (2012). Terminal transport of lytic granules to the immune synapse is mediated by the kinesin-1/Slp3/Rab27a complex. *Blood, 119*(17), 3879-3889. doi:10.1182/blood-2011-09-382556
134. Lai, D., Qin, C., & Shu, Q. (2014). Myeloid-derived suppressor cells in sepsis. *Biomed Res Int, 2014*, 598654. doi:10.1155/2014/598654
135. Lauw, F. N., Simpson, A. J., Hack, C. E., Prins, J. M., Wolbink, A. M., van Deventer, S. J., . . . van Der Poll, T. (2000). Soluble granzymes are released during human endotoxemia and in patients with severe infection due to gram-negative bacteria. *J Infect Dis, 182*(1), 206-213. doi:10.1086/315642
136. Lauw, F. N., Simpson, A. J. H., Erik Hack, C., Prins, J. M., Wolbink, A. M., van Deventer, S. J. H., . . . van der Poll, T. (2000). Soluble Granzymes Are Released during Human Endotoxemia and in Patients with Severe Infection Due to Gram-Negative Bacteria. *The Journal of Infectious Diseases, 182*(1), 206-213. doi:10.1086/315642
137. Lavergne, M., Hernández-Castañeda, M. A., Mantel, P. Y., Martinvalet, D., & Walch, M. (2021). Oxidative and Non-Oxidative Antimicrobial Activities of the Granzymes. *Front Immunol, 12*, 750512. doi:10.3389/fimmu.2021.750512
138. Law, R. H., Lukoyanova, N., Voskoboinik, I., Caradoc-Davies, T. T., Baran, K., Dunstone, M. A., . . . Whisstock, J. C. (2010). The structural basis for membrane binding and pore formation by lymphocyte perforin. *Nature, 468*(7322), 447-451. doi:10.1038/nature09518
139. Lelubre, C., & Vincent, J. L. (2018). Mechanisms and treatment of organ failure in sepsis. *Nat Rev Nephrol, 14*(7), 417-427. doi:10.1038/s41581-018-0005-7
140. Levi, M., Schouten, M., & van der Poll, T. (2008). Sepsis, Coagulation, and Antithrombin: Old Lessons and New Insights. *Semin Thromb Hemost, 34*(08), 742-746. doi:10.1055/s-0029-1145256
141. Levi, M., Schultz, M., & van der Poll, T. (2013). Sepsis and thrombosis. *Semin Thromb Hemost, 39*(5), 559-566. doi:10.1055/s-0033-1343894
142. Levi, M., van der Poll, T., & Schultz, M. (2012). New insights into pathways that determine the link between infection and thrombosis. *Neth J Med, 70*(3), 114-120.
143. Lewis, A. J., Seymour, C. W., & Rosengart, M. R. (2016). Current Murine Models of Sepsis. *Surg Infect (Larchmt), 17*(4), 385-393. doi:10.1089/sur.2016.021
144. Liang, Y., Wang, M.-L., Chien, C.-S., Yarmishyn, A. A., Yang, Y.-P., Lai, W.-Y., . . . Chiou, S.-H. (2020). Highlight of Immune Pathogenic Response and Hematopathologic Effect in SARS-CoV, MERS-CoV, and SARS-Cov-2 Infection. *Frontiers in Immunology, 11*. doi:10.3389/fimmu.2020.01022
145. Lieberman, J. (2010). Granzyme A activates another way to die. *Immunol Rev, 235*(1), 93-104. doi:10.1111/j.0105-2896.2010.00902.x
146. Lin, G. L., McGinley, J. P., Drysdale, S. B., & Pollard, A. J. (2018). Epidemiology and Immune Pathogenesis of Viral Sepsis. *Front Immunol, 9*, 2147. doi:10.3389/fimmu.2018.02147
147. Liu, D., Huang, S.-Y., Sun, J.-H., Zhang, H.-C., Cai, Q.-L., Gao, C., . . . Zeng, L. (2022). Sepsis-induced immunosuppression: mechanisms, diagnosis and current treatment options. *Military Medical Research, 9*(1), 56. doi:10.1186/s40779-022-00422-y
148. Liu, M., Guo, S., Hibbert, J. M., Jain, V., Singh, N., Wilson, N. O., & Stiles, J. K. (2011). CXCL10/IP-10 in infectious diseases pathogenesis and potential therapeutic implications. *Cytokine Growth Factor Rev, 22*(3), 121-130. doi:10.1016/j.cytogfr.2011.06.001

149. Liu, T., Zhang, L., Joo, D., & Sun, S. C. (2017). NF-κB signaling in inflammation. *Signal Transduct Target Ther, 2*, 17023-. doi:10.1038/sigtrans.2017.23
150. Looney, M. R., & Matthay, M. A. (2007). The role of protein C in sepsis. *Curr Infect Dis Rep, 3*(5), 413-418. doi:10.1007/s11908-007-1008-x
151. Lopez, K. E., & Bouchier-Hayes, L. (2022). Lethal and Non-Lethal Functions of Caspases in the DNA Damage Response. *Cells, 11*(12). doi:10.3390/cells11121887
152. Maamar, A., Guillot, P., Joussellin, V., Delamaire, F., Painvin, B., Bichon, A., . . . Gacouin, A. (2023). Moderate to severe ARDS: COVID-19 patients compared to influenza patients for ventilator parameters and mortality. *ERJ Open Research*, 00554-02022. doi:10.1183/23120541.00554-2022
153. MacIntyre, C. R., Chughtai, A. A., Barnes, M., Ridda, I., Seale, H., Toms, R., & Heywood, A. (2018). The role of pneumonia and secondary bacterial infection in fatal and serious outcomes of pandemic influenza a(H1N1)pdm09. *BMC Infectious Diseases, 18*(1), 637. doi:10.1186/s12879-018-3548-0
154. Majumder, R., & Nguyen, T. (2021). Protein S: function, regulation, and clinical perspectives. *Curr Opin Hematol, 28*(5), 339-344. doi:10.1097/moh.0000000000000663
155. Marcinkiewicz, J., Ciszek, M., Bobek, M., Strus, M., Heczko, P. B., Kurnyta, M., . . . Chmielarczyk, A. (2007). Differential inflammatory mediator response in vitro from murine macrophages to lactobacilli and pathogenic intestinal bacteria. *International Journal of Experimental Pathology, 88*(3), 155-164. doi:https://doi.org/10.1111/j.1365-2613.2007.00530.x
156. Markwart, R., Condotta, S. A., Requardt, R. P., Borken, F., Schubert, K., Weigel, C., . . . Rubio, I. (2014). Immunosuppression after sepsis: systemic inflammation and sepsis induce a loss of naive T-cells but no enduring cell-autonomous defects in T-cell function. *PLoS One, 9*(12), e115094. doi:10.1371/journal.pone.0115094
157. Martínez-Lostao, L., Anel, A., & Pardo, J. (2015). How Do Cytotoxic Lymphocytes Kill Cancer Cells? *Clin Cancer Res, 21*(22), 5047-5056. doi:10.1158/1078-0432.ccr-15-0685
158. Matsumoto, H., Ogura, H., Shimizu, K., Ikeda, M., Hirose, T., Matsuura, H., . . . Shimazu, T. (2018). The clinical importance of a cytokine network in the acute phase of sepsis. *Sci Rep, 8*(1), 13995. doi:10.1038/s41598-018-32275-8
159. Matuschak, G. M., & Lechner, A. J. (2010). Acute lung injury and the acute respiratory distress syndrome: pathophysiology and treatment. *Mo Med, 107*(4), 252-258.
160. Mayr, F. B., Yende, S., & Angus, D. C. (2014). Epidemiology of severe sepsis. *Virulence, 5*(1), 4-11. doi:10.4161/viru.27372
161. McBride, M. A., Patil, T. K., Bohannon, J. K., Hernandez, A., Sherwood, E. R., & Patil, N. K. (2020). Immune Checkpoints: Novel Therapeutic Targets to Attenuate Sepsis-Induced Immunosuppression. *Front Immunol, 11*, 624272. doi:10.3389/fimmu.2020.624272
162. McIsaac, S. M., Stadnyk, A. W., & Lin, T.-J. (2012). Toll-like receptors in the host defense against Pseudomonas aeruginosa respiratory infection and cystic fibrosis. *Journal of leukocyte biology, 92*(5), 977-985. doi:10.1189/jlb.0811410
163. Medema, J. P., Toes, R. E. M., Scaffidi, C., Zheng, T. S., Flavell, R. A., Melief, C. J. M., . . . Krammer, P. H. (1997). Cleavage of FLICE (caspase-8) by granzyme B during cytotoxic T lymphocyte-induced apoptosis. *European Journal of Immunology, 27*(12), 3492-3498. doi:https://doi.org/10.1002/eji.1830271250
164. Megha, K. B., Joseph, X., Akhil, V., & Mohanan, P. V. (2021). Cascade of immune mechanism and consequences of inflammatory disorders. *Phytomedicine, 91*, 153712. doi:10.1016/j.phymed.2021.153712

165. Memish, Z. A., Perlman, S., Van Kerkhove, M. D., & Zumla, A. (2020). Middle East respiratory syndrome. *Lancet, 395*(10229), 1063-1077. doi:10.1016/s0140-6736(19)33221-0
166. Mentlik, A. N., Sanborn, K. B., Holzbaur, E. L., & Orange, J. S. (2010). Rapid lytic granule convergence to the MTOC in natural killer cells is dependent on dynein but not cytolytic commitment. *Mol Biol Cell, 21*(13), 2241-2256. doi:10.1091/mbc.e09-11-0930
167. Metkar, S., Menaa, C., Pardo, J., Wang, B., Wallich, R., Freudenberg, M., . . . Froelich, C. (2008). Human and Mouse Granzyme A Induce a Proinflammatory Cytokine Response. *Immunity, 29*(5), 720-733. doi:https://doi.org/10.1016/j.immuni.2008.08.014
168. Metkar, S. S., Marchioretto, M., Antonini, V., Lunelli, L., Wang, B., Gilbert, R. J., . . . Froelich, C. J. (2015). Perforin oligomers form arcs in cellular membranes: a locus for intracellular delivery of granzymes. *Cell Death Differ, 22*(1), 74-85. doi:10.1038/cdd.2014.110
169. Metkar, S. S., Menaa, C., Pardo, J., Wang, B., Wallich, R., Freudenberg, M., . . . Froelich, C. J. (2008). Human and mouse granzyme A induce a proinflammatory cytokine response. *Immunity, 29*(5), 720-733. doi:10.1016/j.immuni.2008.08.014
170. Metkar, S. S., Wang, B., Ebbs, M. L., Kim, J. H., Lee, Y. J., Raja, S. M., & Froelich, C. J. (2003). Granzyme B activates procaspase-3 which signals a mitochondrial amplification loop for maximal apoptosis. *J Cell Biol, 160*(6), 875-885. doi:10.1083/jcb.200210158
171. Metzger, D. W., & Sun, K. (2013). Immune dysfunction and bacterial coinfections following influenza. *J Immunol, 191*(5), 2047-2052. doi:10.4049/jimmunol.1301152
172. Miller, W. D., Han, X., Peek, M. E., Charan Ashana, D., & Parker, W. F. (2021). Accuracy of the Sequential Organ Failure Assessment Score for In-Hospital Mortality by Race and Relevance to Crisis Standards of Care. *JAMA Netw Open, 4*(6), e2113891. doi:10.1001/jamanetworkopen.2021.13891
173. Mirzarahimi, M., Barak, M., Eslami, A., & Enteshari-Moghaddam, A. (2017). The role of interleukin-6 in the early diagnosis of sepsis in premature infants. *Pediatr Rep, 9*(3), 7305. doi:10.4081/pr.2017.7305
174. Morris, D. E., Cleary, D. W., & Clarke, S. C. (2017). Secondary Bacterial Infections Associated with Influenza Pandemics. *Front Microbiol, 8*, 1041. doi:10.3389/fmicb.2017.01041
175. Motyka, B., Korbutt, G., Pinkoski, M. J., Heibein, J. A., Caputo, A., Hobman, M., . . . Bleackley, R. C. (2000). Mannose 6-phosphate/insulin-like growth factor II receptor is a death receptor for granzyme B during cytotoxic T cell-induced apoptosis. *Cell, 103*(3), 491-500. doi:10.1016/s0092-8674(00)00140-9
176. Napoli, A. M., Fast, L. D., Gardiner, F., Nevola, M., & Machan, J. T. (2012). Increased granzyme levels in cytotoxic T lymphocytes are associated with disease severity in emergency department patients with severe sepsis. *Shock, 37*(3), 257-262. doi:10.1097/SHK.0b013e31823fca44
177. Nduka, O. O., & Parrillo, J. E. (2009). The pathophysiology of septic shock. *Critical care clinics, 25 4*, 677-702, vii.
178. Nedeva, C., Menassa, J., & Puthalakath, H. (2019). Sepsis: Inflammation Is a Necessary Evil. *Front Cell Dev Biol, 7*, 108. doi:10.3389/fcell.2019.00108
179. Nemzek, J. A., Hugunin, K. M., & Opp, M. R. (2008). Modeling sepsis in the laboratory: merging sound science with animal well-being. *Comp Med, 58*(2), 120-128.
180. Norooznezhad, A. H., & Mansouri, K. (2021). Endothelial cell dysfunction, coagulation, and angiogenesis in coronavirus disease 2019 (COVID-19). *Microvascular Research, 137*, 104188. doi:https://doi.org/10.1016/j.mvr.2021.104188
181. Osuchowski, M. F., Craciun, F., Weixelbaumer, K. M., Duffy, E. R., & Remick, D. G. (2012). Sepsis Chronically in MARS: Systemic Cytokine Responses Are Always Mixed

Regardless of the Outcome, Magnitude, or Phase of Sepsis. *The Journal of Immunology, 189*(9), 4648-4656. doi:10.4049/jimmunol.1201806

182. Ouyang, W., & O'Garra, A. (2019). IL-10 Family Cytokines IL-10 and IL-22: from Basic Science to Clinical Translation. *Immunity, 50*(4), 871-891. doi:https://doi.org/10.1016/j.immuni.2019.03.020
183. Pardo, J., Aguilo, J. I., Anel, A., Martin, P., Joeckel, L., Borner, C., . . . Simon, M. M. (2009). The biology of cytotoxic cell granule exocytosis pathway: granzymes have evolved to induce cell death and inflammation. *Microbes Infect, 11*(4), 452-459. doi:10.1016/j.micinf.2009.02.004
184. Pardo, J., Wallich, R., Martin, P., Urban, C., Rongvaux, A., Flavell, R. A., . . . Simon, M. M. (2008). Granzyme B-induced cell death exerted by ex vivo CTL: discriminating requirements for cell death and some of its signs. *Cell Death Differ, 15*(3), 567-579. doi:10.1038/sj.cdd.4402289
185. Pawlinski, R., & Mackman, N. (2010). Cellular sources of tissue factor in endotoxemia and sepsis. *Thromb Res, 125 Suppl 1*, S70-73. doi:10.1016/j.thromres.2010.01.042
186. Pennini, M. E., Pai, R. K., Schultz, D. C., Boom, W. H., & Harding, C. V. (2006). Mycobacterium tuberculosis 19-kDa Lipoprotein Inhibits IFN-γ-Induced Chromatin Remodeling of MHC2TA by TLR2 and MAPK Signaling1. *The Journal of Immunology, 176*(7), 4323-4330. doi:10.4049/jimmunol.176.7.4323
187. Perk, Y., & Özdil, M. (2018). Respiratory syncytial virüs infections in neonates and infants. *Turk Pediatri Ars, 53*(2), 63-70. doi:10.5152/TurkPediatriArs.2018.6939
188. Plasman, K., Demol, H., Bird, P. I., Gevaert, K., & Van Damme, P. (2014). Substrate Specificities of the Granzyme Tryptases A and K. *Journal of Proteome Research, 13*(12), 6067-6077. doi:10.1021/pr500968d
189. Plasman, K., Demol, H., Bird, P. I., Gevaert, K., & Van Damme, P. (2014). Substrate specificities of the granzyme tryptases A and K. *J Proteome Res, 13*(12), 6067-6077. doi:10.1021/pr500968d
190. Plasman, K., Maurer-Stroh, S., Ahmad, J., Hao, H., Kaiserman, D., Sirota, F. L., . . . Van Damme, P. (2013). Conservation of the extended substrate specificity profiles among homologous granzymes across species. *Mol Cell Proteomics, 12*(10), 2921-2934. doi:10.1074/mcp.M113.028670
191. Poli-de-Figueiredo, L. F., Garrido, A. G., Nakagawa, N., & Sannomiya, P. (2008). EXPERIMENTAL MODELS OF SEPSIS AND THEIR CLINICAL RELEVANCE. *Shock, 30*(7), 53-59. doi:10.1097/SHK.0b013e318181a343
192. Posma, J. J., Grover, S. P., Hisada, Y., Owens, A. P., 3rd, Antoniak, S., Spronk, H. M., & Mackman, N. (2019). Roles of Coagulation Proteases and PARs (Protease-Activated Receptors) in Mouse Models of Inflammatory Diseases. *Arterioscler Thromb Vasc Biol, 39*(1), 13-24. doi:10.1161/atvbaha.118.311655
193. Praper, T., Beseničar, M. P., Istinič, H., Podlesek, Z., Metkar, S. S., Froelich, C. J., & Anderluh, G. (2010). Human perforin permeabilizing activity, but not binding to lipid membranes, is affected by pH. *Molecular Immunology, 47*(15), 2492-2504. doi:https://doi.org/10.1016/j.molimm.2010.06.001
194. Prescott, J., Falzarano, D., de Wit, E., Hardcastle, K., Feldmann, F., Haddock, E., . . . Munster, V. J. (2018). Pathogenicity and Viral Shedding of MERS-CoV in Immunocompromised Rhesus Macaques. *Front Immunol, 9*, 205. doi:10.3389/fimmu.2018.00205
195. Proklou, A., Soulitzis, N., Neofytou, E., Rovina, N., Zervas, E., Gaga, M., . . . Tzortzaki, E. G. (2013). Granule Cytotoxic Activity and Oxidative DNA Damage in Smoking and Nonsmoking Patients With Asthma. *Chest, 144*(4), 1230-1237. doi:https://doi.org/10.1378/chest.13-0367

196. Rawle, D. J., Le, T. T., Dumenil, T., Bishop, C., Yan, K., Nakayama, E., . . . Suhrbier, A. (2021). An inconvenient association between granzyme A and Nicotinamide Nucleotide Transhydrogenase. *bioRxiv*, 2021.2003.2016.435730. doi:10.1101/2021.03.16.435730

197. Rawle, D. J., Le, T. T., Dumenil, T., Bishop, C., Yan, K., Nakayama, E., . . . Suhrbier, A. (2022). Widespread discrepancy in Nnt genotypes and genetic backgrounds complicates granzyme A and other knockout mouse studies. *Elife, 11*. doi:10.7554/eLife.70207

198. Regan, T., Conway, R., & Bharath, L. P. (2022). Regulation of immune cell function by nicotinamide nucleotide transhydrogenase. *Am J Physiol Cell Physiol, 322*(4), C666-c673. doi:10.1152/ajpcell.00607.2020

199. Reinhart, K., Daniels, R., Kissoon, N., Machado, F. R., Schachter, R. D., & Finfer, S. (2017). Recognizing Sepsis as a Global Health Priority — A WHO Resolution. *New England Journal of Medicine, 377*(5), 414-417. doi:10.1056/NEJMp1707170

200. Remick, D. G., Bolgos, G. E., & Siddiqui, J. (2003). Inflammatory status in sepsis alters efficacy of interleukin-18 binding protein therapy. *Crit Care Med, 31*(8), 2096-2101. doi:10.1097/01.ccm.0000080492.81509.29

201. Richardson, K. C., Jung, K., Pardo, J., Turner, C. T., & Granville, D. J. (2022). Noncytotoxic Roles of Granzymes in Health and Disease. *Physiology (Bethesda), 37*(6), 323-348. doi:10.1152/physiol.00011.2022

202. Rinaldi, I., Sudaryo, M. K., & Prihartono, N. A. (2022). Disseminated Intravascular Coagulation in Sepsis and Associated Factors. *J Clin Med, 11*(21). doi:10.3390/jcm11216480

203. Ripoll, V. M., Meadows, N. A., Bangert, M., Lee, A. W., Kadioglu, A., & Cox, R. D. (2012). Nicotinamide nucleotide transhydrogenase (NNT) acts as a novel modulator of macrophage inflammatory responses. *The FASEB Journal, 26*(8), 3550-3562. doi:https://doi.org/10.1096/fj.11-199935

204. Ripoll, V. M., Meadows, N. A., Bangert, M., Lee, A. W., Kadioglu, A., & Cox, R. D. (2012). Nicotinamide nucleotide transhydrogenase (NNT) acts as a novel modulator of macrophage inflammatory responses. *Faseb j, 26*(8), 3550-3562. doi:10.1096/fj.11-199935

205. Rucevic, M., Fast, L. D., Jay, G. D., Trespalcios, F. M., Sucov, A., Siryaporn, E., & Lim, Y. P. (2007). Altered levels and molecular forms of granzyme k in plasma from septic patients. *Shock, 27*(5), 488-493. doi:10.1097/01.shk.0000246905.24895.e5

206. Rudd, K. E., Johnson, S. C., Agesa, K. M., Shackelford, K. A., Tsoi, D., Kievlan, D. R., . . . Naghavi, M. (2020). Global, regional, and national sepsis incidence and mortality, 1990–2017: analysis for the Global Burden of Disease Study. *The Lancet, 395*(10219), 200-211. doi:10.1016/S0140-6736(19)32989-7

207. Sabir, L., Wharton, L., & Goodacre, S. (2022). Retrospective single-centre descriptive study of the characteristics, management and outcomes of adult patients with suspected sepsis in the emergency department. *Emerg Med J, 39*(4), 272-278. doi:10.1136/emermed-2020-211111

208. Sakabe, S., Iwatsuki-Horimoto, K., Takano, R., Nidom, C. A., Le, M. T. Q., Nagamura-Inoue, T., . . . Kawaoka, Y. (2011). Cytokine production by primary human macrophages infected with highly pathogenic H5N1 or pandemic H1N1 2009 influenza viruses. *J Gen Virol, 92*(Pt 6), 1428-1434. doi:10.1099/vir.0.030346-0

209. Salti, S. M., Hammelev, E. M., Grewal, J. L., Reddy, S. T., Zemple, S. J., Grossman, W. J., . . . Verbsky, J. W. (2011). Granzyme B Regulates Antiviral CD8+ T Cell Responses. *The Journal of Immunology, 187*(12), 6301-6309. doi:10.4049/jimmunol.1100891

210. Sang, Y., Roest, M., de Laat, B., de Groot, P. G., & Huskens, D. (2021). Interplay between platelets and coagulation. *Blood Rev, 46*, 100733. doi:10.1016/j.blre.2020.100733

211. Sankar, J., Arora, S., Joshi, G., & Kumar, R. (2022). Pore-forming proteins and their role in cancer and inflammation: Mechanistic insights and plausible druggable targets. *Chemico-Biological Interactions, 366*, 110127. doi:https://doi.org/10.1016/j.cbi.2022.110127

212. Santiago, L., Castro, M., Sanz-Pamplona, R., Garzón, M., Ramirez-Labrada, A., Tapia, E., . . . Pardo, J. (2020). Extracellular Granzyme A Promotes Colorectal Cancer Development by Enhancing Gut Inflammation. *Cell Rep, 32*(1), 107847. doi:10.1016/j.celrep.2020.107847

213. Santiago, L., Menaa, C., Arias, M., Martin, P., Jaime-Sánchez, P., Metkar, S., . . . Froelich, C. J. (2017). Granzyme A Contributes to Inflammatory Arthritis in Mice Through Stimulation of Osteoclastogenesis. *Arthritis Rheumatol, 69*(2), 320-334. doi:10.1002/art.39857

214. Santos, S. S., Carmo, A. M., Brunialti, M. K., Machado, F. R., Azevedo, L. C., Assunção, M., . . . Salomao, R. (2016). Modulation of monocytes in septic patients: preserved phagocytic activity, increased ROS and NO generation, and decreased production of inflammatory cytokines. *Intensive Care Med Exp, 4*(1), 5. doi:10.1186/s40635-016-0078-1

215. Schaer, D. J., Schaer, C. A., Schoedon, G., Imhof, A., & Kurrer, M. O. (2006). Hemophagocytic macrophages constitute a major compartment of heme oxygenase expression in sepsis. *Eur J Haematol, 77*(5), 432-436. doi:10.1111/j.1600-0609.2006.00730.x

216. Schanoski, A. S., Le, T. T., Kaiserman, D., Rowe, C., Prow, N. A., Barboza, D. D., . . . Suhrbier, A. (2020). Granzyme A in Chikungunya and Other Arboviral Infections. *Frontiers in Immunology, 10*. doi:10.3389/fimmu.2019.03083

217. Schreiber, G. (2020). The Role of Type I Interferons in the Pathogenesis and Treatment of COVID-19. *Front Immunol, 11*, 595739. doi:10.3389/fimmu.2020.595739

218. Schroder, K., Hertzog, P. J., Ravasi, T., & Hume, D. A. (2004). Interferon-γ: an overview of signals, mechanisms and functions. *Journal of leukocyte biology, 75*(2), 163-189. doi:https://doi.org/10.1189/jlb.0603252

219. Schulte, W., Bernhagen, J., & Bucala, R. (2013). Cytokines in sepsis: potent immunoregulators and potential therapeutic targets--an updated view. *Mediators Inflamm, 2013*, 165974. doi:10.1155/2013/165974

220. Semeraro, N., Ammollo, C. T., Semeraro, F., & Colucci, M. (2012). Sepsis, thrombosis and organ dysfunction. *Thromb Res, 129*(3), 290-295. doi:10.1016/j.thromres.2011.10.013

221. Sharma, M., Merkulova, Y., Raithatha, S., Parkinson, L. G., Shen, Y., Cooper, D., & Granville, D. J. (2016). Extracellular granzyme K mediates endothelial activation through the cleavage of protease-activated receptor-1. *Febs j, 283*(9), 1734-1747. doi:10.1111/febs.13699

222. Sharma, M., Merkulova, Y., Raithatha, S., Parkinson, L. G., Shen, Y., Cooper, D., & Granville, D. J. (2016). Extracellular granzyme K mediates endothelial activation through the cleavage of protease-activated receptor-1. *The FEBS Journal, 283*(9), 1734-1747. doi:https://doi.org/10.1111/febs.13699

223. Sherwood, E. R., Lin, C. Y., Tao, W., Hartmann, C. A., Dujon, J. E., French, A. J., & Varma, T. K. (2003). Beta 2 microglobulin knockout mice are resistant to lethal intraabdominal sepsis. *Am J Respir Crit Care Med, 167*(12), 1641-1649. doi:10.1164/rccm.200208-950OC

224. Shrestha, A., Duwadi, D., Jukosky, J., & Fiering, S. N. (2019). Cecropin-like antimicrobial peptide protects mice from lethal E.coli infection. *PLoS One, 14*(7), e0220344. doi:10.1371/journal.pone.0220344

225. Shrum, B., Anantha, R. V., Xu, S. X., Donnelly, M., Haeryfar, S. M., McCormick, J. K., & Mele, T. (2014). A robust scoring system to evaluate sepsis severity in an animal model. *BMC Res Notes, 7*, 233. doi:10.1186/1756-0500-7-233

226. Shrum, B., Anantha, R. V., Xu, S. X., Donnelly, M., Haeryfar, S. M. M., McCormick, J. K., & Mele, T. (2014). A robust scoring system to evaluate sepsis severity in an animal model. *BMC Research Notes, 7*(1), 233. doi:10.1186/1756-0500-7-233

227. Simon, M. M., Prester, M., Nerz, G., Kramer, M. D., & Fruth, U. (1988). Release of biologically active fragments from human plasma-fibronectin by murine T cell-specific proteinase 1 (TSP-1). *Biol Chem Hoppe Seyler, 369 Suppl*, 107-112.

228. Singer, M., Deutschman, C. S., Seymour, C. W., Shankar-Hari, M., Annane, D., Bauer, M., . . . Angus, D. C. (2016). The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA, 315*(8), 801-810. doi:10.1001/jama.2016.0287

229. Skirecki, T., Mikaszewska-Sokolewicz, M., Hoser, G., & Zielińska-Borkowska, U. (2016). The Early Expression of HLA-DR and CD64 Myeloid Markers Is Specifically Compartmentalized in the Blood and Lungs of Patients with Septic Shock. *Mediators Inflamm, 2016*, 3074902. doi:10.1155/2016/3074902

230. Smigiel, K. S., Srivastava, S., Stolley, J. M., & Campbell, D. J. (2014). Regulatory T-cell homeostasis: steady-state maintenance and modulation during inflammation. *Immunol Rev, 259*(1), 40-59. doi:10.1111/imr.12170

231. Smith, A. M., & McCullers, J. A. (2014). Secondary bacterial infections in influenza virus infection pathogenesis. *Curr Top Microbiol Immunol, 385*, 327-356. doi:10.1007/82_2014_394

232. Smyth, M., Kelly, J., Sutton, V., Davis, J., Browne, K., Sayers, T., & Trapani, J. (2001). Unlocking the secrets of cytotoxic granule proteins. *Journal of leukocyte biology, 70*, 18-29. doi:10.1189/jlb.70.1.18

233. Song, J., Park, D. W., Moon, S., Cho, H.-J., Park, J. H., Seok, H., & Choi, W. S. (2019). Diagnostic and prognostic value of interleukin-6, pentraxin 3, and procalcitonin levels among sepsis and septic shock patients: a prospective controlled study according to the Sepsis-3 definitions. *BMC Infectious Diseases, 19*(1), 968. doi:10.1186/s12879-019-4618-7

234. Spaeny-Dekking, E. H., Hanna, W. L., Wolbink, A. M., Wever, P. C., Kummer, J. A., Swaak, A. J., . . . Hack, C. E. (1998). Extracellular granzymes A and B in humans: detection of native species during CTL responses in vitro and in vivo. *J Immunol, 160*(7), 3610-3616.

235. Spaeny-Dekking, E. H., Kamp, A. M., Froelich, C. J., & Hack, C. E. (2000). Extracellular granzyme A, complexed to proteoglycans, is protected against inactivation by protease inhibitors. *Blood, 95*(4), 1465-1472.

236. Spaeny-Dekking, E. H. A., Hanna, W. L., Wolbink, A. M., Wever, P. C., Kummer, A. J., Swaak, A. J. G., . . . Hack, C. E. (1998). Extracellular Granzymes A and B in Humans: Detection of Native Species During CTL Responses In Vitro and In Vivo1. *The Journal of Immunology, 160*(7), 3610-3616. doi:10.4049/jimmunol.160.7.3610

237. Stinchcombe, J. C., Barral, D. C., Mules, E. H., Booth, S., Hume, A. N., Machesky, L. M., . . . Griffiths, G. M. (2001). Rab27a is required for regulated secretion in cytotoxic T lymphocytes. *J Cell Biol, 152*(4), 825-834. doi:10.1083/jcb.152.4.825

238. Stinchcombe, J. C., Bossi, G., Booth, S., & Griffiths, G. M. (2001). The immunological synapse of CTL contains a secretory domain and membrane bridges. *Immunity, 15*(5), 751-761. doi:10.1016/s1074-7613(01)00234-5

239. Stinchcombe, J. C., Majorovits, E., Bossi, G., Fuller, S., & Griffiths, G. M. (2006). Centrosome polarization delivers secretory granules to the immunological synapse. *Nature, 443*(7110), 462-465. doi:10.1038/nature05071

240. Stortz, J. A., Raymond, S. L., Mira, J. C., Moldawer, L. L., Mohr, A. M., & Efron, P. A. (2017). Murine Models of Sepsis and Trauma: Can We Bridge the Gap? *ILAR Journal, 58*(1), 90-105. doi:10.1093/ilar/ilx007

241. Sula Karreci, E., Eskandari, S. K., Dotiwala, F., Routray, S. K., Kurdi, A. T., Assaker, J. P., . . . Azzi, J. (2017). Human regulatory T cells undergo self-inflicted damage via granzyme pathways upon activation. *JCI Insight, 2*(21). doi:10.1172/jci.insight.91599

242. Swieringa, F., Spronk, H. M. H., Heemskerk, J. W. M., & van der Meijden, P. E. J. (2018). Integrating platelet and coagulation activation in fibrin clot formation. *Res Pract Thromb Haemost, 2*(3), 450-460. doi:10.1002/rth2.12107

243. Szabo, P. A., Anantha, R. V., Shaler, C. R., McCormick, J. K., & Haeryfar, S. M. (2015). CD1d- and MR1-Restricted T Cells in Sepsis. *Front Immunol, 6*, 401. doi:10.3389/fimmu.2015.00401

244. Tak, P. P., Kummer, J. A., Hack, C. E., Daha, M. R., Smeets, T. J., Erkelens, G. W., . . . Breedveld, F. C. (1994). Granzyme-positive cytotoxic cells are specifically increased in early rheumatoid synovial tissue. *Arthritis Rheum, 37*(12), 1735-1743. doi:10.1002/art.1780371205

245. Taylor, A. L., & Llewelyn, M. J. (2010). Superantigen-Induced Proliferation of Human CD4+CD25− T Cells Is Followed by a Switch to a Functional Regulatory Phenotype. *The Journal of Immunology, 185*(11), 6591-6598. doi:10.4049/jimmunol.1002416

246. Teo, A. K. J., Singh, S. R., Prem, K., Hsu, L. Y., & Yi, S. (2019). Delayed diagnosis and treatment of pulmonary tuberculosis in high-burden countries: a systematic review protocol. *BMJ Open, 9*(7), e029807. doi:10.1136/bmjopen-2019-029807

247. Tesh, V. L., Ramegowda, B., & Samuel, J. E. (1994). Purified Shiga-like toxins induce expression of proinflammatory cytokines from murine peritoneal macrophages. *Infect Immun, 62*(11), 5085-5094. doi:doi:10.1128/iai.62.11.5085-5094.1994

248. Thangavel, R. R., & Bouvier, N. M. (2014). Animal models for influenza virus pathogenesis, transmission, and immunology. *Journal of Immunological Methods, 410*, 60-79. doi:https://doi.org/10.1016/j.jim.2014.03.023

249. Tibbs, E., & Cao, X. (2022). Emerging Canonical and Non-Canonical Roles of Granzyme B in Health and Disease. *Cancers (Basel), 14*(6). doi:10.3390/cancers14061436

250. Torres, L. K., Pickkers, P., & Poll, T. v. d. (2022). Sepsis-Induced Immunosuppression. *Annual Review of Physiology, 84*(1), 157-181. doi:10.1146/annurev-physiol-061121-040214

251. Tremblay, G. M., Wolbink, A. M., Cormier, Y., & Hack, C. E. (2000). Granzyme Activity in the Inflamed Lung Is Not Controlled by Endogenous Serine Proteinase Inhibitors1. *The Journal of Immunology, 165*(7), 3966-3969. doi:10.4049/jimmunol.165.7.3966

252. Tri Yahya, B., Charis, A., Guruh, P., Ratih, R., Yesika, P., Virgin, K., & Sharoneva, L. (2021, 2021/03/08). *Detection of Coliforms and Enteric Pathogens in Favorite Snack Food Sold in Yogyakarta City.* Paper presented at the Proceedings of the 7th International Conference on Research, Implementation, and Education of Mathematics and Sciences (ICRIEMS 2020).

253. Tubau, C., & Puig, L. (2021). Therapeutic targeting of the IL-13 pathway in skin inflammation. *Expert Review of Clinical Immunology, 17*(1), 15-25. doi:10.1080/1744666X.2020.1858802

254. Tugues, S., Burkhard, S. H., Ohs, I., Vrohlings, M., Nussbaum, K., vom Berg, J., . . . Becher, B. (2015). New insights into IL-12-mediated tumor suppression. *Cell Death & Differentiation, 22*(2), 237-246. doi:10.1038/cdd.2014.134

255. Turner, C. T., Hiroyasu, S., & Granville, D. J. (2019). Granzyme B as a therapeutic target for wound healing. *Expert Opin Ther Targets, 23*(9), 745-754. doi:10.1080/14728222.2019.1661380
256. Turner, C. T., Lim, D., & Granville, D. J. (2019). Granzyme B in skin inflammation and disease. *Matrix Biol, 75-76*, 126-140. doi:10.1016/j.matbio.2017.12.005
257. Turner, C. T., Zeglinski, M. R., Boivin, W., Zhao, H., Pawluk, M. A., Richardson, K. C., . . . Granville, D. J. (2022). Granzyme K contributes to endothelial microvascular damage and leakage during skin inflammation. *British Journal of Dermatology*. doi:10.1093/bjd/ljac017
258. Turner, C. T., Zeglinski, M. R., Richardson, K. C., Zhao, H., Shen, Y., Papp, A., . . . Granville, D. J. (2019). Granzyme K Expressed by Classically Activated Macrophages Contributes to Inflammation and Impaired Remodeling. *J Invest Dermatol, 139*(4), 930-939. doi:10.1016/j.jid.2018.09.031
259. Van Amersfoort, E. S., Van Berkel, T. J., & Kuiper, J. (2003). Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock. *Clin Microbiol Rev, 16*(3), 379-414. doi:10.1128/CMR.16.3.379-414.2003
260. van Daalen, K. R., Reijneveld, J. F., & Bovenschen, N. (2020). Modulation of Inflammation by Extracellular Granzyme A. *Frontiers in Immunology, 11*. doi:10.3389/fimmu.2020.00931
261. van den Boogaard, F. E., van Gisbergen, K. P., Vernooy, J. H., Medema, J. P., Roelofs, J. J., van Zoelen, M. A., . . . van der Poll, T. (2016). Granzyme A impairs host defense during Streptococcus pneumoniae pneumonia. *Am J Physiol Lung Cell Mol Physiol, 311*(2), L507-516. doi:10.1152/ajplung.00116.2016
262. van der Poll, T., & Opal, S. M. (2009). Pathogenesis, treatment, and prevention of pneumococcal pneumonia. *Lancet, 374*(9700), 1543-1556. doi:10.1016/S0140-6736(09)61114-4
263. van Woensel, J. B. M., Biezeveld, M. H., Hack, C. E., Bos, A. P., & Kuijpers, T. W. (2005). Elastase and granzymes during meningococcal disease in children: correlation to disease severity. *Intensive Care Medicine, 31*(9), 1239-1247. doi:10.1007/s00134-005-2720-3
264. Vanden Berghe, T., Demon, D., Bogaert, P., Vandendriessche, B., Goethals, A., Depuydt, B., . . . Vandenabeele, P. (2014). Simultaneous targeting of IL-1 and IL-18 is required for protection against inflammatory and septic shock. *Am J Respir Crit Care Med, 189*(3), 282-291. doi:10.1164/rccm.201308-1535OC
265. Velotti, F., Barchetta, I., Cimini, F. A., & Cavallo, M. G. (2020). Granzyme B in Inflammatory Diseases: Apoptosis, Inflammation, Extracellular Matrix Remodeling, Epithelial-to-Mesenchymal Transition and Fibrosis. *Frontiers in Immunology, 11*. doi:10.3389/fimmu.2020.587581
266. Venet, F., Chung, C.-S., Kherouf, H., Geeraert, A., Malcus, C., Poitevin, F., . . . Monneret, G. (2009). Increased circulating regulatory T cells (CD4+CD25+CD127−) contribute to lymphocyte anergy in septic shock patients. *Intensive Care Medicine, 35*(4), 678-686. doi:10.1007/s00134-008-1337-8
267. Venet, F., & Monneret, G. (2018). Advances in the understanding and treatment of sepsis-induced immunosuppression. *Nature Reviews Nephrology, 14*(2), 121-137. doi:10.1038/nrneph.2017.165
268. Vincent, J. L., Lefrant, J. Y., Kotfis, K., Nanchal, R., Martin-Loeches, I., Wittebole, X., . . . investigators, S. (2018). Comparison of European ICU patients in 2012 (ICON) versus 2002 (SOAP). *Intensive Care Med, 44*(3), 337-344. doi:10.1007/s00134-017-5043-2
269. Vincent, J. L., Sakr, Y., Sprung, C. L., Ranieri, V. M., Reinhart, K., Gerlach, H., . . . Sepsis Occurrence in Acutely Ill Patients, I. (2006). Sepsis in European intensive care

units: results of the SOAP study. *Crit Care Med, 34*(2), 344-353. doi:10.1097/01.ccm.0000194725.48928.3a

270. Voskoboinik, I., Whisstock, J. C., & Trapani, J. A. (2015). Perforin and granzymes: function, dysfunction and human pathology. *Nat Rev Immunol, 15*(6), 388-400. doi:10.1038/nri3839
271. Wang, L., Bastarache, J. A., & Ware, L. B. (2008). The coagulation cascade in sepsis. *Curr Pharm Des, 14*(19), 1860-1869. doi:10.2174/138161208784980581
272. Ward, N. S., Casserly, B., & Ayala, A. (2008). The compensatory anti-inflammatory response syndrome (CARS) in critically ill patients. *Clin Chest Med, 29*(4), 617-625, viii. doi:10.1016/j.ccm.2008.06.010
273. Ward, P. A., & Gao, H. (2009). Sepsis, complement and the dysregulated inflammatory response. *Journal of Cellular and Molecular Medicine, 13*(10), 4154-4160. doi:https://doi.org/10.1111/j.1582-4934.2009.00893.x
274. Weiss, S. L., Fitzgerald, J. C., Pappachan, J., Wheeler, D., Jaramillo-Bustamante, J. C., Salloo, A., . . . Thomas, N. J. (2015). Global epidemiology of pediatric severe sepsis: the sepsis prevalence, outcomes, and therapies study. *Am J Respir Crit Care Med, 191*(10), 1147-1157. doi:10.1164/rccm.201412-2323OC
275. Wensink, A. C., Hack, C. E., & Bovenschen, N. (2015). Granzymes Regulate Proinflammatory Cytokine Responses. *The Journal of Immunology, 194*(2), 491-497. doi:10.4049/jimmunol.1401214
276. Wensink, A. C., Kemp, V., Fermie, J., Garcia Laorden, M. I., van der Poll, T., Hack, C. E., & Bovenschen, N. (2014). Granzyme K synergistically potentiates LPS-induced cytokine responses in human monocytes. *Proc Natl Acad Sci U S A, 111*(16), 5974-5979. doi:10.1073/pnas.1317347111
277. Wensink, A. C., Kemp, V., Fermie, J., García Laorden, M. I., van der Poll, T., Hack, C. E., & Bovenschen, N. (2014). Granzyme K synergistically potentiates LPS-induced cytokine responses in human monocytes. *Proceedings of the National Academy of Sciences, 111*(16), 5974-5979. doi:doi:10.1073/pnas.1317347111
278. Wensink, A. C., Kok, H. M., Meeldijk, J., Fermie, J., Froelich, C. J., Hack, C. E., & Bovenschen, N. (2016). Granzymes A and K differentially potentiate LPS-induced cytokine response. *Cell Death Discovery, 2*(1), 16084. doi:10.1038/cddiscovery.2016.84
279. Wensink, A. C., Kok, H. M., Meeldijk, J., Fermie, J., Froelich, C. J., Hack, C. E., & Bovenschen, N. (2016). Granzymes A and K differentially potentiate LPS-induced cytokine response. *Cell Death Discov, 2*, 16084. doi:10.1038/cddiscovery.2016.84
280. Wieckowski, E., Wang, G. Q., Gastman, B. R., Goldstein, L. A., & Rabinowich, H. (2002). Granzyme B-mediated degradation of T-cell receptor zeta chain. *Cancer Res, 62*(17), 4884-4889.
281. Willers, A., Arens, J., Mariani, S., Pels, H., Maessen, J. G., Hackeng, T. M., . . . Swol, J. (2021). New Trends, Advantages and Disadvantages in Anticoagulation and Coating Methods Used in Extracorporeal Life Support Devices. *Membranes (Basel), 11*(8). doi:10.3390/membranes11080617
282. Wilson, J. A. C., Prow, N. A., Schroder, W. A., Ellis, J. J., Cumming, H. E., Gearing, L. J., . . . Suhrbier, A. (2017). RNA-Seq analysis of chikungunya virus infection and identification of granzyme A as a major promoter of arthritic inflammation. *PLOS Pathogens, 13*(2), e1006155. doi:10.1371/journal.ppat.1006155
283. World Health, O. (2020). *Global report on the epidemiology and burden of sepsis: current evidence, identifying gaps and future directions*. Geneva: World Health Organization.
284. Wu, Z., Deng, J., Zhou, H., Tan, W., Lin, L., & Yang, J. (2022). Programmed Cell Death in Sepsis Associated Acute Kidney Injury. *Frontiers in Medicine, 9*. doi:10.3389/fmed.2022.883028

285. Yang, H., Antoine, D. J., Andersson, U., & Tracey, K. J. (2013). The many faces of HMGB1: molecular structure-functional activity in inflammation, apoptosis, and chemotaxis. *J Leukoc Biol, 93*(6), 865-873. doi:10.1189/jlb.1212662

286. Zeerleder, S., Hack, C. E., Caliezi, C., van Mierlo, G., Eerenberg-Belmer, A., Wolbink, A., & Wuillenmin, W. A. (2005). Activated cytotoxic T cells and NK cells in severe sepsis and septic shock and their role in multiple organ dysfunction. *Clin Immunol, 116*(2), 158-165. doi:10.1016/j.clim.2005.03.006

287. Zeerleder, S., Voves, C., & Wuillemin, W. A. (2005). Comment on hemostatic markers and the sepsis-related organ failure assessment score in patients with disseminated intravascular coagulation in an intensive care unit by Okabayashi et al. *Am J Hematol, 79*(4), 344. doi:10.1002/ajh.20343

288. Zenobia, C., & Hajishengallis, G. (2015). Basic biology and role of interleukin-17 in immunity and inflammation. *Periodontol 2000, 69*(1), 142-159. doi:10.1111/prd.12083

289. Zhang, Y., Yao, Y. M., Huang, L. F., Dong, N., Yu, Y., & Sheng, Z. Y. (2011). The potential effect and mechanism of high-mobility group box 1 protein on regulatory T cell-mediated immunosuppression. *J Interferon Cytokine Res, 31*(2), 249-257. doi:10.1089/jir.2010.0019

290. Zhang, Y. Y., & Ning, B. T. (2021). Signaling pathways and intervention therapies in sepsis. *Signal Transduct Target Ther, 6*(1), 407. doi:10.1038/s41392-021-00816-9

291. Zhao, C.-z., Fang, X.-c., Wang, D., Tang, F.-d., & Wang, X.-d. (2010). Involvement of type II pneumocytes in the pathogenesis of chronic obstructive pulmonary disease. *Respiratory Medicine, 104*(10), 1391-1395. doi:https://doi.org/10.1016/j.rmed.2010.06.018

292. Zhao, T., Zhang, H., Guo, Y., Zhang, Q., Hua, G., Lu, H., . . . Fan, Z. (2007). Granzyme K cleaves the nucleosome assembly protein SET to induce single-stranded DNA nicks of target cells. *Cell Death & Differentiation, 14*(3), 489-499. doi:10.1038/sj.cdd.4402040

293. Zhong, C., Li, C., Wang, X., Toyoda, T., Gao, G., & Fan, Z. (2012). Granzyme K inhibits replication of influenza virus through cleaving the nuclear transport complex importin α1/β dimer of infected host cells. *Cell Death & Differentiation, 19*(5), 882-890. doi:10.1038/cdd.2011.178

294. Zhou, Z., He, H., Wang, K., Shi, X., Wang, Y., Su, Y., . . . Shao, F. (2020). Granzyme A from cytotoxic lymphocytes cleaves GSDMB to trigger pyroptosis in target cells. *Science, 368*(6494), eaaz7548. doi:doi:10.1126/science.aaz7548

295. Zhu, Z., Lian, X., Su, X., Wu, W., Marraro, G. A., & Zeng, Y. (2020). From SARS and MERS to COVID-19: a brief summary and comparison of severe acute respiratory infections caused by three highly pathogenic human coronaviruses. *Respiratory Research, 21*(1), 224. doi:10.1186/s12931-020-01479-w

296. Zorov, D. B., Juhaszova, M., & Sollott, S. J. (2006). Mitochondrial ROS-induced ROS release: An update and review. *Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1757*(5), 509-517. doi:https://doi.org/10.1016/j.bbabio.2006.04.029