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The impact of penetrating abdominal trauma on gut-associated lymphoid tissue and the influence of HIV-infection

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WITWATERSRAND,
JOHANNESBURG

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfillment of the requirements for the degree

of

Doctor of Philosophy

Johannesburg, 2021

1 Declaration

I, Martin Ernst Mauser declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



7th of April 2021

2 Publications of scientific papers

I. Mauser M, Kruger D, Pather S, Plani F

Compromised gut associated lymphoid tissue is a risk factor for postoperative septic complications after trauma surgery

World Journal of Surgery (2021) 45:1006-1013, published.

II. Mauser M, Kruger D, Pather S, Plani F

Trauma results in immune cell-induced intestinal epithelial damage with subsequently increased sepsis rate

Journal of Trauma and Acute Care Surgery (2021) 90(3):565-573, published.

III. Mauser M, Kruger D, Pather S, Plani F

Posttraumatic natural killer cell decrease is associated with septic complications

Journal of Surgical Research, submitted and awaiting response from journal

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4 Abstract

Background:

The gut associated lymphoid tissue (GALT) of the bowel with different lymphocyte subpopulations as an important pillar of the human immune system has never been investigated in the context of trauma; neither the short-term histological trauma-induced changes in the GALT, nor its impact on the outcome after trauma surgery. Furthermore, it has never been investigated whether the preexisting altered GALT of HIV-seropositive trauma patients compromises their postoperative course.

Methods:

This prospective, longitudinal proof-of-concept study included patients who required damage control surgery after abdominal gunshot wounds with small bowel involvement. Bowel specimens were obtained during the index and relook operations, and the T-lymphocytic quantity therein analysed via immunohistochemistry. We investigated how the lymphocyte structure and numbers of the GALT altered, and whether the extent and nature of these changes had an impact on the postoperative outcome with regard to septic and surgical complications.

Results:

In total, 62 patients were included in the study of which 38 patients were HIV-seronegative and 24 were HIV-seropositive. HIV-seropositive patients had a significantly lower quantity of CD4+ T cells in the GALT compared to the HIV-seronegative patients (p = 0,0001), which was also associated with a significantly higher rate of septic complications in the postoperative course. The main histological changes between the index and relook specimen was a shift of CD8+ T cells from the lamina propria (LP) into the epithelium and a decrease of T-

lymphocytes in the LP. The significant increase of the intraepithelial CD8+ T cells was associated with a more extensive enterocyte apoptosis, and correlated significantly, positively with the number of postoperative septic complications.

Conclusions:

The compromised GALT in HIV-seropositive patients may predispose these patients to postoperative septic complications. Antiretroviral therapy does not result in an adequate immune reconstitution in this tissue. Hence, safe surgery and vigilant postoperative care with early, aggressive diagnostic investigations and a low threshold for relook operations if the postoperative course does not progress as expected, is of paramount importance for these patients.

Trauma induces an immune cell-driven impairment of the intestinal epithelium, as well as an increased apoptosis of lymphocytes in the LP which is associated with a worse clinical outcome. The underlying mechanism suggests that a therapeutic approach to minimize apoptosis in the intestine may impact the outcome of severely injured trauma patients.

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5 List of important abbreviations

AAST	American Association for Surgery and Trauma
AD	apoptotic density
AICD	activation induced cell death
AIDS	acquired immunodeficiency syndrome
ART	antiretroviral therapy
ARDS	acute respiratory distress syndrome
BT	bacterial translocation
CARS	compensatory anti-inflammatory response syndrome
CCR	chemokine receptor
CD	cluster of differentiation
CHBAH	Chris Hani Baragwanath Academic Hospital
CRP	c-reactive protein
DCS	damage control surgery
FDC	fixed-dose combination
GALT	gut associated lymphatic tissue
GIT	gastro-intestinal tract
GSW	gunshot wound
GvHD	graft versus host disease
HAART	highly active antiretroviral therapy
HIF	hypoxia-inducible factor
H&E	haematoxylin and eosin
ICU	intensive care unit
IEL	intraepithelial lymphocytes
Ig	immunoglobulin

IHC	immunohistochemistry
IL	interleukin
IED	immune enhancing diet
ISS	injury severity score
LBP	Lipopolysaccharide-binding protein
LOS	length of (hospital) stay
LP	lamina propria
LPL	lamina propria lymphocytes
M-cells	microfold cells
MLN	mesenteric lymph node
MOF	multi organ failure
NK cells	natural killer cells
NO	nitric oxide
PATI	penetrating abdominal trauma index
PP	Peyer’s patches
SIRS	systemic inflammatory response syndrome
SA	South Africa
SSI	surgical site infection
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TNF	tumour necrosis factor
TPN	total parenteral nutrition
VAP	ventilator associated-pneumonia

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8 Literature review and Background

8.1 The human gut immune system and the gut mucosal barrier

The gastrointestinal tract is constantly in contact with food- and bacterial antigens of the commensal bacterial flora and other pathogens. Confronted with this constant antigen exposure, the intestine including the gut associated lymphoid tissue (GALT) plays a crucial role in the immune system containing the majority of the body's T cell population (1).

The key structures of the intestinal immune system are the intestinal epithelium, the lamina propria (LP), organized lymphoid aggregates also known as the Peyer's patches (PP) in the small bowel and the colonic patches in the large bowel, and the mesenteric lymph nodes (MLNs). These main structures build up a barrier that prevents the invasion of luminal microorganism into the systemic circulation. In the following paragraphs these main structures and their interactions will be further elucidated.

8.1.1 Intestinal epithelium

The intestinal epithelium, consisting of a single-cell layer, forms a large surface that is one and a half times the size of a tennis court comprising almost 400 m^2; the epithelium covers the crypts and villi, facing towards the lumen of the gut. The villus structure is more pronounced in the proximal small bowel than the distal small bowel and is not present in the colon, thus increasing the surface area in the proximal part of the lower gastrointestinal tract more markedly than in the distal part (2).

The majority of the intestinal epithelial cells have absorptive function and are known as enterocytes. The epithelial layer is persistently in contact with the external

environment, fulfilling two main functions: first to absorb nutrients, second to maintain a barrier function. The intestinal epithelial cells interact and adapt to changes of the gut microbiota. Furthermore, they are in a constant interplay with immune cells and modulate the immune response of the bowel by producing cytokines and chemokines (3).

8.1.2 The intraepithelial lymphocytes (IEL)

The most frequently encountered immune cells in the epithelium are T-lymphocytes. Located in the front line of the epithelium, they play an important role in the initial response to antigen exposure (4).

Around 80% of the intraepithelial T-lymphocytes are CD8+, long-lived and reside permanently in the gut. Two major T cell lineages can be differentiated: the thymic or natural lineage, and the peripheral or induced lineage. The programming of the natural intraepithelial T cells occurs during the development in the thymus; they express the T cell receptor (TCR) $\gamma\delta$ or TCR $\alpha\beta$. The majority of human intestinal IEL are CD8 $\alpha\beta^+$ TCR$\alpha\beta^+$ cells, which move after thymic maturation directly from the thymus to the gut epithelium (5). Induced IEL are activated in response to peripheral antigens. After up-regulation of homing receptors triggered by transforming growth factor (TGF)-β, the T cells move to and reside in the epithelium. The lymphocyte receptor responsible for homing of the IEL is the integrin $\alpha_E\beta_7$ that binds to the E-cadherin ligand, which is located on the basolateral surface of the enterocytes (6).

The numbers of IELs in relation to the enterocytes lessen from the proximal to the distal lower gastrointestinal tract. Three times as many IELs can be found in the small bowel as compared to the colon. An average of 13 to 22 per 100 enterocytes

are encountered in the healthy duodenum, and around 5 per 100 enterocytes in the colon. In the rectum, in turn, they increase to 20 per 100 enterocytes (7).

The differences in the distribution are not yet fully understood but the diversity in antigen exposure might be an important determinant, being most intense in the small bowel.

Natural as well as induced IELs secure the stability of the epithelial barrier and prevent the invasion of pathogens by constantly interacting with surrounding cells (8). The $\gamma\delta$-T cell subtype of the IEL is described as a highly mobile cell, travelling through the intestinal epithelium and able to cross the basement membrane, thereby surveying the intraepithelial compartment and the superficial LP (9,10). With their cytolytic capacity they remove damaged cells and contribute to the epithelial homeostasis. On activation these cells are able to produce keratinocyte growth factor (KGF) and help to repair the damaged mucosa of the bowel (11). Furthermore, the IEL seem to play an important role in maintaining the robustness of the intercellular tight junctions. In IEL-depleted mice an incomplete synthesis of the tight junction proteins occludin and claudin was observed leading to a higher penetration rate of pathogens (12).

By producing inflammatory transmitters, the IELs may induce inflammation and destruction of the bowel epithelium, hence they are thought to play a role in auto-immune bowel disorders such as celiac disease (13).

In reviewing the latest literature about IEL and their function, it becomes apparent that the majority of new findings derive from animal research, mostly murine models (14,15). This novel human research project offered the opportunity to analyse the quantity of human IEL and changes thereof in different parts of the small bowel which complemented the knowledge gained in animal experiments.

8.1.3 Other epithelial barrier supporting structures

The tight junctions are structures in between epithelial cells, supporting the integrity of the gut mucosal barrier and regulating the paracellular permeability. These structures are composed of so-called transmembrane proteins which connect the cytoskeleton of neighboring epithelial cells, only allowing the transport of specific substances to cross the membrane. The three most important transmembrane proteins are occludin, claudin, and the junctional adhesion molecules; of these, claudin seems to play the most important role for the maintenance of an intact barrier (16,17). Different enteric bacteria have developed mechanisms to damage the tight junction complexes, for example Clostridium difficile impairs the cytoskeleton which is bound to the transmembrane proteins, Bacteroides fragilis secretes proteases that sever the tight junctions, and the Clostridium perfringens toxin causes damage by binding directly to claudin. Notably, Escherichia coli and Salmonella are able to disrupt the tight junction via all the above described mechanisms (18).

The Paneth cells are secreting cells, situated at the base of the intestinal crypts of the bowel. These cells release lysozymes and defensins from their eosinophilic granules which have an antimicrobial effect, thereby defending the intestinal crypts from bacterial invasion (19).

Another cell type in the epithelium of the gut with an important immunological function is the microfold cell (M cell). These specialized epithelial cells are localized in the area of PP and other lymphoid aggregates and they sample antigens and microorganisms from the gut lumen (20). Incorporated in vesicles, the

microorganisms are then transported from the lumen to the other side of the epithelial lining and delivered to the lymphocytes of the organized lymphoid tissue where an immune response is induced. The disadvantage of this antigen sampling system is that it can be used by pathogens to traverse the epithelial barrier and subsequently get access to the systemic circulation (21).

8.1.4 Lamina propria (LP) of the bowel and Peyer's patches (PP)

The LP, situated between the epithelial layer and the submucosa, is the effector site of the intestinal immune system. Cells of the adaptive and innate immune defense such as macrophages, plasma cells, mast cells, eosinophils, dendritic cells, and lymphocytes are found in this layer of the bowel. In total, 40 to 90% of the LP lymphocytes (LPL) are T lymphocytes, around 65 to 80% of them are CD4+ (22). β_7-containing integrins on these lymphocytes are responsible for the migration to and homing of these lymphocytes in the gut (23). After antigen-priming in the PP, the T-lymphocytes are drained via the MLNs and thoracic duct and gain access to the bloodstream. Recirculation and accumulation back in the LP and mucosa are directed via the interaction of the $\alpha_4\beta_7$-integrin on the lymphocytes and its ligand mucosal addressin cell-adhesion molecule 1 (MAD-CAM1) that is found on the venules of the intestinal mucosa (24).

Up to date no absolute numbers of LPL in different regions of the small bowel are published. One important reason for the lack of data about the small bowel is the difficulty to access this organ. Only the duodenum and terminal ileum are accessible via standard endoscopy. Tauschmann *et al.* obtained specimens from sixteen healthy volunteers and observed a CD4+ T cell percentage of 40% and a CD8+ T

cell percentage of 13% of total T-lymphocytes with a CD4/CD8 ratio of 3.0 in the terminal ileum (25).

In our research project small bowel specimens between the ligament of Treitz and the ileocecal valve were sampled and examined, depending on the level of the injury. This has given insight about distribution and absolute numbers of T-cells in regions that has not been published before.

LPL receive signals from epithelial cells, as well as from stroma cells, and interact with other surrounding immune cells. They play an important role in the maintenance of the protective immunity of the gut via a Th-1 like immune response against intracellular pathogens. Against extracellular pathogens they mount a Th-2 like humoral response by supporting mucosal B cells and plasmablasts (26).

Cytokine production of the LPL, already in response to a low level pathogen stimulus, helps to protect the mucosal barrier effectively against bacterial invasion and translocation (15). Cytokine-induced activation of macrophages by LPL is an important mechanism to clear commensal bacteria that traverse the gut epithelium. In a functioning immune system, macrophages with their bactericidal capacity phagocytose these bacteria before they reach the systemic circulation (27). Furthermore, the interaction between LPL and epithelial cells is important for the homeostasis and differentiation of the epithelium; interruption of this balance for instance in inflammatory bowel disease, results in an acceleration of the differentiation of the epithelial cells (28).

Mainly in the distal small bowel and appendix, the lymphocytes form organized aggregates of lymphoid tissue in the form of Peyer's patches (29); to a lesser extent, they form colonic patches in the colon (30). Almost 50% of the PP which are linked with the MLNs via lymphatic vessels, are localized in the last 25 cm of the ileum

(31). Together with the M-cells, the follicle-associated epithelium of the PP is the preferential place for the uptake of antigens and microorganisms which are subsequently sampled in the PP as an essential step in the mucosal immune response.

8.1.5 Regeneration and apoptosis of the epithelium

Under physiological conditions, the complete small bowel epithelium is renewed within three to five days (32). The high turnover rate of epithelial cells in the gut with the differentiation and maturation on the one hand and the programmed cell death on the other hand is important to maintain a healthy tissue homeostasis and the integrity of the gut mucosal barrier.

The crypt stem cells play the main role in the regeneration of the epithelium. They differentiate either into Paneth cells or enteroendocrine cells, both of which stay at the base of the crypt. Crypt stem cells may also develop into absorptive epithelial cells and goblet cells which migrate upwards towards the tip of the villus where they shed off into the lumen later (33). The maturation process of the epithelial cell from crypt to villus is regulated by growth factors such as TGF-α (34).

Certain triggers like immune-cell derived causes, drugs, and physical stressors lead to an increased apoptosis rate in the gut and thereby impair the integrity of the gut mucosal barrier. Irreversible cell shrinkage, condensed nuclei (pyknosis), nuclear fragmentation (karyorrhexis) and formation of apoptotic bodies are the features of the programmed cell death, called the apoptosis (35). Apoptosis needs to be distinguished from necroptosis and autophagy: apoptosis is mediated by a group of aspartic-acid-specific proteases called caspases, and necrobiosis is based on a caspase-independent process based on proteins of the receptor-interacting protein

kinase family (36). Conversely to apoptosis, necroptosis induces inflammation due to rupture of the cell membrane and release of cytoplasmatic factors; in the process of autophagy specific dysfunctional cellular components are removed.

There are two main pathways that initiate the apoptosis: the first one is the extrinsic pathway, activated via receptors that interact with ligands such as tumor necrosis factor (TNF)-α, Fas, and Dr3, all members of the TNF group. Caspase 8 is the initiating mediator in this cascade. The second pathway is the intrinsic way, induced by mitochondrial damage and transmitted via Caspase 9. Any triggers that have the ability to impair the mitochondrial membrane potential, such as reactive oxygen species, drugs or radiation, can activate this pathway. The common final step of both ways is activation of caspase 3 which results in cytoskeletal damage of the cell (37).

Several reports have shown that caspase inhibition, especially caspase 3 as final common pathway of the cell death program, may lead to an improved survival, reduced organ dysfunction, decreased lymphocyte apoptosis and blood bacterial count in sepsis, burns and ischemia-reperfusion injury. Caspase inhibition may therefore offer a useful therapeutic approach in the therapy of critical care patients (38,39).

In the intrinsic cell death initiation, the protein Bcl-2 plays an important role in the regulation of the mitochondrial involvement in the apoptosis (40) and functions as an antiapoptotic protein. In two research projects with transgenic mice that overexpress this protein better outcomes in sepsis with reduced mortality was documented (41,42). Hence these two mouse models indicate that apoptosis

inhibition or -reduction may have a beneficial prognostic effect on the outcome in critical illness.

This research project investigated if trauma leads to an increased enterocyte- and lymphocyte apoptosis rate in the intestinal epithelium and LP and if this is associated with increased septic complications. To our knowledge the association of LPL- and IEL-apoptosis and septic complications has never been scrutinized in human trauma patients.

These results might influence and support further work involving therapeutic approaches with apoptosis inhibitors.

8.2 Barrier dysfunction and bacterial translocation

More than 1000 species of bacteria are residing in the gut, of which 95% are obligate anaerobes such as Bifidobacterium, Clostridium, Bacteroides, and Fusobacterium. Facultative anaerobes make up 5% and include Lactobacillus, Escherichia coli, Klebsiella and Pseudomonas aeruginosa (43). The gut mucosal barrier separates this abundance of luminal microorganism from the host and the systemic circulation. Berg and Garlington were the first who came up with the theory of bacterial translocation (BT) in 1979, defining it as a "*passage of viable and non-viable microbes and microbial products, such as endotoxin, from the intestinal lumen through the epithelial mucosa into the mesenteric lymph nodes (MLN) and possibly other organs*" (44).

8.2.1 Measurement methods of bacterial translocation

There are different methods to measure and quantify BT. The Gold Standard is the direct identification and quantification of intestinal bacteria in MLNs (45). However,

experiments with radioactive labelled bacteria have shown that this method vastly underestimates the amount of translocated bacteria because they are eliminated by immune cells before reaching the MLNs (46). Hence, increased quantity of bacteria in the MLN is more the consequence of decreased elimination than increased transepithelial penetration. Another disadvantage of this technique is that only a few selective lymph nodes are sampled and therefore can't provide a representative overall assessment of the entire MLNs. Moreover, the intraoperative sampling of MLNs may be time consuming and cumbersome, hence very difficult to justify in unstable trauma patients.

Identification of intestinal bacteria in aerobe and anaerobe blood cultures from peripheral blood serves as an indirect method of measuring BT (47). Disadvantages of this test are the difficult timing, interference with antibiotics, and contamination, often leading to diagnostic confusion (48).

Another indirect method is the identification of endotoxin or lipopolysaccharide (LPS) in blood; endotoxin, or LPS, is an element of the cell wall of all gram-negative bacteria. The most commonly used test therefore is the Limulus amoebocyte lysate assay (49).

The strict scientific proof of BT can only be done in a clean abdomen. Hollow viscus injuries with subsequent contamination of the peritoneal cavity as often encountered in penetrating abdominal trauma, may lead to bias via transperitoneal absorption of bacteria.

The routinely used method to assess *intestinal permeability* of the bowel per se is to measure the urine for orally given non metabolizable sugars, such as mannitol and lactulose. The amount of absorption and excretion gives an idea of the gut

permeability (50). It is important to emphasize that intestinal permeability cannot be equated with BT.

8.2.2 Mechanism of bacterial translocation

BT can take place either through the paracellular or transcellular pathway, or the combination of both. In the more common transcellular pathway, the microbes pass the epithelium through the enterocytes via specific enterocyte channels and membrane pumps. Alexander *et al*. directly observed transcellular migration of ^{14}C glucose-labeled Candida albicans and Escherichia coli through undamaged enterocytes with transmission electron microscopy (46). In the paracellular pathway, the translocation occurs in between the enterocytes due to impairment of the tight junctions (51).

The three main causes inducing BT are: first, changes in the normal microflora of the gastrointestinal tract, leading to bacterial overgrowth; second, damage to the gut mucosa from direct injury to the enterocytes or reduced blood perfusion to the gut and its epithelium; and third, a compromised local and systemic immune system.

8.2.2.1 Pathogenic bacteria and bacterial overgrowth

Of all intestinal bacteria certain species, such as Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa and certain families such as Enterobacteriaceae, Enterococci and Streptococci are more commonly involved in BT than others (52); these bacteria are the main microbial culprits for septic complications in critically ill patients. It seems that these strains have a higher ability to adhere to the epithelium than non-pathogenic strains which is the first important step of getting past the gut mucosal barrier (53).

Throughout the intestinal tract the proportion of anaerobe bacteria is 100- to 1000-fold higher than that of the aerobe bacteria. The anaerobe bacteria cover the mucosa densely, controlling and limiting colonization of facultative invasive strains. Elimination of anaerobes may result in bacterial overgrowth of pathogens and promote BT (54). A disturbance of the intestinal microflora is particularly encountered in mechanical bowel obstruction or paralytic ileus. The stasis of the bowel contents disrupts the composition of the physiologic bacterial flora, hence putting the patient at a higher risk of BT. Mucosal damage due to overdistension of the bowel aggravates this complication resulting in a higher rate of septic complications (55,56).

8.2.2.2 Epithelial barrier dysfunction

Shock, trauma and thermal injury reduce blood perfusion to the gastrointestinal tract at an early stage, while the blood supply to the central organs is still preserved. Salzman *et al.* observed that an increasing mucosal acidosis during ischemia causes an increased intestinal permeability; already elevated arterial carbon dioxide levels induce gut hyperpermeability even without mucosal ischemia (57). Acidosis-induced stress mediated by hydrogen peroxide and superoxide radicals damage the cytoskeleton of the epithelial cells and subsequently increase the gut permeability (58).

The critical intestinal blood supply is enhanced by the unique vascular architecture in the intestinal villi: only a single arteriole perfuses each villus, along the villus the oxygen tension decreases, hence, a relative hypoxia is detected at the villus tip even under normal physiological circumstances (59). Furthermore, this central arteriole takes off at a 90 degree angle from the parent artery which results due to rheological

dynamics in a hemodilution and concomitant reduced oxygen carrier capacity in the blood perfusing the villus (60). Ischemia is considered as the common pathway in shock and trauma leading to epithelial barrier dysfunction. Deitch describes this theory in a “three hit model”: first hit is the splanchnic hypoperfusion, followed by a gut ischemia-reperfusion injury considered as the second hit; this impairs the gut mucosal barrier and induces the third hit, the production of inflammatory mediators in the bowel that reaches the systemic circulation via the intestinal lymphatics and thoracic duct and contribute to systemic inflammatory response syndrome (SIRS), acute respiratory distress syndrome (ARDS) and multi organ failure (MOF) (61).

8.2.2.3 The immune system in BT

The important role of a functioning immune system is shown by the fact that BT is observed in healthy hosts without overt clinical signs of sepsis or infection. A small amount of intestinal bacteria can be identified in MLNs in healthy humans and animals (62) inducing an activation of immune cells and antigen presenting cells. This physiological process has importance for the regulation of the local and systemic immunity and tolerance for antigens that are in touch with the epithelium.

The importance of the T cell mediated immunity for the defense of BT was demonstrated impressively in several animal models. These were milestone experiments with importance for our research project and therefore they are presented in more detail:

In 1980 Owens and Berg noticed spontaneous BT of E. coli to the MLN, spleen and liver in athymic mice which was reduced after nude mice were grafted with thymus (63).

Gautreaux *et al*. (1994) destroyed CD4+- and CD8+ T-lymphocytes by monoclonal antibodies. Subsequently, T-cell destruction in the epithelium, LP and MLN was confirmed by flow cytometry. T-cell depleted mice had a significantly increased translocation of E. coli, S. typhimurium and other indigenous microorganism, such as Staphylococcus species, Proteus mirabilis and Lactobacillus species, from the gut to MLNs. They speculated that the intraepithelial CD8+ T lymphocytes may play a central role in preventing BT by impeding the passage of the bacteria through the epithelium (64).

A year later in 1995, Gautreaux *et al*. transferred CD4+- and CD8+-T lymphocytes from donor mice to mice that were T cell depleted by thymectomy plus injection of anti-mouse T-cell monoclonal antibodies (65). The transferred T cells migrated to MLN and spleen and reduced the quantity of translocated E. coli from the gut hence confirming the central role of the T cells in the prevention of BT.

Xu *et al*. examined mice after laparotomy and hemorrhagic shock and observed an increased apoptosis of lymphocytes in the PP leading to intestinal immune dysfunction (66). More recently in 2018, Choudhry *et al*. used a rodent model and observed an increased BT after antibody-induced T cell depletion in the GALT in animals that were exposed to alcohol and burn injury. They detected a bacterial accumulation in MLNs as well as dissemination of bacteria to the spleen and systemic circulation, thus again manifesting the deleterious consequences of a compromised T cell system (67).

These animal experiments demonstrated that an appropriately functioning T-lymphocytic response is essential to maintain an intact gut mucosal barrier and prevent translocated microorganisms from spreading beyond the MLN. Immunosuppression with an impaired T cell function and cytokine production seem

to lead to an inadequate recruitment and activation of neutrophils and macrophages, and thus to an impairment of the local immune response.

The influence of lymphocytic quantity in LP and the epithelium on the clinical outcome has never been explored in humans. It therefore still needs to be elucidated whether the findings from animal experiments can be simply extrapolated to humans.

In this research project it was investigated whether the quantity of LP- and intraepithelial T-lymphocytes and their quantitative changes, HIV- as well as trauma-induced, have an impact on postoperative complications in trauma patients. One of the primary aims of this research was to determine if patients with fewer T-lymphocytes in the GALT are more prone to septic complications, hence indirectly indicating that they are predisposed to BT which may subsequently cause more septic problems.

8.2.3 Route and site of bacterial translocation

There are multiple ways for intraluminal microorganism to reach extra-intestinal sites; well described is the retrograde migration of bacteria to the lungs which is especially observed during aspiration in ventilated patients. Transmural migration was never proven to have clinical relevance.

To further explore the way of systemic bacterial dissemination, Mainous *et al.* induced systemic inflammation in rats and documented almost 12 000 times greater number of bacteria in the portal vein compared to the mesenteric lymph. Because translocated bacteria were detected in almost 90% of the portal vein blood samples in contrast to only 25% in the efferent lymph (68), they postulated that the main way of translocation is hematogenous. The main difference between the hematogenous

and lymphatic routes of translocation is that in the latter the liver, with its important function of detoxifying and neutralizing toxic metabolites and bacteria, is circumvented. Microorganisms in the lymph are draining directly via the thoracic duct into the blood circulation.

There are only a few studies that have investigated which site of the lower gastrointestinal tract is more prone to BT. While the upper gastrointestinal tract is relatively sterile, the density of bacteria increases towards the large bowel, having almost the same species of microorganisms in the ileocecal region as in the colon. In a burn model with guinea pigs ^{14}C-labelled E.coli had the same translocation rate throughout the lower gastrointestinal tract, the only difference being that more viable bacteria were found in the MLN of the jejunum than the colon (69). Different permeability characteristics of the colon compared to the small bowel, including a higher electrical resistance and a lower permeability to the passive movement of ions seems to make the colon less vulnerable to BT (70).

8.3 Clinical relevance of bacterial translocation

8.3.1 Bacterial translocation in trauma

One of the best proven causes of BT is thermal injury. Desal *et al.* noted in the autopsy of burn patients that 50% had intestinal mucosal damage, 80% were septic at the time of death with endogenous intestinal microorganisms identified as cause of sepsis (71). Within one day after sustaining burn injury, elevated levels of endotoxin can be identified. The level of the measured endotoxin correlates with the percentage of burnt total body surface area (72).

Data is not as clear for patients who sustained severe non-thermal trauma, especially in the first acute phase before septic complications occur. Peitzman *et al.* examined the MLN of 25 blunt trauma patients and they did not find a single positive MLN culture even in patients who were re-operated on three to five days after their injury (73). However, Nieves *et al.* cultured bacteria in MLN as evidence of BT in one third of his trauma patients. These patients had significantly more infectious complications than the patients with the negative MLN bacterial cultures (74). In this series of 36 patients, 15 patients (42%) had bowel injuries with subsequent contamination of the abdominal cavity. As explained earlier, proof of BT is not definite in a contaminated abdomen as, even after a thorough washout, traces of faeces containing enteric bacteria may be present in the abdominal cavity. These bacteria may traverse the peritoneum, and not the mucosa, to reach the MLN and hence give a positive MLN-culture, potentially biasing the result.

Moore *et al.* tested portal vein blood via a portal vein catheter and peripheral blood on day one, two and five of 20 trauma patients, of which 12 patients were in shock at the time of admission. They could neither detect bacteria nor endotoxin in the blood of these severely injured patients of which one third went into MOF (75). This important result from a well-designed study negates the hematogenous way as the main route of BT in human trauma patients, as well as contradicts the abovementioned results from a rat model from Mainous' group supporting the hematogenous way as the dominant one (68).

Roumen *et al.* noted an increased *intestinal permeability* in patients with trauma and hemorrhagic shock, detected by an increased lactulose-mannitol excretion rate, but there was no significant correlation between the increased gut permeability and septic complications or MOF score (76).

Cumulatively, from these aforementioned studies, controversies remain with regard to the causes, extent and clinical importance of BT in non-thermal trauma. The discrepancies indicate how difficult it is to prove a causal relationship between BT and septic complications. This has led to the hypothesis that rather than bacteria or particles of bacteria themselves, inflammatory products, such as proinflammatory cytokines, produced by the gut might be the driving factor for sepsis and MOF (77). Certainly this hypothesis was supported by animal studies which showed that proinflammatory cytokines are transported from the gut to distal organs via mesenteric lymph, causing, amongst other things, shock-induced lung injury (78,79).

All abovementioned trauma studies recruited relatively small patient numbers which might be partly the reason for the contradictory observations. Furthermore, a mismatch in the time-point of sampling and injury severity might also have contributed to the inconsistent results.

Increased intestinal permeability seems to be a crucial factor for BT but it appears that a functional immune system may have the capability to prevent the microorganisms and endotoxins from spreading into the systemic circulation, showing the importance of the GALT and its immune cells.

The assessment of the lymphocytic structure and quantity in the GALT with regard to the clinical outcome might give us a better understanding of whether these host factors play a role in the clinical outcome of trauma patients.

8.3.2 Prevention and therapy for bacterial translocation

In experimental models and in clinical practice multiple therapeutic strategies to reduce BT have been explored. A well-documented finding is the influence of diet

on the gut mucosal barrier. Starvation and total parenteral nutrition (TPN) in animal research showed several negative effects on gut homeostasis such as bacterial overgrowth, reduced mucus and IgA production, mucosal atrophy with intestinal hyperpermeability and impaired function of GALT lymphocytes. Deitch *et al*. compared enterally fed rats with rats receiving TPN and documented a significantly higher rate of translocated E.coli in the latter, hence demonstrating the importance of enteral feeds (80).

A meta-analysis with data from eight prospective randomized trials including more than 200 high-risk, surgical patients showed significantly fewer septic complications in patients receiving enteral feeds compared to those receiving TPN. A further subgroup analysis revealed that trauma patients receiving enteral feeds benefitted especially by having the most significant decrease in postoperative septic complications (81). Blaser *et al*. concluded in their guidelines that early enteral nutrition within 48 hours of ICU admission resulted in a reduction of infectious complications in critically ill patients, in patients with severe acute pancreatitis and in patients who underwent surgery of the gastrointestinal tract. Based on these guidelines, the group suggested that the only contraindications for early enteral feeding are severe shock, uncontrolled ventilatory problems, severe upper gastrointestinal-bleeding, mechanical bowel obstruction, ischemia of the bowel, high gastric aspirates as sign of paralytic ileus and abdominal compartment syndrome (82). So-called enteral immune enhancing diets may possibly be favorable; Kudsk *et al*. noted fewer septic complications in trauma patients receiving an enteral diet enriched with glutamine, arginine and omega 3 fatty acids. Most septic complications were observed in starved patients (83).

Probiotics are thought to decrease BT by suppression of bacterial overgrowth, particularly by Gram-negative microorganisms. Moreover, they protect the gut epithelium against penetration of virulent bacteria by promoting the secretion of IgA (84). Mattar *et al*. demonstrated in an enterocyte cell culture model that probiotic Lactobacillus casei inhibited the translocation of E. coli (85). However, the clinical relevance of probiotics is still controversial. In a recent meta-analysis including 23 randomized controlled trials, probiotics showed no clear effect on infection risk, hospital mortality and hospital length of stay. Clear recommendations therefore cannot be made without further research (86). Additionally, the fact that there are different types of probiotics, such as Lactobacillus, Bifidobacterium and Saccharomyces to only mention the most common ones, contributes to the heterogenous results of their role in hindering BT.

Non-absorbable antimicrobials such as tobramycin, polymyxin E, amphotericin B and cefotaxime are used for the selective gut decontamination mainly targeted at Pseudomonas, Enterobacter, Acetobacter and yeast. The aim of selective gut decontamination is the eradication of these strains and the prevention of colonization by these potentially pathogenic organisms which are responsible for the majority of septic complications in critical care patients. Recent studies have shown that selective gut decontamination is reducing hospital acquired infections, especially ventilator associated pneumonia. In addition, the duration of ventilation and ICU length of stay is reduced (87,88).

8.4 Impact of trauma on the immune system

8.4.1 SIRS response in trauma and damage associated molecular patterns

Improved resuscitation strategies and early hemorrhage control have resulted in an improved survival of trauma patients. However many trauma patients who survive the initial bleeding succumb later due to the consequences of the SIRS, sepsis and MOF, all of which are indicators of a dysregulated immune system (89).

Severe injury may trigger a SIRS response in less than an hour and is caused by tissue and cell damage rather than infection. The cause is endogen mediators, called damage associated molecular patterns (DAMPs), produced by activated immune cells and necrotic cells (90,91). Mitochondrial peptides and DNA from bacteria play an important role in this process (92). DAMPs activate neutrophils, monocytes and the complement cascade (93), which in turn generate inflammatory factors such as interleukins (ILs), that contribute to the systemic reaction.

The impact of trauma also triggers the coagulation system by enhancing the platelet function (94) which leads to the formation of platelet-leucocyte aggregates. These aggregates further activate immune cells and damage endothelial cells (95).

Activated neutrophils traverse the damaged endothelium and infiltrate so-called bystander organs (96). Reactive oxygen species and proteases released by the neutrophils lead to tissue damage of these organs, which is the underlying patho-mechanism for organ dysfunction, such as the ARDS. Notably, the neutrophils which are activated by the SIRS seem to have an impaired antibacterial response (97).

In the theoretical model, the compensatory anti-inflammatory response syndrome (CARS) follows the SIRS and is driven by increased levels of anti-inflammatory cytokines, such as TGF-β and IL-10, as well as cytokine antagonists, e.g. IL-1Ra (98). Interplay between SIRS and CARS will either lead the immune system back to baseline or further impair the immune system and lead to ongoing inflammation and

catabolism which may, in turn, finally end in sepsis and MOF (99). Organ damage and sepsis triggers the liberation of more DAMPs which completes the vicious cycle of the SIRS response.

8.4.2 Trauma induced changes of the endocrine system

Trauma-induced elevated glucocorticoid levels have an anti-inflammatory function depressing the innate and adaptive immune system. Cortisol causes lymphocyte apoptosis and releases neutrophils from the bone marrow (100). Dehydroepiandrosterone sulphate (DHEAS), another adrenal hormone, strengthens the immunity; after trauma DHEAS levels decrease while cortisol synthesis rises. The subsequent elevated cortisol-to-DHEAS ratio seems to weaken the immune system and predisposes trauma patients to infections (101). Glucocorticoid-induced changes in the cytokine pattern affect and further suppress the Th1-axis which orchestrates mainly the cellular mediated immunity and to a lesser extent the Th2-axis which has a greater effect on the humoral immunity (102). Stress related release of epinephrine, produced by the adrenal medulla, and noradrenaline, liberated by the postganglionic nerve endings, generate a shift of the T helper cells towards the T-helper 2 subtype, with a reduced profile of proinflammatory cytokines (103).

8.5 Epithelial cell and lymphocyte apoptosis in the gut due to trauma

Research about the influence of trauma on quantitative changes of the T-lymphocytes in the gastrointestinal tract in humans is scarce. Hotchkiss *et al*. was one of few research groups to investigate apoptosis of lymphocytes and epithelial cells of the gut in patients who sustained trauma, sepsis and MOF (104,105). This

group explored the gut lymphocytes and epithelial cells of 10 trauma patients and compared these to non-injured control patients using four different methods: a) light microscopy with hematoxylin/eosin stained specimens, b) immunohistochemical staining for cytokeratin 18, which is a filament component of crypt epithelial cells that is exposed during apoptosis, c) immunohistochemical staining for active caspase 3, a reliable marker for lymphocyte apoptosis, and d) laser scanning confocal microscopy. In all four methods, trauma patients evidently had increased apoptosis of the crypt epithelial cells compared to the non-injured control group and, moreover, the crypt epithelial cell apoptosis was more extensive in the more severely injured trauma patients. Furthermore, apoptotic changes of lymphocytes could be detected in the trauma patients but not in the control group. Hotchkiss' group only commented on LPL as a whole and did not distinguish between CD4+ and CD8+ T cells in the LP, nor did they investigate the IEL specifically. The small sample size of only 10 patients was not powered for statistical analyses and the study was therefore of a descriptive nature.

Important to mention is that the trauma patients in Hotchkiss' study were not in prolonged shock and had only brief periods of hypotension. However, the splanchnic blood flow might be decreased despite normal peripheral blood pressure or only moderate hypotension as the blood is shifted away early from the bowel to enhance perfusion of the central organs such as brain, heart and kidney. The rapidity of the apoptotic changes within two to three hours after trauma is worth noting and these findings were confirmed by Noka *et al*. in a rat model where they observed the apoptosis peak one hour after reperfusion following ischemia (106). Where Hotchkiss' group concluded that loss of epithelial cells and lymphocytes in the gut may increase the risk of sepsis due to BT, he did not follow-up these patients

to determine whether the apoptotic changes observed had an influence on their clinical outcome.

In our research project CD4+ and CD8+ T cells of the LP were distinguished and analysed via immunohistochemistry. Further focus was put on changes of the CD8+ IEL to achieve a more complete picture of the lymphocyte structure of the GALT and its trauma-induced changes.

8.6 Trauma induced lymphocyte changes in the peripheral blood

Much research has focused on the phenomenon of trauma-induced apoptosis of immune cells in the blood as the cause of posttraumatic immunosuppression. Apoptosis of monocytes, macrophages, natural killer (NK)-cells, and T- and B-lymphocytes may be one of the determining causes of increased infection rates after trauma.

The naïve CD4+ T helper (Th) cell differentiates depending on the cytokine pattern into a Th1- or Th2-type (107). Th1-type plays the main role in the delayed immune response and is responsible for the proliferation of macrophages and neutrophils, production of opsonizing antibodies and the maturation of cytotoxic CD8+ T-cells; IL-2, interferon-γ and TNF-α are the principal secreted inflammatory factors.

Th2-type is the driver of the immediate immune response and triggers the production of non-opsonizing antibodies, such as IgE by activating mast cells and eosinophil leucocytes. Th2-type releases anti-inflammatory cytokines such as IL-4, IL-5, IL10, IL-13 (108). Severe trauma and hemorrhage shift the Th1:Th2 ratio towards a dominance of the Th2-subtype, partially induced by the endocrine system as explained above under section 8.4.2.

Several research groups documented the impact of trauma on the cellular adaptive immune system analysing blunt and penetrating trauma patients (109–112). CD4+- and CD8+ T lymphocytes were found to be affected in a similar pattern and had their lowest numbers on postinjury day three, while B-lymphocytes had their lowest level on postinjury day five. While these research groups observed an association between lower posttraumatic lymphocyte numbers and increased rate of major infection, increased mortality as well as MOF, others described posttraumatic lymphocyte apoptosis as a double-edged sword being either positive by controlling an overshooting T cell response, or negative by inducing immunosuppression via an inappropriate downregulation of lymphocytes. They were not able to document a correlation between injury severity and lymphocytic apoptosis rate and considered genetic factors and the cytokine pattern as critical factors for this balance (113).

The impact of major surgical procedures alone without the impact of trauma on T cell count was detected by Sasajima *et al*., this group noted a significant lymphocyte apoptosis until day five after surgery in patients after esophagectomy (114).

Importantly, all quantitative CD4+ T cell analyses are age-, sex- and ethnicity-dependent. Lower mean CD4+ T cell counts are observed in the black population and in the male sex (115–118). Furthermore, the timing of the blood sampling has an impact on the CD4+ T cell count with highest numbers in the afternoon hours (119).

8.6.1 NK cells in trauma

Almost 15% of circulating lymphocytes are NK cells which play a central role in the innate immune response. These cells are described as large granular lymphocytes; in the flow cytometric analysis they are characterized by the absence of the cell

surface marker CD3 and the presence of CD56. Depending on the density, or expression, of the cell surface marker CD56 they are divided into $CD56^{bright}$ and $CD56^{dim}$ cells. NK cells are crucial early in the defense process and are able to lyse target cells without preceding sensitization. The main function of the $CD56^{bright}$ NK-cell is to produce cytokines of which IFN-γ is the predominant one. $CD56^{dim}$ NK-cells have more of a cytotoxic function with the potential to destroy infected target cells (120). $CD56^{bright}$ NK-cells are mainly situated in the secondary lymphoid tissue and are the more immature cells compared to the $CD56^{dim}$ NK-cells which are to a large extent located in blood, spleen and bone marrow (121).

Joshi *et al*. observed a reduced NK-cell activity after trauma as a consequence of reactive oxygen metabolites, but the quantity of the cells was not diminished (122). Correspondingly Blazar *et al*. documented in burns patients a suppressed NK-cell activity for 40 days and in trauma patients a reduced NK-cell activity from day three to day six after injury (123).

Further research is needed to gain more insight into the role and function of NK-cells in trauma. Evidence from murine research projects in sepsis and trauma showed a reduction of mortality, lymphocyte apoptosis and inflammatory events after induced NK-cell depletion and demonstrated that NK-cells are partly responsible for a diminished T cell-proliferation (124,125). Given the above, NK cells might even be harmful in the context of trauma and partly the reason for septic events by impeding the clearance of infection.

In our research project quantitative changes of the immune cells in the blood were analysed and it was investigated whether these changes had an impact on patients' outcomes. Special focus was put on the NK cells and we investigated whether

changes in the NK cell quantity had an influence on the CD8+-T cells dynamic and septic complications after surgery in severely injured trauma patients.

8.7 Indication and principle of damage control surgery

Trauma patients who are physiologically severely compromised and require surgery undergo damage control surgery (DCS). DCS in trauma is an established approach of abbreviated surgery in severely injured patients who are not stable enough to undergo definitive surgery (126). It is applied to patients who are experiencing a combination of hypothermia, acidosis and coagulopathy, and are therefore unable to tolerate lengthy surgical procedures in their index operation. As these patients are often hemodynamically compromised due to severe hemorrhage, they usually require perioperative inotropic support and blood products. Only bleeding and contamination are controlled in the initial operation; after resuscitation in the ICU, the anatomical reconstruction of the injured organs is completed during the relook operation that usually takes place 24-48 hours later (127). This approach has led to a significant increase in survival of seriously injured patients (128).

In our study, the index operation and relook procedure provided the opportunity for two bowel specimens to be sampled at two different occasions and to investigate the manner in which the T-lymphocytic population of the gut changed between the index and relook operations. Novel insight into the rapidity and the extent of the T-lymphocytic changes of the bowel in the first days of trauma and the possible impact on the clinical outcome was gained.

8.8 HIV in South Africa (SA)

HIV-1 and HIV-2 are retroviruses of the Retroviridae family, Lentivirus genus. They are enveloped, diploid, single-stranded, positive-sense RNA-viruses with a DNA intermediate, which is an integrated viral genome that persists within the host cell DNA. Responsible for the world-wide epidemic is the subtype HIV-1 (129).

The estimated number of HIV-seropositive people in SA grew from 4,94 million people in 2002 to 7,52 million people in 2018 which shows the enormous challenge the country is facing; while the overall prevalence rate in 2018 was around 13.1% of the South African population, 19% of the adults aged between 15 and 49 years are HIV-infected. A quarter of the deaths in 2017 was acquired immunodeficiency syndrome (AIDS)-related (130).

Antiretroviral treatment (ART) of HIV-infected people was initially started in middle- and high-income countries in 1996. The problem that sub-Saharan Africa, having the highest numbers of HIV-infected people in the world, didn't have access to this medication became more and more evident. An important step in improving this situation was the declaration of an agreement between the Joint United Nations Programme on HIV and AIDS (UNAIDS) and five pharmaceutical companies at the XIIIth International AIDS Conference in Durban in 2000: the companies gave their approval to start supplying ART at massively reduced prices to low-income countries (131). Further progress for better supply and structured distribution of ART in poor countries was achieved by the World Health Organization (WHO) initiative '*3 by 5*' launched in 2003 with the aim of having 3 million people on treatment by 2005 (132). By September 2005, only 85 000 people had been started on treatment in the public health sector in SA, so the intended number was not nearly reached regardless of the fact that all South African provinces were able to offer ART with 199 health care facilities involved nationwide (133).

In 2010 the National Department of Health renewed the treatment guidelines, expanding the treatment to children of less than a year old, all pregnant women irrespective of CD4+T cell-count, and all HIV-patients coinfected with tuberculosis and a CD4+T cell-count less than 350 cells/μl. By 2013, around 2,3 million of the 6,4 million HIV-seropositive people were on treatment.

The START (Strategic Timing of Antiretroviral Therapy)-study compared HIV-seropositive patients who were started on treatment with a CD4+T cell-count of more than 500 cells/μl to those whose treatment was delayed until the CD4+T cell-count dropped below 350 cells/μl and showed a clear decrease of AIDS-related complications in the patients who were started at higher CD4+T cell-counts (134). This landmark study resulted in a change of the treatment guidelines in 2014 with the objective of starting ART as soon as possible after establishing the diagnosis of HIV, irrespective of the CD4+ T cell-count.

8.9 HIV in trauma

8.9.1 Prevalence of HIV in trauma patients

The percentage of HIV-seropositive patients treated in South African trauma units is higher compared to the seroprevalence in the general population in Gauteng, SA. Urban location, intravenous drug use, younger age group below 50 years and high-risk sexual behaviour are the predisposing factors for the higher prevalence of HIV-infection in this local patient population. Patients who sustain penetrating trauma or assault tend to have higher HIV rates than patients with blunt trauma (135).

In an analysis of trauma patients from Johannesburg from 2002, 27% of the patients who survived and 47% of the non-survivors were HIV-seropositive (136). Another study of an urban teaching hospital in Johannesburg in which 23% of their acute

orthopaedic trauma patients were HIV-infected (137) confirmed these high numbers.

8.9.2 Impact of HIV on the outcome of trauma patients

The impact of HIV-infection on trauma patients has mostly been evaluated in retrospective studies. Differences in methodology make it difficult to compare the studies and in many of the research projects conducted, HIV-positive patients were not stratified with regards to the severity of their immunosuppression (138).

Stawicki *et al*. evaluated 559 HIV-positive trauma patients retrospectively and observed a higher rate of pre-existing morbid conditions such as psychiatric disease, alcohol/drug abuse, liver, renal and pulmonary disease in this population. The examined cohort had heterogeneous injury patterns consisting of blunt, penetrating, and orthopedic injuries. They observed that HIV-positive patients had a significantly longer hospital and ICU length of stay, had more pulmonary, septic and renal complications and a higher likelihood to undergo mechanical ventilation. There was no statistically significant difference in the mortality between the HIV-positive and negative trauma patients (139).

Similar results were observed in critical care patients, more than half of them trauma patients, who were admitted to an ICU unit in Durban, SA. HIV-seropositive patients had significantly higher rates of organ failure and septic shock without difference in mortality. The authors highlighted the urgent need to elucidate the causes for the higher incidence of these complications (140).

Where some smaller retrospective studies reported on higher mortality rates, significant complications and/or infections in HIV-seropositive patients with lower

CD4+ T cell-counts (141,142), others found no associations with a decreased CD4+ T cell-count (143).

To summarize, the trauma studies of HIV-seropositive patients are plagued by a broad spectrum of injury severity and -patterns. Furthermore, blunt and penetrating trauma are often not subcategorized which makes it almost impossible to gain valid insights and to evaluate the real impact of this disease on trauma outcome and complications.

In our study, we aimed to address this shortcoming in the trauma research literature by investigating only one injury type, namely penetrating abdominal trauma. To our knowledge this study is the first project in which the impact of HIV and the risk of trauma surgery will be evaluated prospectively.

8.10 HIV and CD4 lymphocyte count in surgery

Outcome differences between HIV-seropositive and -seronegative patients after elective surgery have been investigated extensively. In the era before the availability of ART, surgery was associated with a high rate of complications and other morbidities (144). Compared to the ART-era, many patients pre-ART suffered from opportunistic infections and most HIV-seropositive patients progressed to advanced stages of AIDS, including surgical pathologies. The situation has changed considerably now that a higher percentage of HIV-seropositive patients has access to free medication. Further advances were achieved with the change of the treatment guidelines in 2014 by starting ART immediately after diagnosing the disease. Reconstitution of the immune system after starting ART resulted in an increased life expectancy (145) and a reduced complication rate after surgical procedures compared to the pre-ART time (146).

Unfortunately, a considerable number of HIV-seropositive patients are not fully “optimized” at the time of surgical intervention. Most problematic are the patients who have to undergo urgent surgery and are not on treatment yet or newly diagnosed. Defaulting of treatment and experience of only an incomplete immune recovery after starting ART are further factors that put patients who need surgery, especially emergency operations, at higher risk.

From the literature, the postoperative percentage CD4 lymphocyte count and the decrement of percentage CD4 lymphocyte count (preoperative to day one) in surgical patients are reportedly risk factors for postoperative complications, infections and/or longer hospital stay, although higher viral load has not been shown to impact on postoperative problems (147–150). Additionally, higher mortality rates have been reported in HIV-infected patients on ART at all CD4+T cell-count levels, with hypoalbuminemia also identified as a risk factor for mortality (151). The latter highlights the importance of the nutritional status in the surgical treatment of HIV-seropositive patients (152).

To summarize, these studies demonstrate the importance of the CD4+ T cell-count as a predictor for the outcome of patients on ARVs. Since HIV is a systemic disease, virus-induced changes are not only found in the peripheral blood but also in other organs such as the intestine. To date, it has not been investigated whether intestinal changes have an influence on the outcome after surgery and this objective has been addressed in our research project.

8.11 Intestinal changes, GALT and bacterial translocation in HIV-seropositive patients

8.11.1 Morphologic changes and CD4+ T cell depletion of the GALT

Untreated, the intestine of a HIV-seropositive person undergoes histologic changes such as crypt hyperplasia, villous blunting and atrophy which was already described early on in the era of HIV as “HIV-enteropathy” (153). Direct virotoxic effects on enterocytes with impairment of the glucose uptake were described, being held partly responsible for the HIV-related malnutrition (154). Severe functional changes of the ileum with reduced absorption of vitamin B12 and bile acids are observed in AIDS patients that potentially lead to neurologic disorders and diarrhoea. The discrepancy between severe bowel dysfunction and often only minimal morphologic changes is remarkable (155).

Citrulline is a useful biomarker to assess the loss of enterocytes in the small bowel as it is solely produced by the enterocytes as part of the glutamine metabolism. Decreased citrulline plasma levels are measured in HIV-seropositive patients, indicating a decrease of the number of functional enterocytes which is associated with an impairment of the gut epithelium and malabsorption (156).

Moreover, increased levels of intestinal fatty-acid binding protein (I-FABP) are observed even in treated chronic HIV-seropositive patients. I-FABPs are expressed and released from enterocytes in the event of epithelial damage and indicate ongoing mucosal destruction (157).

Another factor that contributes to the damage of the intestinal epithelium is the accumulation of HIV-infected macrophages on the mucosal surface. These infected macrophages induce an inflammation of the mucosa and show a reduced phagocytic function compared to healthy macrophages, thus they predispose to an increased translocation of microorganisms (158).

CD4 was found to be the principal cellular receptor for HIV fusion and entry (159). In the past decade it has been discovered that additional to CD4, a chemokine co-receptor, usually either chemokine receptor four (CXCR4) or five (CCR5), is essential for viral entry into target cells.

After infection the virus moves rapidly to regional lymph nodes and subsequently spreads via the bloodstream to other lymphoid tissues of the body. The GALT is a major target where the virus replicates extensively with the consequence of a massive depletion of CD4+ T cells in the gut early after infection (160). Most CD4+T cells in the GALT are activated memory cells, also called effector memory cells, and express CCR5, which functions as the co-entry-receptor for HIV, making them the preferred target cells (161). Moreover, the HIV-envelope glycoprotein GP120 binds to the integrin $\alpha_4\beta_7$. This integrin, expressed on T cells, serves as homing receptor and is responsible for the migration of the lymphocytes to the GALT which may further explain the affinity to and massive replication of the virus in this lymphoid tissue (162).

The underlying cause for the massive depletion of the mucosal CD4+ T cells during the acute infection phase is direct cell infection and, subsequently, a virus-induced immune response followed by elimination of the cell (163). This takes place early in the course of infection; almost two thirds of memory CD4+ T cells are infected when the peak levels of viremia are reached, and within four days 80% of CD4+ T cells are destroyed (164). This observation carries even more importance due to the fact that the mucosal CD4+ T cell compartment contains the majority of the T-cell population in the body.

In untreated HIV-seropositive patients, the CD4+ T cell depletion is most pronounced in the gastrointestinal mucosa during all disease phases (160). As the decrease of the CD4+T cells is more distinct in the GALT than in the blood (165), the assessment of the disease and its progression by examining blood alone most likely does not give us the complete picture of the extent of immunosuppression.

Isolation of specific T cell subsets via flow cytometry with subsequent identification of viral DNA with polymerase chain reaction is able to quantify the number of infected cells within cell populations of interest. This technique confirmed that many more T cells in the gastrointestinal tract, the CCR5+ CD4+ T cells, are virus-infected than the CD4+ T cells in the blood; actually a ten-fold higher infection rate was reported which, in turn, mirrors the huge extent of the viral reservoir in the gut compartment (166,167). Notably, the depletion of these CCR5+ CD4+ T cells is observed all along the gastrointestinal tract, including the colon and rectum (168), hence the HIV-infection leads to a pronounced destruction of the gut's effector cells, the LPL (164).

CD4+ T cell loss is further promoted by HIV-induced changes of the lymph node tissue with subsequent disruption of the physiological processes that usually take place herein. Peripheral lymph nodes are organized in such a way that facilitates interaction between antigens, lymphocytes and chemokines and which triggers an immunologic response that controls the balance between CD4+ and CD8+ T-cell population and their proliferation (169). The response of the innate and adaptive immune system to the HIV replication induces inflammation in the lymphoid tissue leading to deposition of collagen, thus changing the structure of the lymph node architecture (170). As CD4+ T cells depend on a working lymph node environment

these changes contribute to the loss and decreased life time of this T cell subset (171).

Interaction of T cells with follicular dendritic cells is also sustained by a functional lymph node structure; in fibrosed lymph nodes this important interplay is impaired, hence, the immune system suffers partial loss of an important site of antigen presentation.

In summary, the destruction of CCR5+CD4+T cells in the gut together with structural damage of lymphoid tissue leads to a significant reduction of the quantity of CD4+T cells in the body with a reduced memory T-cell pool (160).

Architectural changes and collagen deposition is not only limited to the peripheral lymph nodes. The immune-inductive sites of the GALT, namely the PP, are also affected by collagen deposition leading to permanent changes in this compartment. Notably, CD4+ T cell reduction reportedly correlates with the amount of collagen deposition in the secondary lymphatic tissue (170).

8.11.2 BT and activation of the immune system

There is evidence that the depletion of the mucosal CD4+T cells together with structural changes of the tight junctions in the intestine contributes to the impairment of the gut mucosal barrier in HIV-patients, opening the way for BT. Brenchley *et al.* detected significantly increased levels of endotoxin in chronic HIV-patients compared to healthy individuals; as mentioned above, endotoxin levels are used as measurement for BT. Increased endotoxin levels correlated with increased quantity of CD8+T cells with activated CD38+ HLA-DR+ phenotype, with increased levels of proinflammatory cytokines as well as with macrophage activation; these

phenomena are interpreted as signs of an activated immune system as result of continuous BT (172).

Special attention was paid to IL-18, a proinflammatory cytokine of which increased levels are found in HIV-seropositive patients. The latest research in human colonic epithelial cell lines showed that IL-18 leads to apoptosis of intestinal epithelial cells via the caspase-1 and -3 pathways, impairs integrity of the tight junctions, and reduces electrical resistance in the epithelium. All of the above mentioned factors disturb the intestinal epithelial integrity and promote BT (173). Since these results originate from laboratory findings of intestinal cell lines, the clinical relevance and the translation into human intestinal epithelium is yet to be evaluated.

Further causes for ongoing activation of the immune system in HIV-seropositive individuals is a continuing HIV-replication on a very low level in tissues such as lymph nodes and the gastrointestinal tract. Replication persists despite ART and this can only be detected with specialized laboratory methods rather than with routine clinical tests. The HIV viral protein components are believed to trigger the immune system further (174,175). In addition, inflammatory lipids which are observed at higher levels in HIV-seropositive people, specifically oxidized low-density lipoproteins, may trigger the immune system and are associated with parameters of monocyte activation (176).

Another factor that may contribute to the immune system activation is the altered gut microbiome in HIV-seropositive patients: their gut microbiota are in general less diverse, and bacteria regarded as commensals are especially less frequent. Potentially pathogenic bacterial taxa, which are often the source for bacteraemia, such as Pseudomonas, Acinetobacter, Campylobacter, and Escherichia on the

other hand, seem to be more abundant compared to the microbiota of healthy individuals (177).

Alterations in the mucosal-adherent bacteria, especially a depletion of Bacteroides species and expansion of the mucosal bacteria Prevotella are associated with intestinal T cell activation, increased levels of proinflammatory cytokines, chronic mucosal inflammation and higher mortality in HIV-seropositive patients (178–180). These results provide solid evidence for the interplay between altered gastrointestinal microbiome and immune dysfunction and inflammation. However, it stills needs to be further elucidated whether there is a causal relationship between HIV-induced dysbiosis and immune system activation. Whether the altered gut microbiome drives the immune dysfunction or vice versa is yet to be revealed.

Mehandru *et al*. noted a dramatic expansion of activated memory cells (CD45R0+/CD38+) as sign of a triggered immune system as early as the acute phase of infection. Almost 70% of the intestinal CD4+ T cells are fully differentiated activated memory cells, which are more prone to apoptosis compared to naïve Th cells in the blood. This suggests that the immune activation induces a greater pool of vulnerable CD4+ T cells in the gut (167).

A possible beneficial effect of the immune system activation is the proliferation and delivery of CD4+T cells to the gut mucosa, re-establishing some sort of immune competence. However, the activation of the immune system seems to be the driver for the progression of the disease with deleterious consequences, such as fibrosis and structural changes of the lymph nodes, exhaustion of the naïve T cell pool, functional derangements of the thymus, and withholding of effector-type T-lymphocytes in the lymph nodes (170,181).

Furthermore an association was found between increased endotoxin levels and hypertriglyceridemia, reduced insulin sensitivity and atherosclerosis. Hence, the impairment of the gut mucosal barrier seems to contribute to non-AIDS related disorders that are regularly encountered in treated, chronic HIV-seropositive patients and becomes a new challenge in the era of ART (182). HIV-induced inflammation appears to be playing a crucial role in the pathogenesis of coronary artery disease, and increased levels of inflammatory markers seem to result in a higher prevalence of coronary stenosis in HIV-seropositive men (183).

Endotoxin levels decrease after starting ART and, in turn, reduced endotoxin levels are associated with increased CD4+T cell reconstitution, but they don't return to normal values observed in the healthy population (184). Despite ART, persistent high biomarkers are observed: LBP (Lipopolysaccharide-binding protein), CrP, and sCD14 are elevated and suggest an ongoing monocyte/macrophage activation, BT and inflammation (185).

In summary, the impairment of the gut mucosal barrier, especially influenced by the decrease of the mucosal CD4+T cells under a certain level, is thought to be one of the determining factors for the disease progression (186). Although the important role of the CD4+ T cells in maintaining the integrity of the gut mucosal barrier seems to be established, it must be taken into consideration that other cell populations, such as the M-cells, intraepithelial lymphocytes, goblet cells, intestinal macrophages, and Paneth cells also contribute to the prevention of BT.

8.11.3 Restauration of the GALT under ART

The CD4+ T cell-count in the blood is usually used to monitor the efficacy of ART. While it is established that ART leads to an immune reconstitution in the blood, there

is still an ongoing debate as to the extent to which ART is able to restore the immunity in the GALT.

In the chronic infection state only one study in the literature observed an almost complete restoration of CD4+ T cells in the gut after initiation of ART (187), while other studies noted only a minimal or incomplete reconstitution of CD4+ T cells in the GALT of chronic HIV patients. Chun *et al.* took biopsies of the ileum after a mean antiretroviral treatment duration of 8.4 years and detected that only 11.3% of the lymphocytes in the gut mucosa are CD4+, compared to 40% in healthy individuals (175). Guadalupe *et al.* followed up acutely infected and chronic HIV-seropositive patients with jejunal biopsies up to two years after ART initiation and, correspondingly, there was no effective restoration of the T-cell pool in the GALT of the chronic patients. Notably, Guadalupe's group observed an almost complete restoration of CD4+ T cells in patients who started ART early and reported that patients with a more severe CD4+ T cell depletion during the acute phase of infection had a more delayed treatment response (188).

It is still not fully understood why the recovery of the CD4+ T cells in the GALT is less complete compared to that in the blood. Collagen deposition in the GALT and structural changes in the architecture of the lymph nodes may be one reason for an incomplete CD4 T+ cell reconstitution in patients with long running disease. Analysis of homing receptors and cell proliferation markers suggest that T-cell restoration is more the result of cell-trafficking to the GALT than of local proliferation (188), although others have found that a defect of one of the lymphocyte homing receptors accounts for an impaired lymphocyte trafficking to the gut and thus incomplete restoration of the GALT (189).

Starting ART early in the course of the disease seems to result in a more complete and efficient but still not full recovery of the CD4+ T-cells in the GALT (164,190), supporting an early initiation of treatment after diagnosing the disease.

We investigated the impact of the compromised GALT of HIV-infected patients on the outcome after trauma. The T-lymphocytic quantity in the intestinal mucosa and GALT was documented and compared between HIV-seropositive and -seronegative patients. Patients were followed up postoperatively to investigate whether HIV-induced changes had an influence on septic and surgical complications.

The study provided a unique platform and gave us the opportunity to further study the GALT from different parts of the small bowel in HIV-seropositive adults and to compare the findings with those who are HIV-negative. These results might influence decision making in the treatment of HIV-seropositive trauma patients.

9 Study objectives

The main hypothesis of this study is that trauma leads to quantitative changes of immune cells in the GALT with an increased apoptosis of immune and epithelial cells in the gut mucosa which has an influence on the clinical outcome of trauma patients.

The aim was to determine the impact of trauma on the cell-mediated adaptive immune system in the blood and in the gut. A subgroup analysis of patients who underwent DCS enabled us to get an impression of the extent of these changes.

The analysis of HIV-seropositive patients gave us the opportunity to observe whether a preexisting compromised GALT in these patients has an impact on the clinical course. This leads us to the four objectives in this PhD research project:

1. The Quantification of T cell subsets (CD4+ and CD8+ T-lymphocytes) and lymphocyte apoptosis in full thickness bowel specimens assessed by immunohistochemistry and assessment of gut-epithelial cell apoptosis in context of penetrating trauma and comparison to a non-trauma control group. Documentation whether the quantity in the T-lymphocytes of the GALT is associated with septic, surgical and non-surgical complications in the postoperative course.
2. To compare the T-lymphocytic quantity as well as the lymphocyte apoptosis in the GALT of HIV-seropositive and -negative patients and follow up whether these differences impact the clinical outcome.
3. The Quantification of T cell subsets (CD4+ and CD8+ T-lymphocytes), lymphocyte apoptosis and enterocyte apoptosis in a second full thickness bowel specimen that is sampled during the relook laparotomy in damage control patients. Documentation of the extent of the changes of the immune cells and analysis of the impact of the changes on the clinical outcome.
4. To document changes in T-, B-lymphocytes and NK cells in the peripheral blood after penetrating trauma and subsequent surgery and investigate the impact of these changes on the clinical outcome. Special focus was placed on the role and clinical relevance of NK cells.

10 Methods

10.1 Study design

In this prospective study we recruited HIV-seropositive and -negative adult patients from December 2018 to March 2020 at the Chris Hani Baragwanath Academic Hospital (CHBAH) Trauma Unit in Johannesburg, South Africa, who required surgery for abdominal gunshot injuries with bowel involvement.

The protocol was approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (clearance number M180914). Informed consent was obtained from all patients or, if the patient had a decreased level of consciousness on admission, consent was signed by the patient's next of kin.

A full thickness, non-contused specimen of the small intestine, at least 5 mm in size, was obtained during index laparotomy from the segment of the bowel requiring resection due to the sustained injuries. The entirety of the sustained intra-abdominal injuries was graded according to the penetrating abdominal trauma index (PATI) (191) [Appendix B, Table 1] which is a commonly used and validated score for these injuries (192). These specimens were analysed and compared to bowel specimens of a control group who underwent resection for non-traumatic reasons.

A peripheral blood specimen for assessment of CD4+T-, CD8+T-, B- lymphocytes and NK cells was sampled on admission and 48 hours postoperatively after index surgery.

The focus of the study was on the GALT of the *small bowel* only in order to facilitate interpatient comparability.

10.1.1 Subgroup of damage control patients

Physiologically severely compromised patients underwent DCS. In these patients a first bowel specimen was taken in the index operation and a second one in the relook operation approximately 48 hours later. Blood specimens were taken on admission, 48 hours after the index surgery and again 48 hours after the first relook operation.

10.2 Study group

Inclusion criteria:

- Age≥18 years of age
- Patients undergoing surgery due to penetrating abdominal trauma (gunshot) with small bowel injury which requires bowel resection
- HIV-seropositive and -negative patients.
- Patients undergoing definitive surgery and DCS

Exclusion criteria:

- Organ failure or dysfunction prior to the sustained trauma such as chronic renal failure, asthma, uncontrolled diabetes mellitus (A1C level > 7%), chronic obstructive airways disease, hepatic dysfunction and/or cardiac dysfunction.
- Death within 72 hours of hospital admission

10.3 Control group

Inclusion criteria:

- Age≥18 years of age

- Elective surgery with bowel resection for non-traumatic indications, such as neoplasm or reversal of stoma.

Exclusion criteria:

- Pre-existing organ failure or dysfunction, such as chronic renal failure, asthma, uncontrolled diabetes mellitus (A1C level > 7%), chronic obstructive airways disease, hepatic dysfunction and/or cardiac dysfunction.
- Recent chemo- or radiotherapy within the last six months
- Physiologic derangements such as metabolic acidosis
- Signs of sepsis or shock
- HIV-infection

Eleven control patients were recruited for the research project.

10.4 Sample size and statistical tests

Sample size estimation was based on the key research question/s to be answered, this is for objective 1 and 2 whether there is a difference in the lymphocyte quantity of the GALT between HIV-negative and positive patients and whether this difference has an impact on the outcome (septic and surgical complications). The Chi-squared test or Fisher's exact test was used, as appropriate, to assess the relationships between categorical variables according to the patients' HIV-status.

The relationship between the continuous variables and HIV status was assessed by the t-test. Where the data did not meet the assumptions of these tests, a non-parametric alternative, the Wilcoxon rank sum test was used. The relationship between two continuous variables was assessed by Pearson's correlation coefficient or, where indicated, the Spearman's rank correlation coefficient was

used. A minimum sample size of 54 patients was required for determination of a large size effect.

To test the first part of the 3rd objective that trauma leads to changes in GALT lymphocytes with epithelial impairment, a minimum of 15 patients were required for determination of a large size effect. To test the second part of the 3rd objective that these changes following trauma influence the outcome and complications in patients, a minimum of 54 patients were required. Statistical tests were applied as described above.

The sample size calculation for hypothesis 4 was identical to hypothesis 3 and statistical tests were used as mentioned above.

Descriptive analysis of the data was carried out as follows: Categorical variables were summarised by frequency and percentage tabulation, and illustrated using bar charts. Continuous variables were summarised by the mean, standard deviation, median and interquartile range, and their distribution illustrated by histograms.

Sample size calculations were carried out in G*Power (193). Data analysis was carried out using STATA version 15.1 for Windows (StataCorp LLC, Texas, USA). A 5% significance level was used.

10.5 Surgical treatment and follow-up

All patients with penetrating abdominal trauma due to gunshot wounds with indication for surgery were operated on. Hemodynamically stable patients with little blood loss on admission had definitive surgery that entails resection of the damaged part of the bowel and primary anastomosis. Patients who were hemodynamically unstable and sustained significant blood loss preoperatively, had with regards to the bowel injury only a resection of the injured part without performing an anastomosis

(“clip and drop”). Subsequently the abdomen was closed temporarily, and the definitive surgery was performed approximately 48 hours later, after the patient was in a more stable condition.

The extent of the sustained bowel injury was classified according to the American Association for the Surgery of Trauma (AAST)-classification [Appendix B, Table 2]. The severity of abdominal fecal contamination was reported and classified as minimal (localized, minimal contamination), moderate (< 2 quadrants) and severe (> 2 quadrants and/or colonic stool contamination).

Postoperative care was conducted according to general surgical standards and not dictated by the patient’s participation in the study.

The follow-up of the patients was restricted to the in-hospital course and a weekly review in our trauma clinic for the period of one month after discharge from the hospital [Appendix C, Form 1: Follow-up template]. Septic and postoperative surgical complications were used to analyse the clinical outcome of the patients. By nature, septic and surgical complications are often overlapping, causally related events; the aim was to summarize and target with the septic complications all problems involving infectious processes. With the surgical complications a stronger emphasis was put on the requirement of interventions and the need for organ support due to postoperative complications.

Septic complications included superficial and deep surgical site and wound infections, bacteraemia with positive blood cultures, fungemia, pneumonia, septic intraabdominal collections and urinary tract infections. Superficial surgical site infection was defined as an infection of the skin incision site with purulent drainage within 30 days of surgery involving the skin and subcutaneous tissue while the deep

surgical site infection involved muscle and fascial layers with purulent drainage and spontaneous dehiscence of the fascia (194).

The postoperative complications and interventions (anastomotic leakage, intraabdominal collections, number of relooks, requirement of organ support etc.) were classified according to the Clavien-Dindo classification which is a commonly used and validated tool in the follow-up of surgical complications (195) [Appendix D]. Grades 1-2 were categorized as minor, whereas grades 3-5 were major complications. The latter division was based on grade 3 complications and higher needing invasive interventions, either surgically, endoscopically or radiologically.

Anastomotic leakage was defined as a defect at the site of the anastomosis resulting in a communication between extra- and intraluminal compartment which became clinically symptomatic requiring a therapeutic intervention (196).

10.6 Tissue/blood sampling and laboratory investigations

a) Standard laboratory blood tests of haematological, renal function, liver function, blood glucose, routine inflammatory markers (CRP) and arterial blood gas was performed as part of the standard management of trauma patients.

b) HIV sero-status was assessed after consent by laboratory HIV ELISA and by routine rapid testing which involves a second rapid confirmatory test for all those testing positive on their first assay. A viral load and CD4+ T cell-count in plasma was determined in all HIV-seropositive patients and the immunosuppression was classified in accordance to the Centre for Disease Control guidelines (197) [Appendix B, Table 3].

c) CD4+T cell-, CD8+T cell-, B cell-, NK cell- count, and CD4/CD8 ratio was assessed for all patients via flow cytometry. For the flow cytometric assays EDTA peripheral blood (5 mL) was drawn on admission (A-blood), 48 hours after the index operation (P1-blood) and for damage control patients 48 hours after their first relook (P2-blood). In all patients with fever or raised inflammatory markers aerobe and anaerobe blood cultures were taken following the principles of aseptic technique.

The blood for the lymphocyte analysis was stored at room temperature and processed at the latest within 72 hours as the robustness of the T-cell subset enumeration is maintained within that time (198). Whenever logistically possible, blood analysis was done within 24 hours of sampling. Specimens were sent via courier, on the next working day after retrieval, to the SANAS accredited Charlotte Maxeke Johannesburg Academic Hospital National Health Laboratory Service flow cytometry laboratory (Facility accreditation number M0109) where the lymphocyte subset testing was conducted.

The flow cytometric analysis was performed as follows:

Single platform CD3+ total T-lymphocytes, CD3+/CD4+ Helper T-lymphocytes, CD3+/CD8+ Suppressor T-lymphocytes, CD19+ B-lymphocytes and CD3-/CD56+ Natural Killer lymphocytes were prepared using the Immunoprep™ lyse-no-wash method and Cyto-stat multi-colour antibody cocktails as follows; CD45-FITC/CD4-PE/CD8-ECD/CD3-PC5 and CD45-FITC/CD56-PE/CD19-ECD/CD3-PC5 (Beckman Coulter Life Sciences Inc., Indianapolis, USA). Post preparation, Beckman Flow Count ™ beads were added and samples acquired and analysed on a Beckman Navios™ flow cytometer (Beckman Coulter Ireland, Inc., Lismeehan). Briefly,

10 µL of the multi-colour antibody cocktails were added to 100 µL of whole blood. Samples were incubated for 10 minutes in the Beckman T-Q-Prep™ automated sample preparation system during which time the sample reacts with fluorochrome labelled monoclonal antibodies that are specific for the cell surface antigens, in this instance CD3, CD4, CD8, CD19, CD56 and CD45. Post incubation, Immunoprep™ reagents (lysing agent, stabiliser and fixative) are automatically added to haemolyse red blood cells while maintaining the integrity of the white blood cells and the white blood cell surface antigens. Lastly, 100 µL of Flow Count ™ beads were added to each sample prior to acquisition and analysis on the flow cytometer. Cells that had bound the fluorochrome labelled antibody were identified on the basis of their fluorescence emission by the specific fluorochromes attached to either the CD3, CD4, CD8, CD19, CD56 or CD45 antigens on the cell surface. A sequential automated gating strategy was used, CD45 (bright) with CD3 to identify T cells, to determine CD4 and CD8 subsets subsequently. Hereby the lymphocyte subsets expressing the fluorochromes labelled antibodies of interest were isolated. During acquisition and analysis, the flow cytometer software automatically calculated the CD3, CD4, CD8, CD19 and CD3-/CD56+ absolute counts by comparing the number of fluorochromes labelled cells to the bead events incorporating the bead calibration factor supplied with each bottle of Flow Count ™ beads. The formula used was the number of positive events/ number of bead events x calibration factor with the final result reported as the number of cells per µL.

Daily quality control on the Navios flow cytometer included background and carryover assessment as well as running Beckman Flow Check PRO™

fluorospheres to verify optical alignment and fluidics. To monitor sample processing, acquisition (including fluorescence staining/detection) and analysis Beckman Immunotrol™ process controls were tested daily. Further patient sample specific internal quality control procedures comprised inclusion of two common markers in each tube (namely CD45 and CD3) to ensure inter-tube reproducibility of the total white cell and lymphocyte counts and CD3 positive lymphocytes. In addition, the laboratory participated in two external proficiency testing schemes (PTS) for lymphocyte subsets, namely United Kingdom National External Quality Assessment Scheme (UKNEQAS), and the South African National Health Laboratory Service Proficiency Testing Scheme (NHLS PTS).

For the blood cultures BACTEC™-blood culture bottles were used and the specimens were sent to the NHLS Laboratory where they were incubated routinely for a period of five days at 37^0C under constant monitoring. In case a blood culture flagged positive for growth, a Gram stain was conducted. Biochemical standard protocols, growth characteristics and antibiotic susceptibility were used for further identification of bacteria and resistance.

d) Biopsies and sampling for histopathological assessment:

Full thickness bowel specimens, measuring at least 5mm in size, were sampled from all patients from macroscopically healthy bowel. To clarify further, the specimens were sampled from the segment of the bowel that required resection due to the sustained injuries. Enough distance between the penetrating wounds and the place where the specimens were sampled

was respected to guarantee that only non-contused bowel was taken for analysis.

The sampling occurred at the time of initial surgery and in DC-patients additionally during the relook as part of the anastomotic procedure. Tissue specimens were placed into 10% buffered formalin fixative solution to facilitate routine processing at the Division of Histopathology, NHLS at CHBAH.

Hematoxylin and Eosin (H&E)-staining, as well as CD4 and CD8 immunohistochemistry (IHC) was performed on all bowel specimens. For the IHC staining CONFIRM anti-CD4 (SP35) Rabbit Monoclonal Primary Antibody and CONFIRM anti-CD8 (SP57) Rabbit Monoclonal Primary Antibody (Ventana Medical Systems, Inc., Arizona) were used.

The antibodies were stored at 2-8 ^{0}C and required detection kits for use on a Ventana BenchMark XT automated slide stainer. The Ventana diaminobenzidine (DAB) detection kit is an indirect biotin-free system for detecting the rabbit primary antibody (anti-CD4/CD8) in this setting.

The staining process is based on a horseradish peroxidase (HRP) multimer (hapten) detection system in which the enzyme of the multimer molecule is directly conjugated to a secondary antibody by long-arm linkers.

Microtomy was performed to obtain tissue sections 2-4 mm in thickness from the paraffin embedded tissue blocks of the test tissue. The sections were set afloat on a water bath, the optimal temperature of which is 45^{0}C. Mounting on positively charged/adhesive slides that were appropriately labelled with the specimen number/s was followed by incubation for 60 minutes at a temperature of 60^{0}C.

The automated staining procedure entailed deparaffinisation and cell conditioning/antigen unmasking, a heat-induced antigen retrieval method was used. The primary antibody incubation occurred for 16 minutes at 37^0C. Thereafter amplification and counterstaining with haematoxylin ll occurred for four minutes, followed by post counterstaining with bluing agent for four minutes. Insertion of coverslips occurred by means of an automated procedure. Positive control tissue (tonsil) was included on each slide.

Light microscopic examination was performed by the researcher and one histopathologist in a blinded manner without having any clinical information. Histological presentation of cell death was used to assess the bowel specimens. The presence of lymphocytic apoptosis (pyknosis and karyorrhexis) of the intraepithelial lymphocytes as well as the LPL was documented. If lymphoid aggregates were present in the specimen, follicular lysis was reported if present. In addition to the lymphocyte analysis, the enterocyte apoptosis was documented.

As an apoptotic cell disintegrates into several particles, a cluster of particles within one nuclear diameter was reported as one apoptotic event (199). For comparison amongst the study patients an apoptotic density was defined and reported as number of apoptotic events per mm^2 as suggested by other research groups (200). Therefore 10 high power fields (hpf) were evaluated and the number of apoptosis divided by 2,37 (10 hpf=2,37 mm^2).

The number of CD4+ and CD8+ lymphocytes were assessed at X400 magnification within 10 non-overlapping high-power fields of the LP by manual counting with the aid of Zeiss (Zeiss Inc.; Jena, Germany) Axioscope and software. The lymphocytic counts within the tissue sections were

expressed as number of cells per mm^2. The number of intraepithelial CD8+ T-lymphocytes was documented per 100 epithelial cells within the small intestine. The method suggested by Goldstein and Underhill (2001) was applied in which the IEL within the topmost 20 enterocytes of five randomly selected villi tips are counted (201). [Appendix C, Form 2: Histopathology datasheet].

11 Results of objectives 1 and 2 (paper 1)

Title Paper 1: Compromised gut associated lymphoid tissue is a risk factor for postoperative septic complications in HIV-seropositive trauma patients

Status: published – World Journal of Surgery (2021) 45:1006-1013 (Appendix E).

11.1 Patient recruitment, demographics and perioperative factors

During the study period, 167 patients required a laparotomy for abdominal gunshot wounds, of which 93 patients were excluded because they had no small bowel injuries and 12 were excluded due to demise within 72 hours of admission. In total, 62 patients were recruited. The demographics, injury severity, other relevant perioperative factors in the study population, as well as the injured organs, are listed in Table 11-1. The HIV-seropositive patients were significantly older than the HIV-seronegative patients.

Of the 24 HIV-seropositive patients, 12 patients were newly diagnosed, while 12 patients knew their HIV-status. Of those who knew their HIV-status, 10 (83%) were taking ART. The mean (±SD) treatment duration in the latter group was 5.5 (4.1) years. The median CD4+ T lymphocyte count of the entire HIV-seropositive study population on admission was 338 cells/μl (IQR 204-471). The median viral load was

8 010 copies/mL (IQR 368-64 450) which is equivalent to a mean (±SD) of 3.7 (1.6) Log copies/mL. No significant difference in the CD4+ T lymphocyte count between HIV-naïve and patients on ART was detected in the admission blood of the HIV-seropositive patients (HIV naïve: 320; IQR 196-440 vs. HIV treatment: 390; IQR 238-482, p = 0.58; data not shown). Only two HIV-seropositive patients showed clear signs of AIDS-defining conditions, both of them were treatment-naïve.

The main aim of the control group was to document lymphocyte quantity in subjects that are as close as possible to normal physiology. All operations were elective procedures, no control patient had emergency surgery. 7 specimens were taken from the distal small bowel during reversal of an ileostomy, 4 specimens were collected from the proximal small bowel, 3 patients had a Whipple's operation for pancreatic neoplasms, one a Frey procedure due to chronic pancreatitis.

The amount of blood products, including red blood cells, fresh frozen plasma and platelets did not differ between the groups.

Table 11-1. Demographics, perioperative factors and injured organs of study patients according to HIV-serostatus

Variable	HIV-seronegative patients (n=38)	HIV-seropositive patients (n=24)	P-value
Age, years	31 (25-40)	41 (32-47)	0.013
Sex			
Male	36 (95%)	22 (92%)	0.64
Female	2 (5%)	2 (8%)	
Time injury-to-operation (h)	4 (3-6)	5 (4-6)	0.51
PATI	27 (17-33)	27 (16-32)	0.79
pH, on admission	7,34 (7,26-7,43)	7,33 (7,26-7,36)	0.32
Lactate (mmol/L), on admission	4,5 (3,2-6,6)	4,7 (3,1-5,4)	0.82
Bicarb (mmol/L), on admission	21,7 (16,3-23,2)	21,9 (17,8-23,8)	0.59
BE (mmol/L), on admission	-4,1 (-8,8 to -1,0)	-4,0 (-7,4 to -1,6)	0.85
Fecal contamination			
Mild	5 (13%)	3 (13%)	
Moderate	10 (26%)	6 (25%)	>0.99
Severe	23 (61%)	15 (63%)	
Colonic injury	25 (66%)	13 (54%)	0.43
Liver injury	3 (8%)	4 (17%)	0.42
Stomach injury	5 (13%)	5 (21%)	0.49
Vascular injury	11 (29%)	4 (17%)	0.37
Involvement of other organs	10 (26%)	9 (38%)	0.40
Inotrope requirement	23 (61%)	15 (63%)	>0.99
Inotrope duration (h)	41 (7-66)	50 (24-96)	0.21
Hypotension (SBP<90mmHg), on admission	10 (26%)	7 (29%)	>0.99
Damage control surgery	19 (50%)	12 (50%)	>0.99

Continuous numerical variables presented as median (interquartile range [IQR]). Categorical variables expressed as absolute and relative frequencies (in brackets). *Abbreviations*: RBC, red blood cell; FFP, fresh frozen plasma; SBP, systolic blood pressure.

11.2 Comparison of lymphocyte quantity in the GALT in relation to HIV-infection and treatment

The LP of the HIV-seropositive patients showed a severe depletion of CD4+ T cells, with the median number (IQR) of CD4+ LPL significantly lower compared to the HIV-seronegative patients at 252 (176-413) cells/mm^2 vs. 905 (813-997) cells/mm^2, respectively (p = 0.0001). Conversely, the quantity of CD8+ LPL was significantly higher in HIV-seropositive (1 143; IQR 826-1 235 cells/mm^2) vs. HIV-seronegative (512; IQR 410-572 cells/mm^2) patients (p = 0.0001). In the HIV-seropositive patient group a significantly lower CD4+LPL count and a significantly higher CD8+LPL count was associated with a significantly higher rate of septic complications compared to the HIV-seronegative patients (p = 0.002); data shown in Figure 11-1 and Table 11-2. In four (17%) of the HIV-seropositive patients villous blunting as a typical feature of HIV-enteropathy was observed.

Within the group of HIV-seropositive patients, there was no significant difference between the treatment-naïve patients and the patients on ART in the number of the CD4+ LPL [treatment-naïve 229 (IQR 166-260) vs. treated 349 (IQR 225-425) cells/mm^2; p = 0.18] and CD8+ LPL [treatment-naïve 1 175 (IQR 878-1 307) vs. treated 979 (IQR 778-1 231) cells/mm^2; p = 0.27; data not shown].

The inclusion and analysis of the non-trauma, HIV-seronegative control group provides reference values for CD4+ LPL and CD8+ LPL. These reference values are as follows [median(IQR)]: CD4+ LPL: 1 121 (720-1 199); and CD8+ LPL: 414 (371-612) cells/mm^2.

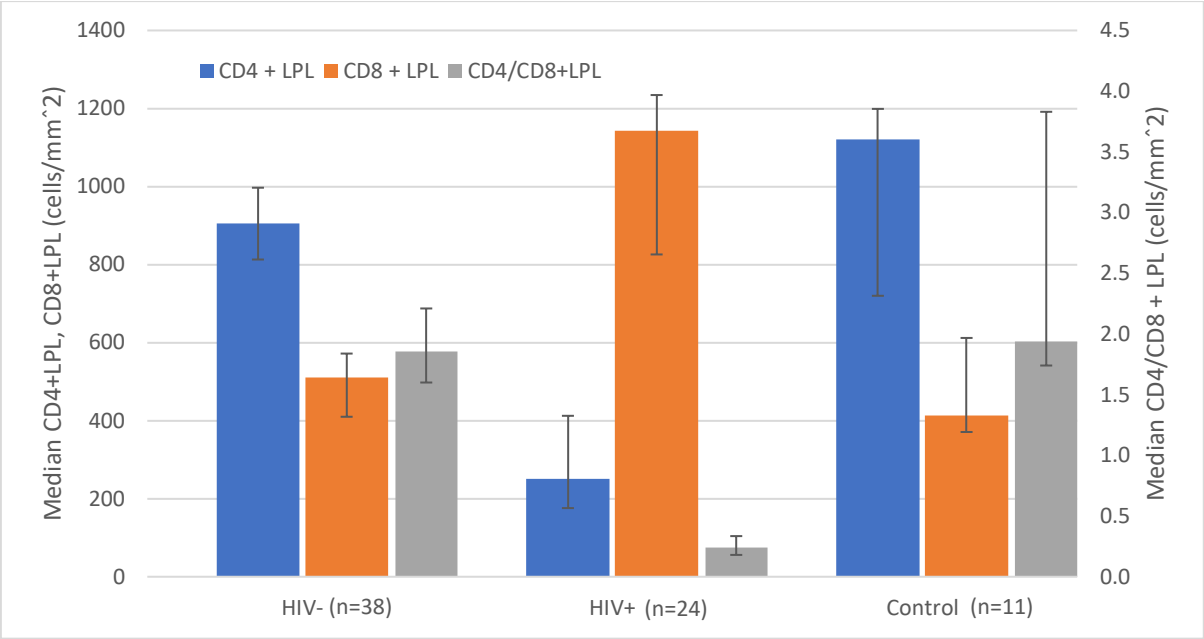


Figure 11-1. Comparison of CD4+ and CD8+ lamina propria lymphocyte quantity and CD4+/CD8+ ratio in GALT between study and control groups

Abbreviations: LPL, lamina propria lymphocytes.

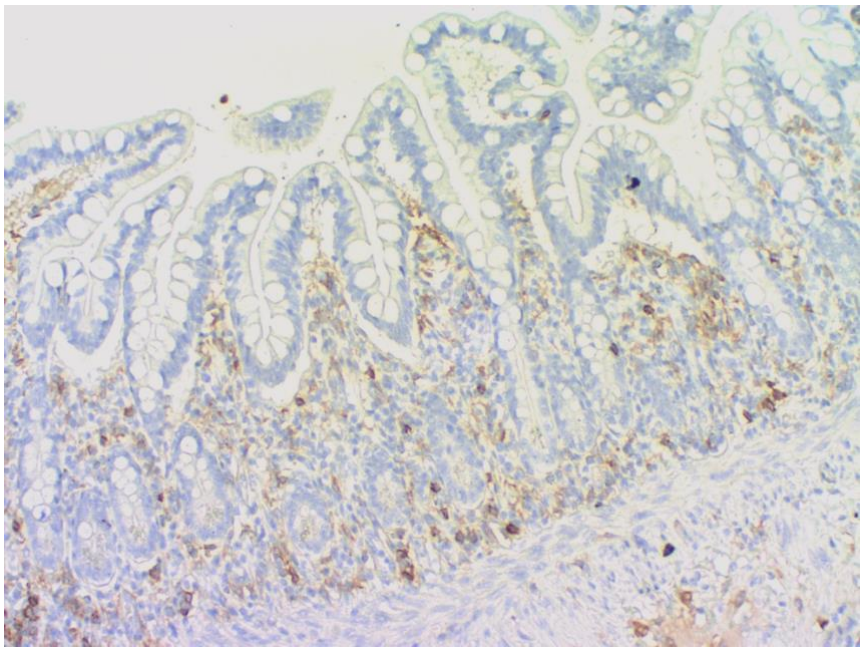
Table 11-2. Comparison of quantity of CD4+LPL and CD8+LPL between HIV-seronegative and HIV-seropositive patients in relation to septic complications

	HIV-seronegative patients (n=38)	HIV-seropositive patients (n=24)	p-value
CD4+LPL (cells/mm^2)	905 (813-997)	252 (176-413)	0.0001
CD8+LPL (cells/mm^2)	512 (410-572)	1 143 (826-1 235)	0.0001
Patients with septic complications	20 (53%)	22 (92%)	0.002

Continuous numerical variables presented as median (interquartile range [IQR]). Categorical variables expressed as absolute and relative frequencies (in brackets). Abbreviations: LPL, lamina propria lymphocytes; mm, Millimeter

Figure 11-2 displays microscopic images following IHC and shows the difference of the CD4+ LPL between a treatment-naïve HIV-seropositive patient (Figure a) and a HIV-seronegative control patient (Figure b), with CD4+ T cells embrowned.

a



b

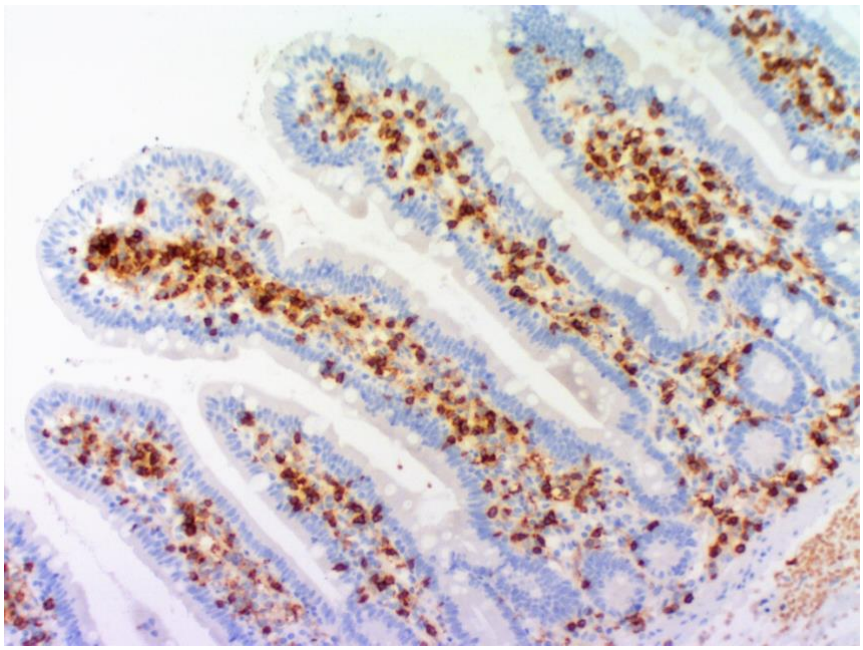


Figure 11-2. Immunohistochemistry analysis of CD4+ T cells in the lamina propria of a treatment-naïve HIV-seropositive patient (a) and a HIV-seronegative patient (b). *Specification: small bowel, x100 magnification.*

11.3 Clinical course and complications

In the postoperative follow-up of the study patients, significantly more patients with septic complications (p = 0.002; Table 11-3) were observed in the HIV-seropositive group compared to the HIV-seronegative group. The higher complication rate was primarily due to superficial surgical site infections that were significantly more common in the HIV-seropositive patients (p = 0.005; Table 11-3). HIV-seropositive patients had an anastomotic leak rate of 38%, HIV-seronegative patients leaked in 18% of the cases; this difference did not reach statistical significance (p = 0.14). With regard to the time of diagnosing the anastomotic leak, the mean (±SD) time to notice this complication was significantly later in the HIV-seropositive patients with 10.4 (1.4) days, compared to the HIV-seronegative patients [6.9 (1.6) days; p = 0.0003].

A subanalysis between the treatment-naïve HIV-seropositive patients and patients on ART showed a similarly high complication rate in both groups, and the patients on ART did not have significantly fewer septic problems (Table 11-4).

Table 11-3. Comparison of clinical course and complications according to HIV-serostatus

Parameter	HIV-seronegative patients (n=38)	HIV-seropositive patients (n=24)	P-value*
Septic complications	20 (53%)	22 (92%)	**0.002**
Superficial SSI	17 (45%)	18 (75%)	**0.005**
Deep SSI	8 (21%)	8 (34%)	0.22
Bacteraemia (positive BC)	14 (37%)	14 (58%)	0.28
Pneumonia			
No pneumonia	29 (76%)	16 (67%)	
VAP	5 (13%)	5 (21%)	0.13
Postop pneumonia (not ventilator-associated)	4 (11%)	3 (13%)	
Septic intraabdominal collection	16 (42%)	14 (58%)	0.30
Anastomotic leak	7 (18%)	9 (38%)	0.14
Repair break down	3 (8%)	3 (13%)	0.66
Number of relooks	1 (0-3)	1 (0-4)	0.40
ICU-LOS (in days, censored for death)	30 (15-ne)	18 (5-33)	0.78
Ventilatory days	6 (3-15)	4 (4-18)	0.93
Renal failure			
Dialysis required	5 (13%)	4 (17%)	0.67
Dialysis not required	5 (13%)	1 (4%)	
Clavien-Dindo classification			
0-2, minor	19 (50%)	7 (29%)	0.12
3-5, major	19 (50%)	17 (71%)	
Mortality	7 (18%)	9 (38%)	0.14

*__Bold__ values are statistically significant. Continuous numerical variables presented as median (interquartile range [IQR]). Categorical variables expressed as absolute and relative frequencies (in brackets). *Abbreviations*: BC, blood culture; ICU-LOS, intensive care unit-length of stay; SSI, surgical site infections; VAP, ventilator-associated pneumonia; ne, not estimable

Table 11-4. Sub-analysis for septic and surgical complications of HIV-seropositive patients according to treatment status

	HIV-seropositive naïve (n=14)	HIV-seropositive on treatment (n=10)	p-value
Number of septic complications			
0	0 (0%)	2 (20%)	0,16
1 or more	14 (100%)	8 (80%)	
Clavien-Dindo classification			
0-2 minor	4 (29%)	3 (30%)	>0,99
3-5 major	10 (71%)	7 (70%)	

Categorical variables expressed as absolute and relative frequencies (in brackets).

(see statistical raw data in Appendix G)

12 Discussion of objectives 1 and 2 (paper 1)

This study determined the T-lymphocytic quantity in the LP of the GALT in the small bowel and investigated whether it has an influence on the postoperative course after penetrating abdominal trauma. Moreover, special emphasis was to investigate whether HIV-infection and, specifically, preexisting HIV-induced changes of the GALT had an impact on postoperative septic complications.

The demographic profile, perioperative factors, injury pattern and severity showed no significant differences between HIV-seropositive and HIV-seronegative patients, hence the groups were well matched, except for a significant difference in age between the two groups.

In the analysis of the GALT, we observed that HIV-seropositive patients had significantly less CD4+ T cells and significantly more CD8+ T cells in the LP compared to the HIV-seronegative study patients and the non-trauma, HIV-seronegative control group. Lower CD4+ T cells and higher CD8+ T cells were also associated with an increased rate of septic complications in this study population.

Our finding reflects Brenchley *et al.'s* observation in which he described a severe depletion of the mucosal CD4+ T cells after primary HIV-infection that persists for the further course of disease resulting in a severe lymphopenia (186). The cause for this massive decrease of CD4+ LPL is still debatable and is most likely multifactorial; virus-induced Fas/FasL-mediated apoptosis, virus triggered cytolysis, lymphocyte destruction by cytotoxic T lymphocytes and NK-cells as well as activation-induced cell death of lymphocytes seem to play a role (202).

Among the HIV-infected patients in our study, we did not find a significant quantitative difference of CD4+ T cells in the GALT of treatment-naïve patients and patients on ART. The patients on treatment showed only a slightly higher CD4+ T cell count on average, indicating a partial recovery, but their numbers fell far short of the HIV-negative study members, which confirms results of current HIV-research (190).

We observed a significantly higher quantity of CD8+ T cells in the LPL of the HIV-seropositive patients compared to the HIV-seronegative patients. Despite the observation that CD8+ T cell-induced responses are essential for viral control and limit the viral replication in the acute and chronic disease phase (203,204), the accumulation of activated CD8+ T cells in the LP also seems to have a negative effect on the reexpansion of the CD4+ T cell pool (188,190).

In line with the findings of the CD4+ cell count in the HIV-seropositive patients, we also did not detect a significant difference in the CD8+ T cell count between treatment-naïve and treated patients, showing again the insufficient efficacy of the ART in restoration of lymphocytes in the LP of the GALT, a finding broadly supported by current HIV research (205–207).

Significantly more patients in the HIV-seropositive group suffered septic complications, especially superficial surgical site infections played a major role. More than a third of the HIV-infected patients was affected by an anastomotic leak which is an unacceptably high number and makes intrinsic, systemic causes, such as an impaired GALT, likely.

Fascial as well as anastomotic healing are reparative processes involving fibroblasts that are essential for remodeling the extracellular matrix and collagen synthesis. The fibroblast function is regulated by cytokines derived from monocytes/macrophages as well as from CD4+ T-lymphocytes (208). The severe depletion of T-lymphocytes in the GALT may result in an altered fibroblast activity which affects the collagen and proteoglycan synthesis and influences wound strength and resilience. The latter is further supported by the finding that patients who suffered from an anastomotic leak had a significant lower CD4+ T cell count in their GALT compared to patients without a leak. The observation of impaired wound- and anastomotic healing is further supported by a rat model showing that a severe decrease of CD4+ T cells resulted in weaker wounds measured with several mechanical properties (209).

The analysis of the T-lymphocytic quantity of the GALT revealed a significant association between a decreased quantity of CD4+ LPL and more septic postoperative complications. The loss of these immune cells in the GALT may therefore be a predisposing factor for propagation of enteric bacteria into the blood stream with subsequent septic events. Supporting this presumption is the

impressive mouse model from Gautreaux *et al.* in which T cell depletion resulted in a significantly higher translocation of E. coli from the small bowel (64).

However, the interpretation of positive blood cultures as proof of bacteraemia is difficult as the source of bacteraemia in trauma patients may originate from multiple sites, i.e. infected bullet tracts/muscles, or septic abdominal/intrathoracic collections. Hence it is not possible to attribute a positive blood culture to BT.

Identically low CD4+ T cell quantity in the GALT was documented as a common denominator in treatment-naïve as well as treated HIV-seropositive patients. There is no doubt about the beneficial role of the ART in the treatment and prognosis of HIV-infected patients. However, the very limited efficacy on the reconstitution of the GALT may be the “Achilles heel” in the perioperative phase. Several reports about the influence of HIV on surgical treatment documented that the treatment with antiretrovirals did not result in better outcomes or less complications which highlights the significance of this problem (150,210,211).

There is a paucity of data regarding the impact of age on the GALT. Quantity of LPL does not seem to be affected, but functional changes, especially decreased IL-2 production, was observed in higher age groups (212) which, in turn, could have contributed to the higher rate of septic complications in the HIV-seropositive study group that was also significantly older.

This part of our research project was not without limitations. The number of 62 patients only had the statistical power to determine large size effects. Hence, more subtle significant associations might have been missed, not because there is no significant relationship but because there is insufficient evidence due to the small sample size. Consecutive patient recruitment resulted in groups that were not age-

matched which could have led to biased results in the comparison between HIV-seropositive and -seronegative patients.

Furthermore, the vast majority of our patients were male patients, and only four female patients were recruited. This sex imbalance makes it difficult to generalize our results as the new insights gained related almost exclusively to men.

No considerations were given to the nutritional status of the patients. It is documented that hypoalbuminemia is a risk factor for impaired wound and anastomotic healing (213), hence inclusion of this variable would have improved the study design.

Reference values for the comparison with the study patients were derived from the control patients. For this purpose, we tried to select patients as close as possible to normal physiology, immune status and health. For the distal small bowel specimens, we recruited patients who underwent stoma reversal with practically no comorbidities. For the specimens of the proximal bowel however, we recruited three patients who had surgery for malignancies. It needs to be considered that all control patients were exposed to anaesthesia and surgical procedures of different duration before the specimen was obtained, which might have already resulted in changes compared to healthy individuals. In the three patients with neoplasms the values might have been influenced further due to the impact of the malignancy on the immune system.

13 Conclusion of objectives 1 and 2 (paper 1)

We documented in HIV-seropositive patients a significantly higher septic complication rate compared to the HIV-seronegative patients. HIV-seropositive patients had a significantly lower CD4+ T cell quantity and a significantly higher

CD8+ T cell quantity in their GALT which may predispose them to postoperative septic complications. The alterations of the GALT in HIV-seropositive patients on ART were persistent and almost unchanged in terms of CD4+ T cell recovery.

14 Results of objective 3 (paper 2)

Title Paper 2: Trauma results in immune cell-induced intestinal epithelial damage with subsequently increased sepsis rate

Status: published – Journal of Trauma and Acute Care Surgery (2021) 90(3):565-573 (Appendix E).

14.1 Demographics and perioperative factors

Patient recruitment is summarized again in Fig.14-1. Altogether 31 patients with severe physiological compromise on presentation were recruited, of which 61% were HIV-seronegative and 39% were HIV-seropositive. The patient demographics and other relevant perioperative factors are listed in Table 14-1 and show no significant differences in these DC-patients when grouped according to HIV-serostatus.

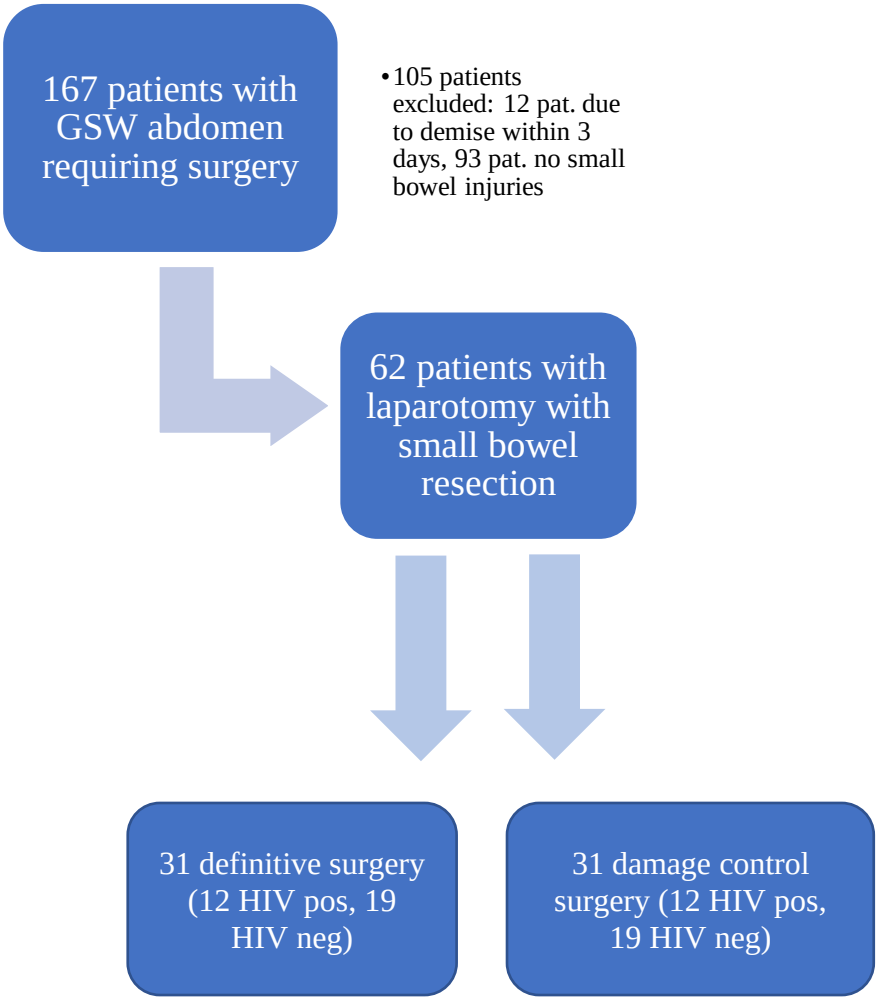


Figure 14-1. patient recruitment during study period

Table 14-1. Demographics and perioperative factors of the damage control (DC)-patients

Variables	HIV-seronegative patients (n=19)	HIV-seropositive patients (n=12)	P-value
Age, years	30 (23-40)	39 (28-45)	0.14
Sex			
Male	19 (100%)	11 (92%)	0.39
Female	0 (0%)	1 (8%)	
Time injury-operation (h)	4 (3-6)	5 (3-5)	0.83
Time index operation-relook (h)	54 (44-67)	52 (40-63)	0.76
PATI	33 (30-38)	32 (29-37)	0.70
pH	7.36 (7.25-7.43)	7.27 (7.23-7.36)	0.17
Lactate (mmol/L)	4.5 (3.4-6.6)	4.9 (4.3-6.3)	0.81
Bicarbonate (mmol/L)	20.0 (14.6-22.3)	19.5 (16.7-23.4)	0.69
BE (mmol/L)	-5.1 (-9.5 to -1.3)	-5.2 (-12.0 to -3.5)	0.75
Fecal contamination			0.69
Mild	2 (11%)	0 (0%)	
Moderate	4 (21%)	3 (25%)	
Severe	13 (61%)	9 (75%)	
Colonic injury	14 (74%)	9 (75%)	>0.99
Liver injury	2 (11%)	4 (33%)	0.17
Stomach injury	4 (21%)	5 (42%)	0.25
Vascular injury	11 (58%)	3 (25%)	0.14
Involvement of other organs	5 (26%)	5 (42%)	0.45
Duration of postop. Inotropes (h)	42 (8-72)	50 (24-60)	0.54
Hypotension (SBP<90mmHg), on admission	9 (47%)	6 (50%)	>0.99
Blood products			
RBC			0.63
0	0 (0%)	0 (0%)	
1-2	6 (32%)	6 (50%)	
3-4	9 (47%)	4 (33%)	
5 or more	4 (21%)	2 (17%)	
FFP			0.56
0	1 (5%)	2 (17%)	
1-2	7 (37%)	3 (25%)	
3 or more	11 (58%)	7 (58%)	
units of platelets			>0.99
0	12 (63%)	8 (67%)	
1 or more	7 (37%)	4 (33%)	

Continuous variables presented as median (interquartile range [IQR]. Categorical variables expressed as absolute and relative frequencies (in brackets). *Abbreviations*: RBC, red blood cell; FFP, fresh frozen plasma; SBP, systolic blood pressure.

All DC-patients had a resection of the injured part of their small bowel during the index surgery and the bowel was left in discontinuity until the first relook procedure when the anastomosis was performed; median (IQR) time between index and first relook operation was 54 (42-63) h. With regard to the management of colonic injuries, nine patients (29.0%) had a primary repair of the colon, and 14 patients (45.2%) had a resection of the injured part of the colon in the index operation.

The mean (±SD) CD4+ T lymphocyte count in the blood of the HIV-seropositive patients in this subgroup was 353 (±217) cells/µL, with a median (IQR) viral load of 8780 (750-46700) copies/mL. A third of these patients (n=4) was on antiretroviral treatment.

14.2 Changes of the lymphocyte quantity and apoptotic density in the GALT between index and relook operation

The first bowel specimen, referred to as the index specimen, was taken during the first operation from the section of bowel that required immediate resection due to the trauma-induced injuries. The second specimen, referred to as relook specimen, was taken during the relook operation from the distal part of the afferent loop related to the resected area. The extent to which the afferent loop was dilated depended on the timing of the relook procedure, as the bowel was temporarily left in discontinuity after being stapled off during the index procedure.

Both specimens were taken in close proximity in relation to the distance from the duodeno-jejunal flexure. The two specimens offered the opportunity to investigate the rapidity and the extent of the changes of the LPL and the IEL. In addition, the apoptotic density of enterocytes, LPL and IEL were compared for these two specimens.

The overall results for the whole DC-group are presented in Figures 14-2 and 14-3. The apoptotic density of enterocytes ($p < 0.0001$) and LPL ($p = 0.0008$), as well as the median number of CD8+ IEL ($p < 0.0001$) increased significantly (Figure 14-2), while the CD4+ LPL ($p = 0.0001$) and CD8+LPL ($p < 0.0001$) decreased significantly when the relook specimen was compared to the index specimen (Figure 14-3).

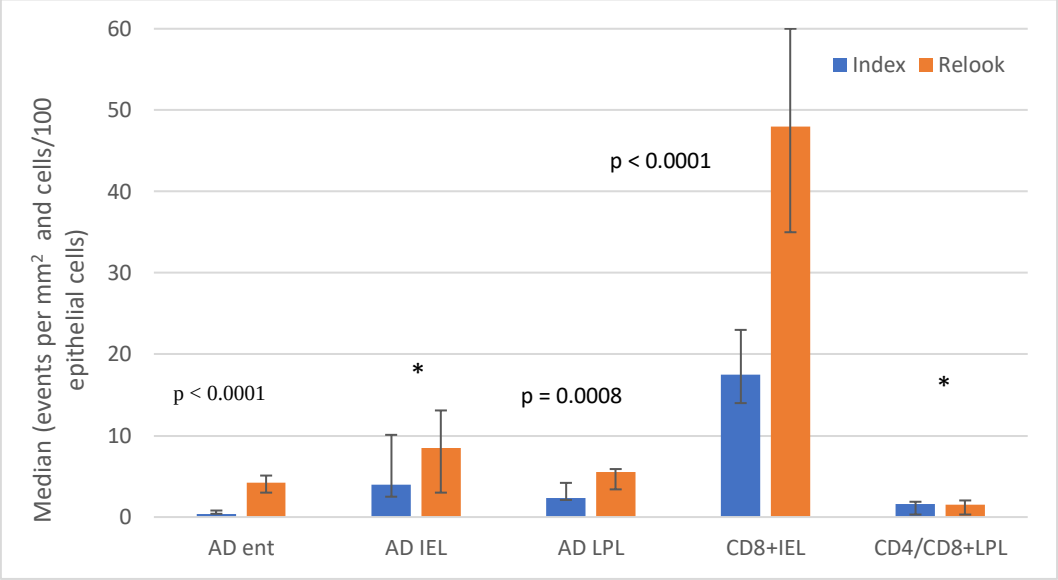


Figure 14-2. Comparison of apoptotic density (AD) and lymphocyte quantity (CD8+ IEL and CD4/CD8 ratio) in index and relook specimen

Abbreviations: AD: apoptotic density; ent: enterocytes; IEL: intraepithelial lymphocytes; LPL: lamina propria lymphocytes, * non-significant

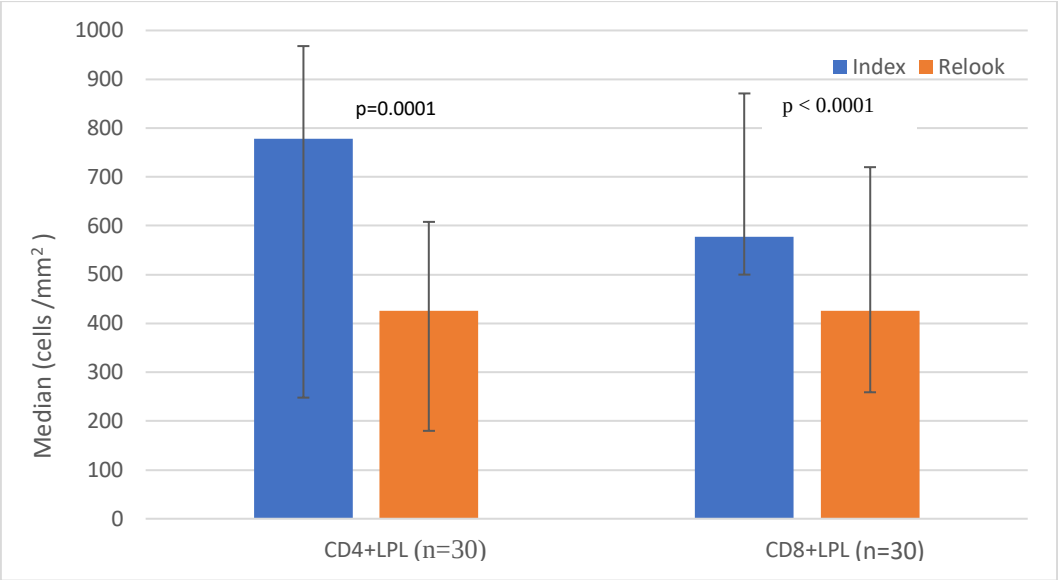


Figure 14-3. Comparison of the quantity of the lamina propria lymphocytes (CD4+LPL and CD8+ LPL) in the index and relook specimens

Abbreviations: LPL: lamina propria lymphocytes

The subgroup analysis of HIV-seronegative and -seropositive patients is shown in Table 14-2. The changes documented in HIV-seronegative and HIV-seropositive patients were comparable: the median number of CD8+ IEL in the relook specimen was significantly higher than the index specimen ($p = 0.0001$ and $p = 0.0033$, respectively), while the median numbers of CD4+ and CD8+ LPL significantly decreased (CD4+ T cells: $p = 0.0022$ and $p = 0.0041$, respectively; CD8+ T cells: $p = 0.0001$ and $p = 0.0033$, respectively). Furthermore, HIV-seronegative and HIV-seropositive patients had in the relook specimen a significantly higher apoptotic density of enterocytes ($p = 0.0001$ and $p = 0.0033$, respectively) and LPL ($p = 0.012$ and $p = 0.036$, respectively) compared to the index specimen.

Table 14-2. Changes of lymphocyte quantity and apoptotic density in index and relook specimens according to HIV-serostatus

Lymphocyte quantity, cells/mm^2 AD, events/mm^2	**Index specimen**	**Relook specimen**	**Difference (Relook – Index)**	**p-value**
CD4 LPL				
HIV-	925 (801-1091)	544 (423-774)	-351 (-553 to -151)	0.0022
HIV+	176 (155-400)	140 (98-310)	-63 (-79 to -10)	0.041
CD8 LPL				
HIV-	515 (421-572)	295 (223-446)	-162 (-281 to -122)	0.0001
HIV+	1148 (842-1206)	752 (673-932)	-248 (-425 to -86)	0.0033
CD4/CD8 ratio				
HIV-	1.8 (1.6-2.0)	1.9 (1.5-2.4)	0.0 (-0.3-0.6)	0.52
HIV+	0.2 (0.1-0.4)	0.2 (0.1-0.4)	0.0 (-0.1-0.0)	0.79
CD8 IEL				
HIV-	15 (3-24)	41 (31-60)	22 (15-35)	0.0001
HIV+	18 (15-23)	57 (47-61)	29 (26-43)	0.0033
AD enterocyte				
HIV-	0.4 (0.0-0.8)	4.2 (2.1-5.5)	3.0 (0.9-4.7)	0.0001
HIV+	0.4 (0.4-0.8)	4.2 (3.8-5.1)	3.4 (2.5-4.7)	0.0033
AD IEL				
HIV-	3.4 (2.1-10.5)	4.6 (2.5-10.1)	1.2 (-1.7-6.8)	0.33
HIV+	4.6 (2.5-10.1)	10.1 (3.8-18.1)	3.0 (-0.4-5.5)	0.29
AD LPL				
HIV-	2.1 (2.1-3.8)	5.5 (3.4-5.9)	2.1 (0.0-3.4)	0.012
HIV+	3.4 (1.7-5.5)	5.9 (4.2-8.9)	2.1 (0.8-2.9)	0.036

All variables presented as median (interquartile range [IQR]). *Abbreviations*: LPL, lamina propria lymphocytes; IEL, intraepithelial lymphocyte; AD, apoptotic density.

The comparison of LPL numbers of the control specimen and the index specimen of the HIV-seronegative study patients revealed lower CD4+LPL numbers for the HIV-seronegative patients but did not reach a significant level (p = 0.061, data not shown). Likewise, no significant difference was detected with regard to the apoptotic density. The relook specimens were then compared to the control group's specimens, further reaffirming the extensive trauma induced changes of the GALT (Table 14-3).

Table 14-3. Comparison of lymphocyte quantity and apoptotic density in control and relook specimen (overall)

LPL quantity, cells/mm^2; IEL quantity, cells/100 enterocytes; AD, events/mm^2	**Relook**	**Control**	**P-value**
CD4 LPL	426 (180-608)	1121 (720-1199)	0.0001
CD8 LPL	427 (259-720)	414 (371-612)	0.72
CD4/CD8 ratio	1.5 (0.3-2.0)	1.9 (1.7-3.8)	0.017
CD8 IEL	48 (35-60)	17 (11-26)	0.0002
AD enterocyte	4.2 (3.0-5.1)	0.4 (0-0.4)	<0.0001
AD IEL	8.5 (3.0-13.1)	3.4 (2.1-5.1)	0.038
AD LPL	5.5 (3.4-5.9)	2.1 (1.7-3.4)	0.0072

All variables presented as median (interquartile range [IQR]). Abbreviations: LPL, lamina propria lymphocytes; IEL, intraepithelial lymphocyte; AD, apoptotic density.

Picture 14-4 shows a normal epithelium of the index specimen with few interspersed CD8+ T lymphocytes in the epithelium. As already mentioned, the most obvious change in the relook specimen was the shift of the CD8+ T cells from the LP into the epithelium as shown in Picture 14-5.

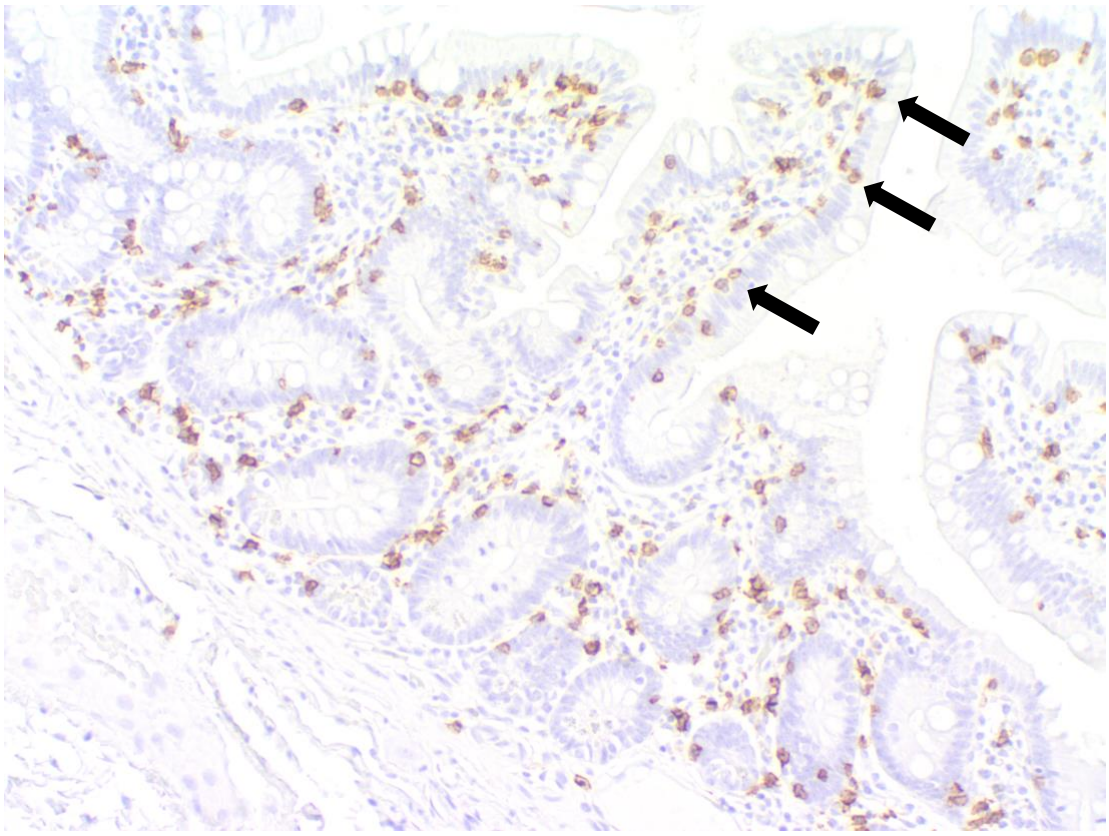


Figure 14-4. normal epithelial structure, with few intraepithelial CD8+T lymphocytes in index specimen (control patient).

Abbreviations: IHC CD8, small bowel specimen, x100 magnification

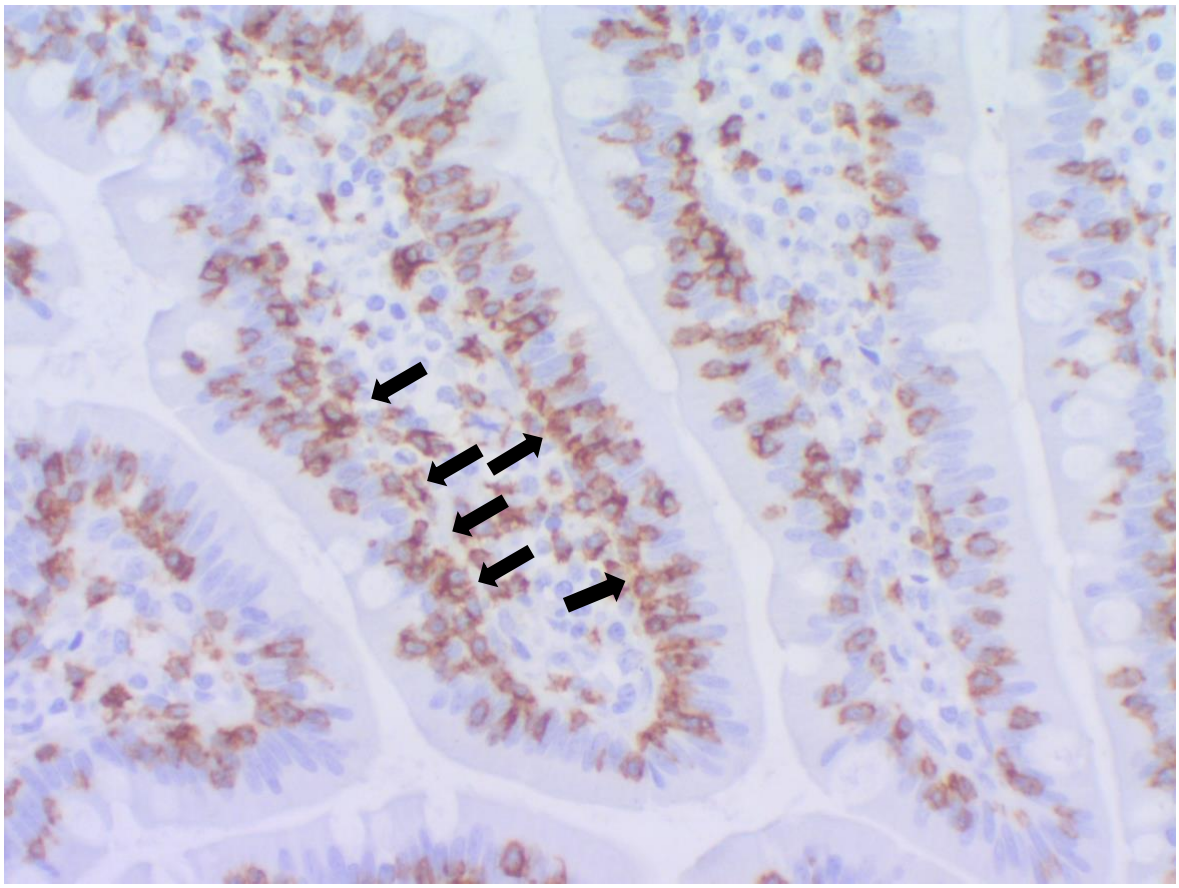


Figure 14-5. Epithelium densely packed with CD8+ T lymphocytes (arrows) in the relook specimen

Abbreviations: IHC CD8, small bowel specimen, x200 magnification

The absolute differences of the CD8+ LPL and the CD8+ IEL between index and relook specimen correlated significantly and negatively which confirms this observation (rho = -0.43; p = 0.017).

The decline of CD8+ T cells was more pronounced in the area of the villi than in the area of the base region of the muscularis mucosae. The majority of CD8+ T cells seemed to have crossed the basement membrane and invaded the epithelium.

With regard to the CD4+ T cells, we noted a decrease of these cells in the LP of the relook specimen, also more pronounced in the villi than in the muscularis mucosae-directed area. Almost no CD4+ T cells were noted in the epithelium.

To determine if and which physiologic alterations had an influence on the quantitative changes of LPL and IEL between index and relook specimen, we investigated the impact of the following clinical variables on the GALT: PATI, time from index operation to relook, duration of inotropic requirement, initial pH, Lactate, hypotension on admission and blood transfusion requirement in the first 48 hours . None of the examined variables had a significant impact on the quantitative percentage change of LPL and IEL (data not shown).

14.3 Correlation of IEL quantity and enterocyte apoptotic density

To investigate the impact of the epithelial invasion by the CD8+ lymphocytes on the epithelium, the absolute number of CD8+ IEL in the relook specimen as well as the increase of the CD8+ IEL between index and relook specimen was correlated with the apoptotic density of the enterocytes. A higher number of CD8+ IEL in the epithelium of the relook specimen was associated with a significantly higher apoptotic density of enterocytes ($r = 0.52$; $p = 0.0033$). Correspondingly, a more severe influx of CD8+ T cells into the epithelium was correlated with a higher enterocyte apoptotic density ($r = 0.47$; $p = 0.0089$). The apoptotic density of the enterocytes may be interpreted as a marker of epithelial damage.

14.4 Clinical course and complications of DC-patients

As seen in Table 14-4, no significant differences with regard to septic and surgical postoperative complications were detected between the HIV-seropositive and -seronegative groups. The requirement for organ support, i.e. ventilation and dialysis, was also comparable. Despite having a similar complication profile, the

percentage mortality of almost 60% in the HIV-seropositive group was considerably higher than in the HIV-seronegative group, although this did not reach statistical significance, probably due to small numbers.

In total, 13 DC-patients suffered from an anastomotic leak. Seven leaks resulted from small bowel, five from colonic anastomosis and one patient leaked from small bowel- and colonic anastomosis.

Table 14-4. Clinical course and complications of DC-patients

Parameters	HIV-seronegative patients (n=19)	HIV-seropositive patients (n=12)	p-value
Septic complications (total)	17(89%)	12 (100%)	0.51
Superficial SSI			0.14
no	5 (28%)	0 (0%)	
yes	13 (72%)	9 (100%)	
Deep SSI			>0.99
no	10 (59%)	4 (50%)	
yes	7 (41%)	4 (50%)	
Bacteraemia (pos. BC)			>0.99
no	5 (26%)	2 (18%)	
yes	14 (74%)	9 (82%)	
Pneumonia/VAP			0.70
no pneumonia	11 (58%)	5 (42%)	
VAP	5 (26%)	5 (42%)	
Postoperative pneumonia (not ventilator-associated)	3 (16%)	2 (17%)	
Septic intraabdominal collection	15 (79%)	10 (83%)	>0.99
Anastomotic leak			0.71
no	11 (61%)	6 (50%)	
yes	7 (39%)	6 (50%)	
Repair break down			>0.99
no	5 (63%)	6 (75%)	
yes	3 (38%)	2 (25%)	
Number of relooks	3 (1-6)	4 (1-4)	0.88
ICU-LOS (in days, censored for death)	30 (4 - not est)	18 (5 - not est)	0.70
ventilatory days (n=29)	8 (4-15)	7 (4-17)	0.88
Renal failure			0.65
no renal failure	11(58%)	9 (75%)	
requiring dialysis	5 (26%)	2 (17%)	
Renal dysfunction not requiring diaysis	3 (16%)	1 (8%)	
Clavien-Dindo classification			0.63
0-2 minor	2 (11%)	2 (17%)	
3-5 major	17 (89%)	10 (83%)	
mortality	7 (37%)	7 (58%)	0.29

Continuous variables presented as median (interquartile range [IQR]). Categorical variables expressed as absolute and relative frequencies. *Abbreviations*: BC, blood culture; ICU-LOS, intensive care unit-length of stay; SSI, surgical site infections; VAP, ventilator-associated pneumonia; not est, not estimable.

14.5 Impact of the lymphocyte quantity of the GALT in the relook specimen on the clinical outcome

The analysis of the relook specimen with regards to postoperative surgical complications revealed a significantly higher median number [IQR] of CD8+ IEL in patients with major postoperative complications compared to those with minor complications (52 [38-60] per 100 enterocytes versus 21 [20-35] per 100 enterocytes, p = 0.020).

A significant association was observed between the epithelial variables and septic complications: the higher the number of epithelial-invading CD8+ T cells, the higher the number of septic complications (r = 0.57; p = 0.0009). Higher apoptotic density of enterocytes and IEL apoptosis were also significantly and positively correlated with the number of septic complications (enterocyte apoptosis: r = 0.65; p = 0.0001, IEL apoptosis: r = 0.45; p = 0.013).

A sub-analysis was performed to check whether more extensive changes of the GALT-lymphocytes were associated with more septic or surgical postoperative complications. Patients with major surgical complications had a significantly higher percentage decrease of CD8+ LPL compared to the patients with minor complications according to Clavien-Dindo classification (major complications -36%; IQR -44% to -24% vs. minor complications -7%; IQR -14% to -5%, p = 0.0034).

The percentage change in the number of CD8+ LPL was significantly negatively correlated with the number of septic complications (r = -0.43; p = 0.016), while the CD8+ IEL changes were significantly positively correlated with those (r = 0.42; p = 0.020).

(see statistical raw data in Appendix G)

15 Discussion of objective 3 (paper 2)

This study investigated the extent to which trauma affects the GALT of the small intestine and the factors having the strongest impact on these alterations. We also explored whether the extent and nature of the lymphocytic changes influence the postoperative course with respect to septic and surgical complications.

The main changes between the index and relook specimen were a shift of CD8+ T cells from the LP into the epithelium and a decrease of T-lymphocytes in the LP. The significant increase of the intraepithelial CD8+ T cells was associated with a more extensive enterocyte apoptosis as well as apoptosis of the IEL.

The majority of the LPL are terminally differentiated effector memory cells which is a distinctive phenotype encountered in the gastrointestinal tract. These cells develop from central memory T cells which in turn derive from naïve T cells after antigen exposure (214). Effector memory cells are characterized by a very low activation threshold. An already low quantity of antigens originating from mucosal pathogens or their intracellular components that breach the mucosa, may reactivate these immune cells to respond quickly and specifically within 12 to 24 hours (215,216). This explains the extensive changes that were observed in the relook specimen within 48 hours.

Notably, up to 85 IEL per 100 enterocytes were observed in the relook specimen, numbers that far exceed the reported norm values of IEL which are 10-20 per 100 enterocytes (217). This demonstrated that trauma with tissue destruction and inflammation triggers a pronounced response of CD8+LPL. Once activated, they

traverse the basement membrane and invade the epithelium, and hence become intraepithelial lymphocytes.

High mobility of T cells described in murine models has shown that the LP and epithelium are not compartments that are closed off from each other by the basement membrane. Memory CD8+ T cells that are located in the LP, shift in response to pathogen invasion into the epithelium (10,218), findings that we have now observed in the bowel specimens of trauma patients.

The CD8+ IEL have primarily a protective function for the gut mucosal barrier. Located at the basement membrane and interacting with enterocytes, they constantly monitor the epithelium and peripheral LP (219) in order to counteract intruding pathogens (220). Expression of cytolytic genes and the cytolytic capacity of the IEL help remove damaged and infected cells (13).

However, in the relook specimen, the extensive influx of CD8+ T cells into the epithelium was accompanied by a significantly higher enterocyte apoptosis compared to the index specimen. This suggests a T cell triggered damage to the enterocytes which is also supported by the proximity of the IEL and enterocytes. The main property of activated CD8+ T cells is cytotoxicity, which is enabled by a huge quantity of perforin carried in these effector memory cells (214). The primarily protective function of CD8+ IEL turns into a detrimental response in the acute phase of trauma as a downregulation of these activated cells does not occur timeously.

The apoptotic density of the IEL was significantly higher in the relook specimen compared to that of the control group. Apoptosis on the maximum and subsequent downphase of the immune response is described as activation induced cell death (AICD) and most likely explains the high apoptotic density of the CD8+ IEL that we

observed in the relook specimen. Activated effector memory cells have a high propensity for apoptosis; more than 90% of activated CD8+ T cells undergo apoptosis, thus the majority of them are susceptible to AICD (221). AICD is a T cell receptor-mediated type of apoptosis which aims to decrease the number of reactive and overactivated cells, thus trying to control the immune response (222).

A significant numeric decrease of CD4+ LPL was observed in the relook specimen compared to the index- as well as to the control specimen. After the index surgery the decrease in the CD4+ LPL was less extensive in the HIV-seropositive patients. The percentage of CD4+ T cells with the most vulnerable effector memory phenotype becomes smaller in the GALT in the chronic HIV disease state (157) which may contribute to the less severe decrease compared to the HIV-seronegative patients.

In human tissue, an extensive upregulation of the apoptotic receptor FasL on lymphocytes has especially been observed in the intestine, driven by TNFα that is liberated by T cells early on in trauma (223,224). The TNFα levels correlate with the extent of the post-traumatic SIRS response (225,226) and might also contribute to the intestinal increased lymphocyte apoptosis.

The investigation which perioperative factors induce the most severe changes in the GALT did not reveal any significant results. In other words, we were unable to identify which stimulus is the strongest contributor to intestinal lymphocyte apoptosis. Factors such as injury- and shock severity with concomitant tissue destruction which are known to have an impact on the posttraumatic SIRS response (227), had no significant impact on lymphocyte apoptosis and -decrease in the GALT. Since no external factor could be identified, it is suggestive that genetically

determined elements, such as gene expression on immune cells, contribute to the extent of lymphocytic changes (228).

The complication profile and rate were similar among the recruited DC-patients, irrespective of the HIV-status. While the mortality rate in the HIV-group was considerably higher, it did not reach a significant level. Extreme physiological compromise in DC-patients seems to raise an extreme challenge even for the patients without comorbidities, often leading to dismal outcomes.

With standard techniques in DC surgery, such as stapling off and leaving the bowel in discontinuity until the relook, an iatrogenic bowel obstruction is created which may further compromise the physiologic status of these patients. Bacterial overgrowth has been noted in animal research as early as six hours after the onset of bowel obstruction, accompanied with an extensive rise of enteric bacilli (56). In the postoperative course, the bowel flora dysbiosis might be further aggravated from a prolonged paralytic ileus due multiple relooks.

Our study supports that DC patients are affected by the three main predisposing factors of bacterial translocation: firstly, bacterial overgrowth due to stasis of the faeces after stapling off the small bowel; secondly, epithelial damage due to invasion of CD8+ T cells; and thirdly, insufficiency of the local immune system as result of the decreasing LPL quantity.

Invasion of the epithelium by CD8+ T cells with a subsequent higher apoptotic density of enterocytes was associated with a worse clinical outcome. Patients who had a more severe influx of these cells suffered more septic complications in their postoperative course. Likewise, it was documented that patients with major postoperative complications, including organ failure, had a significantly higher level

of CD8+ IEL, compared to those with minor complications. These two associations might indirectly show that the immune cell-driven epithelial damage may promote bacterial translocation and warrants further investigation.

After observing the decrease of LPL and the accelerated activation of CD8+ LPL followed by apoptosis, apoptosis-based therapeutic agents may offer an attractive therapeutic medical approach. Specifically, slowing down the lymphocyte activation and the immune cell driven enterocyte apoptosis may stabilize the gut mucosal barrier and thus reduce septic complications.

A research project with a murine model in which segments of the terminal ileum were devascularized simulated the scenario of abdominal injury and demonstrated the possible value of antiapoptotic agents. Histologically documented bowel damage and apoptosis correlated positively with increasing ischemia time, and were significantly decreased with a broad-spectrum caspase inhibitor (229).

Different classes of apoptosis-based agents are under ongoing investigation, the first clinical trials were performed in the early 2000s. The three most intensively studied drug classes are: firstly, drugs that influence the synthesis of Bcl-2, which functions as an antiapoptotic protein; secondly, drugs that inhibit death receptor binding (e.g. Fas); and lastly, drugs that block the caspases.

Therapeutic approaches should ideally be guided by laboratory observations whereby an extensively high apoptotic index of LPL and IEL may comfortably be diagnosed via a simple HE-staining. This might be a valuable marker in identifying high risk patients.

This part of our research project was not without limitations. Light microscopy after HE-staining was used in this project as an accepted method for the visualization of apoptotic events based on morphological changes such as karyorrhexis and pyknosis. As this is a fairly basic method, a confirmation with a second method such as caspase-3 staining (230) is recommended, but was not performed here. This could have led to missed apoptoses. We tried to minimize this problem by having the same experienced histopathologist analyse the apoptosis in all specimens.

This research project focused on acute quantitative changes in the first line of defense in the GALT which comprises the epithelium and LP. While GALT structures such as Peyer Patches and mesenteric lymph nodes were not included in the analysis, they might have played a part in explaining the complex pathophysiological phenomena in this lymphoid tissue.

Consecutive patient recruitment resulted in the participation of HIV-seropositive patients which are highly prevalent in the South African trauma patient population (231). While this gave us further insight into this special patient group, it might have also complicated the analysis of the data and discussion.

In the analysis of the clinical outcome, we did not achieve the patient number of the sample size calculation. We therefore might have missed significant associations, not because there was no significant relationship, but because there was insufficient evidence due to the small sample size. Since this project was a proof-of-concept study, the results will serve as basis for further investigation.

16 Conclusion of objective 3 (paper 2)

Our study shows that trauma-induced changes of the GALT include an extensive migration of CD8+ T cells from the LP into the epithelium, followed by significant

increases in enterocyte apoptosis. These epithelial changes are significantly correlated with the number of postoperative septic complications. A significant decrease of the quantity of LPL seems to further compromise the local gastrointestinal immune system and potentially opens the way for bacterial translocation.

While observed pathophysiological changes in the gut mucosa give support to the use of antiapoptotic agents, their real clinical value in the treatment of acute trauma patients still needs further investigation.

17 Results of objective 4 (paper 3)

Title Paper 3: Posttraumatic natural killer cell decrease is associated with septic complications

Status: submitted to Journal of Surgical Research October 2020, awaiting response

17.1 Patient recruitment, demographics and perioperative factors

Patient recruitment to investigate hypothesis four was identical to that of hypothesis one and two. The study population demographics, injury severity, other relevant perioperative factors, as well as the injured organs are shown in Table 11-1. Except for HIV-seronegative patients being younger ($p = 0.013$), no significant differences were seen for these variables when grouped according to HIV-serostatus.

17.2 Postoperative changes in lymphocytic quantity

Absolute numbers and decrease of CD3+ T cells, CD4+ cells, CD8+ cells, B-cells and NK cells were analyzed via flow cytometry and are presented in Table 17-1 and

17-2. For the control patients, blood samples were only taken on admission and 48 hours postoperatively. Blood results on admission reflected the common HIV-induced changes with a significantly lower CD4+ T cell count ($p = 0.0001$) and CD4/CD8 ratio ($p = 0.0001$) in the HIV-seropositive patients compared to the HIV-negative patients. The median NK cell count was significantly higher in the HIV-negative compared to the HIV-seropositive patients ($p = 0.037$).

In a subgroup analysis of the HIV-seropositive patients, no significant difference in the CD4+ T cell count between HIV-treatment naïve and patients on ART was detected on admission (HIV naïve, 320 (IQR 196-440) vs. HIV treatment, 390 (IQR 238-482), $p = 0.58$; data not shown).

Between A and P2 blood, the HIV-seronegative patients had a more significant decrease in CD4+ T cells than the HIV-seropositive patients (HIV-seronegative $p=0.0005$ vs. HIV-seropositive, $p=0.0069$). NK cell decrease was extensive, independent of the HIV-serostatus and resulted nearly in a depletion of this lymphocyte subtype in the P2-blood. Lymphocyte numbers differ in Table 17-1 and 17-2 as only study patients with a full set of A, P1, and P2-blood values were included in Table 17-2.

It was investigated whether there is an association between the postoperative NK-cell and CD8+ T cells changes which showed a significant, positive correlation between the NK-cell decrease and the CD8+ T cell decrease ($rho = 0.547$; $p < 0.0001$, data not shown). The B-lymphocytes were the only lymphocyte subtype that showed non-significant changes of all examined immune cells in study and control group during the postoperative course.

Table 17-1. Quantity of lymphocytes at admission (A-blood), 48 hours after the index operation (P1-blood) and 48 hours after the first relook operation (P2-blood) according to HIV-serostatus

Quantity, (cells/μL)	Control group	HIV-seronegative patients (n=38)	HIV-seropositive patients (n=24)	P-value
CD3+				
A-blood	1999 (1609-2550)	1425 (787-2257)	1187 (646-1813)	0.058
P1-blood	1058 (497-1716)[a]	533 (420-740)[b]	578 (383-777)[b]	0.029
P2-blood		317 (263-444)	331 (274-594)	0.47
CD4+				
A-blood	1216 (892-1340)[a]	816 (481-1151)[a]	338 (204-471)[b]	0.0001
P1-blood	582 (335-794)[a]	304 (235-408)[b]	164 (106-288)[c]	0.0001
P2-blood		180 (131-276)	118 (80-183)	0.039
CD8+				
A-blood	793 (618-1214)	471 (288-907)	571 (321-1298)	0.13
P1-blood	424 (172-658)[a]	186 (120-304)[b]	334 (247-525)[a]	0.0005
P2-blood		121 (77-172)	198 (149-450)	0.012
B-cell				
A-blood	258 (140-471)[a]	206 (132-335)[a]	120 (79-179)[b]	0.0029
P1-blood	199 (117-281)[a]	171 (132-244)[a]	110 (61-155)[b]	0.012
P2-blood		139 (101-264)	91 (43-125)	0.021
NK-cells				
A-blood	183 (146-276)	314 (121-535)[a]	165 (79-264)[b]	0.037
P1-blood	155 (59-169)[a]	63 (37-84)[b]	38 (20-91)[b]	0.015
P2-blood		29 (16-49)	19 (9-39)	0.40
CD4/CD8 ratio				
A-blood	1.14 (1.01-1.43)[a]	1.50 (0.97-1.96)[a]	0.40 (0.32-0.68)[b]	0.0001
P1-blood	1.34 (1.30-1.87)[a]	1.45 (1.14-2.17)[a]	0.43 (0.34-0.71)[b]	0.0001
P2-blood		1.61 (1.16-2.27)	0.46 (0.33-0.70)	0.0002

Variables presented as median (interquartile range [IQR]), different lowercase superscripts denote significantly different groups (where three groups were compared), same letters indicate no significant difference

Table 17-2. Lymphocyte decrease between A and P2-blood according to HIV-serostatus

Quantity, (cells/μL)	HIV-seronegative patients (n=16)	HIV-seropositive patients (n=10)
CD3+		
A-blood	1370 (925-2152)	1193 (888-2309)
P2-blood	317 (239-453)	331 (274-594)
P-value	0.0004	0.0051
CD4+		
A-blood	747 (444-1130)	320 (240-407)
P2-blood	188 (127-279)	118 (80-183)
P-value	0.0005	0.0069
CD8+		
A-blood	469 (315-852)	824 (486-1823)
P2-blood	110 (74-168)	198 (149-450)
P-value	0.0004	0.0051
B-cell		
A-blood	189 (114-317)	104 (67-149)
P2-blood	154 (118-280)	91 (43-125)
P-value	0.11	0.33
NK-cells		
A-blood	412 (213-556)	133 (83-258)
P2-blood	38 (19-52)	19 (9-39)
P-value	0.0004	0.0051
CD4/CD8 ratio		
A-blood	1.23 (0.77-1.85)	0.29 (0.18-0.58)
P2-blood	1.76 (1.33-2.43)	0.46 (0.33-0.70)
P-value	0.0035	0.15

Variables presented as median (interquartile range [IQR])

17.3 Perioperative clinical factors and quantitative changes in circulating immune cells

To determine whether any perioperative factors influence the quantitative changes of the circulating immune cells between index and relook operations, we investigated the impact of the following clinical variables: PATI, time from index operation to relook, duration of inotropic requirement, pH and lactate on admission, hypotension on admission, and blood transfusion requirements. Of these perioperative factors, only the duration of inotropes had a significant impact on the quantities of circulating immune cells. Specifically, patients who required inotropic support for more than 48 hours had a significantly lower decrease of CD3+ T cells and CD8+ T cells, as well as B-cells. The increased transfusion requirement did not result in any significant quantitative lymphocyte changes, i.e. the dilution effect of the transfused blood did not play any notable role overall.

17.4 HIV status, immune cells and postoperative outcome

In the overall analysis of the study group, including all HIV-seronegative and HIV-seropositive patients, septic complications were significantly associated with a lower median CD4+ T cell and B cell count, and a lower CD4/CD8 ratio on admission (Table 17-3). The subgroup analysis in the HIV-seropositive and HIV-seronegative patients did not reveal any significant associations between immune cell quantity on admission and postoperative septic complications.

Table 17-3. Influence of lymphocyte quantity on admission on septic complications

	No septic complications (n=20)	Septic complications (n=42)	P-value
CD3+ T cells	1815 (787-2301)	1280 (727-2152)	0.22
CD4+ T cells	816 (369-1328)	450 (256-772)	0.0094
CD8+ T cells	721 (316-907)	523 (285-1092)	0.96
CD4/CD8 ratio	1.30 (0.97-2.02)	0.73 (0.37-1.37)	0.0044
B-cells	226 (142-272)	135 (82-252)	0.049
NK-cells	261 (108-579)	196 (106-422)	0.46

Variables presented as median (interquartile range [IQR])

In the postoperative follow-up, significantly more patients with septic complications were observed in the HIV-seropositive group compared to the HIV-negative group (HIV+: 92% vs. HIV-: 53%; p = 0.002). Similarly, surgical site infections were significantly more common in HIV-seropositive group (HIV+: 86% vs. HIV-: 46%; p = 0.005). Even though the percentage of anastomotic leaks was twice as high as that of the HIV-negative patients, this did not reach statistical significance (HIV+: 38% vs. HIV-: 19%; p = 0.14). Organ support requirements and ICU-length of stay (LOS) did not show marked differences according to HIV-serostatus, and where the percentage of in-hospital mortality was almost double in the HIV-seropositive patients, this did not reach statistical significance (HIV+: 38% vs. HIV-: 18%; p = 0.14; data not shown).

To document whether patients with a more pronounced immune cell decrease within the first 48 hours are more prone to septic and surgical complications, we categorized the study patients in 2 groups, one group with an extensive decrease of 50% or more, the other with a decrease of less than 50%. Septic and surgical postoperative complications were also categorized as binary variables and results

of these analyses are shown in Table 17-4. Notably, a decrease of 50% more than in NK cells resulted in a significantly higher level of septic and surgical postoperative complications.

Table 17-4. The extent of lymphocyte decrease on postoperative outcome

% decrease in cellular subtype	Number of septic complications			Clavien-Dindo classification		
	0 or 1 (n=25)	2 or more (n=33)	p	0-2 (n=25)	3-5 (n=33)	p
CD3+ T cells			0.59			0.59
<50%	8 (38%)	13 (62%)		8 (38%)	13 (62%)	
≥50%	17 (46%)	20 (54%)		17 (46%)	20 (54%)	
CD4+ T cells			0.78			>0.99
<50%	10 (48%)	11 (52%)		9 (43%)	12 (57%)	
≥50%	15 (41%)	22 (59%)		16 (43%)	21 (57%)	
CD8+ T cells			0.79			0.79
<50%	9 (39%)	14 (61%)		9 (39%)	14 (61%)	
≥50%	16 (46%)	19 (54%)		16 (46%)	19 (54%)	
B-cells			>0.99			0.50
<50%	20 (43%)	27 (57%)		19 (40%)	28 (60%)	
≥50%	5 (45%)	6 (55%)		6 (55%)	5 (45%)	
NK-cells			0.042			0.006
<50%	8 (73%)	3 (27%)		9 (82%)	2 (18%)	
≥50%	17 (36%)	30 (64%)		16 (34%)	31 (66%)	

Categorical variables expressed as absolute and relative frequencies.

(see statistical raw data in Appendix G)

18 Discussion of objective 4 (paper 3)

In this study we determined how the main circulating immune cells change in the acute phase following penetrating abdominal trauma and whether there is a difference in the immune cell profile between HIV-seropositive and -negative

patients. Special focus was put on the role of the NK cells. Furthermore, it was investigated whether the extent of these changes affected the rate of postoperative septic and surgical complications.

Regardless of HIV-status, CD4+ T cells, CD8+ T cells and NK cells had the most severe decrease between admission and relook operation while the B cell numbers were only affected marginally. CD4+ T cell changes were more extensive in HIV-negative- than in HIV-seropositive patients. Of all examined immune cells, only a more severe decrease in NK cells within the first 48h was associated with more postoperative septic complications.

In addition to the trauma burden, HIV-seropositive patients are further jeopardized by their preexistingly compromised immune system. This was reflected in a significantly lower CD4+ T cell count in their admission blood compared to the HIV-negative patients.

The CD4+ T cell count in the seropositive group was only slightly higher for the patients on ART compared to the treatment-naïve patients. The numbers in a range between 300 and 400 cells/µL are comparable to numbers derived from an evaluation of 1310 HIV- patients of rural KwaZulu-Natal in South Africa (232). Notably, the study patients of our research project were almost exclusively male, who on average have a significantly lower CD4-counts compared to females, as analysed in the above mentioned study. Moreover, there is evidence that a high percentage of South African patients on ART have an inadequate rise of their CD4 count in their peripheral blood despite full viral suppression. The main reasons identified for the incomplete reconstitution in the peripheral blood are advanced age and low CD4 cell count nadir on treatment initiation (115). Since our patients were

not of advanced age, the main reason for the low admission CD4+ T lymphocyte count in the patients on ART may have been a delayed start of the antiretroviral treatment.

In the admission blood, the NK cell numbers of the HIV-negative patients were higher than those of the control- and the HIV-seropositive patients, reaching a significant level between the HIV-negative and HIV-seropositive groups. NK cells are predominantly activated by interferon and IL-12. They are also strongly affected by catecholamines, to a much higher extent than other lymphocyte subsets. There is evidence that trauma initially leads to a catecholamine-induced mobilization of NK cells from the spleen, bone marrow, lung, lymphatic tissue and marginal blood pool into the circulating blood, which might explain this early increase among the HIV-seronegative patients (233,234). Lower initial NK cell numbers in the HIV-seropositive patients might be due to HIV-induced quantitative alterations, especially of the CD56+ NK cell subset. Together with a disturbed cytokine pattern, this might lead to a reduced capacity to mobilize these cells in the initial trauma phase in response to the increased catecholamines (235,236).

In the P1- blood, a significant decrease of more than half of the T-lymphocytes and NK-cells was documented in the HIV-negative patients, while the B-lymphocytes declined only marginally. Notably the drop of T-lymphocytes was significantly higher in the HIV-negative compared to the HIV-seropositive patients. In the P2-blood that was taken 4 days after injury we demonstrated an almost complete depletion of NK-cells irrespective of the HIV-status. This early severe decrease after trauma is not supported by previous studies that describe only a reduced NK-cell *activity* (122) and not this substantial drop in absolute numbers. In homeostasis NK cells monitor lymphoid and non-lymphoid organs; a possible explanation for the severe decrease

might be a sequestration of these cells to the site of injury and inflammation (237). It has also been described that activated NK cells become more adherent to the vascular epithelium which may also have contributed to the massive drop of circulating and detectable cells in the blood stream (238).

In determining which perioperative factors have the strongest influence on quantitative changes in the immune cells, we report that only the duration of inotropes had a significant impact for the majority of the analyzed immune cells. Patients who required ongoing inotropic support for more than 48 hours had a significantly lower decrease of T- and B-lymphocytes. The less pronounced lymphocyte fall in the acute phase might be explained by adrenaline-induced mobilization of lymphocytes from a marginated pool into the peripheral blood. This influx is thought to derive mainly from the spleen, bone marrow, and walls of high-endothelial venules (239).

Injury severity did not result in significant changes in the immune cell quantity, hence results from previous research could not be confirmed (111).

The investigation which immune cells have a prognostic impact on the outcome revealed for the admission blood the importance of the CD4+ T lymphocyte count: patients with no postoperative complications had significantly higher CD4+ T cell counts on admission than those with complications.

During the postoperative course a more extensive decrease of NK cells in the study group was significantly associated with more septic and surgical postoperative complications. The main function historically attributed to NK cells has been protection against virus-infected- and tumor cells. NK cells may either stimulate and promote differentiation of T cells or may inhibit T cell activation and even induce apoptosis of these immune cells. This effect takes place via direct cell to cell

interaction or is cytokine-mediated (240). The importance of NK cells for fungal immunity as well as their antibacterial properties has become more evident over the last years (241,242), but is not without controversy.

While Ebbo *et al.* demonstrated that patients with low NK cell counts are at higher risk for invasive bacterial infections (243), there is evidence from a murine model that show improved outcomes after induced depletion of the NK cells: Barkhausen *et al.* demonstrated this impressively by having a decreased mortality after induced NK cell depletion in a murine trauma study. An improved outcome after depletion was associated with a lower IL-6 level, which is a central cytokine in the inflammatory response, and less lymphocyte apoptosis (125).

Moreover it has been documented that NK cell depletion results in higher numbers of CD8+ T cells which correlates with an improved function of antigen presenting cells and therefore a quicker clearance of an evolving infection (124). However, our data demonstrated the opposite: the NK cell decrease correlated significantly, positively with the CD8+ T cell decrease.

Current HIV research has provided evidence that NK-cells are involved in HI-virus control and suppression and play as important a role as CD8+ T cells. Via crosstalk with dendritic and other effector cells and via secretion of certain chemokines, NK cells are able to destroy HIV-infected target cells and suppress viral replication (244,245), hence the posttraumatic NK-cell decrease might affect HIV-seropositive patients more severely losing one more pillar of their armamentarium in the fight against HIV-infection.

Since we observed in this research project that patients with a more severe decrease of NK cells had more septic complications, we are not able to confirm the results of the abovementioned murine models and doubt whether they can simply

be translated into the human and trauma context. In fact, our findings encourage an approach of boosting the NK cell function with the aim of strengthening the immune system to control bacterial infections.

There are various options to enhance the NK cell function; either directly by targeting active ligands on the cell itself, such as the NKG2D-ligand, by the blockade of inhibitory receptors or indirectly via cytokine-driven activation. Immunotherapy with re-transfusion of externally expanded NK cell populations or transfusion of cryopreserved NK cells is under clinical investigation and may be especially beneficial for immunocompromised patients (246,247). The main problem of this therapeutic approach is a possible graft-versus-host-disease and the long-term storage of these cells (248,249).

This part of the research project is not without limitations. In the flow cytometric analysis all the lymphocytes that are CD3 negative and CD56 positive were counted and reported as NK cells; subanalysis and differentiation into $CD56^{bright}$ and $CD56^{dim}$ NK-cells were not conducted. Nonetheless, with the antibodies that were used, a correct reflection of the total quantity of circulating NK cells in the blood was achieved and the changes in the perioperative phase could be documented.

The patient number of this research project was only powered to determine large size effects, in the subgroup analysis we might have missed significant associations, not because they did not exist but due to the small numbers. Furthermore, the HIV-seropositive patients were not age-matched with the HIV-seronegative patients, the HIV-seropositive patients being significantly older than the HIV-seronegative patients, which needs to be considered in the interpretation of our data since the factor age itself results in a higher immune risk profile with lower circulating immune

cells (250). However clinical significance is only to be expected in higher age groups and hence should not have biased our results since our patients weren't of advanced age.

19 Conclusion of objective 4 (paper 3)

Our study shows that the impact of penetrating abdominal trauma leads to an almost complete depletion of NK cells in the blood. Patients with a more extensive NK cell decrease had a significantly higher rate of septic and surgical complications, suggesting the important role of these cells for antibacterial immunity. Strengthening the NK cell function or limiting their decrease in the postoperative course might be of therapeutic value in severe trauma patients and is worth exploring in future research.

20 Overall conclusion

The research project has shown that the GALT plays an important, complementing role in the immune system influencing the prognosis of HIV-seropositive as well as HIV-seronegative trauma patients.

The compromised GALT with a decreased quantity of CD4+ LPL in HIV-seropositive patients was associated with an increased rate of postoperative septic complications. HIV-seropositive patients on ART did not show an adequate immune reconstitution of their GALT indicating a persistently increased risk for postoperative complications despite being on antiretroviral medication.

The GALT of the relook specimens of patients who required DCS showed an extensive migration of CD8+ T cells from the LP into the epithelium followed by significant increases in enterocyte apoptosis. These trauma-induced changes of the GALT correlated with septic complications during the postoperative course. This correlation suggests that the immune cell-driven epithelial damage may promote bacterial translocation and warrants further investigation.

With regards to the cellular immunity, we observed a severe posttraumatic decrease of NK cells and T cells while B cells were not affected to that extent. Only the decrease of NK cells was associated with a significantly increased rate of septic complications, suggesting an important function of these cells in the antibacterial immunity.

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22 Appendices

Appendix A (Clearance certificate)

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R14/49 Dr ME Mauser, et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M180914

NAME: (Principal Investigator)	Dr ME Mauser, et al
DEPARTMENT:	School of Clinical Medicine Department of Surgery Chris Hani Baragwanath Academic Hospital
PROJECT TITLE:	The impact of penetrating abdominal trauma on gut-associated lymphoid tissue and the influence of HIV-infection
DATE CONSIDERED:	28/09/2018
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Professor F Plani
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	14/11/2018

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on 3rd floor, Phillip V Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.
I/We fully understand the conditions under which I am/we are authorised to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to resubmit to the Committee. I agree to submit a yearly progress report. When a funder requires annual re-certification, the application date will be one year after the date of the meeting when the study was initially reviewed. In this case, the study was initially reviewed in September and will therefore reports and re-certification will be due early in the month of September each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature _____ Date _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix B (PATI, small bowel injury scale, CD4 classification)

Table 1:

Penetrating Abdominal Trauma Index

The risk associated with injury to each organ is graded from 1 to 5
The estimated severity of each type of injury is graded from 1 to 5

Organ Injured	Risk Factor	Injury	Injury Estimate
duodenum	5	single wall	1
		≤ 25% wall	2
		>25% wall	3
		duodenal wall and blood supply	4
		pancreaticoduodenectomy	5
pancreas	5	tangential	1
		through-and-through (duct intact)	2
		major debridement or distal duct injury	3
		proximal duct injury	4
		pancreaticoduodenectomy	5
liver	4	nonbleeding peripheral	1
		bleeding, central or minor debridement	2
		major debridement or hepatic artery ligation	3
		lobectomy	4
		lobectomy with caval repair or extensive bilobar debridement	5
large intestine	4	serosal	1
		single wall	2
		≤ 25% wall	3
		>25% wall	4
		colon wall and blood supply	5
major vascular	4	≤ 25% wall	1
		>25% wall	2
		complete transection	3
		interposition grafting or bypass	4
		ligation	5
spleen	3	nonbleeding	1
		cautery or hemostatic agent	2
		minor debridement or suturing	3
		partial resection	4
		splenectomy	5
kidney	3	nonbleeding	1
		minor debridement or suturing	2
		major debridement	3
		pedicle or major calyceal	4

		nephrectomy	5
extrahepatic biliary	2	contusion	1
		cholecystectomy	2
		≤ 25% common duct wall	3
		>25% common duct wall	4
		biliary enteric reconstruction	5
small bowel	2	single wall	1
		through-and-through	2
		≤ 25% wall or 2-3 injuries	3
		>25% wall or 4-5 injuries	4
		wall and blood supply or >5 injuries	5
stomach	2	single wall	1
		through-and-through	2
		minor debridement	3
		wedge resection	4
		>35% resection	5
ureter	2	contusion	1
		laceration	2
		minor debridement	3
		segmental resection	4
		reconstruction	5
bladder	1	single wall	1
		through-and-through	2
		debridement	3
		wedge resection	4
		reconstruction	5
bone	1	periosteum	1
		cortex	2
		through-and-through	3
		intra-articular	4
		major bone loss	5
minor vascular	1	nonbleeding small hematoma	1
		nonbleeding large hematoma	2
		suturing	3
		ligation of isolated vessels	4
		ligation of named vessels	5

- The percent injury to an organ wall probably indicates the portion of the entire circumference involved.
- Organ-score=(risk factor) x (injury estimate)
- Penetrating abdominal trauma index (PATI) = SUM (all injured organs)
- Minimum PATI: 0
- Maximum PATI: 200

Table 2:

Small bowel injury scale (AAST)

Grad	Type of injury	Description of injury
I	Hematoma	Contusion or hematoma without devascularization
	Laceration	Partial thickness, no perforation
II	Laceration	Laceration $<$ 50% of circumference
III	Laceration	Laceration $\geq$ 50% of circumference without transection
IV	Laceration	Transection of small bowel
V	Laceration	Transection of small bowel with segmental tissue loss
	Vascular	Devasculralized segment

Table 3:

The CD4 –lymphocyte classification system for Human Immunodeficiency Virus (HIV) infection

Category	CD4 lymphocyte counts (cells/μl)
1	$>$500
2	200-499
3	$<$200

Appendix C (clinical evaluation sheet, histology sheet)

Form 1:

study number:

Patient name, sex, age:

Admission date and initial operation date:

Discharge date and HLOS (days):

Comorbidities:

Time from injury to theater (in hours):

HIV-status (pos=1, neg=0), new diagnosis (yes=1, no=0)
HIV pos diagnosed since (years)
HAART since (months)+drug combination
AIDS-defining conditions (yes=1, no=0):

Viral load:
Injuries sustained:

PATI:

Grade of small bowel/Grade of colon injury:

Resection and anastomosis done=1, primary repair only=2, stoma=3, clip and drop=4:

Location of biopsy in the small bowel (proximal=1, mid-small bowel=2, distal=3):

Location of colon anastomosis (right hemicolon=1, left hemicolon=2):
hand-sutured=1 or stapled=2:
Initial surgery performed:

Severity of contamination (minimal, localized=0, <2 quadrants=1, >2 quadrants=2, if colonic contamination add A):

Damage control approach (yes=1, no=0):
Inotropes (yes=1, no=0), start and duration (in hours):

Initial ABG: pH: pO2: pCO2: BE: HCO3: Sats:
Hb: Lactate:
ICU-days:
BP/pulse rate on admission:
APACHE score on admission:

Postoperative ventilation (yes=1, no=0), indication and number of vent days:

VAP (yes=1, no=0):
Renal failure (yes=1, no=0) and dialysis days:
Number of blood products: RBC FFPs platelets

Time from index operation to relook (in hours):

Anastomosis done in first relook (yes=1, no=0):

Indication for relook and procedure in relook:

Anastomotic leak (yes=1, no=0):

Management of complication:

Intraabdominal collections (yes=1, no=0), diagnosed with:

Invasive fungal infection (yes=1, no=0), fungitell/BDG:

Culture of intraabdominal fluid:

Blood cultures:

Closure of abdomen on day:

Dehiscence of fascia (yes=1, no=0):

Subcutaneous wound infection (yes=1, no=0):

Open abdomen (yes=1, no=0):

Form 2:

Specimen number:

Morphological features of apoptosis: condensed nuclei (pyknosis) and nuclear fragmentation (karyorrhexis).

Light microscopic examination of the tissue section will occur at X200 and X400 magnification. Apoptotic index is defined as the number of apoptosis (clusters) per mm^2. Number of clusters per 10 HPF divided by 2,37 will give the apoptotic index

enterocyte apoptosis:

Present – number of clusters/10 high power fields (HPF)
Absent - apoptotic index 0/10 high power fields (HPF)

1	2	3	4	5	6	7	8	9	10	total	AI

Lymphocytic apoptosis:

⇒Intraepithelial lymphocyte apoptosis:

Present, number of clusters/10 HPF

Not identified/10 HPF

1	2	3	4	5	6	7	8	9	10	total	AI

⇒Lymphoid nodules /aggregate/s within the tissue section:

Present=1 Not identified=0

- ***Lymphocyte apoptosis in lymphoid aggregate/s:***

  Present, number of clusters in number of lymphoid aggregates in the section examined

  Not identified in section examined Not applicable

- ***Follicular lysis:*** Present=1 Not identified=0 Not applicable

⇒Lamina propria: lymphocyte apoptosis

Present, number of clusters/10 HPF

Not identified/10 HPF

1	2	3	4	5	6	7	8	9	10	total	AI

Appendix D (Clavien-Dindo classification)

Grade	
1	Any deviation from the normal postoperative course without the need for pharmacological treatment or surgical, endoscopic and radiological interventions. Acceptable therapeutic regimens are: drugs as antiemetics, antipyretics, analgetics, diuretics and electrolytes and physiotherapy. This grade also includes wound infections opened at the bedside.
2	Requiring pharmacological treatment with drugs other than such allowed for grade I complications. Blood transfusions, antibiotics and total parenteral nutrition are also included.
3	Requiring surgical, endoscopic or radiological intervention
3a	Intervention under regional/local anesthesia
3b	Intervention under general anesthesia
4	Life-threatening complication requiring intensive care/intensive care unit management
4a	Single organ dysfunction
4b	Multi-organ dysfunction
5	Patient demise

Appendix E (published/submitted articles)

[Compromised gut associated lymphoid tissue is a risk factor for postoperative septic complications after trauma surgery]

[Trauma results in immune cell-induced intestinal epithelial damage with subsequently increased sepsis rate]

[Posttraumatic natural killer cell decrease is associated with septic complications]

Appendix F (Turnitin report)

https://drive.google.com/file/d/1zBlNTpcPM0Pm8_qENy0m9NRh0zleSSKg/view?usp=sharing

Appendix G (Raw data)

Variable	Category	Overall		HIV- study		HIV+ study		Control		p-value for between-group comparison		
		n	%	n	%	n	%	n	%			
n		73		38		24		11				
Age (y)	median (IQR)	37 (27-42)		31 (25-40)		41 (32-47)		44 (31-59)		0,0047	Kruskal-Wallist test	Post-hoc test: HIV-<HIV+, Control (p=0.027, 0.0061)
Gender	Female	6	8	2	5	2	8	2	18	0,29	Fisher's exact test	
	Male	67	92	36	95	22	92	9	82			
CD4+LPL	median (IQR)	813 (425-986)		905 (813-997)		252 (176-413)		1,121 (720-1,199)		0,0001	Kruskal-Wallist test	Post-hoc test: HIV-<HIV+, Control (p<0.0001, <0.0001)
CD8+LPL	median (IQR)	572 (428-860)		512 (410-572)		1,143 (826-1,235)		414 (371-612)		0,0001	Kruskal-Wallist test	Post-hoc test: HIV->HIV+, Control (p<0.0001, <0.0001)
CD8+IEL (n=71)	median (IQR)	17 (13-26)		17 (13-27)		18 (14-23)		17 (11-26)		0,98		
CD4/CD8+LPL	median (IQR)	1.6 (0.4-2.1)		1.9 (1.6-2.2)		0.2 (0.2-0.3)		1.9 (1.7-3.8)		0,0001	Kruskal-Wallist test	Post-hoc test: HIV-<HIV+, Control (p<0.0001, <0.0001)
Enterocytes	median (IQR)	0.4 (0.0-0.8)		0.4 (0.0-0.8)		0.6 (0.0-0.8)		0.4 (0.0-0.4)		0,28		
IEL	median (IQR)	4.2 (2.5-8.4)		4.4 (2.1-9.3)		4.4 (3.0-7.9)		3.4 (2.1-5.1)		0,44		
LPL	median (IQR)	2.5 (1.7-4.2)		2.1 (1.7-4.2)		3.4 (1.9-4.6)		2.1 (1.7-3.4)		0,56		

Comparison of all three groups w.r.t. age, gender, CD4+LPL, CD8+LPL, CD4/CD8 ratio lamina propria, CD8+IEL at index operation

Variable	Category	Overall		HIV+ VL not suppressed		HIV+ VL suppressed		p-value for between-group comparison	
		n	%	n	%	n	%		
n		24		20		4			
Age (y)	median (IQR)	41 (32-47)		40 (29-44)		47 (43-52)		0.027 (r=0.45)	Wilcoxon Rank Sum test
Gender	Female	2	8	2	10	0	0	>0.99	Fisher's exact test
	Male	22	92	18	90	4	100		
CD4+LPL	median (IQR)	252 (176-413)		252 (176-413)		272 (163-488)		>0.99	Wilcoxon Rank Sum test
CD8+LPL	median (IQR)	1,143 (826-1,235)		1,175 (875-1,273)		708 (627-980)		0.037 (r=0.43)	Wilcoxon Rank Sum test
CD8+IEL (n=22)	median (IQR)	18 (14-23)		18 (15-25)		12 (10-16)		0,061	Wilcoxon Rank Sum test
CD4/CD8+LPL	median (IQR)	0.2 (0.2-0.3)		0.2 (0.2-0.3)		0.3 (0.2-0.6)		0,28	Wilcoxon Rank Sum test
Number of septic complications	0	2	8	1	5	1	25	0,31	Fisher's exact test
	1 or more	22	92	19	95	3	75		
Number of septic complications	0-1	4	17	3	15	1	25	0,54	Fisher's exact test
	2 or more	20	83	17	85	3	75		
Dindo classification (grouped)	0-2	7	29	6	30	1	25	>0.99	Fisher's exact test
	3-5	17	71	14	70	3	75		
Septicemia	No	9	39	8	42	1	25	>0.99	Fisher's exact test
	Yes	14	61	11	58	3	75		

Comparison of the HIV+ treated suppressed and unsuppressed sub-groups w.r.t. age, gender, CD4+LPL, CD8+LPL, CD4/CD8 ratio lamina propria, CD8+IEL at index operation

Variable	Category	Overall		Number of septic complications: 0		Number of septic complications: 1 or more		p-value for between-group comparison
		n	%	n	%	n	%	
n		62		20		42		
CD4+LPL	median (IQR)	799 (331-947)		892 (835-965)		482 (238-940)		0.0061 (r=0.35)
CD8+LPL	median (IQR)	585 (464-932)		531 (368-684)		731 (508-1,181)		0.0061 (r=0.35)
CD8+IEL (n=60)	median (IQR)	17 (13-26)		19 (14-31)		17 (13-24)		0,25
CD4+LPL	<500	23	37	1	5	22	52	<0.0001 (phi=0.46)
	>=500	39	63	19	95	20	48	
CD8+LPL	<250		0		0		0	
	>=250		0		0		0	
CD8+IEL (n=60)	<20	35	58	10	50	25	63	0,41
	>=20	25	42	10	50	15	38	

Within the study group, comparison of CD4+LPL (uncategorised, and cut-off 500), CD8+LPL (uncategorised, and cut-off 250), CD8+IEL (uncategorised, and cut-off 20) at index operation vs. number of septic complications, Dindo classification

Whole group (n=31)

variable	variable	N	mean	sd	median	IQR		min	max
AD ent	enterocy~p_c	30	0,7	0,9	0,4	0,4	0,8	0,0	4,2
AD IEL	intraepith~t	30	7,7	8,3	4,0	2,5	10,1	1,7	36,7
AD LPL	lymphocyte~i	30	3,4	2,3	2,3	2,1	4,2	1,3	10,5
CD4+LPL	cd4_ihc_nu~_	30	694,1	411,7	778,5	248,0	968,0	119,0	1616,0
CD8+LPL	cd8_ihc_nu~_	30	717,0	335,0	577,5	500,0	871,0	257,0	1692,0
CD8+IEL	cd8_ihc_nu~p	30	19,3	9,3	17,5	14,0	23,0	7,0	55,0
CD4/CD8+LPL	cd4cd8_rat~x	30	1,3	0,9	1,6	0,3	1,9	0,1	3,5
RL_AD ent	enterocy~1_c	30	4,3	2,1	4,2	3,0	5,1	0,8	10,1
RL_AD IPL	fi	30	9,0	6,8	8,5	3,0	13,1	0,8	27,8
RL_AD LPL	fl	30	5,3	2,5	5,5	3,4	5,9	0,8	12,2
RL_CD4+LPL	cd4_ihc_l~re	30	474,2	335,3	425,5	180,0	608,0	76,0	1697,0
RL_CD8+LPL	cd8_ihc_l~re	30	493,5	274,2	426,5	259,0	720,0	173,0	1170,0
RL_CD8+IEL	cd8_ihc~100_	30	47,8	17,8	48,0	35,0	60,0	18,0	85,0
RL_CD4/CD8+LPL	cd4cd8_rat~o	30	1,4	1,2	1,5	0,3	2,0	0,1	4,7

variable	variable	N	mean	sd	median	IQR		min	max	p-value for test: median = 0
diff_AD ent	diff_AD_ent	30	3,5	2,2	3,4	2,1	4,7	0,0	10,1	<0.0001
percCH_AD ent	percCH_AD_~t	30	1428%	2313%	544%	207%	1180%	0%	10100%	<0.0001
diff_AD IPL	diff_AD_IPL	30	1,3	9,5	1,9	-0,9	5,9	-26,2	24,8	0,18
percCH_AD IPL	percCH_A~IPL	30	108%	199%	36%	-16%	174%	-91%	827%	0,0085
diff_AD LPL	diff_AD_LPL	30	1,9	2,6	2,1	0,8	3,4	-5,0	8,0	0,0008
percCH_AD LPL	percCH_A~LPL	30	91%	92%	100%	12%	162%	-73%	324%	<0.0001
diff_CD4+LPL	diff_cd4_l~1	30	-219,8	276,9	-128,0	-483,0	-68,0	-648,0	657,0	0,0001
percCH_CD4+LPL	c~4_lami_p~r	30	-29%	25%	-36%	-46%	-18%	-62%	63%	0,0001
diff_CD8+LPL	diff_cd8_l~1	30	-223,5	155,6	-180,0	-320,0	-119,0	-671,0	-45,0	<0.0001
percCH_CD8+LPL	change_per..	30	-32%	17%	-32%	-42%	-21%	-65%	-5%	<0.0001
diff_CD8+IEL	difference~e	30	28,5	17,1	26,0	19,0	38,0	0,0	75,0	<0.0001
percCH_CD8+IEL	change_per~i	30	187%	166%	137%	69%	256%	0%	750%	<0.0001
diff_CD4/CD8+LPL	diff_CD4_C~L	30	0,1	0,7	0,0	-0,1	0,2	-1,8	2,9	0,58
percCH_CD4/CD8+LPL	percCH_CD4~L	30	7%	41%	1%	-18%	27%	-52%	157%	0,73

HIV-negative (n=19)

variable	variable	N	mean	sd	median	IQR		min	max
AD ent	enterocy~p_c	19	0,8	1,0	0,4	0,0	0,8	0,0	4,2
AD IEL	intraepith~t	19	7,0	7,0	3,4	2,1	10,5	1,7	28,7
AD LPL	lymphocyte~i	19	3,1	1,7	2,1	2,1	3,8	1,7	8,4
CD4+LPL	cd4_ihc_nu~_	19	953,5	265,7	925,0	801,0	1091,0	486,0	1616,0
CD8+LPL	cd8_ihc_nu~_	19	516,3	127,4	515,0	421,0	572,0	257,0	860,0
CD8+IEL	cd8_ihc_nu~p	19	19,5	10,4	15,0	13,0	24,0	9,0	55,0
CD4/CD8+LPL	cd4cd8_rat~x	19	1,9	0,6	1,8	1,6	2,0	0,9	3,5
RL_AD ent	enterocy~1_c	19	4,2	2,5	4,2	2,1	5,5	0,8	10,1
RL_AD IPL	fi	19	7,7	5,9	4,6	2,5	10,1	2,1	21,1
RL_AD LPL	fl	19	4,7	1,7	5,5	3,4	5,9	0,8	7,2
RL_CD4+LPL	cd4_ihc_l~re	19	632,4	311,4	544,0	423,0	774,0	338,0	1697,0
RL_CD8+LPL	cd8_ihc_l~re	19	322,2	108,3	295,0	223,0	446,0	173,0	504,0
RL_CD8+IEL	cd8_ihc~100_	19	44,4	17,8	41,0	31,0	60,0	18,0	79,0
RL_CD4/CD8+LPL	cd4cd8_rat~o	19	2,0	0,9	1,9	1,5	2,4	0,9	4,7

variable	variable	N	mean	sd	median	IQR		min	max	p-value for test: median = 0
diff_AD ent	diff_AD_ent	19	3,4	2,7	3,0	0,9	4,7	0,0	10,1	0,0001
diff_AD IPL	diff_AD_IPL	19	0,7	9,1	1,2	-1,7	6,8	-26,2	16,5	0,33
diff_AD LPL	diff_AD_LPL	19	1,7	2,4	2,1	0,0	3,4	-5,0	4,2	0,012
diff_CD4+LPL	diff_cd4_l~1	19	-321,1	303,8	-351,0	-553,0	-151,0	-648,0	657,0	0,0022
diff_CD8+LPL	diff_cd8_l~1	19	-194,2	116,0	-162,0	-281,0	-122,0	-453,0	-45,0	0,0001
diff_CD8+IEL	difference~e	19	24,9	16,0	22,0	15,0	35,0	0,0	64,0	0,0001
diff_CD4/CD8+LPL	diff_CD4_C~L	19	0,1	0,9	0,0	-0,3	0,6	-1,8	2,9	0,52

HIV-positive (n=12)

variable	variable	N	mean	sd	median	IQR		min	max
AD ent	enterocy~p_c	11	0,6	0,4	0,4	0,4	0,8	0,0	1,3
AD IPL	intraepith~t	11	8,9	10,5	4,6	2,5	10,1	2,1	36,7
AD LPL	lymphocyte~i	11	4,0	3,1	3,4	1,7	5,5	1,3	10,5
CD4+LPL	cd4_ihc_nu~_	11	245,9	124,9	176,0	155,0	400,0	119,0	450,0
CD8+LPL	cd8_ihc_nu~_	11	1063,5	296,0	1148,0	842,0	1206,0	617,0	1692,0
CD8+IEL	cd8_ihc_nu~p	11	18,9	7,5	18,0	15,0	23,0	7,0	35,0
CD4/CD8+LPL	cd4cd8_rat~x	11	0,2	0,1	0,2	0,1	0,4	0,1	0,5
RL_AD ent	enterocy~1_c	11	4,3	1,2	4,2	3,8	5,1	2,5	6,3
RL_AD IPL	fi	11	11,2	8,0	10,1	3,8	18,1	0,8	27,8
RL_AD LPL	fl	11	6,4	3,3	5,9	4,2	8,9	2,1	12,2
RL_CD4+LPL	cd4_ihc_l~re	11	201,0	147,7	140,0	98,0	310,0	76,0	549,0
RL_CD8+LPL	cd8_ihc_l~re	11	789,4	211,9	752,0	673,0	932,0	369,0	1170,0
RL_CD8+IEL	cd8_ihc~100_	11	53,6	17,1	57,0	47,0	61,0	19,0	85,0
RL_CD4/CD8+LPL	cd4cd8_rat~o	11	0,3	0,2	0,2	0,1	0,4	0,1	0,6

variable	variable	N	mean	sd	median	IQR		min	max	p-value for test: median = 0
diff_AD ent	diff_AD_ent	11	3,7	1,2	3,4	2,5	4,7	2,1	5,9	0,0033
diff_AD IPL	diff_AD_IPL	11	2,4	10,7	3,0	-0,4	5,5	-18,6	24,8	0,29
diff_AD LPL	diff_AD_LPL	11	2,4	3,0	2,1	0,8	2,9	-3,0	8,0	0,036
diff_CD4+LPL	diff_cd4_l~1	11	-44,9	56,2	-63,0	-79,0	-10,0	-105,0	99,0	0,041
diff_CD8+LPL	diff_cd8_l~1	11	-274,2	203,9	-248,0	-425,0	-86,0	-671,0	-45,0	0,0033
diff_CD8+IEL	difference~e	11	34,7	17,9	29,0	26,0	43,0	4,0	75,0	0,0033
diff_CD4/CD8+LPL	diff_CD4_C~L	11	0,0	0,1	0,0	-0,1	0,0	-0,1	0,2	0,79

Comparison of CD4+LPL, CD8+LPL, CD4/CD8 ratio lamina propria, CD8+IEL, enterocytes, IPL, LPL between index and relook operations: overall and split by HIV status

Group	n	Variable vs.	Variable	Correlation coefficient	p-value (for sig diff to zero)	
DCS study	30	Number of septic compl	percCH_CD4+LPL	-0,30	0,11	Spearman's correlation coefficient
DCS study	30	Number of septic compl	percCH_CD8+LPL	-0,43	0,016	Spearman's correlation coefficient
DCS study	30	Number of septic compl	percCH_CD8+IEL	0,42	0,020	Spearman's correlation coefficient

			percCH_CD4+LPL	p-value for between-group test	percCH_CD8+LPL	p-value for between-group test	percCH_CD8+IEL	p-value for between-group test
Variable	Category	n	median (IQR)		median (IQR)		median (IQR)	
PATI	<=25	3	-16 (-25 to -13)	0,12	-24 (-38 to -10)	0,48	138 (43 to 200)	0,70
	>25	27	-37 (-47 to -20)		-33 (-44 to -21)		135 (69 to 271)	
Time from index to relook op	<=48h	14	-32 (-44 to -20)	0,51	-27 (-42 to -19)	0,84	121 (76 to 200)	0,90
	>48h	16	-37 (-48 to -17)		-34 (-42 to -22)		157 (42 to 266)	
Duration of inotropic requirement	<=48h	11	-41 (-53 to -18)	0,12	-40 (-55 to -14)	0,28	135 (47 to 400)	0,62
	>48h	8	-24 (-44 to -6)		-25 (-30 to -23)		228 (142 to 273)	
pH at admission	<=7.25	9	-36 (-44 to -12)	0,40	-24 (-29 to -14)	0,15	161 (104 to 271)	0,54
	>7.25	21	-37 (-48 to -20)		-37 (-44 to -23)		135 (69 to 240)	
Lactate at admission	<5 mmol/L	16	-35 (-48 to -19)	0,48	-34 (-43 to -21)	0,84	103 (54 to 248)	0,17
	>=5 mmol/L	14	-36 (-44 to -14)		-28 (-40 to -19)		171 (105 to 271)	
Hypotension	No	16	-38 (-48 to -27)	0,11	-37 (-54 to -23)	0,081	142 (72 to 239)	>0.99
	Yes	14	-21 (-46 to -12)		-25 (-38 to -14)		137 (47 to 271)	
Faecal contamination in index op	Mild/moderate	8	-28 (-46 to -11)	0,57	-31 (-40 to -23)	0,96	90 (42 to 165)	0,075
	Severe	22	-37 (-46 to -20)		-33 (-42 to -19)		157 (94 to 275)	
Dindo classification (grouped)	0-2	4	-17 (-27 to -14)	0,10	-7 (-14 to -5)	0,0034	45 (35 to 104)	0,059
	3-5	26	-38 (-47 to -23)		-36 (-44 to -24)		146 (94 to 271)	
Anastomotic leak (n=29)	No	16	-36 (-50 to -17)	0,43	-36 (-53 to -16)	0,860	122 (45 to 364)	0,60
	Yes	13	-27 (-44 to -20)		-31 (-37 to -23)		154 (113 to 240)	
Demised	No	17	-39 (-48 to -23)	0,14	-24 (-53 to -19)	0,79	129 (47 to 240)	0,440
	Yes	13	-27 (-43 to -6)		-35 (-38 to -26)		138 (104 to 256)	

Comparison between CD4+LPL, CD8+LPL, CD8+IEL (% change between index and relook), and number of septic complications, anastomotic leak, Dindo classification, mortality

Variable	Label	n		Overall	HIV-study	HIV+study	Control	p-value for between-group comparison		
				n	n	n	n			
n				73	38	24	11			
A_CD3	number of CD3 positive lymphocytes on admission	70	median (IQR)	1466 (847-2300)	1425 (787-2257)	1187 (646-1813)	1999 (1609-2550)	0,058	Kruskal-Wallist test	
A_CD4	number of CD4 positive lymphocytes on admission	70	median (IQR)	604 (336-1066)	816 (481-1151)	338 (204-471)	1216 (892-1340)	0,0001	Kruskal-Wallist test	Post-hoc test: HIV-, Control > HIV+ (p<0.0001, <0.0001)
A_CD8	number of CD8 positive lymphocytes on admission	70	median (IQR)	599 (328-1098)	471 (288-907)	571 (321-1298)	793 (618-1214)	0,13	Kruskal-Wallist test	
A_B	number of B-cell lymphocytes on admission	70	median (IQR)	158 (112-266)	206 (132-335)	120 (79-179)	258 (140-471)	0,0029	Kruskal-Wallist test	Post-hoc test: HIV-, Control > HIV+ (p=0.0041, 0.0082)
A_NK	number of NK cells on admission	70	median (IQR)	212 (121-420)	314 (121-535)	165 (79-264)	183 (146-276)	0,037	Kruskal-Wallist test	Post-hoc test: HIV- > HIV+ (p=0.016)
A_CD4/CD8	CD4/CD8 ratio of blood on admission	70	median (IQR)	1.02 (0.58-1.56)	1.50 (0.97-1.96)	0.40 (0.32-0.68)	1.14 (1.01-1.43)	0,0001	Kruskal-Wallist test	Post-hoc test: HIV-, Control > HIV+ (p<0.0001, 0.0002)
P1_CD3	number of CD3 positive lymphocytes on P1	72	median (IQR)	540 (422-910)	533 (420-740)	578 (383-777)	1058 (497-1716)	0,029	Kruskal-Wallist test	Post-hoc test: Control > HIV-, HIV+ (p=0.013, 0.037)
P1_CD4	number of CD4 positive lymphocytes on P1	72	median (IQR)	291 (157-414)	304 (235-408)	164 (106-288)	582 (335-794)	0,0001	Kruskal-Wallist test	Post-hoc test: Control > HIV- > HIV+ (p=0.021, 0.0032)
P1_CD8	number of CD8 positive lymphocytes on P1	72	median (IQR)	264 (159-431)	186 (120-304)	334 (247-525)	424 (172-658)	0,0005	Kruskal-Wallist test	Post-hoc test: HIV+, Control > HIV- (p=0.0009, 0.0078)
P1_B	number of B-cell lymphocytes on P1	72	median (IQR)	151 (102-238)	171 (132-244)	110 (61-155)	199 (117-281)	0,012	Kruskal-Wallist test	Post-hoc test: HIV-, Control > HIV+ (p=0.0094, 0.036)
P1_NK	number of NK cells on P1	72	median (IQR)	65 (34-98)	63 (37-84)	38 (20-91)	155 (59-169)	0,015	Kruskal-Wallist test	Post-hoc test: Control > HIV- > HIV+ (p=0.048, 0.0054)
P1_CD4/CD8	CD4/CD8 ratio of blood on P1	72	median (IQR)	1.17 (0.69-1.61)	1.45 (1.14-2.17)	0.43 (0.34-0.71)	1.34 (1.30-1.87)	0,0001	Kruskal-Wallist test	Post-hoc test: HIV-, Control > HIV+ (p<0.0001, <0.0001)
A-P1_CD3	number of CD3 positive lymphocytes (diff A to P1)	69	median (IQR)	-737 (-1282 to -316)	-965 (-1494 to -459)	-672 (-903 to -153)	-853 (-1209 to -283)	0,13	Kruskal-Wallist test	
A-P1_CD4	number of CD4 positive lymphocytes (diff A to P1)	69	median (IQR)	-272 (-599 to -130)	-416 (-827 to -218)	-174 (-272 to -36)	-491 (-881 to -41)	0,0004	Kruskal-Wallist test	Post-hoc test: HIV+ > HIV-, Control (p=0.0002, 0.037)
A-P1_CD8	number of CD8 positive lymphocytes (diff A to P1)	69	median (IQR)	-338 (-576 to -161)	-319 (-591 to -170)	-372 (-631 to -104)	-322 (-440 to -249)	0,91	Kruskal-Wallist test	
A-P1_B	number of B-cell lymphocytes (diff A to P1)	69	median (IQR)	-25 (-93 to 21)	-27 (-129 to 31)	-4 (-40 to 12)	-29 (-64 to 23)	0,65	Kruskal-Wallist test	
A-P1_NK	number of NK cells (diff A to P1)	69	median (IQR)	-118 (-315 to -39)	-269 (-492 to -76)	-101 (-186 to -47)	-68 (-118 to 1)	0,013	Kruskal-Wallist test	Post-hoc test: Control > HIV- (p=0.013)
P2_CD3	number of CD3 positive lymphocytes on P2	28	median (IQR)	317 (265-453)	317 (263-444)	331 (274-594)		0,47	Wilcoxon rank sum test	
P2_CD4	number of CD4 positive lymphocytes on P2	28	median (IQR)	166 (115-236)	180 (131-276)	118 (80-183)		0,039	Wilcoxon rank sum test	
P2_CD8	number of CD8 positive lymphocytes on P2	28	median (IQR)	137 (89-206)	121 (77-172)	198 (149-450)		0,012	Wilcoxon rank sum test	
P2_B	number of B-cell lymphocytes on P2	28	median (IQR)	125 (86-182)	139 (101-264)	91 (43-125)		0,021	Wilcoxon rank sum test	
P2_NK	number of NK cells on P2	28	median (IQR)	24 (15-49)	29 (16-49)	19 (9-39)		0,40	Wilcoxon rank sum test	
P2_CD4/CD8	CD4/CD8 ratio of blood on P2	28	median (IQR)	1.28 (0.67-1.96)	1.61 (1.16-2.27)	0.46 (0.33-0.70)		0,0002	Wilcoxon rank sum test	
P1-P2_CD3	number of CD3 positive lymphocytes (diff P1 to P2)	28	median (IQR)	-175 (-282 to -46)	-196 (-317 to -34)	-161 (-272 to -130)		0,81	Wilcoxon rank sum test	
P1-P2_CD4	number of CD4 positive lymphocytes (diff P1 to P2)	27	median (IQR)	-61 (-123 to 4)	-104 (-171 to -7)	-16 (-88 to 32)		0,15	Wilcoxon rank sum test	
P1-P2_CD8	number of CD8 positive lymphocytes (diff P1 to P2)	28	median (IQR)	-108 (-147 to -21)	-78 (-120 to -13)	-129 (-182 to -103)		0,19	Wilcoxon rank sum test	
P1-P2_B	number of B-cell lymphocytes (diff P1 to P2)	28	median (IQR)	-15 (-99 to 10)	-36 (-102 to 8)	-3 (-27 to 12)		0,57	Wilcoxon rank sum test	
P1-P2_NK	number of NK cells (diff P1 to P2)	28	median (IQR)	-16 (-33 to -5)	-18 (-37 to -9)	-11 (-16 to 8)		0,21	Wilcoxon rank sum test	
A%_CD3	percentage of CD3 positive lymphocytes of total lymphocytes	70	median (IQR)	72 (65-77)	69 (59-75)	73 (67-81)	75 (73-82)	0,020	Kruskal-Wallist test	Post-hoc test: Control > HIV- (p=0.015)
A%_CD4	percentage of CD4 positive lymphocytes of total lymphocytes	70	median (IQR)	32 (21-41)	36 (30-42)	19 (16-23)	43 (35-46)	0,0001	Kruskal-Wallist test	Post-hoc test: HIV-, Control > HIV+ (p<0.0001, <0.0001)
A%_CD8	percentage of CD8 positive lymphocytes of total lymphocytes	70	median (IQR)	33 (25-44)	27 (20-32)	46 (42-54)	33 (31-36)	0,0001	Kruskal-Wallist test	Post-hoc test: HIV+ > HIV-,Control (p<0.0001, 0.011)
A%_B	percentage of B-cell lymphocytes of total lymphocytes on adm	70	median (IQR)	9 (6-14)	10 (7-14)	8 (4-13)	8 (6-18)	0,24	Kruskal-Wallist test	
A%_NK	percentage of NK cells of total lymphocytes on admission	70	median (IQR)	11 (7-19)	15 (9-20)	9 (6-17)	7 (6-11)	0,020	Kruskal-Wallist test	Post-hoc test: HIV- > Control (p=0.016)
P1%_CD3	percentage of CD3 positive lymphocytes of total lymphocytes	72	median (IQR)	71 (60-78)	63 (56-75)	73 (66-82)	76 (68-80)	0,0087	Kruskal-Wallist test	Post-hoc test: HIV+, Control > HIV- (p=0.0090, 0.045)
P1%_CD4	percentage of CD4 positive lymphocytes of total lymphocytes	72	median (IQR)	34 (23-42)	37 (28-44)	21 (15-26)	44 (37-49)	0,0001	Kruskal-Wallist test	Post-hoc test: HIV-, Control > HIV+ (p<0.0001, <0.0001)
P1%_CD8	percentage of CD8 positive lymphocytes of total lymphocytes	72	median (IQR)	30 (23-40)	24 (18-29)	46 (39-55)	29 (26-34)	0,0001	Kruskal-Wallist test	Post-hoc test: HIV+ > HIV-,Control (p<0.0001, 0.0021)
P1%_B	percentage of B-cell lymphocytes of total lymphocytes on P1	72	median (IQR)	17 (10-22)	18 (15-29)	16 (8-20)	12 (9-18)	0,061	Kruskal-Wallist test	
P1%_NK	percentage of NK cells of total lymphocytes on P1	72	median (IQR)	7 (4-11)	8 (5-11)	5 (2-12)	8 (6-10)	0,27	Kruskal-Wallist test	
P2%_CD3	percentage of CD3 positive lymphocytes of total lymphocytes	28	median (IQR)	68 (58-75)	61 (54-70)	73 (68-84)		0,039	Wilcoxon rank sum test	
P2%_CD4	percentage of CD4 positive lymphocytes of total lymphocytes	28	median (IQR)	32 (23-39)	36 (30-40)	21 (19-30)		0,012	Wilcoxon rank sum test	
P2%_CD8	percentage of CD8 positive lymphocytes of total lymphocytes	28	median (IQR)	30 (19-40)	22 (14-31)	46 (33-58)		0,0003	Wilcoxon rank sum test	
P2%_B	percentage of B-cell lymphocytes of total lymphocytes on P2	28	median (IQR)	21 (17-32)	24 (19-36)	19 (8-24)		0,039	Wilcoxon rank sum test	
P2%_NK	percentage of NK cells of total lymphocytes on P2	28	median (IQR)	5 (3-7)	5 (4-7)	4 (2-7)		0,30	Wilcoxon rank sum test	

Comparison of all three groups w.r.t. CD3, CD4, CD8, B-cell, NK-cell, CD4/CD8 ratio at A, P1, difference between A and P1, difference between P1 and P2

Variable	septic compl: 0 or 1		septic compl: 2 or more		p-value for between-group comparison	Dindo 0-2		Dindo 3-5		p-value for between-group comparison
	n	row %	n	row %		n	row %	n	row %	
	25		33			25		33		
%chA-P1_CD3	8	38	13	62	0,59	8	38	13	62	0,59
	17	46	20	54		17	46	20	54	
%chA-P1_CD4	10	48	11	52	0,78	9	43	12	57	>0.99
	15	41	22	59		16	43	21	57	
%chA-P1_CD8	9	39	14	61	0,79	9	39	14	61	0,79
	16	46	19	54		16	46	19	54	
%chA-P1_B	20	43	27	57	>0.99	19	40	28	60	0,50
	5	45	6	55		6	55	5	45	
%chA-P1_NK	8	73	3	27	0,042	9	82	2	18	0,0060
	17	36	30	64		16	34	31	66	

In the study group, comparison of CD3, CD4, CD8, B-cell, NK-cell (categorised % change from A to P1) w.r.t. number of septic complications, Dindo classification