Aus dem Institut für Immunologie und Transfusionsmedizin
(Direktor/in Univ.- Prof. Dr. Christine Schütt)
der Medizinischen Fakultät der Ernst-Moritz-Arndt-Universität Greifswald

# Effect of surgical intervention on the activation status of circulating monocytes and T-cells

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## ABBREVIATIONS

| | |
|---|---|
| APC | Antigen presenting cell |
| 7-AAD | 7-amino actinomycin D |
| aaUTP | Amino allyl UTP |
| APC | Antigen-presenting cell |
| aRNA | Anti-sense RNA |
| BSA | Bovine serum albumin |
| CASP | Colon ascendens stent peritonitis |
| CCR | Chemokine receptor |
| CD | Cluster of differentiation |
| cDNA | Complementary DNA |
| CLIP | Class-ii-associated invariant chain peptide |
| CLP | Caecal ligation and puncture |
| COX | Cytochrome oxidase |
| CRP | C-reactive protein |
| Ct | Threshold cycle |
| CX3CL1 | Chemokine (C-X3-C motif) ligand 1 |
| Cy | Cyanin |
| DCs | Dendritic cells |
| DDT | Dithiothretol |
| DEPC | Diethyl pyrocarbonate |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleic acid triphosphate |
| EDTA | Ethylendiamine tetraacetic acid |
| EN-RAGE | Extracellular newly identified rage-binding protein |
| ER | Endoplasmic reticulum |
| ESL-1 | E-selectin ligand 1 |
| FACS | Fluorescence-activated cell sorter |
| FCS | Fetal calf serum |
| FITC | Fluorescein isothiocyanate |
| FSC | Forward scatter |
| GFP | Green fluorescent protein |

| | |
|---|---|
| GM-CSF | Granulocyte monocyte colony stimulating factor |
| HA | Hyaluronan |
| HLA | Human leucocyte antigen |
| ICAM | Intercellular adhesion molecule |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| kb | Kilobase |
| kDa | Kilodalton |
| LAD | Lymphocyte adhesion deficiency |
| LIP | Leupeptin-induced peptide |
| LPS | Lipopolysaccharide |
| LTA4 | Leukotriene A4 |
| MACS | Magnetic activated cell sorting |
| MAdCAM-1 | Mucosal addressin cellular adhesion molecule-1 |
| MAPK | Mitogen-activated protein kinase |
| MCP-1 | Monocyte chemoattractant protein-1 |
| MEF | Molecules of equivalent fluorochrome |
| MFI | Mean fluorescence index |
| MHC | Major histocompatibility complex |
| MIP | Macrophage inflammatory protein |
| MOPS | 3-(n-morpholino) propan sulfonic acid |
| MP | Mononuclear phagocytes |
| mRNA | Messenger ribonucleic acid |
| MW | Molecular weight |
| NaN3 | Sodium azide |
| NF-κB | Nuclear factor-κb |
| NK cells | Natural killer cells |
| PBMC | Peripheral blood mononuclear cells |
| PCR | Polymerase chain reaction |
| PE | Phycoerythrin |
| PECAM-1 | Platelet/endothelial cell adhesion molecule |
| PerCP | Peridinin chlorophyll protein |

| | |
|---|---|
| PMT | Photomultiplier tube |
| PSGL-1 | P-selectin glycoprotein ligand-1 |
| RAGE | Receptor for advanced glycation end products |
| RNA | Ribo nucleic acid |
| rpm | Rounds per minute |
| rRNA | Ribosomal RNA |
| RT | Room temperature |
| SDS | Sodium dodecyl sulphate |
| sec | Second |
| SIRS | Systemic inflammatory response syndrome |
| SSC | Side scatter |
| TCR | T cell receptor |
| TLR | Toll like receptor |
| TNF | Tumor necrosis factor |
| TSST | Toxic shock syndrome toxin |
| U | Unit |
| UV | Ultraviolet |
| VCAM | Vascular cell adhesion molecule |

# 1 Introduction

Disturbances of normal physiology caused by accident or disease must be quickly brought under control and to achieve this the organism requires homeostatic mechanisms that will recognise and deal with the problem. One major set of homeostatic regulators ensure that activation of the immune defence system is maintained within safe limits under normal physiological conditions. Ideally, when the immune system detects a pathogen or some other sign of danger it should respond appropriately and, having done so, return to its initial state. However under certain circumstances the immune system may be stimulated to overreact to such a degree that the normal homeostatic controls become inadequate. Hyper inflammation or allergic responses are examples of this sort of situation (1-3). This type of excessive activation is one side of the coin, the other involves the situation in which immunity suffers an incapacitating degree of down-regulation leaving it unable to fulfil its essential defence role. An example of this sort of disturbance of immune homeostasis is the so-called "immune paralysis" evident in many patients after major traumatic events, as a result of which they appear to be unable to mount an adequate response against otherwise harmless infections. Typically, paralysis is thought to follow an initial phase of over activation referred to as "systemic hyper inflammation". The majority of patients manage to bring this inflammatory response under control but in a minority of cases the homeostatic regulation fails. The system appears to over compensate and is driven into a non responsive "hypo-inflammatory" state (4). This scheme is probably oversimplified and it has been suggested that the clinical condition of many patients may reflect the simultaneous actions of both hyper and hypo inflammatory events (5).

## 1.1 The inflammatory response

A localised bacterial infection is initially detected by the tissue macrophages which use innate immune system receptors such as the Toll Like Receptors (TLRs) to detect traces of bacterial products. Having bound the ligands the TLRs transmit an activation signal into the cell via a pathway which has been worked out in great detail over the last ten years (6, 7). This signal transduction pathway leads to the activation of the transcription factor NFkB and of the p38 kinase which in turn are required for the activation of genes coding for pro-inflammatory cytokines including TNF α, IL-1 and IL-6 (8). These cytokines have important roles in the development of an inflammatory reaction.

Macrophage and endothelial cell derived cytokine IL-6 acts like a hormone in that it is released from the site of infection and is carried by the circulation to the liver where it induces the synthesis in hepatocytes of acute phase proteins (9). These include C-reactive protein and serum amyloid A both of which help to combat an infection by binding to common repeating elements of bacterial polysaccharides and, by doing so, to opsonise the pathogen. The synthesis of these acute phase proteins is rapidly induced and the serum concentration of CRP for example can rise over one thousand fold within 24-48 hours (10).

In contrast to IL-6, one important function of the pro-inflammatory mediators TNF-α and IL-1 released from activated tissue macrophages is to act directly on the endothelial cells which line the neighbouring blood vessels. Tight junctions between these endothelial cells allow them to form a barrier which prevents leakage of fluid through the capillary wall. Once activated by pro-inflammatory mediators the endothelial cells reduce their tight adhesion to each other. This in turn reduces the barrier function of the vessel (11-13) and permits blood fluid to leak out into the surrounding tissue. This fluid contains natural antibodies, complement and acute phase proteins which together will initiate an attack on the pathogens. Pro-inflammatory mediators released from tissue macrophages are not the only means of activating endothelium. Uric acid, produced as a breakdown product of RNA released from necrotic cells rapidly reaches saturation in the tissue fluid and crystallises out. Crystalline uric acid is a powerful activator of endothelium and may thus serve an endogenous danger signal in response to traumatic tissue damage (14, 15).

## 1.2 Leucocyte extravasation

In addition to reducing their barrier function the activated endothelial cells also play a central role in initiating the process by which leucocytes extravasate from the blood. In the first step the leucocytes adhere loosely to the vascular endothelium and are swept across the vessel wall by the current, a process referred to as “rolling”. On activated endothelium this rolling is mediated largely by selectin - selectin ligand interactions. Selectins are a family of lectins with specificity for oligosaccharides on their cell bound glycoprotein ligands. L-selection is expressed on most leucocytes in blood, E-selectin is expressed on endothelium at sites of inflammation while P-selectin is largely restricted to activated platelets and endothelial cells. The selectin ligands identified so far are cell bound glycoproteins. All three selectins bind the tetrasaccharide sialyl-LewisX motif (16, 17). Nevertheless the different selectins do have preferences for different ligands: L-selectin shows

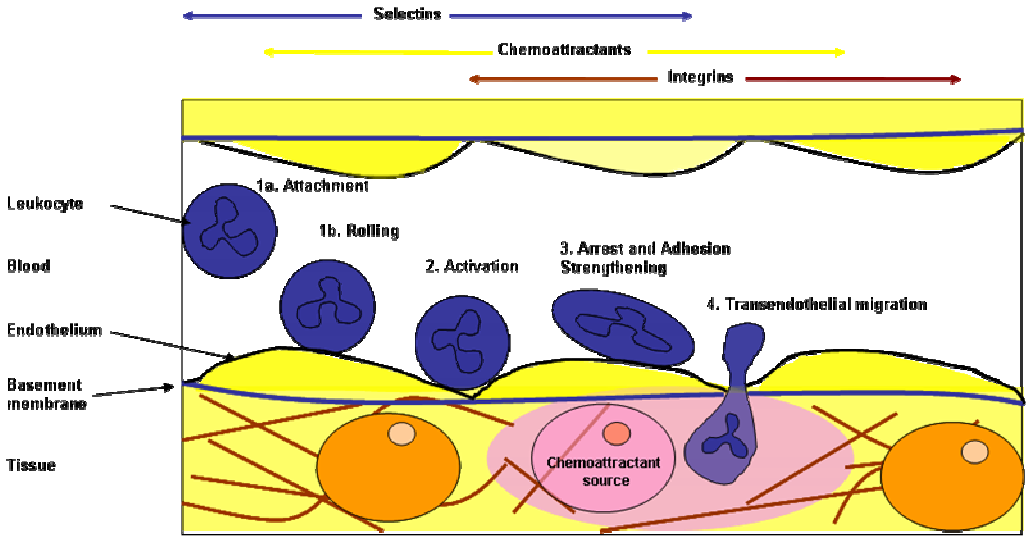



**Figure 1: Sequential steps in leukocyte extravasation.** 1. Interaction between selectins and their carbohydrate ligands results in rolling and attachment of leucocytes. 2. Chemokines expressed at the endothelial surface bind their leucocyte receptors resulting in up-regulation of integrin. 3. Integrin binding to endothelial cell adhesion molecules results in arrest of the leucocyte. 4. Morphological changes are followed by transmigration across the endothelial barrier. (Picture adopted from Springer 1994 (32))

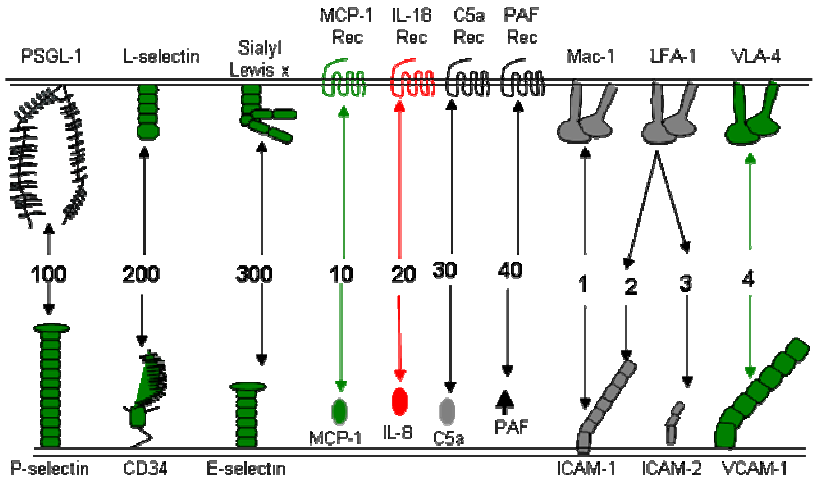
preferences for MAdCAM-1, P-selectin for PSGL-1 and E-selectin for ESL-1 (18, 19). The basis for the difference in binding preference is not yet clear.

The acutely released inflammatory mediators histamine and thrombin stimulate endothelial surface expression of P-selectin from the preformed pool stored within the endothelial cell's Weibel-Palade bodies (20). IL-1β and TNFα regulate the expression of endothelial E-selectin by increasing its de novo synthesis (21, 22). The reversible selectin-selectin ligand interactions which promote leucocyte rolling on the endothelium are the first part of a three stage process required to achieve firm recruitment of leukocytes from the blood to the endothelial surface (Figure 1).

The second stage of attachment to endothelium involves the activation of the leucocyte by a chemokine tethered to the endothelial cell surface. Some chemokines, including MIP1 α, and IL-8 are released from endothelial cells and then tethered on the endothelial cell surface by their adhesion to heparin sulphate proteoglycans (23-25). Others, such as CX3CL1 (fractalkine) are solidly fixed as transmembrane proteins in the endothelial cell membrane (26). Tethering is essential for otherwise the chemokine would be rapidly swept away in the blood flow and its local concentration could not be maintained. The leucocyte receptors for chemokines are seven membrane spanning G-protein coupled cell

surface molecules (27). Only if the leucocyte expresses the appropriate chemokine receptor will the third stage – integrin activation - be initiated.

Integrins are a major class of cell adhesion molecule. They are heterodimers made up of one α and one β chain. Since there are 18 genes for alpha subunits and 8 for the beta subunits a large number of different combinations are in principle possible and 24 of them have been identified as *in vivo* products (28, 29). Different cell types differ in the integrins which they express (30). Integrins are coupled on the cytoplasmic side of the cell membrane to structural elements within the cell and thus serve as a bridge between the cytoskeleton and the extracellular matrix to permit cell mobility. However the integrin heterodimers are initially expressed on the cell surface in an inactive conformation with low ligand affinity. A signal generated inside the cell is required to cause a change of conformation which results in a large increase in the integrins affinity for its ligand (31). In leucocyte extravasation this signal is generated by the cell's chemokine receptor once it has engaged its ligand.




| | |
|---|---|
| Monocyte Area Codes | 111,211,311,112,212,312,113,213,313,114,214,314, 134,234,334,144,244,344 |
| Neutrophil Area Codes | 121,221,321,122,222,322,123,223,323 |
| Monocyte and Neutrophil Area Codes | 131,231,331,132,232,332,133,233,333, 141,241,341,142,242,342,143,243,343 |
| Null Area Codes | 124,224,324. |

**Figure 2: Area code model for selective extravasation of leucocytes.** Specific combination of adhesive interactions and chemokine signals from endothelium directs leucocyte migration into specific tissue sites. Selectin and integrin interactions are shown in hundreds. Chemoattractants are shown in the tens place. Specific interactions form monocytes and neutrophils are shown in green and red respectively. (Picture adopted from Springer 1994 (32))

This tripartite interaction (Figure 2) serves as the basis for directed leucocyte extravasation since only when the interacting leucocytes and endothelial cells possess appropriate molecular partners for all three steps will firm adhesion be achieved. The availability of selectin/ selectin ligands, chemokines / chemokine receptors and integrin / integrin ligand pairs provides an "area code" that allows for selective extravasation of the different leucocyte subsets from the blood (32).

Once the leucocyte is firmly attached to the endothelial cell surface it extends filopodia and feels its way towards a junction between two adjacent endothelial cells. The junction between the endothelial cells is maintained in part by homotypic interactions between cell adhesion molecules expressed by adjacent cells. The leukocyte expresses some of the same molecules and by "exchanging" leucocyte-endothelial interactions for the interactions of two adjacent endothelial cells it initiates the process of diapedesis. Homophilic interactions with PECAM 1 (CD31) and of CD99 on leucocytes and endothelial cells are important in this respect (33, 34)

Local inflammation in response to an infection permits the recruitment of phagocytic cells (in particular granulocytes) which act to control the infection. An inflammatory response is therefore an essential component of an innate response to infection. In patients in whom this process fails to function properly due to a dysfunction of the β2 integrin chain, leucocytes may roll but fail to attach firmly and hence cannot extravasate. The consequences are serious and these Lymphocyte Adhesion Deficiency (LAD-1) patients suffer from constant infections which they are unable to control and most die within the first few years (35, 36). A second much milder form of LAD is caused by loss of activity of the enzyme which converts mannose to fucose. Fucose is a component of the ligands of L, P and E-selectins and in LAD-2 patients the synthesis of the selectin ligands is reduced by 97-98% though not entirely abolished.

## 1.3 Systemic inflammation

If an inflammatory response is not spatially restricted but instead disseminated then a systemic inflammation may ensue. The Systemic Inflammatory Response Syndrome (SIRS) describes a clinical situation in which inflammation – useful and necessary as a local defence measure – converts into a pathological systemic inflammation. It may arise in response to a wide variety of traumatic insults and is clinically defined by the occurrence of two or more of the following conditions:

- Temperature greater than 38°C or less than 36°C
- Heart rate greater than 90 beats per minute
- Respiratory rate greater than 20 breaths per minute or $PaCO_2$ less than 32 mm Hg
- White blood cell count greater than 12,000/µL, less than 4000/µL, or 10% immature (band form) granulocytes.

Those patients who fail to get the SIRS under control may progress to sepsis (SIRS plus evidence of infection) or finally to septic shock (sepsis plus a requirement for catecholamines to maintain blood pressure) (37).

Sepsis is a major health problem that is responsible for ~250,000 deaths a year in the United States and its incidence continues to increase at an estimated rate of ~1,5% per year. Despite more than 20 years of intensive research the incidence of sepsis and the number of sepsis-related deaths continues to rise (38, 39). Mortality rates in septic patients range from 30% to 70%. Systemic inflammation is a situation which involves many cell types and organs. Because of this it is difficult to adequately model it in it's entirety in *in vitro* experiments and much of what we know about the pathophysiology of systemic inflammation comes from work on animal models.

## 1.4 Animal models of systemic inflammation

The classical inflammatory model in mice involves treating the animals with a bolus injection of purified LPS. This results in an immediate hyper-inflammatory response accompanied by the release of pro-inflammatory mediators including TNF alpha and IL-1 (40, 41). That the mediator release is causally associated with the inflammation was shown by eliciting the hyper-inflammatory response directly by injection of TNF or of IL-1. As one might expect from this the inflammatory response can be abrogated if these mediators are sequestered in vivo by injection of large amounts of specific antibodies, of soluble forms of their receptors or by using knock out mice models (e.g. $TNFRp55^{-/-}$ mice). This promising approach in the animal models led to the development of such anti-mediator therapies for human patients. However all attempts to control dysregulated inflammation in this way have so far failed (42, 43). Though the analysis of the murine response to LPS has been the basis of much of our understanding of the processes involved in hyper-inflammation, it is now widely

accepted that it fails to adequately model the range of immune processes taking place in patients with inflammatory complications.

For this reason attempts have been made to develop models which may more fully reflect the complexities of the inflammatory processes in patients. The most generally used model is Caecal Ligation and Puncture (CLP) (44, 45). This involves ligation of the caecum which is then punctured once or more using a needle of known gauge. A small amount of the caecal contents is then squeezed out before the surgical wound in the abdomen is closed. The hole in the caecum is usually quite quickly closed by sticking to the mesentery after which a localised ulceration of the punctured caecum develops. CLP thus causes an acute flare of peritonitis followed by a longer period of ulceration. More recently a second animal model of peritonitis has been developed in which a stent is inserted into the ascending colon. This Colon Ascendens Stent Peritonitis (CASP) differ from CLP in that the stent cannot be readily blocked and so in place of the ulceration typical of CLP this model results in a continual leakage of gut contents into the peritoneum (46). The models differ not only in their clinical course but also show differences in the pattern of cytokines involved (47). Neither in the LPS model nor in CLP or CASP do the animals show evidence of immune paralysis sufficiently extensive to result in a failure to suppress secondary infections. For these reasons the work in this thesis was directed towards directly assessing parameters of immune paralysis in human patients.

## 1.5 Monocyte response to injury

Patients with systemic inflammation exhibit an initial hyper-inflammatory response which is followed by the development of a sustained anti-inflammatory or immunosuppressive state that has been termed immune paralysis. In this regard, Munford and Pugin hypothesized that local inflammation is often accompanied by systemic anti-inflammatory responses (48). The advantage of coordinating local inflammation with systemic anti-inflammation is that it may allow the immune system to focus its efforts on containing the local inflammation while preventing potentially injurious inflammation in unaffected sites. However, these normally protective systemic responses may become immunosuppressive.

Immune suppression has been shown to affect not only the innate but also the adaptive arm of the immune system. In the innate system the suppression in systemic inflammation is associated with circulating monocytes. These mononuclear leukocytes are derived from

common myeloid progenitor stem cells in the bone marrow and released into blood where they circulate with a half life of 1-3 days (49). Following recruitment to tissues, monocytes can differentiate into macrophages or certain classes of myeloid dendritic cells (DCs) (50).

## 1.6 Monocyte differentiation

Monocyte homing and differentiation have been studied *in vivo* by using adoptive precursor cell transfers into mononuclear phagocytes (MP) depleted mice. The cells to be grafted are isolated from transgenic donor mice in which the CX3CR1 chemokine receptor gene is replaced with a green fluorescent protein gene (GFP) and harbor a specific GFP label in MP precursors. Using this animal model, it was shown that macrophage and DC progenitors (MDPs) are *in vivo* precursors of Gr-1$^{low}$CX$_3$CR1$^{high}$ and Gr-1$^{high}$CX$_3$CR1$^{low}$ bone marrow (BM) and blood monocytes in mice. Once released into the blood, Gr1$^{high}$ monocytes shuttle back to the BM in the absence of inflammation, differentiate into Gr1$^{low}$ monocytes and can return into blood to replenish peripheral mononuclear phagocytes. Thus, the blood monocyte is not a "one-way intermediate" from the BM to the periphery and inflammatory status of the host influences the differentiation of monocytes (51).

Based on their chemokine receptor expression Gr-1$^{high}$ and Gr-1$^{low}$ murine monocytes correlate to human CD14$^{++}$CD16$^{-}$ and CD14$^{+}$CD16$^{+}$ monocytes, respectively (52, 53). CD14$^{+}$CD16$^{+}$ cells have characteristic patterns of cell surface molecules when compared with the classical monocytes, and this includes higher amounts of MHC class II molecules, epidermal growth factor module-containing mucinlike receptor 2 (EMR2), Ig-like transcript 4 (ILT-4), CD43, and CD45RA expression and it has been suggested that these cells may more resemble tissue macrophages (52, 53). Distinct chemokine-receptor expression profiles are also found between these subsets: for example, CCR5 positive cells are found predominantly among the CD14$^{+}$CD16$^{+}$ monocytes pool (54) which also expresses higher levels of the receptor for fractalkine, CX3CR1. In contrast the CD16$^{-}$ population expresses lower levels of CX3CR1 but high levels of the receptor CCR2 which recognises MCP-1, the principle chemokine released by inflamed tissue (55).

Adoptive transfer of CX$_3$CR1$^{low}$ or CX$_3$CR1$^{high}$ into naïve mice revealed different migratory properties of these cell populations. The CX$_3$CR1$^{high}$ cells homed to a wide range of tissues and this pattern was not significantly altered when a peritoneal inflammation was induced in the recipients by intraperitoneal application of thioglycollate. This monocyte population may represent the precursors of tissue resident macrophages and DCs.

Interestingly, the monocytes expressing CCR2, CD62L, and low levels of $CX_3CR1$ were almost undetectable in peripheral tissues in healthy animals but in thioglycollate treated mice these cells preferentially homed to the inflamed peritoneum where they acquired the DC markers CD11c and MHC class II as well as the functional capacity to prime naïve T cells. Moreover, these two main blood monocyte subsets differ in their potential to give rise to pulmonary mononuclear phagocytes. Under inflammatory and noninflammatory conditions, both Gr-$1^{high}$ and Gr-$1^{low}$ monocyte subsets give rise to pulmonary dendritic cells. In contrast, under inflammatory conditions, only Gr-$1^{low}CX_3CR1^{high}$ monocytes, but not Gr-$1^{high}CX_3CR1^{low}$ cells, had the potential to differentiate into lung macrophages. The latter can, however, gain the potential to become lung macrophages by conversion into Gr-$1^{low}$ monocytes (53, 56).

## 1.7 Monocyte activation by injury

Experimental and clinical studies have shown that systemic inflammation affects monocytes. Detection of *ex vivo* LPS stimulated blood cytokine responses and measurements of monocytic HLA-DR expression have been widely used as indicators of compromised host defense mechanisms following injury. Monocytes of healthy donors exposed to LPS under *in vitro* conditions release the pro-inflammatory cytokines TNFα, IL-1β, IL-6. However, monocytes of post surgical patients and patients with systemic inflammation are characterised by diminished responsiveness to LPS, as assessed by a reduced capacity to produce pro-inflammatory mediators (57, 58). This hyporesponsiveness to endotoxin correlated with the severity of sepsis (59) and injury (60, 61) and it resembles the phenomenon of endotoxin tolerance (62, 63) which is refractoriness to endotoxin stimulation induced *in vitro* by exposure of monocytes to LPS several hours or days prior to re-challenge (63). Despite a large number of studies dealing with monocyte hyporesponsiveness in response to LPS pretreatment, the exact mechanism of suppression of cytokine production has not been identified yet (64, 65).

Another modified parameter is the expression of surface HLA-DR on monocytes in sepsis. Monocytes from post surgical patients and septic patients are characterized by a markedly reduced HLA-DR expression (66-73). In line with this, diminished monocytic HLA-DR expression correlates with disease severity and outcome (68, 74-76). Thus strongly diminished monocytic HLA-DR expression could identify patients with temporary immune depression (77). Moreover, monocytes from septic patients showed a reduced capacity to

produce IL-12, a key cytokine in immune regulation (78). This immune suppression also has been shown to affect the adaptive arm of the immune system. The effective development of a coordinated immune response to a pathogen depends on a carefully regulated interplay between innate and adaptive immunity.

## 1.8 T cell response to injury

Studies of patients with systemic inflammation showed that there is a biphasic immunologic response: an initial hyperinflammatory phase is followed by a hypoinflammatory one (Figure 3). The initial phase is driven by proinflammatory cytokines and the latter phase is associated with a shift of host immune response to an anti-inflammatory or anergic state in which the patient is effectively immune suppressed (4). Many patients at this phase acquire secondary infections, often caused by organisms which would typically be unable to infect hosts with a normal immune system. This failure to mount an effective inflammatory response against secondary infections may contribute to the increased morbidity and mortality of this patient group.

Most of the patients with systemic inflammation do not die from the primary insult but rather as a result of a secondary infection. To explain this an inflammatory "two hit" model

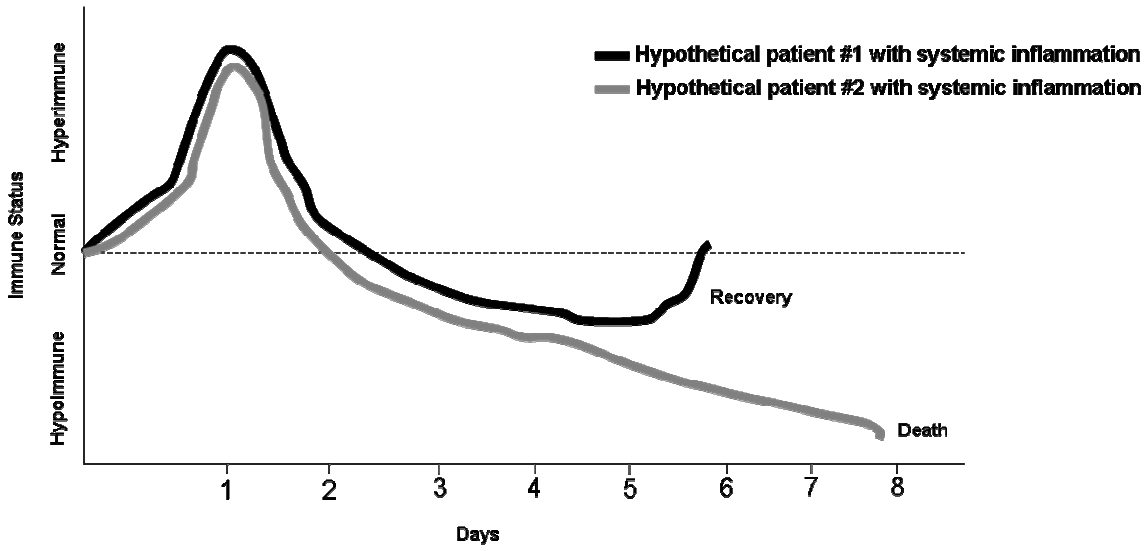



**Figure 3. Inflammatory response during systemic inflammation.** Various stimuli such as trauma, major surgery can lead to excessive production of pro-inflammatory cytokines. This initial hyperinflammatory response can rapidly progress to prolonged hypoinflammatory or immune suppressive state. This results in increased susceptibility to infection and treatment may improve the likelihood of survival in hypothetical patient#1. Failure to respond to the treatment may cause death in hypothetical patient #2. (Picture adopted from Hotchkiss and Karl 2003 (3))

has been proposed to account for the development of inflammatory complications in trauma and critically ill surgical patients (79). According to this model, the first "hit" is the initial tissue trauma which leads to generalized inflammation within hours after injury followed by a second "hit" such as a bacterial infection. Thus this model reflects the late onset of immune suppression and subsequent susceptibility to secondary infection (80, 81).

In some cases the requirement for two hits may be bypassed as happens in the case of toxic shock syndrome. Toxic shock results from superantigens produced by *Staphylococcus aureus* or *Streptococcus pyogenes*. The family of superantigens includes staphylococcal enterotoxins SEA-SEU, toxic shock syndrome toxin 1 (TSST-1) and the streptococcal pyrogenic exotoxins SPEA and SPEC (2, 82). These superantigens have the ability to circumvent the mechanisms of conventional, MHC-restricted, antigen processing. Whilst conventional antigenic peptide requires uptake and processing by antigen presenting cells (APCs), superantigens require no processing once secreted, in order to interact with the APC. Conventional antigenic peptides are then presented externally within the antigen groove of specific MHC class II molecules and undergo a highly specific interaction with T cells that is restricted by both the class II molecule and peptide, classically resulting in T helper cell activation. Bacterial superantigens bypass the need for processing by antigen-presenting cells by directly binding to major histocompatibility complex class II molecules on the surface of these cells and stimulate almost all T cells bearing particular domains in the variable portion of the β chain (Vβ) of the T cell receptor (TCR), without need for processing by APCs. In contrast, normal peptides stimulate only a few T cells (<1 in 1000) (83). However, the superantigens typically activate 5 to 30% of the entire T-cell population leading to a massive release of proinflammatory cytokines, such as TNF-α, IL-6, and IFN-γ. This disproportionate proinflammatory activity is the cause of toxic shock syndrome (84, 85).

In the adaptive system, immune suppression in patients with systemic inflammation is associated with a high level of apoptosis. The key role of apoptosis was shown in a study of lymphocytes in the spleens of patients who died of sepsis compared with patients in intensive-care units who died of non-septic causes. The autopsies revealed extensive apoptosis of lymphocytes and gastrointestinal epithelial cells in the septic patient group but not in the non septic controls (86). The analysis of the spleen revealed a decrease in the number of B cells and $CD4^+$ T cells (87). These post-mortem findings of the septic patients have been supported by new studies of circulating blood leucocytes from patients in intensive care units (88). An increased level of lymphocyte apoptosis was evident in patients in septic shock compared

with healthy volunteers. These studies indicate that lymphocyte apoptosis is rapidly increased in the blood of patients with septic shock, and that this leads to a profound and persistent lymphocytopenia that is associated with poor outcome (89). Thus apoptosis is a key pathophysiological process in sepsis and leads to a striking loss of lymphocytes. Moreover, numerous immune functions are known to be impaired in patients after major surgery (66, 69, 72, 73, 90).

Clinical studies have shown reductions in the peripheral lymphocyte counts particularly of the $CD4^+$ T cells with a consequent skewing of the CD4:CD8 ratio in post surgical patients (91). T cells from these patients exhibited decreased proliferation following mitogen stimulation (92-94). In addition, Oka et al demonstrated a significantly higher frequency of apoptosis of circulating lymphocytes after surgery (95-97). The Th1/Th2 balance is also affected by major surgery (93). $CD4^+$ T helper cells are generally divided into two subsets, Th1 and Th2 on the basis of their cytokine secretion profiles. Th1 subsets are characterized by the regulation of cell mediated immunity and the production of interferon (IFN)-γ. In contrast, Th2 cells secrete IL-4 which is important for B cell proliferation and antibody production (98, 99). However, an inappropriate differentiation of Th1 and Th2 cells during an immune response may result in severely impaired defense against diverse pathogens. Hensler and co-workers demonstrated that major surgery results in a severe defect in proliferative response after ligation of CD3 and CD28 receptors of T cells and these cells showed reduced secretion of cytokines characteristic of both Th1 and Th2 cells, including IFN-γ, IL-2, TNF-α and IL-4 (72). Thus surgical trauma profoundly affects the immune system.

Recent studies demonstrated that minimization of the surgical trauma results in less pronounced depression of immune response. Studies comparing major colorectal surgery and minor surgery (i.e. Laparoscopic surgery) found a decrease in IL-2, IFN-γ production by T cells and fall in lymphocyte count was less pronounced in minor surgical interventions (100, 101). Similarly, the ability of T lymphocytes to respond to mitogenic activation i.e., concanavalin A and phytohemagglutinin was suppressed 15 days after major operation, whereas no suppressive effect was found after minor surgical intervention (102). These results suggest that minor surgical intervention is associated with less disturbance of systemic immune function.

## 1.9 Objectives of the study

The objectives of the study were to assess the degree of immune impairment induced in a group of 101 patients undergoing major elective surgery and 17 patients undergoing minor surgery. As indicators of changes in the innate immune system, monocyte HLA-DR expression and monocyte gene expression were monitored in post surgical patients. At the same time, T-cell numbers, apoptosis and changes in the T-cell gene expression patterns were examined as markers of adaptive immune system modulation in the perioperative period.

# 2 Materials and Methods

## 2.1 Materials

### 2.1.1 Instruments

| | |
|---|---|
| ABI PRISM 7000 Sequence Detection System | Applied Biosystems, Foster City, CA, USA |
| Analytical balance | Sartorius AG, Goettingen |
| Black-Quarz-Cuvette | Hellma GmbH & Co KG, Müllheim |
| Cell Counter K-4500 | Sysmex Deutschland GmbH, Norderstedt |
| Centrifuges: | |
| Biofuge fresco | Heraeus Instruments, Hanau |
| Multifuge 3 S-R | Heraeus Instruments, Hanau |
| EBA 12 R | Hettich Centrifugen GmbH & Co KG, Tuttlingen |
| Vacuum-centrifuge | Johan GmbH, Unterhaching |
| Digital balance | Sartorius AG, Goettingen |
| Digital Graphic Printer UP-D890 | Sony, Köln |
| FACSCalibur | Becton Dickinson GmbH, Heidelberg |
| FACScan | Becton Dickinson GmbH, Heidelberg |
| Freezer(-20$^{o}$C) | Liebherr-International AG, Bulle, Schweiz |
| Freezer(-80$^{o}$C) | Forma Scientific, Thermo Electron Corporation, Waltham, MA, USA |
| Gel Electrophoresis Chamber | BioRad, München |
| Hot block (QBT1) | Grant Instruments, Grants Pass, Oregon, USA |
| Incubator | Heraeus Instruments, Hanau |
| Laminar Flow Station | Nunc GmbH, Wiesbaden-Biebrich |
| Magnetic stirrer | Heidolph Instruments, Schwabach |
| Micropipette | Eppendorf, Hamburg |
| Microwave | Bosch, Gerlingen - Schillerhöhe |
| Multistep-Pipette | Eppendorf, Hamburg |
| Multi-channel pipette | Brand, Wertheim/Main |
| MiniMACS™ Separator | Mitenyi Biotech GmbH, Bergisch-Gladbach |
| $P_H$-meter | Hanna Instruments, Kehl am Rhein |
| Photometer (GeneQuant) | Amersham Pharmacia Biotech, Piscataway, USA |

| | |
|---|---|
| Scanner (Genepix 4000B) | Axon Instruments, Union City, CA, USA |
| Shake incubator | New Brunswick Scientific, Nürtingen |
| Shaker | Vibrax, Janke&Kunkel |
| Thermal cycler | Biometra, Goettingen |
| Vortexer (Genie 2) | Scientific Industries, Bohemia, NY, USA |
| Gel Documentation System | MWG Biotech AG, Ebersberg |
| Waterbath | |
| RM6 Lauda | DRR Wober GmbH & Co, Lauda-Königshofen |
| Julabo SW21 | Seelbach, Germany |

### 2.1.2 PC programmes and Online- Data Bank

| | |
|---|---|
| ABI PRISM® 7000 Software | Applied Biosystems, Foster City, CA, USA |
| Genepix 4 software | Axon Instruments, Foster City, California |
| NCBI | http.\\www.ncbi.nlm.nih.gov |
| TallyCal Software | DAKO cytomation, Hamburg |
| Primer Express® Software v2.0 | Applied Biosystems, Foster City, CA, USA |
| PhotoFinish v3.0 | WorldStar Atlanta Technology Center Inc., Atlanta, GA, USA |
| EndNote v9.0.1 | Thomson Corporation, Stamford, CT, USA |
| SPSS for Windows Student v11.0.0 | SPSS Inc., Chicago, IL, USA |
| WinMDI 2.8 and 2.9 | Joseph Trotter |

### 2.1.3 Reagents

| | |
|---|---|
| 5-(3-aminoallyl)-UTP | Ambion, Huntingdon, UK |
| 7-AAD | BD Biosciences, Heidelberg |
| Agarose | Applichem, Darmstadt |
| Ammonium Chloride Lysing Reagent | Becton Dickinson GmbH, Heidelberg |
| Bromo Phenol Blue | SERVA, GMBH, Heidelberg |
| BSA | Sigma-Aldrich, Steinheim |
| Calcium Binding Buffer | Becton Dickinson GmbH, Heidelberg |
| dATP, dCTP, dGTP, dTTP | Promega, Mannheim |
| DEPC | Sigma-Aldrich, Steinheim |
| DMSO | Sigma-Aldrich, Steinheim |

| | |
|---|---|
| DNA 100 bp ladder | MBI Fermentas GmbH, St. Leon-Rot |
| DNA 1kb ladder plus | MBI Fermentas GmbH, St. Leon-Rot |
| Ethidium bromide | Boehringer, Mannheim |
| FACS lysing solution | Becton Dickinson GmbH, Heidelberg |
| Fetal Calf Serum (FCS) | Sigma-Aldrich, Steinheim |
| Ficoll density 1.077 | Biochrom AG, Berlin |
| Fragmentation Buffer | Ambion, Huntingdon, UK |
| Glycogen | Roche Applied Science, Mannheim |
| Hybridisation buffer | Ambion, Huntingdon, UK |
| MOPS (10x buffer) | Ambion, Huntingdon, UK |
| NHS-Cy3 | Amersham Bioscience, Freiburg |
| NHS-Cy5 | Amersham Bioscience, Freiburg |
| PBS (phosphate buffered saline) | BioChrom AG, Berlin |
| RNA ladder | Boehringer, Mannheim |
| TÜRK's Solution | Merck, Darmstadt |

### 2.1.4 Materials

| | |
|---|---|
| 20 G Needle | Dispomed, Witt oHG, Gelnhausen |
| ABI PRISM Optical tubes and caps | Applied Biosystems, Foster City, California |
| Adhesive film for PCR Plate | ABgene House, UK |
| Blood collection tube (Vacutainer, EDTA) | BD biosciences, Heidelberg |
| Centrifuge tube (15 ml, 50 ml) | Falcon BD GmbH, Heidelberg |
| Cover glasses (24 × 60 mm) | Erie Scientific, Erlangen |
| Hybridization chamber | Corning, NY, USA |
| Leukocyte seperation tubes (50 ml) | Greiner Bio-One GmbH |
| MiniMacs Separation Column | Miltenyi Biotec GmbH, Bergisch-Gladbach |
| PCR Tubes | PEQLAB biotechnologie GmbH, |
| RNase free reaction tube (0.5 ml, 1.5 ml) | Eppendorf, Hamburg |
| TruCount Tubes | BD Biosciences, Heidelberg |

All other chemicals were of reagent grade.

### 2.1.5 Buffers and solutions

| | |
|---|---|
| DNA Gel Loading Buffer(5X) | EDTA (0.1M)<br>Bromophenol blue (0.25%)w/v<br>Xylene Cyanol FF (0.25%)w/v<br>Glycerol (30%)v/v<br>Water |
| DEPC $H_2O$ | DEPC 0.001% in $H_2O$ stirred overnight and then autoclaved |
| FACS buffer | FCS 1 %<br>$NaN_3$ 0. 1%<br>in PBS and sterile filtered. |
| Fragmentation buffer (5×) | Tris-acetate pH 8.1(20 mM)<br>Magnesium acetate (15 mM)<br>Potassium acetate (50 mM)<br>DEPC $H_2O$ |
| MACS buffer | $NaN_3$ 0. 1% w/v<br>BSA 0. 5% w/v<br>in PBS |
| MOPS (10×) | MOPS 104.7 g<br>added to 400 ml DEPC $H_2O$<br>pH adjusted to 7.0 with NaOH<br>Filled up to 500 ml with DEPC $H_2O$<br>Autoclaved and stored in the dark at room temperature |
| 5 M NaCl | 29.2 g of NaCl in 100 ml final volume of $dH_2O$ |
| 5 M NaOH | 20 g of NaOH was added and make up to 100 ml with $dH_2O$ |

| | | |
|---|---|---|
| RNA loading buffer | EDTA | 1mM |
| | EtBr | 60µg/ml) |
| | Bromophenol blue | 0.25% w/v |
| | Xylene Cyanol | 0.25% w/v |
| | Glycerol | 50% v/v |
| RNA Gel running buffer (5×) | MOPS 1M | 25.0 ml |
| | Sodium Acetate 3M | 3.33 ml |
| | DTA 0.35M pH 8.0 | 2.5 ml |
| | DEPC $H_2O$ | 219.17 ml |
| 0.1% Sodium Azide ($N_aN_3$) | 1 mg $NaN_3$ /ml PBS | |
| SSC (20 ×) | NaCl | 175.3 g |
| | Sodium citrate | 88.2 g |
| | $dH_2O$ to 1 L pH adjusted to 7.0 | |
| TBE (10 ×) | Tris Base | 108 g |
| | Boric acid | 55 g |
| | EDTA (0.5 M) | 7.2 g |
| | $dH_2O$ to 1 L | |
| RNA Gel (1%) | Agarose | 0.5 g |
| | DEPC water | 40 ml |
| | MOPS buffer (10x) | 5 ml |
| | Formaldehyde,37% v/v | 5 ml |
| | EtBr(10mg/ml) | 1.5 µl |
| DNA Gel (2%) | Agarose | 3 g |
| | TBE buffer, 1x | 150 ml |
| | EtBr(10mg/ml) | 15 µl |

### 2.1.6 Primers

| Name | Accession No | Primer (5´→3´) | Amplicon Size (bp) |
|---|---|---|---|
| CD63 antigen | BC002349 | F-CCCGAAAAACAACCACACTGC | 347 |
| | | R-GATGAGGAGGCTGAGGAGACC | |
| Dopamine receptor D5 | BC009748 | F-GTCGCCGAGGTGGCCGGTTAC | 362 |
| | | R-CTGGAGTCAGAATTCTCTGCAT | |
| HLA-B associated transcript 5 | AF129756 | F-TGATGGCAATGAGATTGACACC | 100 |
| | | R-AAAACCCAGCATTCCCCTCAC | |
| Hypothetical protein (C8ORF16) | AJ312026 | F-TCAGTTAGCAGTGACCAAGGCA | 400 |
| | | R-AGTGTGTTCCCTCCATGTAGGC | |
| IL-21 | AF254069 | F-TGTGAATGACTTGGTCCCTGAA | 84 |
| | | R-CAGGAAAAAGCTGACCACTCA | |
| Interleukin 13 receptor, alpha | BC020739 | F-AGTTAAACCTTTGCCGCCAGT | 100 |
| | | R- ACCTTGCTGGAATAGGTCCCA | |
| Muscarinic acetylcholine receptor M5 | AF385591 | F-GGAAACAGAGAAGCGAACCAA<br>R-AGCACAACCAATAGCCCAAGT | 800 |
| S100A12 | NM_005621 | F-TTGAAGAGCATCTGGAGGG | 269 |
| | | R-CTACTCTTTGTGGGTGTGG | |
| S100A8 | BC005928 | F-TGTCAGCTGTCTTTCAGAAG | 286 |
| | | R-ACGCCCATCTTTATCACCAG | |
| Stat5B | U47686 | F-GTAAACCATGGCTGTGTGGA | 300 |
| | | R-AAATAATGCCGCACCTCAAT | |
| 18s rRNA | X003205 | F-CGGCTACCACATCCAAGGAA | 177 |
| | | R-GCTGGAATTACCGCGGCT | |

### 2.1.7 Kits

| | |
|---|---|
| Ambion hybridisation buffer II | Ambion, Huntingdon, UK |
| Annexin V : FITC Apoptosis Detection Kit I | BD Biosciences, Heidelberg |
| Fluorochrome coupled calibration beads | DAKO cytomation, Hamburg |
| GoTaq® DNA polymerase | Promega, Madison WI, USA |
| Human 40K whole genome array | MWG Biotech, Ebersberg, Germany |
| MessageAmp™ aRNA Kit | Ambion, Huntingdon, UK |
| Pan T Cell Isolation Kit II | Miltenyi Biotech GmbH, Bergisch-Gladbach |
| Qiagen OneStep RT-PCR Kit | QIAGEN GmbH, Hilden |

| | |
|---|---|
| qPCR™Mastermix Plus for SYBR Green I | Eurogentec Deutschland GmbH, Köln |
| RNeasy® Mini Kit | QIAGEN GmbH, Hilden |
| TaqMan Reverse Transcription reagents | Applied Biosystems, Foster City, CA, USA |

### 2.1.8 Magnetic Microbeads

| Specificity | Company |
|---|---|
| anti-human CD14 | Miltenyi Biotech GmbH, Bergisch-Gladbach |
| anti-human CD3 | Miltenyi Biotech GmbH, Bergisch-Gladbach |
| anti-human CD4 | Miltenyi Biotech GmbH, Bergisch-Gladbach |
| anti-human CD8 | Miltenyi Biotech GmbH, Bergisch-Gladbach |

### 2.1.9 Antibodies

| Specificity | Species | Isotype | Clone | Format | Company |
|---|---|---|---|---|---|
| anti- huCD14 | Mouse | $IgG_{2a},\kappa$ | Tük4 | PE-Cy5 | CA |
| anti- huHLA-DR | Mouse | $IgG_{2a},\kappa$ | L243 | FITC | BD |
| anti- huCD3 | Mouse | $IgG_1,\kappa$ | SK7 | PerCP | BD |
| anti- huCD4 | Mouse | $IgG_1,\kappa$ | SK3 | FITC | BD |
| anti- huCD8 | Mouse | $IgG_1,\kappa$ | SK1 | PE | BD |
| anti- huCD3 | Mouse | $IgG_1,\kappa$ | SK7 | PE | BD |
| anti- huCD45 | Mouse | $IgG_1,\kappa$ | 2D1 | FITC | BD |
| anti- huCD45 | Mouse | $IgG_1,\kappa$ | 2D1 | PerCP | BD |
| anti- huCD4 | Mouse | $IgG_1,\kappa$ | SK3 | PE | BD |
| anti- huCD8 | Mouse | $IgG_1,\kappa$ | SK1 | FITC | BD |
| unknown | Mouse | $IgG_{2a},\kappa$ | X39 | FITC | BD |
| unknown | Mouse | $IgG_1,\kappa$ | X40 | FITC | BD |
| unknown | Mouse | $IgG_1,\kappa$ | X40 | PE | BD |

Company:
CA: Caltag laboratories, Hamburg
BD: BD biosciences, Heidelberg

## 2.2 Methods

### 2.2.1 Study design and patient collective

The study was a collaborative effort of the Departments of Surgery and Immunology of the University hospital in Greifswald, Germany. The study group consisted of 101 patients undergoing major elective thoracic or visceral surgery and 17 patients undergoing minor surgery. Patients undergoing elective surgery were recruited after giving written informed consent. Patients with acute infectious disease, those undergoing emergency or palliative operations as well as those in ASA category 4 or higher were excluded from the analysis. Venous blood samples were collected in Vacutainer tubes containing EDTA (BD Biosciences, Heidelberg) from each patient on the day before the operation and once daily on the three days following surgical intervention. The study protocol was approved by the Institutional Ethics Commission and was carried out in accordance with the provisions of the Helsinki declaration. Inflammatory complications (systemic inflammatory response syndrome, sepsis and severe sepsis) following surgery were defined according to the consensus conference definitions as modified by the American College of Chest Physicians/Society of Critical Care Medicine (37, 103).

### 2.2.2 FACS analysis

#### 2.2.2.1 Measurement of HLA-DR expression on monocytes

HLA-DR expression on patient monocytes was determined in whole blood anticoagulated with EDTA. 100µl of blood was incubated with saturating amounts of anti-CD14-PE-Cy5, clone Tük4 (5 µl) (Caltag, Hamburg) and of anti-HLA-DR-FITC antibodies, clone L243 (20 µl) (BD Biosciences, Heidelberg). Isotype-matched fluorochrome-conjugated monoclonal antibodies were used as controls. A second sample was incubated in parallel with 5 µl of anti-CD14-PE-Cy5 and 20 µl of isotype control IgG2a-FITC, clone X39 (BD Biosciences, Heidelberg) to assess the non-specific binding and to set the threshold between positively and negatively stained cell populations. After vortexing gently for 3 seconds, both tubes were incubated in the dark for 15 minutes at room temperature. 2 ml of FACS Lysing Solution (BD Biosciences, Heidelberg) was added to each tube followed by gentle vortexing for 5 seconds and the cells were incubated for 10 minutes in the dark at room temperature to allow for erythrocyte lysis. The tubes were then centrifuged at 300xg for 5 minutes at room temperature. The supernatant was aspirated without disturbing the pellet. To wash the cells, 3

ml of FACS buffer (PBS-NaN3 -1%FCS) was added to each sample tube. Cells were resuspended and then pelleted once more by centrifugation at 300xg for 5 minutes. The supernatant was discarded and the cells were resuspended in 200 µl of FACS buffer. Measurements were performed on a FACSCalibur (BD Immunocytometry Systems, Heidelberg) instrument. Multivariate data of 30,000 events were collected in list mode.

The fluorescence data expressed as Mean Fluorescence Index (MFI) was converted into the number of molecules of fluorochrome bound per cell using fluorescent calibration beads (Fluorospheres, DAKO cytomation, Hamburg) which were measured in parallel. To circumvent the day to day variation in MFI values and to make measurements comparable we used a calibration procedure with fluorescent beads of known fluorochrome coupling density to allow us to convert measured MFI values into "Molecules of Equivalent Fluorochrome" (MEF). The standardized fluorescent beads were mixed vigorously by vortexing. 0.5 ml of dilution buffer (Phosphate-buffered saline, pH 7.2) was added to each of two test tubes. One drop of the resuspended blank beads (beads without fluorochome) was added to the first tube and one drop of the suspension of fluorochrome coupled calibration beads was added to the second tube (Fluorospheres, DAKO cytomation, Hamburg). The contents of each tube were mixed by vortexing to ensure an even suspension of the beads. Prior to data acquisition, logarithmic amplification for the fluorescence detectors was selected, and the fluorescence compensation for all parameters set to zero. The FSC (Forward Scatter) and SSC (Side Scatter) characteristics of the blank beads were then measured on the flow cytometer. PMT voltages were adjusted to allow the peak of the blank beads to appear in the first decade in the histogram. Forward scatter versus side scatter data was collected and a live gate was set on bead singlets. A minimum of 5000 gated events of the blank beads were acquired. The tube with calibration beads was applied on the flow cytometer and data was acquired for a minimum of 5000 gated events. Six different fluorescence intensity peaks were visible in the fluorescence channel. These fluorescence intensities (MEF) were used to construct a standard curve with corresponding MFI and MEF values. Using this standard curve and the TallyCal software for Windows (DAKO cytomation, Hamburg), the arbitrary MFI values can be converted to calibrated MEF values.

Comparisons of the HLA-DR expression level on monocytes of different patient groups were analyzed using the Mann-Whitney test.

### 2.2.2.2 Determination of CD3, CD4 and CD8 cell numbers using TruCount beads

Determination of absolute cell numbers was carried out using the TruCount procedure. 50µl of whole blood was added to a TruCount tube which contains a freeze dried pellet with an accurately known number of particles. Staining for CD3, CD4 or CD8 was carried out as described for the HLA-DR staining of monocytes. Samples were examined in the FACS using fluorescence triggering to measure the number of labeled lymphocytes. This number was then related to the number of bead events registered in the same run to yield the number of labeled lymphocytes per ml of whole blood. The procedure used was as follows: 50 µl blood anticoagulated with EDTA and 20 µl of TriTest CD4/CD8/CD3 three-color antibodies (BD Biosciences, Heidelberg) were added to bead-containing TruCount tubes (BD Biosciences, Heidelberg). Samples were vortexed briefly to resuspend the beads and then incubated for 20 minutes at room temperature after which 450 µl of FACS Lysing Solution was added. The tubes were then incubated in the dark for 15 minutes at room temperature and measurements were performed on a FACSCalibur (BD Immunocytometry Systems, Heidelberg) instrument. Multivariate data of 20,000 events were collected. The absolute count using TruCount tubes was calculated from the appropriate dot plot values entered into a spreadsheet that was formatted to use the formula:

$$\frac{\text{Events in quadrant containing cell population}}{\text{Events in absolute-count bead region}} \times \frac{\text{Total no. of absolute-count beads}}{\text{Test volume (whole blood)}}$$

### 2.2.2.3 T cell apoptosis assay

The fraction of circulating T cells undergoing apoptosis was determined by measuring the fraction of intact cells (7-Amino-Actinomycin D (7-AAD)-negative) expressing Annexin V on the surface. The procedure used was as follows: 100 µl of anticoagulated blood (EDTA) was incubated at $4^0$C with phycoerythrin labeled anti CD3 antibody, clone SK7 (BD Biosciences, Heidelberg). Erythrocyte lysis was carried out by adding 2.0 ml of 1X PharM Lyse (BD Biosciences, Heidelberg) lysing solution and incubating in the dark at room temperature for 15 minutes. The cells were pelleted by centrifuging at 200xg for 5 minutes. To wash the cells, 1 ml of PBS was added and cells were pelleted once again by centrifuging at 200xg for 5 minutes. After discarding the supernatant the pellet was resuspend in 100 µl of 1x calcium binding buffer. 5 µl of Annexin V- FITC (BD Biosciences, Heidelberg) and 5 µl

of 7-AAD (BD Biosciences, Heidelberg) were added, vortexed briefly and incubated for 15 minutes at room temperature. Then 400 µl of 1x calcium binding buffer was added and cells were analysed by FACSCalibur flow cytometer (BD Immunocytometry Systems, Heidelberg).

### 2.2.3 Cell isolation

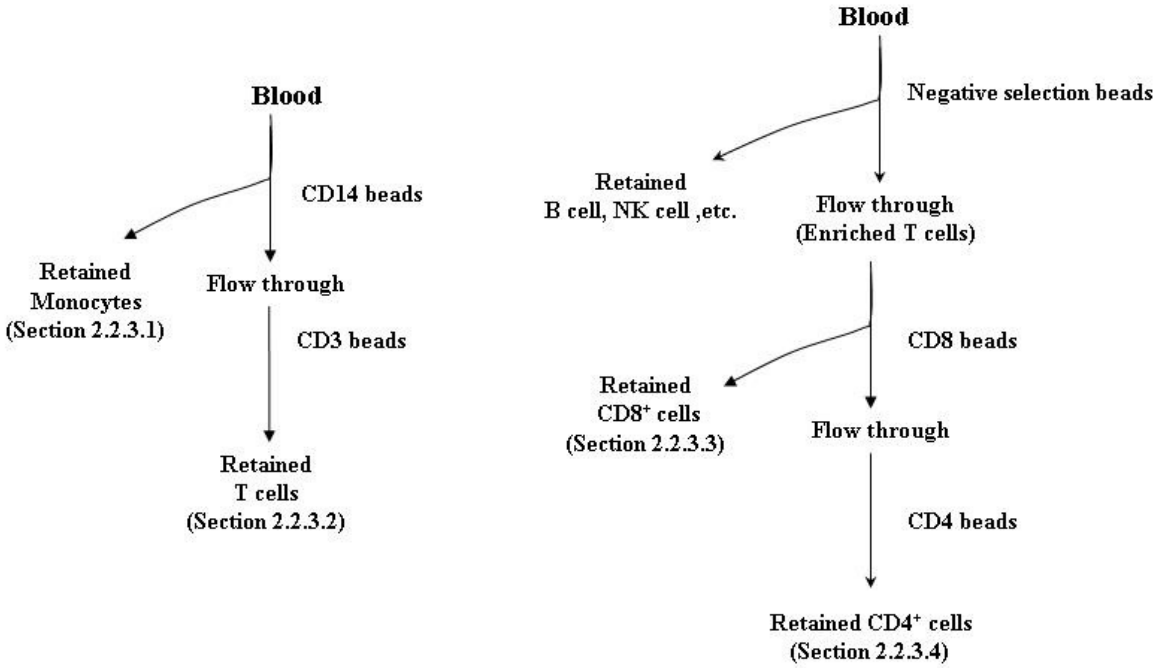



**Figure 4. Flow chart showing isolation of cells.** (A) Monocytes and T cells (B) $CD8^+$ and $CD4^+$T cells from patient blood.

#### 2.2.3.1 Isolation of monocytes from patient blood

15 ml Ficoll (density 1.077, Biochrom AG, Berlin) was added to a 50 ml tube (Leucosep, Greiner Bio-One GmbH) fitted with a sintered filter at the 15 ml mark. Ficoll was collected below the filter by centrifuging at 320xg at room temperature for 1 minute. The excess of Ficoll above the filter was then discarded. 12 ml peripheral blood was drawn into Vacutainer blood collection tubes containing EDTA. The anticoagulated blood was diluted with an equal volume of PBS and was poured onto the filter. After centrifugation at room temperature at 1500xg for 20 minutes, the visible band of mononuclear cells was collected. The red blood cells and most of the granulocytes are centrifuged into the Ficoll layer so that this population of peripheral blood mononuclear cells (PBMC) consists mainly of lymphocytes and monocytes. PBMC were washed twice at 4°C with PBS containing 0.1% sodium azide (NaN3). The cell final pellet was carefully resuspended in a final volume of 1

ml of PBS containing 0.1% sodium azide (NaN3). 5 µl of this suspension was mixed with 95 µl of TÜRK's solution (Merck, Darmstadt) for cell counting. The remaining cells were pelleted by centrifugation and resuspended in 80 µl MACS buffer (PBS containing 0.1 % NaN3, 0.5 % BSA). 20 µl anti-CD14 antibody conjugated magnetic beads (Miltenyi Biotech GmbH, Bergisch-Gladbach) were added per $10^7$ cells in a final volume of 80 µl and incubated with shaking at 4°C for 15 minutes. The magnetic sorting column (Miltenyi Biotech GmbH, Bergisch-Gladbach) was fixed into a magnetic support (MiniMACS Separator, Milteny Biotech GmbH, Bergisch-Gladbach) and equilibrated with 2 × 0.5 ml of MACS buffer. The cell suspension was vigorously vortexed and then loaded on the magnetic column. The cell suspension were allowed to slowly run through the column. It was then washed six times with 0.8 ml MACS buffer. Cells which had bound the magnetically labelled antibodies are retained; cells lacking CD14 molecule are washed through. To elute the bound cells, the column was removed from the magnet and washed with 1 ml of MACS buffer. The entire column volume and wash buffer was recovered by blowing air through the column. The recovered cells were counted and their purity was determined by FACS analysis after labelling a 5µl sample of the cells with anti-CD14 PE and anti-CD45 FITC (BD bioscience, Heidelberg).

#### 2.2.3.2 Isolation of T cells from patient blood

$CD3^+$ T cells were isolated from the same PBMC preps used to isolate monocytes. The cells which were not labeled with the magnetic beads coupled with anti-human CD14 antibody flowed through the magnetic column. This flow-through and the first three washings, which contain cells other than monocytes, were pooled, centrifuged at 230xg, 4°C for 10 minutes and washed once in 1ml PBS-$NaN_3$. The supernatant was discarded and the cells were resuspended in 80 µl MACS-buffer (PBS-NaN3-0.5% BSA) per $1x10^7$ cells. The sample was vortexed at full speed for 15 seconds to separate cell clumps. 20 µl anti human-CD3 antibody conjugated magnetic beads (Miltenyi Biotech GmbH, Bergisch-Gladbach) per $10^7$ cells was added and the supension was mixed gently in a shaker for 15 minutes at 4 °C. The recovery of the $CD3^+$ cells was then carried out as described for the labelled monocytes in the previous section. A magnetic column Miltenyi Biotech GmbH, Bergisch-Gladbach) was washed twice with 0.5 mL MACS-buffer at 4°C. The sample of labelled T cells was made upto 0.5 ml with MACS-buffer and mixed by vortexing at full speed for 15 seconds. A 5 µl sample was retained for FACS analysis. The rest of the labelled cells were loaded onto the magnetic column, washed with MACS buffer and eluted.

To analyse the purity of isolated T cells, cell suspension obtained before and after the magnetic separation for $CD3^+$ cells incubated with phycoerythrin conjugated anti-CD3 (BD bioscience, Heidelberg) and peridinin chlorophyll protein (PerCP) conjugated anti-CD45 (BD bioscience, Heidelberg). Labelled cells were washed with 1 mL FACScan buffer and recovered by centrifugation at 230xg for 10 minutes. The washed cells were resuspended in 200 µl of FACS buffer. All analyses were performed on a FACScan (BD Immunocytometry Systems, Heidelberg) instrument. Approximately 20,000 gated events were collected in each analysis. Data were compensated and analyzed using WinMDI software.

#### 2.2.3.3 Isolation of $CD8^+$ T cells from patient blood

Cells other than T cells, i.e. B cells (CD19), NK cells (CD56 and CD16), dendritic cells (CD123), monocytes (CD14), granulocytes (CD123), and erythroid cells (Glycophorin A) were magnetically labeled by using a cocktail of biotin-conjugated antibodies. Anti-biotin microbeads were used to deplete these cells. $CD8^+$T cells were isolated by direct sorting using magnetically labelled anti $CD8^+$ antibodies by the following procedure. PBMC were isolated as described for monocyte isolation in the previous section and the cell final pellet was resuspended in 40µl of MACS buffer (PBS-NaN3-0.5% BSA) per $10^7$ total cells. 10µl of Biotin-antibody cocktail (Miltenyi Biotech GmbH, Bergisch-Gladbach) was added and after brief vortexing incubated for 10 minutes at $4^oC$. Cells were then resuspended in 30µl of MACS buffer followed by addition of 20µl of anti-biotin microbeads per $10^7$ cells (Miltenyi Biotech GmbH, Bergisch-Gladbach). After thorough mixing the cells were incubated for an additional 15 minutes at $4^oC$. 1 ml of MACS buffer was then added and centrifuged at 300xg for 10 minutes. The cell pellet was resuspended in final volume of 500µl of MACS buffer. Magnetic sorting was carried out as detailed in section 3.3.1. The magnetic column was washed three times and these effluents contain enriched unlabeled T cell fraction. The purity of enriched cells was determined by FACS analysis after labelling a 5 µl of sample with anti-CD3 PE and anti-CD45 PerCP.

The unlabeled T cells were pooled and centrifuged at 300xg for 10 minutes. The pellet was then resuspended in 80 µl of MACS buffer per $10^7$ cells. 20 µl of anti-human CD8 microbeads per $10^7$ cells was added and after brief vortexing, the cells were incubated for 15 minutes at $4^oC$. The magnetic sorting was done as described in section 3.3.1. The flow-through and the first two washings were collected for isolation of CD4 cells (see section 3.3.4). After elution of the magnetically bound $CD8^+$ cells their purity was determined by

FACS analysis by labelling a 5 µl sample of the cells with anti-CD4 PE and anti-CD8 FITC (BD bioscience, Heidelberg).

#### 2.2.3.4 Isolation of $CD4^+$T cells from patient blood

The fraction of cells which were not labelled with CD8 magnetic beads flowed through the magnetic column. These eluates were pooled and centrifuged at 300xg for 10 minutes. The pellet was resuspended in 80 µl of buffer per $10^7$ cells followed by addition of 20 µl of CD4 magnetic beads. Then the same procedure was followed as detailed for $CD8^+$T cells. The labelled $CD4^+$T cells were then eluted and 5 µl of sample was used for FACS analysis as described for $CD8^+$T cells.

### 2.2.4 RNA isolation

Cells were first lysed and homogenized in the presence of a denaturing buffer containing guanidine isothiocyanate (GITC) (RNeasy MiniKit, QIAGEN GmbH, Hilden) to inactivate RNases. Viscosity was reduced by shearing the DNA and the material loaded onto a silica-gel based membrane in a spin column. The RNA binds to the column. After washing to remove other components, RNA was eluted in a small volume of water (50 µl). The procedure was carried out according to the manufacturer's instructions (RNeasy MiniKit, QIAGEN GmbH, Hilden). Cells were pelleted by centrifugation at 300xg at $4^oC$ for 5 minutes and the supernatant discarded. 10 µl of β-Mercaptoethanol was added to 1ml of the lysis buffer "RLT". Then 350µl of this RLT added to cell pellets containing no more than 5 x $10^6$ cells. 600µl of the RLT was used for samples containing between $5x10^6$-$1x10^7$ cells. The sample was vortexed and mixed vigorously by pipetting. The sample was then sheared by passing 20 times vigorously through a 20G (D 0.9mm) needle. 1 volume of 70% Ethanol was added and the sample was mixed thoroughly by pipetting and transferred to a spin column fitted with a silica-membrane. The column was centrifuged at maximum speed (≥10,000 rpm) for 15 seconds and the flow through discarded. The remaining sheared cell extract (if any) was pipetted onto the column which was again centrifuged at maximum speed (≥10,000 rpm) for 15 seconds. 700µl of low salt wash buffer RW1 was added to column which was centrifuged again at maximum speed (≥10,000 rpm) for 15 seconds. The spin column was then washed with buffers of decreasing ionic strength. After which it was transferred to a RNAse free 1.5 ml tube. 50 µl DEPC- $H_2O$ was applied directly onto the column and incubated at room temperature for 1 minute prior to centrifugation at (≥10,000 rpm) for 1 minute. To determine the RNA concentration, 1 µl of the RNA was diluted in 80 µl DEPC

treated water and the optical density at 260 nm and 280 nm was determined. For samples with ratio of OD260/OD280 of 1.8-2.0 the RNA concentration was calculated using the following formula: Concentration (µg/ml) = OD260 X dilution factor X 40µg/ml. All RNA samples were stored at –80 °C.

### 2.2.5 Denaturing agarose gel electrophoresis

Formaldehyde agarose gels were used for separation and identification of RNA based on charge migration. RNA has a high degree of secondary structure, making it necessary to use a denaturing gel. Formaldehyde in the gel disrupts secondary RNA structure and will protect RNA from RNases. The procedure used was as follows: 100 ml of a 1% agarose gel was prepared by adding 1g of agarose (AppliChem, Darmstadt) in 72 ml water, boiled and cooled to $60^0$C. 10 ml of 10x MOPS running buffer and 18 ml of 37% formaldehyde (12.3M) (AppliChem, Darmstadt) was added to the cooled agarose solution, mixed and then poured into gel-former. The agarose gel was allowed to set and then placed in the electrophoresis tank containing 1x MOPS running buffer. 1000ng-3000ng of total RNA was adjusted to 11 µl of nuclease free water. 5 µl of 10x MOPS running buffer and 9 µl of 12.3 M formaldehyde 25 µl of formamide (Sigma-Aldrich, Steinheim) added and heated to $55^0$C for 15 minutes. 10 µl of formaldehyde loading dye (Formaldehyde Load Dye, Ambion, Huntingdon, UK.) was added, mixed well and loaded into slots of the agarose gel. The samples were electrophoresed at 5V/cm and visualised on UV transilluminator at 320nm.

### 2.2.6 Single Cell PCR

Single monocytes were isolated from major trauma SIRS patient (Dr.Claudia Berek, Deutsche Rheumaforschungs Zentram, Berlin). FACS sorting of PBMC prepared by Ficoll gradient centrifugation and stained with CD14-FITC (BD bioscience, Heidelberg). Cells were individually sorted into PCR tubes containing 20µl of PCR buffer (QIAGEN OneStep RT-PCR Kit, Qiagen, Hilden) and immediately frozen on dry ice. Samples were stored at $-80^0$C till required for PCR analysis. The cell was thawed on ice and 40 µl of the master mix was distributed into each PCR tube. Master mix was prepared which contained per synthesis reaction:

| Component | Volume/reaction | Final Concentration |
|---|---|---|
| RNase-free water | 25.5µl | |
| 5x QIAGEN OneStep RT-PCR Buffer | 8µl | 1x |
| dNTP Mix (10 mM of each dNTP) | 2µl | 400µM of each dNTP |
| Primer FW | 1µl | 0.6µM |
| Primer RW | 1µl | 0.6µM |
| QIAGEN OneStep RT-PCR Enzyme Mix | 2µl | |
| RNase inhibitor | 0.5µl | 10U |
| Single cell in 1XPCR Buffer | 10 µl | |
| Total volume | 50 µl | |

The tubes were placed in the thermal cycler (Biometra, Göttingen) and the following cycling program was started immediately.

| Thermal cycles | Temperature | Time |
|---|---|---|
| Step 1: Reverse transcription | $50^0$C | 30 minutes |
| Step 2: Initial Denaturation | $95^0$C | 15 minutes |
| Step 3: Denaturation | $94^0$C | 0.5 minute |
| Step 4: Annealing | $56^0$C l | 0.5 minute |
| Step 5: Extension | $72^0$C | 0.5 minute |
| Repeat step 3-5 for 50 cycles | | |
| Step 6: Final extension | $72^0$C | 10 minutes |

After finishing PCR, the amplicons were analyzed by agarose gel electrophoresis.

#### 2.2.6.1 Agarose-gel electrophoresis

Agarose was dissolved in boiling 1x TBE buffer, the solution was cooled down to 55°C and the ethidium bromide was added to 0.5 µg/ml final concentration. Before pouring onto a gel platform, the gel was allowed to set at room temperature. DNA samples were mixed with 1/10 volume of 10x DNA loading buffer and loaded onto the gel. The electrophoresis was run at a voltage of 1-5V/cm for 30-60 min in 1x TBE buffer. The DNA was visualized under UV light.

### 2.2.7 Microarray analysis

#### 2.2.7.1 RNA amplification

As the amount of RNA obtained from each cell prep is too small (2-5 µg) for direct microarray analysis, it was necessary to amplify the probe. This was achieved using the T7 promoter-aRNA procedure ( MessageAmp aRNA Kit, Ambion, Huntingdon, UK).

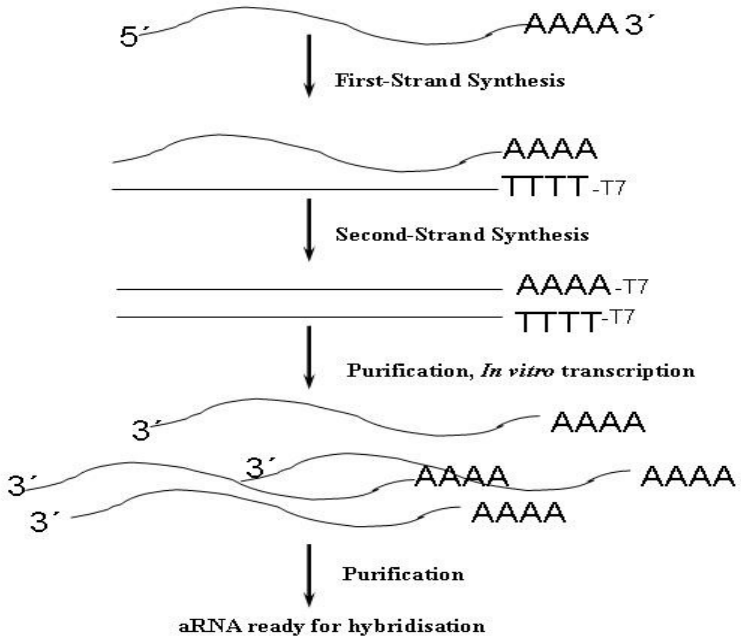



**Figure 5. mRNA Amplification**

The principle of this mRNA procedure is shown in figure 5. The mRNA amplification procedure consists of reverse transcription of RNA with an oligo(dT) primer bearing a T7 promoter and in vitro transcription of the resulting cDNA with T7 RNA Polymerase to generate hundreds to thousands of antisense RNA copies from each mRNA molecule. The antisense RNA is referred to as aRNA .

### 2.2.7.2 First strand cDNA synthesis

For first strand cDNA synthesis 100-1000 ng of total RNA was aliquoted in RNase free tubes and the volume was adjusted to 10 µl. 1 µl of T7 Oligo(dT) primer (Ambion, Huntingdon, UK) was added to each and the volume was adjusted to 12 µl with nuclease-free water. The reaction tubes were incubated for 10 minutes at 70°C and then placed on ice. A first strand master mix was prepared which contained per synthesis reaction:

| Reagent | Amount |
|---|---|
| 10X First Strand Buffer | 2µl |
| Ribonuclease Inhibitor | 1µl |
| dNTP Mix | 4µl |
| Reverse Transcriptase | 1µl |

8µL of the master mix was quickly transferred to each RNA sample. The first strand systhesis was carried out by incubation at 42$^{o}$C for 2 hours in a water bath.

### 2.2.7.3 Second strand cDNA synthesis

Second strand cDNA synthesis converts the single-stranded cDNA with the T7 promoter primer into double-stranded (dsDNA) template for transcription. A second strand synthesis master mix prepared. It contained per reaction:

| Reagent | Amount |
|---|---|
| Nuclease free water | 63µl |
| 10X Second Strand Buffer | 10µl |
| dNTP Mix | 4µl |
| DNA Polymerase | 2µl |
| RNase H | 1µl |

80µl of this master mix was added to each 20µl first strand synthesis reaction and incubated at 16$^{o}$C for 2 hours in a water bath.

### 2.2.7.4 cDNA purification

cDNA purification was carried out by absorption to a silica filter. RNA, primers, enzymes, and salts from the dsDNA that inhibit *in vitro* transcription were removed by washing. A spin column fitted with silica membrane was equilibrated with 50 µl of cDNA binding buffer (Ambion, Huntingdon, UK) for 5 minutes at room temperature, immediately before starting the cDNA purification. 250µl of cDNA binding buffer was added to each cDNA sample, mixed and pipetted to the center of an equilibrated filter cartridge. The cartridge was centrifuged for 1 minute at 10,000xg. The flow-through was discarded. 500µl of wash buffer was added and centrifuged at 10,000xg for 1 minute. Centrifugation was repeated for a further 1 minute to remove traces of the wash buffer. 100µl of nuclease-free water that was preheated to 50$^{0}$C was pipetted onto the center of the column and incubated at room temperature for 2 minutes. Then eluted cDNA was recovered by centrifugation for 1 minute at 10,000xg. The cDNA elution step was repeated using a second 100 µl of preheated water. The pooled cDNA (200 µl) was precipitated by adding 20 µl of 5M NaCl, 1µl of glycogen (20mg/ml) (Roche applied science, Mannheim, Germany) and 600 µl of absolute ethanol. The

tubes were incubated at –80$^0$C for 30 minutes and the cDNA was recovered by centrifugation at 10,000xg for 30 minutes at 4$^0$C. The final cDNA pellet was dried at room temperature and then redissolved in 14 µl of nuclease free water.

### 2.2.7.5 *In vitro* Transcription and aRNA isolation

*In vitro* transcription with aaUTP generates multiple copies of modified aRNA from the double-stranded cDNA templates. The master mix for a single reaction was as follows:

| Component | Amino allyl | Unmodified |
|---|---|---|
| 5-(3-aminoallyl)-UTP(50mM) | 3µl | -- |
| ATP, CTP,GTP Mix(25mM) | 12µl | 12µl |
| UTP Solution (50mM) | 3µl | 6µl |
| T710x Reaction Buffer | 4µl | 4µl |
| T7Enzyme Mix | 4µl | 4µl |

26µl of master mix was added to each tube containing 14 µl of double-stranded cDNA and mixed by pipetting. The samples were then incubated at 37$^o$C for 14 hours. 60 µl nuclease free water was added to each *in vitro* transcription sample bringing the final volume to 100 µl. This aRNA was purified to remove unincorporated aaUTP and Tris from the *in vitro* transcription reactions that would otherwise compete with the aRNA for dye coupling. Purification of aRNA was carried out as described for cDNA purification in the previous section 3.7.4 . The yield of aRNA was 5 µg to 20 µg in total.

### 2.2.7.6 Dye coupling reaction and clean up

The aRNA was labelled using N-hydroxy succinamide derivatives of the dyes Cy3 and Cy5 (Amersham Pharmacia Biotech, Freiburg). Each vial of dye was resuspend in 32 µl of DMSO and kept in dark at room temperature for 30 minutes. 9 µl of the coupling buffer (Ambion, Huntingdon, UK) was added to the 7 µl of aRNA and mixed well. 7 µl of the prepared NHS ester dye was added, mixed well and the reaction incubated at room temperature in the dark for 30 minutes. 4.5 µl of 4M Hydroxylamine (Ambion, Huntingdon, UK) was added to hydrolyze remaining NHS ester dye and the incubation continued at room temperature in the dark for a further 15 minutes. After dye coupling, labelled aRNA was purified as described for purification of cDNA. The purified labelled aRNA was dried by

vacuum centrifugation for approximately 2 hours and resuspended in 8µl Nuclease-free water. 2 µl labeled aRNA was diluted in 78 µl Nuclease-free water. The sample was measured at $A_{260}$ and $A_{dye}$ in a UV spectrophotometer. Here, $A_{dye}$ is aRNA absorbance at 550 nm for Cy3, and 650 nm for Cy5. The coupling efficiency was calculated using the following formula:

$$\frac{\#dye\ molecules}{1000nt} = \frac{A_{dye}}{A_{260}} \times \frac{9010\ M^{-1}cm^{-1}}{dye\ extinction\ coefficient} \times 1000$$

The labeling reactions which by OD measurement contained 20-50 dye molecules incorporated per 1000 nucleotides were used for array hybridization.

#### 2.2.7.7 Hybridization

Labelled aRNA was broken by fragmentation buffer to sizes between 60–200 nucleotides to improve the hybridization kinetics and signal produced on oligonucleotide microarrays. 1 µl of 5x fragmentation buffer (Ambion fragmentation buffer II Ambion, Huntingdon, UK) was added to 4 µl of Cy3 (5 µg) and 4 µl of Cy5 (5 µg) aRNA probes and the contents were heated at 95°C on thermocycler (Biometra, Goettingen) for 5 minutes. The aRNA probes were then pooled in 30 µl of hybridisation buffer (Ambion fragmentation buffer II Ambion, Huntingdon, UK) and immediately hybridizied to duplicate MWG whole genome arrays (MWG Biotech, Ebersberg, Germany) under cover slips in the hybridisation chambers for 24 hours at 42°C. The slides were then washed at room temperature sequentially with 2×SSC (0.3 M NaCl, 30 mM Sodium Citrate, pH 7.0)+0.03% SDS, 2×SSC, 0.2×SSC and 0.2×SSC+20% ethanol, and then centrifuged briefly to dry.

#### 2.2.7.8 Microarray scanning and data analysis

Hybridized arrays were scanned using a Genepix 4000B scanner. The images were saved as Tiff files and analyzed using Genepix 4 software (Axon Instruments, Foster City, California). The local background was calculated using a circular region centered on the spot. This region has a diameter that is three times the diameter of the corresponding spot. After the background intensities are subtracted from the feature intensity, the ratios of median (i.e. the background subtracted median pixel intensity at the wavelength 635 nm (Cy5) to the background subtracted median pixel intensity at wavelength 532 nm (Cy3))are then calculated.

#### 2.2.7.9 Normalization

In order to compare different arrays it is necessary to normalize the data. This is because the dye intensities in the labelling reaction are rarely identical. This bias can stem from a variety of factors including variations of cell numbers, of mRNA concentration, and experimental variability in probe coupling. Global normalisation was used to normalize the microarray data. In this method, the average of the expression distributions across the microarray is set to 1.0. Spots which differed significantly from this value were considered to contain gene sequences which are differently regulated.

A p value $<0.05$ was considered to be statistically significant. Micro-array expression data on up or down regulated genes were analyzed using a one-tailed Fischer t-test. Statistical analyses were carried out using the SPSS package.

### 2.2.8 Quantification of Gene Expression Levels by real time PCR

Target genes were selected from microarray results and real time PCR was carried out to quantify and confirm the degree of differential expression of these genes. Quantification of DNA using real-time, fluorescence-based reverse transcription polymerase chain reaction (RT-PCR) is based on the measurement of amplified products after each cycle of the PCR using fluorescent dyes which interact only with double stranded DNA. The more template present at the beginning of the reaction, the lower the number of cycles it takes to reach a point in which the fluorescent signal is above background. This is the definition of the threshold cycle ($C_T$).

SYBR Green is a double-stranded DNA-binding dye (excitation max. 497nm, emission max. 520nm) whose fluorescence intensity increases 1000-fold in the presence of dsDNA, relative to the free dye or to single stranded DNA. This characteristic allows the detection of PCR products in real time when the dye is added to the amplification reaction. As the amount of double-stranded product increases, so does the fluorescence intensity detected at each amplification cycle.

Because development of SYBR Green dye fluorescence is dependent on the presence of dsDNA, the specificity of the reaction is determined entirely by the specificity of the primers used. Any double-stranded products generated during the PCR amplification will be detected by the instrument. The detection of the target amplicon is not initially distinguishable

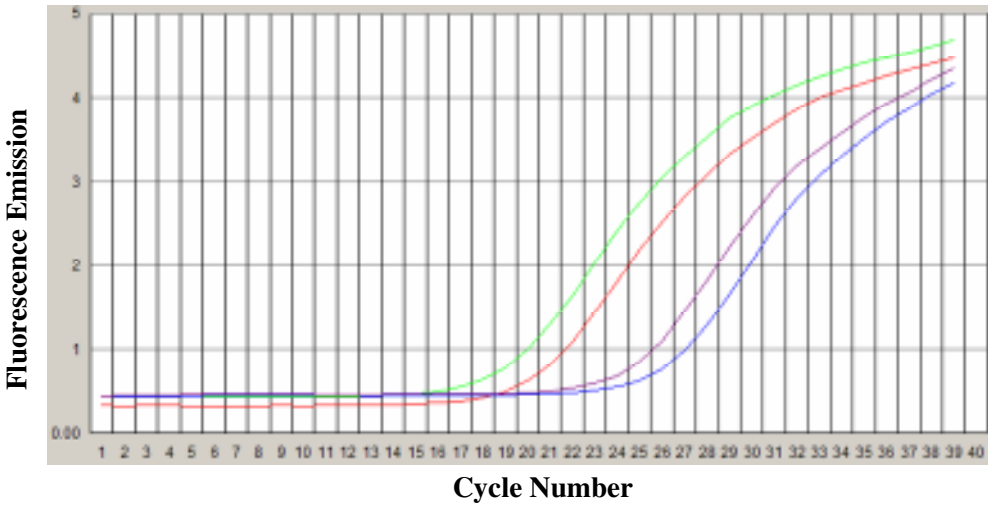



**Figure 6.The PCR amplification curves reflecting the increase of PCR products**

from any spurious PCR products, such as primers bound to non-target DNA sequences, primer-dimer formation, etc. Thus, although an increase in fluorescence is detected, the fluorescence is not necessarily due to increased concentration of the intended PCR product.

To confirm the reliability of the results, the homogeneity of the PCR products was verified by dissociation curve analysis. Melting curves generated by raising the temperature of the PCR product from RT to $95^0$C results in the separation of the DNA strands with consequent loss of syber green fluorescent. Different DNA sequences dissociate at different temperatures. The loss of Syber Green fluorescence was measured at every $0.2^0$C increase in the temperature. A homogenous collection of products gives a complex set of dissociation curves.

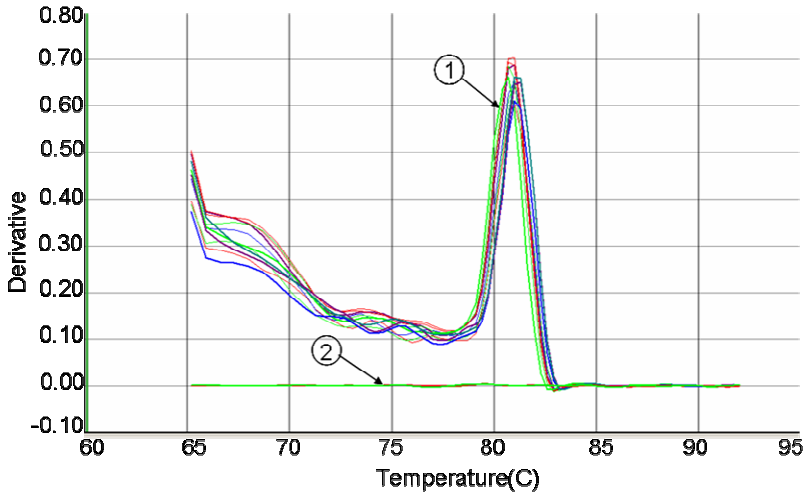



**Figure 7. Dissociation curve of amplification products.** Data is obtained by slowly ramping the temperature of reaction solutions from 60 to 95 °C while continuously collecting fluorescence data. 1:PCR product. 2: NTC

The primers used in this work were all designed using the software Primer Express™ (Applied Biosystems, Foster City, Ca) so that one of the primers crosses a splice junction site. This ensures detection of RNA only and not of contaminating genomic DNA. In addition the primers matched the following criteria:

1. Tm (melting temperature) between 58-60°C. Tm can vary in the range of 52 °C to 65°C. Generally, high Tm is better. However, when Tm of primers is above 65°C-70°C, especially on high GC templates, this can lead to secondary priming artefacts.
2. GC content: 30% to 80%
3. Primer length: 15 to 30 nucleotides
4. Amplicon length: 100 to 200, this produces highest efficiency in real-time PCR using SYBR Green

#### 2.2.8.1 Comparative Ct ($2^{\Delta\Delta Ct}$) method

Generally two quantification types in real-time RT-PCR are possible. (i) A relative quantification based on the relative expression of a target gene versus a reference gene.(ii) An absolute quantification, which determines the copy number per cell or total RNA concentration. Absolute quantitation requires the construction of an absolute standard curve for each individual amplicon to ensure accurate reverse transcription and PCR amplification profiles. During relative quantitation, changes in sample gene expression are measured based on either an external standard or a reference sample, also known as a calibrator. When using a calibrator, the results are expressed as a target/ reference ratio. We used the comparative Ct method as it does not require a standard curve and it is useful when assaying a large number of samples.

The comparative Ct method is a mathematical model that calculates changes in gene expression as a relative fold difference between an experimental and reference sample. While this method includes a correction for nonideal amplification efficiencies, the amplification kinetics of the target gene and reference gene assays must be approximately equal. Consequently, a validation assay must be performed where serial dilutions are assayed for the target and reference gene.

$$\text{Ratio} = 2^{\Delta\Delta Ct} = 2^{\,\Delta Ct \text{ target (control-treated)} - \Delta Ct \text{ ref (control-treated)}}$$

Where, $\Delta Ct_{target}$ is the Ct of control – sample of the target gene transcript and $\Delta Ct_{ref}$ is the Ct of control – sample of reference gene transcript (104).

### 2.2.8.2 Efficiency Analysis

The amplification efficiency values were measured using the CT slope method.The slope was calculated from the plot of log transformation of serial dilution versus Ct (samples), or log transformation of initial target copy number versus Ct (standards). The amplification efficiency (*E*) was determined based on the slope of this graph as $E = 10^{(-1/slope)}$.

### 2.2.8.3 Sybr Green Real time PCR Assay

Two-step real time reverse transcription PCR was carried out to quantify and confirm the degree of differential expression of these genes. In the first step, total RNA was used to generate cDNA by random priming and in the second step specific sequences of cDNA was amplified in the presence of Sybr green. For the cDNA synthesis a master mix was prepared which contained per reaction:

| Component | (µl) | Final Concentration. |
|---|---|---|
| Rnase free water | 15.37 | -- |
| 10×RT buffer | 5 | 1× |
| 25 mM $MgCl_2$ | 11 | 5.5 mM |
| dNTP mixture (2.5 mM) | 10 | 500 µM per dNTP |
| Random Hexamers (50 µM) | 2.5 | 2.5 µM |
| Rnase inhibitor (20 U/µL) | 1 | 0.4 U/µL |
| MultiScribe Reverse Transcriptase | 3.15 | 3.125 U/µL |
| RNA | 2 | -- |
| Total | 50 | -- |

The samples were mixed and centrifuged to remove any air bubbles. The tubes were loaded into a PCR cycler (Biometra, Goettingen) and the following thermal cycling parameters were used for cDNA synthesis. The sample was incubated at 25°C for 10 minutes to allow hexamer binding to RNA. RNA was reverse transcribed into cDNA at 37°C for 1 hour. Then the reverse transcriptase was inactivated at 95°C for 5 minutes. After completion of reverse transcription, all cDNA samples are stored at –20 °C. The second step of the real time PCR is the amplification of the cDNA. For this a master mix was prepared which contained per reaction:

| Component | Volume | Final concentration |
|---|---|---|
| 2 × SYBR green Master Mix | 12.5µl | 1 × |
| Forward primer 10 µM | 1µl (10 pmol) | 0.4µM |
| Reverse primer 10 µM | 1µl (10 pmol) | 0.4µM |
| $H_2O$ | 9.5µl | -- |
| Template (cDNA) | 1µl | |
| Total | 25µl | -- |

Each reaction was transferred into ABI PRISM Optical tubes (Applied Biosystems, Foster city, Ca) and amplified in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster city, Ca).

A common problem with the polymerase chain reaction is the formation of non-specific products. At the lower temperatures before denaturation, primers may bind to targets. These non-specific hybrids may be extended by Taq DNA polymerase, which creates competing targets during subsequent cycles. Hot start PCR methods provide a solution to this lack of specificity by reducing or eliminating non-specific product formation before high-temperature cycling. Thus the Taq DNA polymerase activity was muted before the initial denaturation step, most commonly with a blocking antibody or chemical modification. In this project, anti-Taq DNA polymerase antibody was used to block Taq polymerase activity.

The “hot start” was performed by incubation of samples at 95°C for 10 minutes. The PCR reactions were carried out as follows: denature at 95°C for 15 seconds, annealing at 54°C for 30 seconds and synthesis at 72°C for 30 seconds, repeated 40 cycles.The results were analyzed using ABI PRISM 7000 SDS software(Applied Biosystems, Foster city, Ca).

# 3 Results

## 3.1 Patients

A prospective study was designed to assess the influence of surgical intervention on the immune response in a group of 101 patients undergoing major elective surgery and 17 patients undergoing minor surgery. These patients were recruited over the course of 14 months. From each patient four blood samples were collected: the first at 24 hours prior to the operation and the remaining ones at 24, 48 and 72 hours after surgery. For each patient a clinical data set was established and rapid transport service was organised to deliver patient samples from surgical ward to the analysis laboratory within 30 minutes. The demographic details of the study population are shown in Table 1**.** Subjects' mean age was 57 +/- 17 years for minor operations and 64 **+/-** 12 years for major operations. Ten of the 17 minor operation patients were male (59%) and 63 of the 101 (62.3%) major operation patients included in this study were male. All the patients undergoing minor operations and 81% of those with major operations had an uneventful postoperative recovery. 19 of the patients undergoing major operations suffered from inflammatory complications and 12 of these developed severe sepsis.

**Table 1: Clinical profile of patients recruited**

| | | |
|---|---|---|
| Minor operations | Total | 17 |
| | Age | 57 +/- 17 yrs |
| | Male : Female | 10 : 7 |
| | Inguinal hernia | 7 |
| | Strumectomy | 5 |
| | Lap. Cholecystectomy | 5 |
| Major operations | Total | 101 |
| | Age | 64 +/- 12 yrs |
| | Male: Female | 63 : 38 |
| | Inflammatory complications | 19 |
| | Malignant disease | 75 |
| | Thoracic surgery | 14 |
| | Esophagectomies | 6 |
| | Gastric surgery | 18 |
| | Colorectal surgery | 41 |
| | Liver resection | 4 |
| | Multivisceral surgery | 3 |

## 3.2 Measurement of HLA-DR expression on monocytes

Flow cytometric analysis was performed using a FACSCalibur flow cytometer (BD Immunocytometry Systems, Heidelberg). Monocytes were identified on the basis of their CD14-positive fluorescence and light scatter properties (Figure 8). An HLA-DR histogram and a mouse $IgG_{2a}$ (isotype control) histogram were constructed using a minimum of 1000 monocytes. The HLA-DR expression of monocytes was evaluated by determining the HLA-DR fluorescence intensity of monocytes and the proportion of positively fluorescing monocytes in comparison to isotype control (Figure 8). The median channel number of the isotype control histogram and the median channel number of the respective HLA-DR histogram were converted into MEF (Molecules of Equivalent Fluorochrome) by using DAKO fluorescent calibration beads (Fluorospheres, DAKO cytomation, Hamburg) and TallyCal software (DAKO cytomation, Hamburg) as described in section 3.2.1.

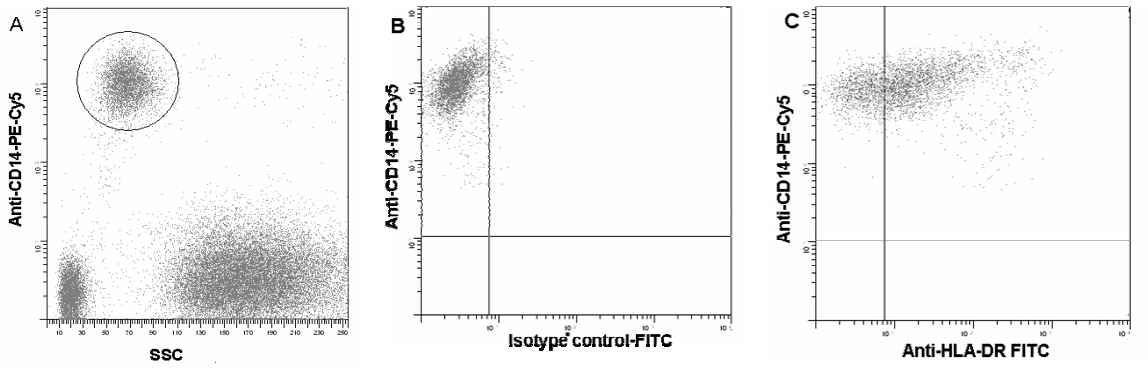



**Figure 8: Measurement of monocytic HLA-DR expression.** (A) Patient's monocytes were gated by their CD14 binding and SSC properties (B) gated monocyte population labelled with the isotype control-FITC (C) gated monocyte population labelled with anti HLA-DR-FITC.

### 3.2.1 Measurement of HLA-DR expression on monocytes in patients undergoing surgery

The effect of surgery on monocytes was analyzed by measuring the median MEF expression of HLA-DR on these cells before operation and on each of the three days following an operation. The effect of the degree of tissue damage on monocyte HLA-DR expression was determined by comparing the results of patients undergoing major surgery (101 patients, 404 HLA-DR determinations) with those of patients undergoing minor surgery (17 patients, 68 HLA-DR determinations). For both major and minor surgery groups there was a profound decrease in HLA-DR expression of monocytes on all three days post

operation. However, minor surgical patients had a significantly lower degree of HLA-DR loss at 24 hours and the HLA-DR expression started to increase already by 48 hours after the operation (Figure 9). In contrast the major surgery patients had a more profound decrease in HLA-DR expression at 24 hours and a recovery was not apparent till 72 hours post surgery. Thus, the extent and the period of decrease of monocyte HLA-DR expression may be related to the degree of surgical trauma.

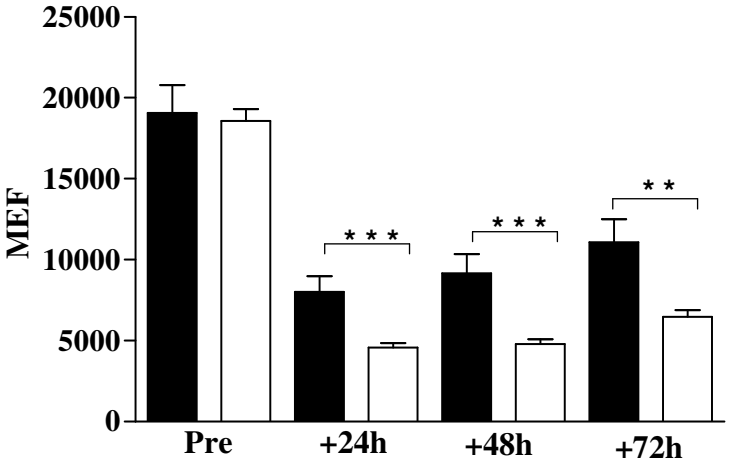



**Figure 9: Monocyte HLA-DR expression as Molecules of Equivalent Fluorochrome (MEF) prior to (Pre) and at 24, 48 and 72 hours after surgery**. The black columns show the pooled data for patients who underwent minor operations; the white columns show the data for patients who underwent major operations. Groups with significant differences in expression level are indicated (** = $p < 0.01$, *** = $p < 0.005$, Mann-Whitney).

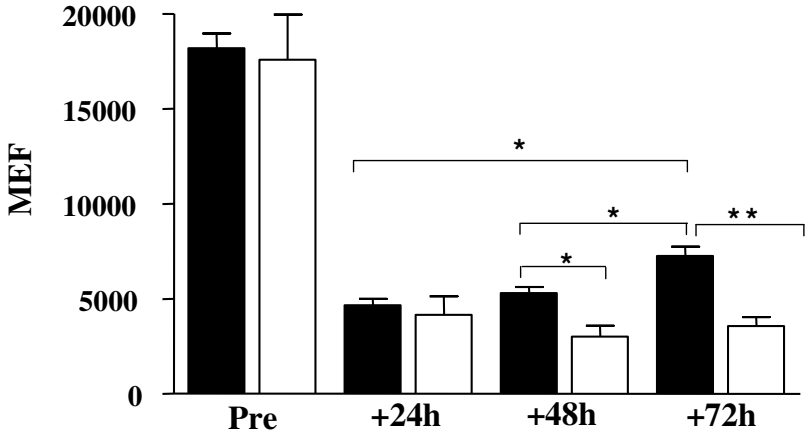



**Figure 10: Comparison of monocyte HLA-DR expression levels in major surgery patients.** The black columns show the pooled data for patients with an uneventful recovery; the white columns show the data for patients who suffered severe septic complications. Groups with significant differences in expression level are indicated (* = $p < 0.05$, Mann-Whitney, ** = $p < 0.01$, Mann-Whitney

To determine the effect of systemic inflammation on the HLA-DR expression of monocytes, its expresssion in patients who subsequently developed septic complications was compared to patients with uneventful recoveries. The degree of HLA-DR down regulation was the same in both of these groups at 24 hrs after operation. However, a further decrease in HLA-DR expression was observed in patients with septic complications at 48 hours after surgery and HLA-DR expression continued to lag in this patient group even at 72 hours post operation. By 72 hours after major surgery a clear increase in expression of HLA-DR was evident in patients who had uneventful recoveries (Figure 10).

## 3.3 Analyses of changes in monocyte gene expression profile following surgery

### 3.3.1 Patients

To explore the early monocyte response to tissue damage caused by surgical interventions, five major surgical patients and one minor surgical patient were selected for microarray analysis. Among the major surgical patients, three developed post surgical complications (Table 2).

**Table 2: Clinical profile of patients selected for monocyte gene expression analysis.**

| Patient | Sex | Age (Years) | Type of surgery | Operation | Complication |
|---|---|---|---|---|---|
| 1 | f | 63 | Major | Stomach | Septic shock |
| 2 | f | 75 | Major | Rectum | No |
| 3 | m | 72 | Major | Stomach | No |
| 4 | f | 74 | Major | Stomach | Sepsis pneumonia |
| 5 | f | 82 | Major | Stomach | Wound infection |
| 6 | f | 50 | Minor | Nodular goiter | No |

### 3.3.2 Isolation of enriched monocytes

The blood samples, collected at 24-hour pre-surgery and 24-hours, 48-hours and 72-hours post-surgery, were processed by Ficoll gradients to yield PBMC and the monocytes were isolated by magnetic cell sorting. The purity of sorted cells was analysed by FACS after labelling the cells with anti CD45 and anti CD14. As an example the monocyte preparation from the pre-operative sample of patient # 2 is shown in Figure 11.

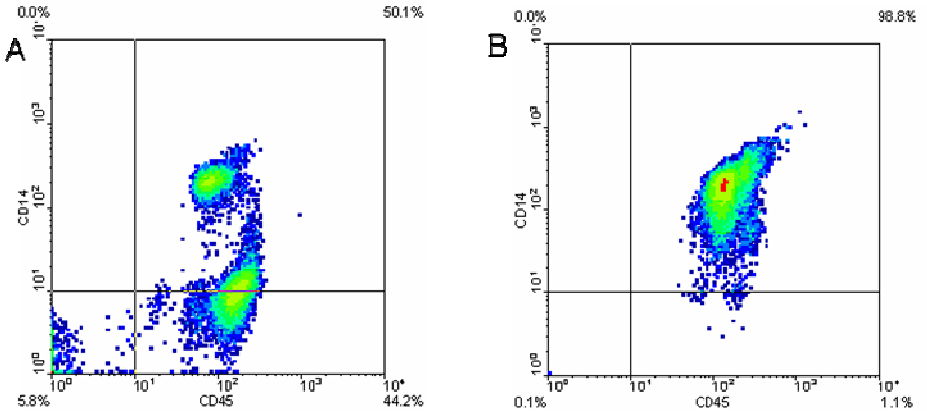



**Figure 11: FACS analysis to asses the purity of monocytes.** A. PBMC B. Cells isolated after magnetic cell sorting using CD14 magnetic beads. The preparation is >95% pure by these criteria.

### 3.3.3 Microarray experimental design and analysis

RNA prepared from each pre-surgical monocyte preparation was amplified as described in section 3.7.1 and labeled with Cy3. Post-surgical RNA was labeled with Cy5. For each patient three hybridisation probes were prepared: (1) pre operation-Cy3 plus 24 hours post operation-Cy5, (2) pre operation-Cy3 plus 48 hours post operation-Cy5 and (3) pre operation-Cy3 plus 72 hours post operation-Cy5. These labeled samples were then hybridized to "trauma cDNA chip" which consists of 1,383 clones representing 315 selected up regulated genes from monocytes of trauma patients (105) (Figure 12). Post processing of the arrays was carried out as detailed in section 3.7. For analysis, house keeping genes used as normalization standards were COX2 and COX3. Ratio of median fluorescence values was chosen to evaluate the up regulation. P values were obtained using Mann-Whitney test. Fold change >1.5 was used as a lower cut off. Table 3 shows up-regulated genes in patient 2 at 24 hours after the operation.

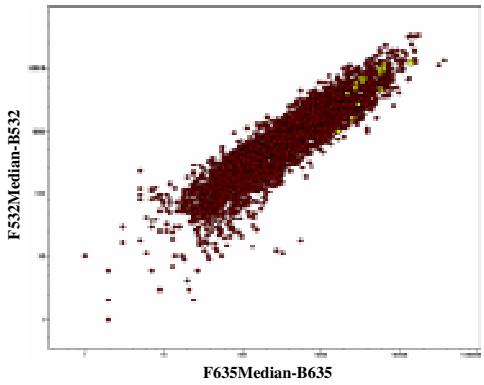



**Figure 12: A scatter plot of the Cy5 vs. Cy3 values for aRNA prepared from patient # 2**. The Cy3 probe was from pre-operation sample while the Cy5 probe was from 24 hours post-operative sample.

**Table 3: Upregulated genes from sample of post-surgical patient # 2 at 24 hours compared with same patient at 24- hours pre-surgery**.

| Gene | Up regulation | SD | Data points | P (1) |
|---|---|---|---|---|
| Beta actin | 2.5 | 1.0 | 300 | < 0.0001 |
| Calgranulin A12 | 12.2 | 4.3 | 81 | < 0.0001 |
| Calgranulin A8 | 17.0 | 8.5 | 807 | < 0.0001 |
| CAP 1 | 1.9 | 0.3 | 12 | < 0.0001 |
| CD163 | 7.8 | 4.2 | 11 | < 0.0001 |
| CD31 | 2.1 | 0.3 | 4 | < 0.03 |
| FLAP | 2.0 | 0.1 | 5 | < 0.01 |
| Gamma actin | 2.9 | 0.4 | 124 | < 0.0001 |
| Leuk A4 hydrolase | 2.0 | 0.3 | 24 | < 0.0001 |
| L-Selectin | 6.3 | 2.2 | 25 | < 0.0001 |
| L-Plastin | 2.5 | 1.7 | 14 | < 0.0001 |
| NOD2 | 4.9 | 0.2 | 4 | < 0.03 |
| RAC 1 | 4.2 | 0.2 | 4 | < 0.03 |
| TM3 | 2.5 | 0.1 | 4 | < 0.03 |
| Versican | 2.7 | 3.4 | 14 | < 0.0001 |
| EDRF | 23.7 | 0.5 | 4 | < 0.03 |
| MAPK6 | 2.7 | 0.2 | 4 | < 0.03 |
| PKCß | 6.9 | 0.9 | 4 | < 0.03 |
| Syk | 2.0 | 0.17 | 4 | < 0.03 |
| ZNF83 | 2.5 | 0.1 | 4 | < 0.03 |
| AASDHPPT | 3.8 | 0.8 | 8 | < 0.001 |
| Cathepsin B | 5.5 | 1.6 | 12 | < 0.0001 |
| ECRP | 2.2 | 1.7 | 9 | < 0.0001 |
| Glutaminyl cyclase | 6.9 | 0.6 | 4 | < 0.03 |
| Haptoglobin | 18.7 | 2.0 | 12 | < 0.0001 |
| Aquaporin 9 | 1.5 | 0.1 | 8 | < 0.0002 |
| TXNIP | 2.0 | 1.5 | 6 | < 0.002 |
| FLJ11131 | 9.7 | 1.0 | 15 | < 0.0001 |
| FLJ22662 | 8.6 | 1.1 | 105 | < 0.0001 |
| FLJ33782 | 12.6 | 0.7 | 4 | < 0.03 |
| FLJ36880 | 10.7 | 0.9 | 4 | < 0.03 |
| FLJ40817 | 1.9 | 0.6 | 12 | < 0.0001 |
| KIAA0386 | 7.5 | 0.4 | 12 | < 0.0001 |
| KIAA0941 | 11.7 | 1.1 | 8 | < 0.001 |

(1) P values calculated by the Mann-Whitney test
SD- standard deviation

### 3.3.4 Kinetics of induction of monocyte injury response genes

Rapid up regulation of a number of genes in monocytes was observed at 24 hours after surgery. Interestingly, many of these genes were also up regulated in monocytes of trauma patients (105). However, the number of up regulated genes quickly declined at later time points (Figure 13). No up-regulation of these genes was seen in the patient #6 who underwent a minor operation. (Data not shown)

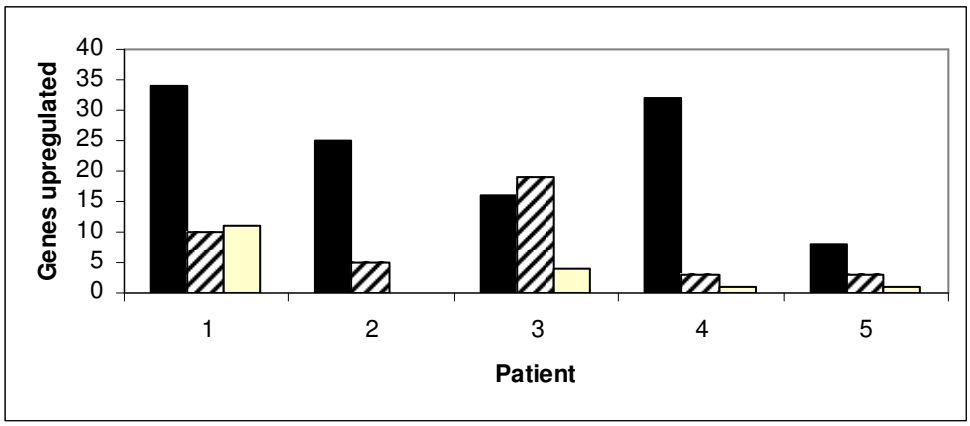



**Figure 13. Kinetics of induction of monocyte injury response genes in major surgery patients.** Monocyte RNA recovered from major surgery patients 24 hours (black bars), 48 hours (hatched bars) and 72 hours (white bars) after surgery were labeled with Cy5 and analyzed on arrays together with Cy3 labeled monocyte RNA from the same patient 24 hours prior to surgery. The number of genes from the injury response which were up regulated in each sample is shown.

## 3.4 Monocytes from trauma patients express Calgranulin A8 and A12

Two genes prominently upregulated in the monocytes after surgery were the Calgranulins A8 and A12. Since both of these genes are expressed at very high levels in granulocytes it was important to rule out the possibility that this result was due merely to a minor contamination of the post surgery monocyte preparations with granulocytes. To do this single cell PCR was carried out for Calgranulins A8 and A12. Single monocytes were sorted from a trauma patient with SIRS (carried out by Dr. Claudia Berek, Deutsches Rheumaforschungszentrum (DRFZ)). Single cells were lysed, and the mRNA was reverse-transcribed, divided into two aliquots and amplified by PCR with primers specific for Calgranulin A8 or Calgranulin A12. Calgranulin A8 and Calgranulin A12 cDNA was successfully amplified from 4 of the 6 cells (66%) as shown in Figure 14. These results confirm that the patients' monocytes express both Calgranulin A8 and A12.

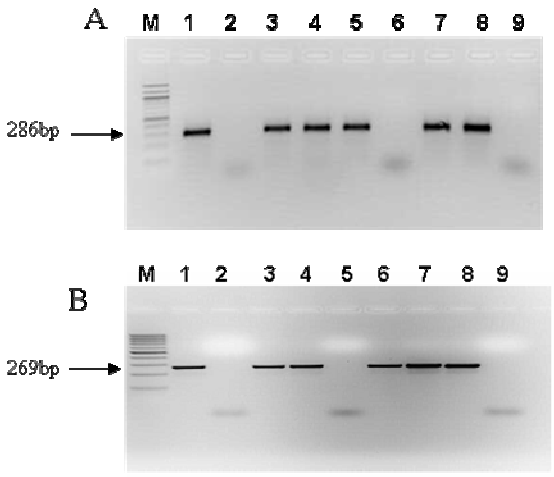



**Figure 14: Amplification of Calgranulin A8 and A12 transcripts from single monocyte.** A 286 nt fragment of Calgranulin A8 (A) and a 269 nt fragment of Calgranulin A12 (B) transcript were amplified from single monocyte cells using RT-PCR. M- Marker, 1-6-Single monocytes, 7- 10 cells, 8-100 cells, 9- Negative control.

To determine the expression level of these Calgranulins in trauma patients, monocyte preparations (>95% purity) were analyzed by real time RT-PCR. In all four patients tested the expression levels of both of the Calgranulins were substantially increased with respect to their level in monocytes from healthy controls (Figure 15).

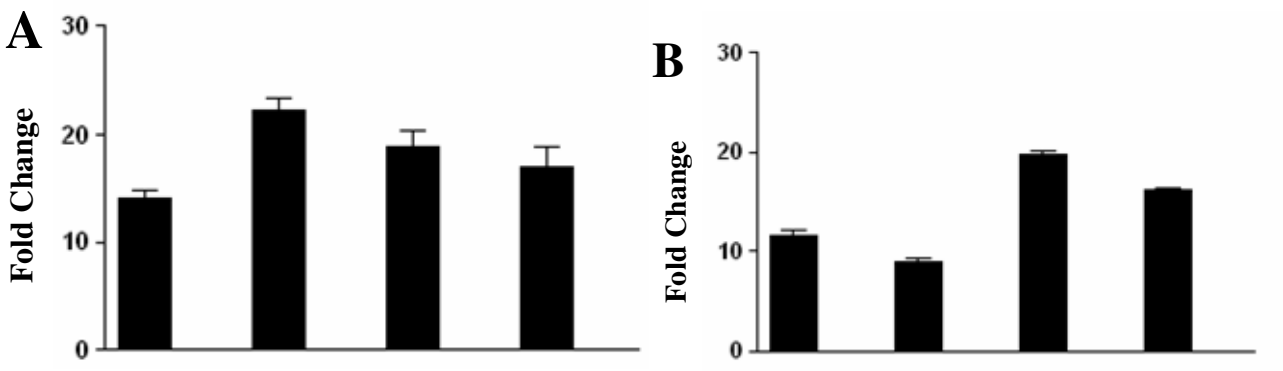



**Figure 15: Real Time-PCR determination of the expression of Calgranulin A8 (A) and Calgranulin A12 (B) in monocytes from four major trauma patients**. For each patient the expression level was normalized using expression of 18S RNA as a standard. Expression levels were determined in triplicate and are shown as the mean+SE.

## 3.5 Changes in peripheral T-lymphocyte count

The effect of surgery on the adaptive immune system was analysed by comparing pre-surgical T-cell counts before and at 24, 48 and 72 hrs after surgery. The recovered T lymphocytes from all 118 patients included in the study were counted using a Neubauer

hemocytometer at each of the four sampling time points. Though the recovery of T-cells was not reduced after a minor operation, a profound reduction in the recovery of circulating T lymphocytes was observed after major operations compared to the pre-operative value.

Automated counting methods such as those based on flow cytometry provide greater accuracy and precision than does manual counting in a haemocytometer. For this reason T cell counts were enumerated by flow cytometry using TruCount beads in eight consecutive major surgery patients. None of these patients suffered post surgical inflammatory complications. As shown in Figure 16, when compared with pre-surgical controls, post surgical patients had mild granulocytosis. In contrast, the absolute numbers of circulating T lymphocytes, showed a marked drop from the pre-surgical values in all patients after major surgery. The decrease in T lymphocyte numbers was generally greatest at 24hrs after surgery (Figure 16). Both $CD4^+$ and $CD8^+$ cells numbers were equally reduced so that the CD4/CD8 ratios were not significantly changed.

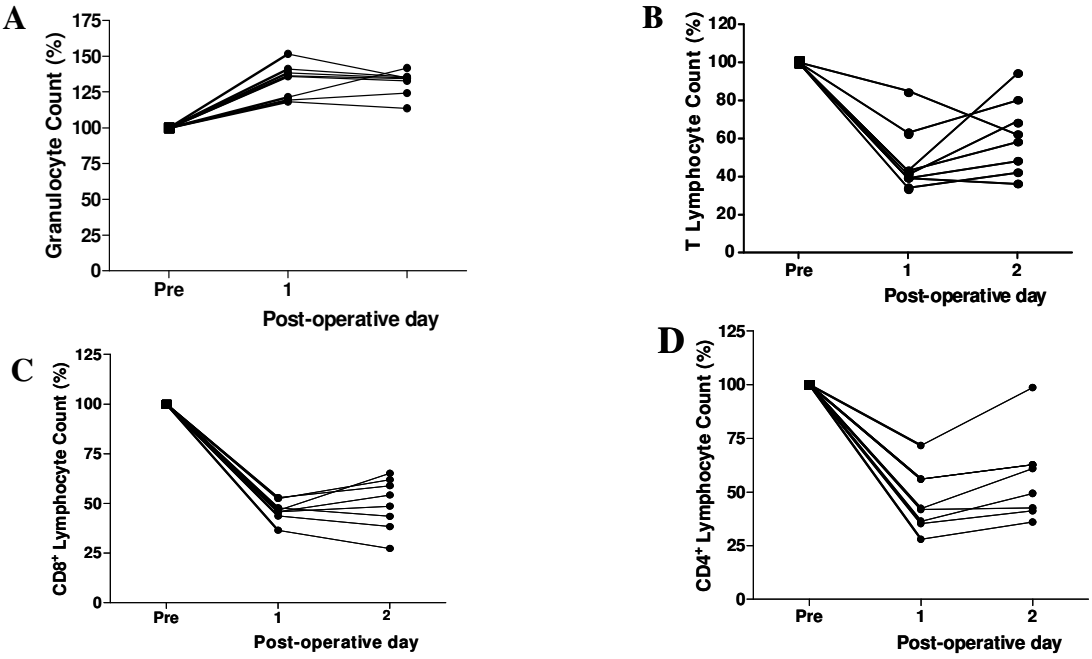



**Figure 16: Kinetics of circulating leucocytes in 8 post surgical patients compared with pre-surgical controls**. A-Granulocytes, B-T lymphocytes, C-$CD8^+$T-lymphocytes and D-$CD4^+$T-lymphocytes.

## 3.6 Apoptosis of peripheral blood T cells

Accelerated lymphocyte apoptosis has been shown to occur in overwhelming infections in humans, polymicrobial animal models of abdominal sepsis and in patients following oesophagectomy (87, 89, 106). We therefore examined the extent of T-cell

apoptosis evident in the circulating T-cell populations before and after surgery. To this end the expression of phosphotidylserine (PS) on circulating T lymphocytes was examined. PS is an early marker of apoptosis which can be detected by its binding of AnnexinV (107).

Peripheral blood samples of five consecutive major visceral surgery patients before and after operation were treated with Annexin V- FITC and 7-amino actinomycin D (7-AAD) 7-AAD binds to nucleic acids, but can only penetrate the plasma membrane when membrane integrity is breached, as occurs in the later stages of apoptosis or in necrosis. Cells that are Anneixn V positive and 7-AAD negative therefore have an intact plasmalemma but are in early apoptosis as PS translocation has occurred. These apoptotic cells were detected with increased frequency in the peripheral blood of patients after major surgery (10.20%±3.6%) relative to their occurrence before surgery (0.22% ± 0.23%) ($P<0.007$). These results indicate that at least a fraction of T cells from patients who had major operation were susceptible to apoptosis (Figure 17). One of the patient's results is shown Figure 18.

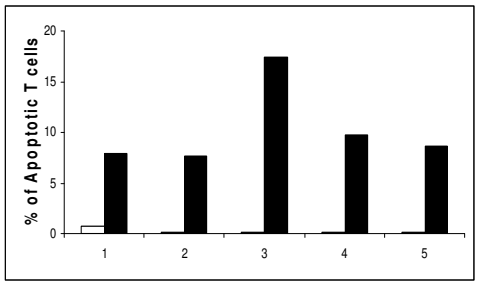



**Figure 17. Fraction of apoptotic cells in the circulating CD3 population in five patients prior to (open columns) or 24 hours after (black columns) major visceral operations.**

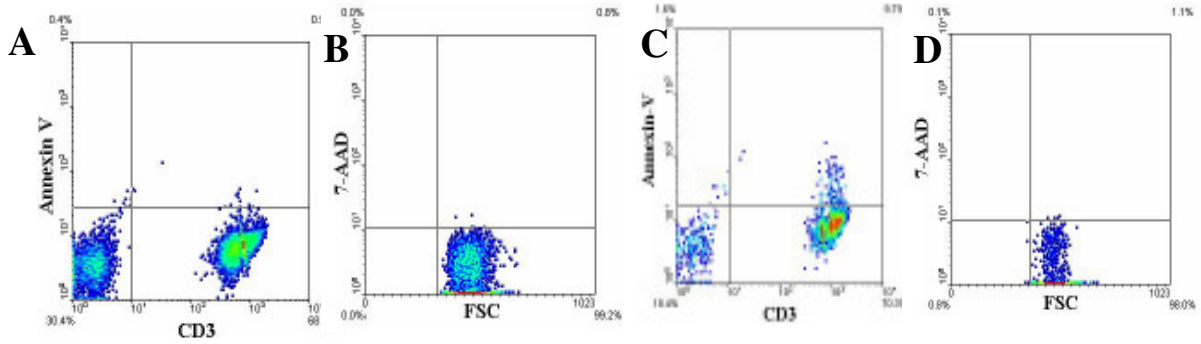



**Figure 18. Characteristic dotblots of Annexin V staining of $CD3^+$ T lymphocytes. A-** Annexin V staining of T cells of a pre-operative patient; B-gate regions identifying 7-AAD positive $CD3^+$ T cells of a pre-operative patient, C- Annexin V staining in T cells of a post-operative patient and D-gate region identifying 7-AAD positive $CD3^+$ T cells of a post-operative patient. There is an increase in the percentages of apoptotic (Annexin V positive) CD3+T lymphocytes.

## 3.7 Microarray analysis of T-cell gene expression

### 3.7.1 Experimental design

The data from the experiments described in section 3.5 and 3.6 indicate that T cells respond rapidly to major surgery. To study this response in more detail, I undertook a microarray analysis of gene expression of T lymphocytes from surgical patients. Because an analysis of gene expression in all 472 T-cell RNA preparations collected from our patient cohert would have been prohibitively costly, I examined RNA sample from two patients with uneventful recoveries and from two patients who developed septic complications within three days of surgery. By pooling RNA from two patients we hoped to reduce the risk of picking up signals derived from individual peculiarities in gene expression.

### 3.7.2 Lymphocyte Preparation and T-cell purification

Peripheral blood $CD3^+$ cells were isolated by Ficoll gradient centrifugation and magnetic sorting as described in section 2.2.3.2. FACS analysis was performed to assess the purity of the isolated T-cells prior to RNA isolation. (Figure 19)

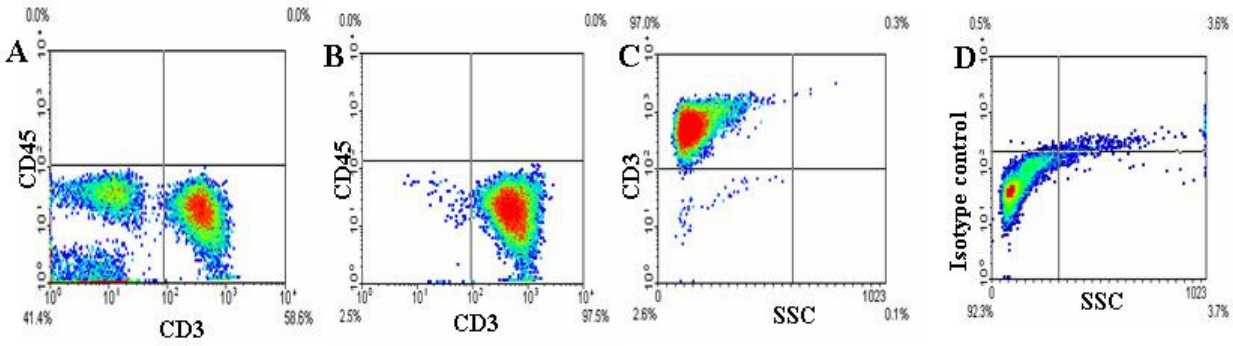



**Figure 19: FACS analysis of enriched T lymphocytes** CD45 was used as a leucocyte specific marker and CD3 was used as T cell marker. A. PBMC. B: Isolated cells showing a puritiy of 97%.C. Gate region identifying isolated T-cells. D. Gate region identifying Isotype control.

RNA isolated from purified cell preparations was evaluated on denaturing agarose gels (Figure 20). Ethidium bromide staining of the RNA reveals the 18S and 28S ribosomal RNA (rRNA) bands. If these bands are discrete (i.e no significant smearing below each band) and the 28S rRNA band is approximately twice as intense as the 18S rRNA band, then the mRNA in the sample is likely to be intact. In Figure 20 one can clearly see the two bands of 28s (4.7 kb) and 18s rRNA (1.8 kb).

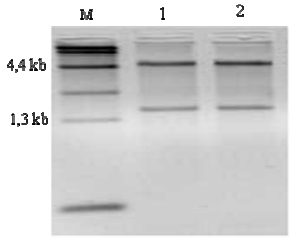



**Figure 20**. **Total RNA was run on a 1% denaturing agarose gel stained with ethidium bromide.** M-RNA Ladder, 1 - Pooled RNA samples of T lymphocytes from patients with uneventful recovery, 2 - Pooled RNA samples of T lymphocytes from patients with septic complications.

### 3.7.3 Microarray Analysis

Pre-operation samples of two patients with an uneventful recovery were pooled and a Cy3 labeled aRNA probe prepared. The post-operation samples from these patients were pooled and used to prepare a Cy5 labeled probe. The two labeled probes were mixed and hybridized to duplicate MWG Human 40K whole genome arrays. In a similar way pre and post operation samples from two patients who developed septic complications were hybridized to microarrays. High density MWG 40K oligonucleotide micro-arrays were used to identify differential expression of genes in T cells. The procedures of cDNA synthesis, subsequent synthesis and the hybridization of the aRNA to the MWG arrays were carried out as described in section 3.7. Figure 21 shows a scatter plot with the intensities of the red channel pixels (635nm) plotted against the intensities of the green channel pixels (532nm). In this scatter plot many features with intensities less than 100 do not lie on the liner part of the distribution. Ratios of median generated from these features are false positive results without biological value. Values below 100 were therefore excluded from the analysis prior to global normalisation.(see also section 3.7.4)

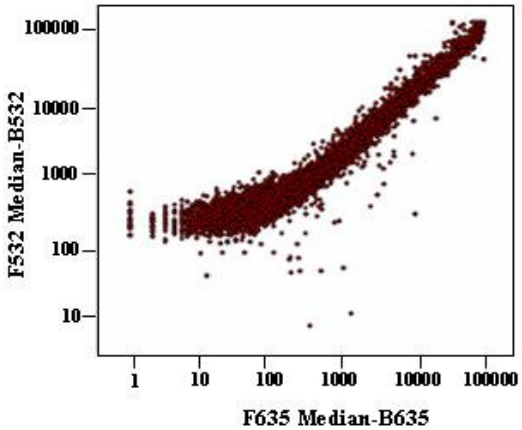



**Figure 21: A scatter plot of the Cy5 vs. Cy3 values obtained for an aRNA**. The Cy3 probe were from pre-operation samples while the Cy5 probe were from post-operative samples

### 3.7.4 Thresholds for Useful Signals

Signals which are real indicators of gene expression must be distinguished from those which simply reflect auto fluorescence. To investigate this issue, signals obtained from the blank spots and the irrelevant (Arabidopsis) sequences were used as controls. All Arabidopsis spots gave very low fluorescence signals. For each array, the 95th percentile of values for these controls was computed to provide a threshold for useful signals. As can be seen in Figure 22 the vast majority of the false positive signals are associated with low intensity signals. Almost all fall below the 100 pixel cut off which marks the end of the linear distribution of array signals (Figure 22) A threshold value of 100 (F635-B635>100) was selected to exclude most of the signal from non-specific binding (Figure 22).

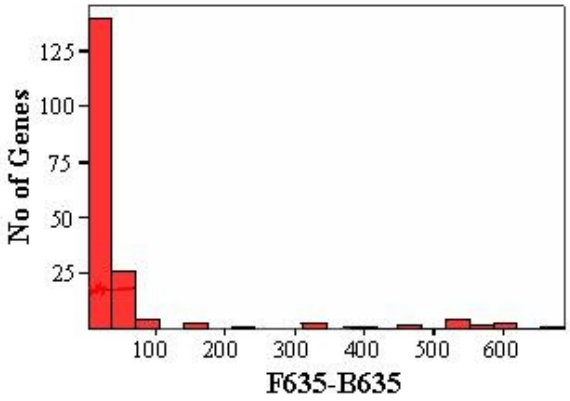



**Figure 22: Fluorescence intensity value distribution of negative control (Arabidopsis) array elements.**

### 3.7.5 Differentially Expressed Genes

After removal of non-specific low intensity signals and global normalisation of array data each array was examined for differential expression of genes using a defined "fold change" cut-off. However setting a "fold change" cut-off is arbitrary and can have quite different significance depending on expression levels. An alternative approach is to use a t-test, for instance on the logarithm of the expression levels to determine significance as this method is gene-specific and it is not affected by heterogeneity in variance across genes because it only uses information from one gene at a time. The following criteria were used to include genes for further analysis:

1. Genes which have expression level difference by a factor of at least x 1.2 from the pre-surgical value.
2. Only genes with p value <0.005 derived by pooling data from 4 arrays were considered for analysis.

Using these criteria, 589 genes were considered to be significantly regulated (Appendix, Table III and IV). 94% of these genes were down regulated (Table 4).

**Table 4: Numbers of genes regulated in patients with sepsis and in those with uneventful recovery.**

| | Number of genes |
|---|---|
| Up in septic and non septic patients<br>Down in septic and non septic patients | 35<br>554 |
| Up only in septic patients<br>Down only in septic patients | 6<br>73 |
| Up only in patients with normal recovery<br>Down in in patients with normal recovery | -<br>2 |

**Table 5: Major functional groups of regulated genes in circulating T-cells after surgery**

| Functional categories | All patients (In both groups) | Uneventful recovery only | Severe sepsis only |
|---|---|---|---|
| Transcriptional regulation | 61 | - | 12 |
| Intermediary metabolism | 253 | - | 32 |
| Immune response | 34 | 1 | 7 |
| Transporter functions | 98 | 1 | 10 |
| Receptors and signaling | 108 | - | 12 |

### 3.7.6 Salient features of different functional groups

- The gene chip experiments identified molecules associated with the immune system that are down regulated in post surgical T lymphocytes. The down regulated expression of T-cell receptor alpha and beta chain was also observed. We also found cytokines and cytokine receptors (IL-13R alpha and beta, IL-1R9, Interferon (alpha and beta) receptor 1 and CCR5) down regulated.
- 37 transcription factors including activating transcription factor, nuclear transcription factor and e4f transcription factor were also down regulated.
- A lower expression level of a number of molecules involved in ion binding and transport was noted, such as potassium channel, Na+, K+ -ATPase alpha subunit, potassium voltage-gated channel and chloride channel.
- Several enzymes involved in intermediary metabolism like 6-phosphofructo-2-kinase, carnitine octanoyltransferase, very long-chain acyl-coa synthetase and tRNA-guanine transglycosylase were down-regulated.
- As mentioned above many receptors involved in the signal transduction pathways like

mitogen-activated protein kinase (MAPK) phosphatase-7, mitogen-activated protein kinase kinase kinase kinase 3 (MAP4K3) and G protein-coupled receptor 37 are down regulated. Table 5 shows a breakdown of the classified genes.

### 3.7.7 Genes differentially expressed only in patients with post surgical septic complications

These hybridization experiments were carried out in duplicate to account for inter-experimental variation and the consistency of hybridizations in replicate experiments was obtained by measuring the signal on the equivalent oligonucleotides across duplicate slides. These two data sets were used to identify differentially expressed genes. The maximum percentage of difference in signal was 12.5 % which was used as a cut-off to exclude oligonucleotides with high signal variance. To identify oligonucleotides that exhibit differential hybridization, the ratios between the averaged Cy5 and Cy3 signals were calculated, and ratios above 3.3 fold are considered to be differentially expressed. This higher fold change was chosen as a stringent measure to determine significant changes in gene expression in these hybridisation experiments where only two independent data sets were available.

A total of 79 genes had differential expression of more than 3.3 fold compared with preoperative controls (Appendix, Table V&VI). 92% of these genes were down regulated. They included IL-21, TCR-beta, calcium/calmodulin-dependent protein kinase II gamma, Muscarinic acetylcholine receptor M5, Dopamine receptor D5 and the TGF beta activated kinase a.

### 3.7.8 Genes differentially expressed only in patients with uneventful recovery

Only 2 genes were found which satisfied our criteria for regulation which did not appear on the list of genes regulated in patients with septic complications (Table II). These are CNOT2 CCR4-NOT transcription complex and cationic amino acid transporter (Appendix , Table VII).

### 3.7.9 Analysis of molecular networks

The network predicition analysis using Ingenuity Pathways Analysis Software (IPAS) was used to generate and rank functional networks of genes. The cellular functions and pathways which were predicted to be influenced by the differentially expressed genes from microarray analysis were listed and ranked in their order of significance. Since the functions

identified by IPAS were below threshold level of significance, we concluded that numerous genes showing differential expression in our study are multiply associated with cellular pathways connected to cellular signalling mechanisms, however these pathways are not centered on any particular function or molecule (Figure 23).

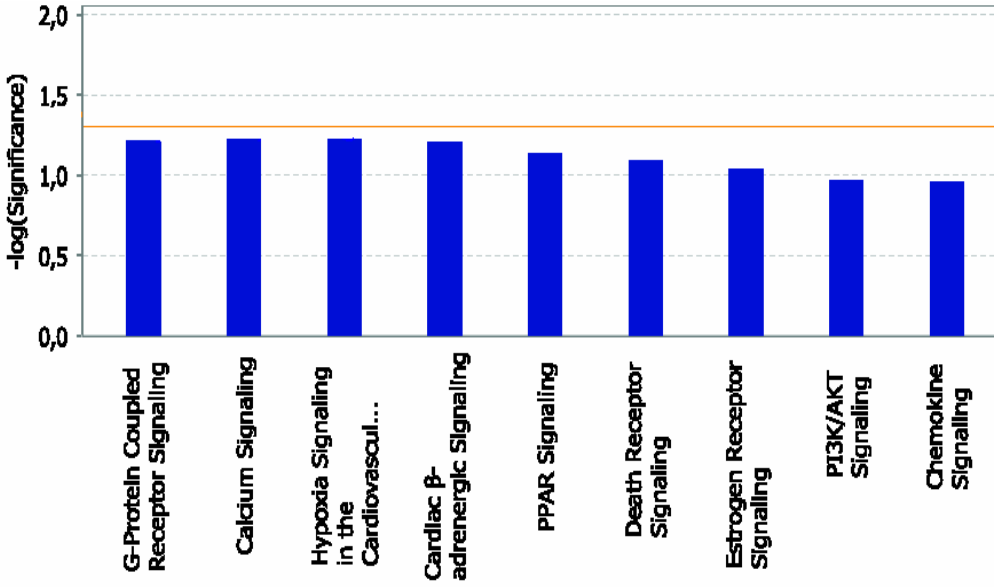



**Figure 23: Canonical pathways generated by Ingenuity Pathway Analysis Suite from the down regulated genes**. Significance calculated for each function is an indicator of the likelihood that the function is associated with the dataset by chance.

## 3.8 Validation of microarray results with real time RT-PCR analysis

To confirm genes identified to be differentially expressed using microarrays, real time PCR was performed on on seven selected regulated genes (Figures 24 and 25). The expression level of the selected genes quantified by real time RT-PCR was similar to the patterns of mRNA regulation as determined by microarray analysis. (Table 6)

**Table 6. Fold change of gene expression between pre and post surgical samples as determined by micro-array and by real time RT-PCR analysis.**

| Gene Name | Array | | Real time PCR | |
|---|---|---|---|---|
| | Sepsis | Non-sepsis | Sepsis | Non-Sepsis |
| CD-63 antigen | 3,8 | 3,5 | 8,5 | 25,6 |
| Interleukin 13 receptor, alpha 2 (IL-13R) | 0,3 | 0,3 | 0,1 | 0,2 |
| Hypothetical protein (C8ORF16) | 0,3 | 0,3 | 0,09 | 0,1 |
| HLA-B associated transcript 5 | 0,3 | 0,3 | 0,1 | 0,1 |
| IL-21 | 0,1 | | 0,06 | |
| Muscarinic acetylcholine receptor M5 | 0,2 | | 0,1 | |
| Dopamine receptor D5 | 0,2 | | 0,1 | |

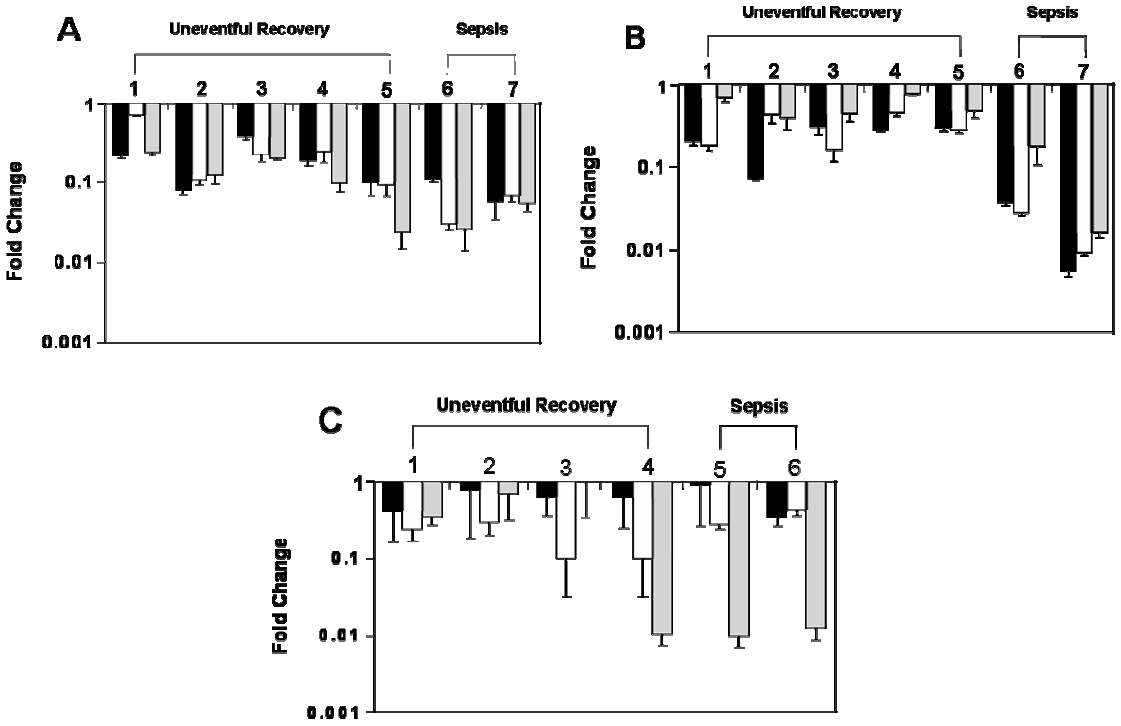



**Figure 24: Real Time-PCR determination of the extent of down regulation of the (A) IL-13 receptor, (B) Hypothetical protein (C8ORF16) and (C) HLA-B associated transcript 5 in major surgical patients with uneventful recovery and in patients who suffered septic complications**. For each patient the expression level prior to surgery was set as 1.0 and the level at 24hours (black bars), 48 hours (white bars) and 72 hours (grey bars) post surgery are shown. RT-PCR determinations on each sample were carried out in triplicate.

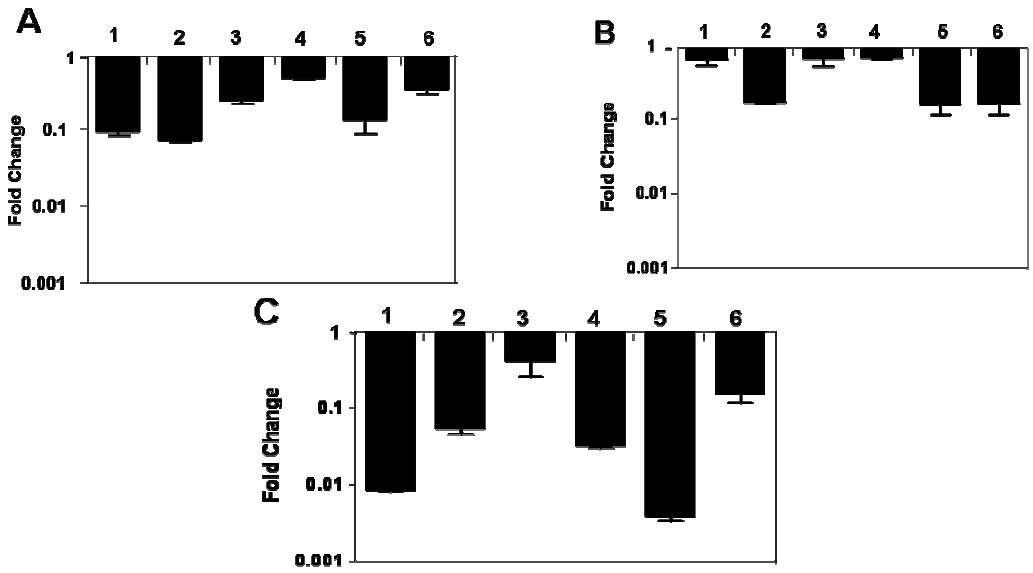



**Figure 25. Real Time-PCR determination of the expression of (A) IL-21, (B) Muscarinic acetylcholine receptor M5 and (C) Dopamine receptor D5 in six major surgical patients with septic complications.** For each patient the expression level prior to surgery was set as 1.0 and the level at 24 hours (black bars) after surgery are shown. RT-PCR determinations on each sample ere carried out in triplicate. Expression levels were determined in triplicates and are shown as the mean+SE.

## 3.9 Both $CD4^+$ and $CD8^+$ T-cells are affected

Different subpopulations of T cells may exhibit distinct programs of transcription. When transcription levels are measured from a population of cells in an experiment, such as by using DNA microarrays, the measured transcription represents the weighted average of these many independent transcriptional programs. Isolated $CD4^+$ and $CD8^+$ T-cells were used to determine whether the observed down regulation is a general feature of the T-cell compartment or whether it is restricted to one of these sub-populations (Figure 26). Total RNA was prepared from $CD4^+$ and $CD8^+$ cells of five major surgical patients who did not suffer inflammatory complications. Real time PCR analyses were carried out to compare the degree of regulation of three of the T- cell specific genes which were down regulated in total T cell. All 3 genes were down regulated in both $CD4^+$ and $CD8^+$ population (Figure 27).

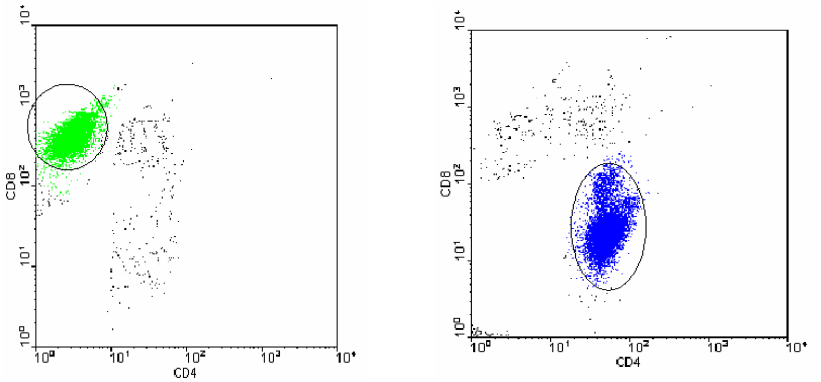



**Figure 26: FACS analysis of purity of $CD4^+$ and $CD8^+$ T cells. Sorted cells had a purity of 85-90%.**

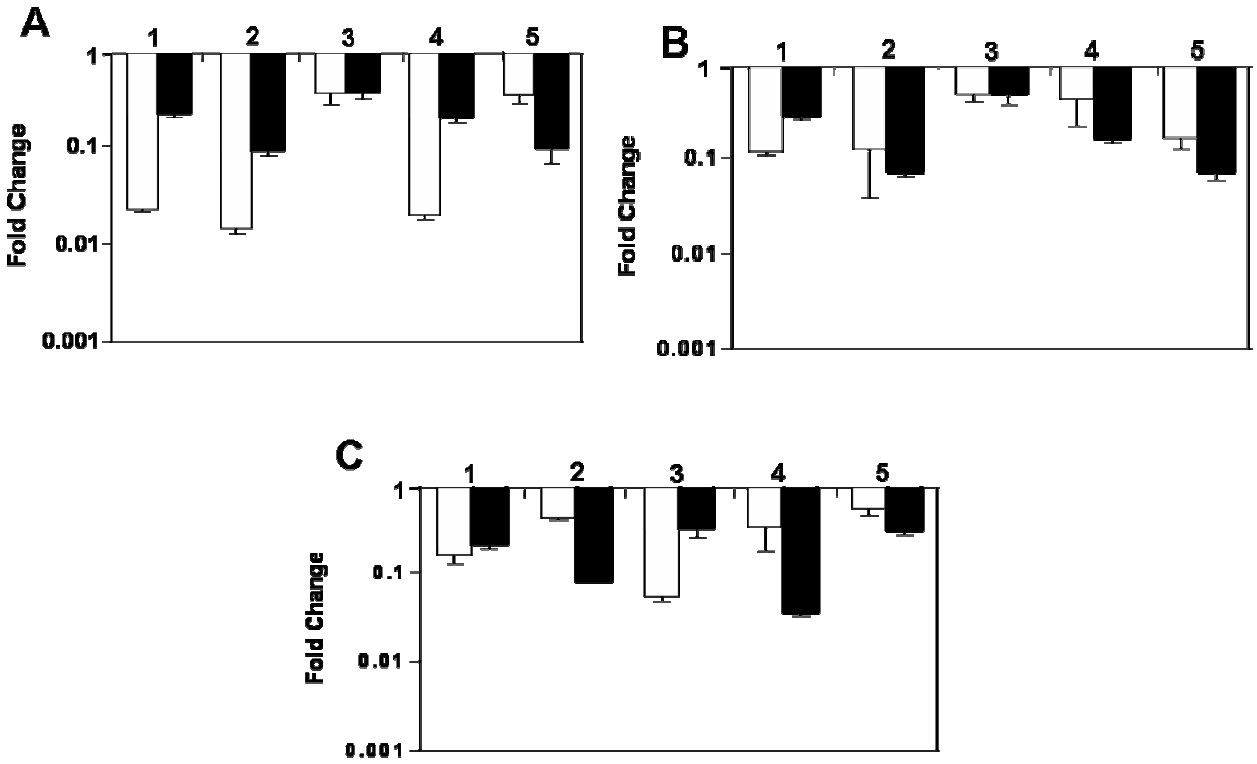



**Figure 27: Real Time-PCR determination of the expression of IL-13 receptor (A), Hypothetical protein (C8ORF16) (B) and HLA-B associated transcript 5 (C) in CD4 and in CD8 cells from five major surgery patients**. For each patient the expression level prior to surgery was set as 1.0 and normalized using expression of 18S RNA as a standard. The expression level 24 hours after surgery is shown for CD4 cells (white bars) and CD8 cells (black bars). Expression levels were determined in triplicate and are shown as the mean+SE.

# 4 Discussion

To assess changes in immune status following surgical intervention, we chose to focus on blood monocytes and T lymphocytes from major surgical patients. Though *in vivo* studies in humans are highly restricted in their extent and difficult to undertake we nevertheless adopted this approach for two reasons. First, extrapolation of immunological responses from animals to patients is difficult because there exist significant differences in the innate and adaptive immune interactions between humans and mice (108, 109). This has been the most dramatically illustrated in the tragic outcome of the recent CD28 therapy trial which gave most promising results in mice and very different results in human volunteers(110). Second, the questions addressed in this work are difficult to approach in rodent models because it is difficult to isolate sufficient numbers of monocytes and T lymphocytes for gene expression studies. For these reasons we concentrated on cells isolated from patient samples in this study.

To carry out these experiments it was first necessary to establish the basic infrastructure. This included patient enrolment after informed consent, creation of a clinical data bank for these patients and the arrangement of a rapid transport service of blood samples from collection site to the analysis laboratory to avoid change in immune parameters due to prolonged storage of blood. Patients enrolled were those undergoing elective major visceral surgery. To reduce possibly confounding extraneous effects those patients with established infections and those with severe systemic disease (ASA category 4 or higher) were excluded from the study. The inclusion criteria were patients undergoing major surgery such as thoracic surgery, esophagectomies, gastric surgery, colorectal surgery, liver resection and multiviceral surgery.

To investigate the perioperative changes of immune functions in these post-surgical patients, the blood sample collected from each patient at 24 hours before an operation was used as a benchmark against which samples from the same patient at 24, 48 and 72 hours after the operation.

## 4.1 Effect of surgical intervention on monocyte HLA-DR expression

The expression of HLA-DR on monocytes is a marker whose determination is readily altered by analytical procedures and sample handling (111). HLA-DR expression can be significantly modulated in blood samples depending on the anti coagulant used and on the storage conditions. It is increased significantly by storage of blood for 2 h at room temperature,

particularly when heparin or citrate are used as anticoagulants. We therefore used EDTA as anticoagulant as it has less effect on the surface expression of HLA-DR and all patient blood samples were analysed within 30 minutes after collection (77).

The measured expression values of HLA-DR determined by FACS are subject to day to day variation due to drift in the cytometer settings. In order to be able to compare different patients it is therefore advantageous to express the results as MEF (Molecules of Equivalence Fluorescence) rather than MFI (Mean Fluorescence Index). The MEF is determined by comparing the measured fluorescence intensity against that of calibration beads. It thus allows a determination of the absolute expression level which is independent of drift in the instrumentation. For this reason, all results are expressed as MEF.

In the current study, surgical intervention resulted in a dramatic change in monocyte HLA-DR expression. All the patients examined in the study had decreased post surgical HLA-DR expression. This is in line with previous work and indicates a considerable change in monocyte phenotype in the immediate post operative period (68, 112). Also in line with previous studies we noted that patients who suffered from inflammatory complications showed a more severe reduction in monocyte class II expression postoperatively than did patients with uneventful recovery (70, 72). More surprisingly, patients who underwent only minor surgery also showed a substantial and prolonged decrease in HLA-DR expression, albeit to a lesser extent than that seen in the major surgery group. This indicates that the decrease of monocyte class II expression is correlated to the degree of surgical trauma. Major surgery patients suffering from inflammatory complications showed a higher degree of HLA-DR suppression than did those without inflammation who in turn showed a higher degree of HLA-DR suppression than did minor surgery patients

## 4.2 Mechanisms affecting HLA-DR expression

The expression of MHC class II is regulated not only at the transcriptional level but also post translationally (113). After a short transit through the trans-Golgi network, HLA heterodimers associated with the invariant chain are transported to the Class II compartment vesicles, where peptide loading occurs (114). Once loaded they are then expressed on the cell surface. Alternatively, immature unloaded HLA-DR molecules may be directly transported to the cell surface, together with the invariant chain (115). In a recycling pathway, loaded and unloaded surface HLA-DR molecules are re-endocytosed to the MHC II compartment , where antigenic

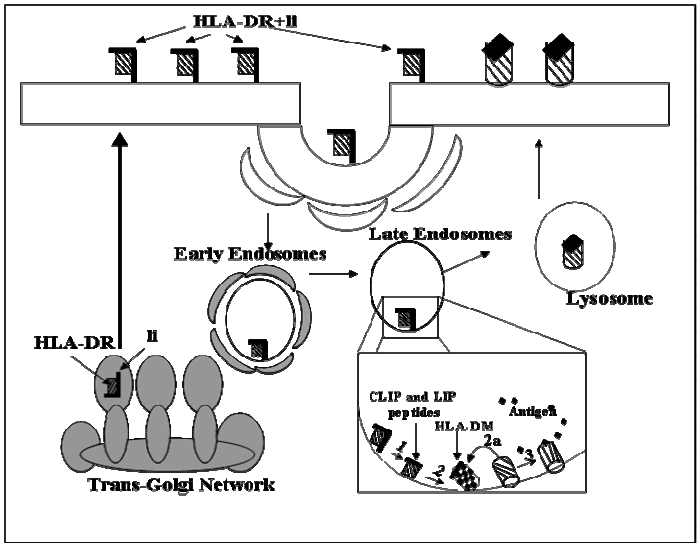



**Figure 28. Intracellular trafficking of HLA-DR and invariant chain (Ii).** HLA-DR form complex with Ii protein associate within the ER before being targeted into endocytic pathway. In endosomes (1) cleavage of Ii is mediated by cathepsin proteases. (2) The CLIP and LIP peptides, proteolytic products of Ii, remain in the antigen binding groove of HLA-DR until they are exchanged for antigen in a reaction catalyzed by HLA-DM (2a). (3) After antigens are loaded into the binding pocket within the late endosomes and lysosomes, HLA-DR is transported to the cell surface. (Picture adopted from Shao and Sperber 2002 (190))

peptides can be loaded or exchanged, before re-expression on the cell surface (Figure 28) (116). The decrease of expression on the cell surface can be modulated by mediators which interfere with the protein recycling system. For example, IL-10 induces *in vitro* the downregulation of HLA-DR surface expression of monocytes with an accumulation of MHC II molecules in intracellular compartments (117, 118). IL-10 also modifies the pH of the MHC Class II compartment vesicles and thus blocks the activity of the cathepsin enzymes required for efficient peptide loading of MHC II molecules and surface expression (119). It has been demonstrated that plasma IL-10 levels increase in the immediate postoperative period in major surgery patients (120, 121) and that they can induce changes in MHC class II expression of monocytes (117). The decreased expression of monocyte membrane MHC II observed in major surgery patients may therefore be at least partly mediated by interleukin 10.

Class II expression may also be regulated at the transcriptional level. Anti-inflammatory mediators including transforming growth factor-β, cortisol, and prostaglandin E2 have been implicated in this regulation. These mediators down regulate MHC II mRNA expression through the transcriptional suppression of the MHC class II specific transcription

factor CIITA (122-124). Thus down regulation of MHC expression observed in surgical patients may be mediated by factors acting both at the transcriptional and post translational levels.

On the other hand HLA-DR expression on monocytes can be increased by exposing the cells to pro-inflammatory mediators such as IFN-γ. In a study by Döcke et al. (68) the decreased HLA-DR expression on monocytes of septic patients was restored *in vivo* by treatment with IFN-γ. Importantly, the monocytes from these treated patients recovered their ability to respond to *in vitro* LPS challenge with the secretion of TNF-α. Similar effects were achieved when GM-CSF was used in place of IFN-γ (125, 126). So far, these studies included only a small number of carefully selected patients. Large randomised, controlled trials will be required to study the possible efficacy of these therapies in the general population of patients suffering from immunesuppression.

## 4.3 Gene expression profiling in monocytes of surgical patients.

The reduction of HLA-DR expression in circulating monocytes demonstrates that these cells rapidly sense the trauma induced by the disruption of tissues which accompanies surgery and change their phenotype accordingly. Monocytes in the blood are normally on their way to become tissue macrophages or DC. They must therefore exit from the blood and this involves interaction with the vascular endothelium - the key event in monocyte extravasation. We therefore decided to ask whether genes involved in extravasation are altered after tissue damage. To do this we employed gene expression profiling techniques.

As blood leukocytes represent a complex mixture of several cellular subpopulations, it is difficult in an array experiment to distinguish changes arising from alterations in gene expression from those which merely reflect alterations in the distribution of individual cell populations (127). Because of this, studies carried out with RNA prepared from whole blood are of limited value (128). We therefore chose to study cell specific immune responses using isolated monocytes.

Monocyte isolation is commonly achieved by Ficoll-Hypaque purification of peripheral blood mononuclear cells (PBMC) followed by adherence of the monocytes to plastic (129). In this procedure lymphocyte contamination in the first hour after adherence may be as high as 40-50% after two washes and 30% even after five washes. Worse still, monocyte isolation by adherence suffers from the disadvantage that it induces activation of IL-1β, TNF-α, macrophage-inflammatory protein-1α (MIP-1α ), IL-1 receptor α (IL-1r α) and

more than 350 genes including NF-kB activators and suppressors (130). To avoid these problems, a selection strategy can be applied in which magnetic separation is used to remove non-monocytes from PBMC. This is a gentle non-activating procedure but it suffers from the drawback that the final monocyte preparation is typically only 80% pure. For these reasons we used positive magnetic sorting of monocytes with paramagnetic beads carrying an anti CD14 antibody. To minimise the possibility of artefactual activation of the cells, the experimental procedures were carried out in the cold and in the presence of sodium azide. By this means highly enriched monocyte populations with purity greater than 95% can be routinely produced. These were then used to study the specific gene expression changes associated with major surgery.

## 4.4 Gene expression changes in monocytes of surgical patients

Trauma induced by mechanical disruption of tissues is unavoidable during surgical procedures and leads to a cascade of events that result in the chemoattraction of inflammatory cells and other cell types to the site of injury. Though we do not know the molecular details, it is clear that monocytes can sense and respond to tissue injury (68). We have carried out microarray analysis to understand the response of circulating monocytes to sugery induced tissue damage. For this, we used a ‘‘trauma cDNA chip’’ which consists of selected genes which are up-regulated in monocytes in response to major trauma (105). To construct this chip, gene expression equalized cDNA libraries were generated from monocytes of trauma patients with SIRS and trauma patients in septic shock. Clones representing 315 genes were pre-selected as being up-regulated in the patients were sequenced and spotted on arrays. This ‘‘trauma cDNA chip’’ were then used to analyse the RNA from individual patients. Surgical intervention also caused a rapid change in the transcriptional profile of these “trauma – induced” genes. However, the extent of gene induction detected in circulating monocytes correlated with the extent of tissue damage. Minor surgical operations resulted in a lesser degree of induction of change in monocyte expression profile than did major surgery. Those major surgical patients with post surgical sepsis showed the largest change in monocyte gene expression profiles. The rapid change in the transcriptional profile observed at 24 hrs was reduced at later time periods following the operations (i.e 48 hrs and 72 hrs).

The functions of the genes regulated in the monocytes give us a hint as to the phenotypic changes which may be expected in the monocyte population. Among the up-regulated transcripts identified by microarray analysis, genes involved in reorganization of the

cytoskeleton, adhesion and migration constitute the largest functional family (Appendix, Table II). These include Sl00A12, S100A8, beta actin (131), L-selectin, platelet/endothelial cell adhesion molecule (CD31 antigen), Tropomyosins (132) and Syk tyrosine kinase. The involvement of these genes in various aspects of monocyte extravasation or activation has been previously established:

The calgranulins are a family of small $Ca^{++}$ binding proteins. S100A12 is a natural ligand for the receptor on endothelial cell which was first identified as a receptor for advanced glycation end products (RAGE). Engagement of RAGE by its ligand results in activation of the NFkB pathway in the process of inflammation (133). Intracellular S100A8/A9 complexes are involved in myeloid cell maturation, cell trafficking, and arachidonic acid (AA) metabolism (134) and these proteins can also be expressed on the cell surface where they are beliveved to enhance the transendothelial migration (135). Single cell PCR analysis of monocyte confirmed the presence of transcripts of S100A8 and S100A12 and real time PCR analyses showed the up regulation of these genes in trauma patients monocytes. However, further experiments will be required to identify the expression of genes at the protein levels in monocytes.

Other upregulated genes encode the cell adhesion molecules CD31 and L-selectin. CD 31 is expressed on the surface of monocytes and has a significant role in leukocyte transendothelial migration. Interaction between CD31 on extravasating leukocytes and CD 31 on endothelial cells guide the leukocytes through the endothelial cell junctions (136). L-selectin plays a major role in the regulation of the inflammatory response by mediating the initial attachment of leukocytes along endothelial cells lining postcapillary venules (137).

PKS, SyK and Rac are all upregulated in circulating monocytes of surgical patients. PKC is part of the signaling pathways controlling the respiratory burst (138) and chemotaxis (139). PKC has also been shown to associate with cytoskeletal elements. Recently, it was established that PKC-ß is involved in the regulation of macrophage function involved in host defense and is a crucial factor that controls chemotaxis (140). Syk and Rac are essential for the activation of certain actin-dependent processes particularly of integrin-mediated adhesion (141) which are essential events required to trigger cellular locomotion and transmigration (142).

A further member of the upregulated group of genes codes for tropomyosins. Tropomyosins bind along the side of actin filaments, stabilise the filament against

spontaneous depolymerisation. Furthermore, tropomyosin has a protective effect against gelsolin severing and ADF/cofilin-mediated actin filament depolymerisation (132).

Further upregulated genes are leukotriene B4 hydrolase, NOD2 and CD163. Leukotriene B4 hydrolase converts LTA4 to LTB4 and LTB4 is a potent trigger of monocyte adhesion (143). The Nod2 protein is an activator of NF-κB via its interaction with RIP2/RICK/CARDIAK through its CARD domain (144, 145). CD163 is exclusively expressed by cells of monocyte/macrophage lineage (146) and it can bind and internalize haptoglobin-hemoglobin complexes (147). Heme, which is mainly derived from hemoglobin (Hb), is a strong oxidant and has potent pro-inflammatory properties. Rapid sequestration of heme by CD163 is necessary to limit pathogen access to host iron (148-151). CD163 expression is strongly induced by glucocorticoids (147) and by the anti-inflammatory cytokine interleukin-10 (152).

## 4.5 Effect of surgical intervention on T lymphocytes

Major surgery has been shown by others to affect the T-cell compartment (153, 154). We therefore followed the effects of surgery on the T-cells in our patient cohort. In these patients surgery was associated with a significant decrease in the numbers of circulating T cells, both of the $CD4^+$ and of the $CD8^+$ subpopulation. In contrast, there was no significant change in lymphocyte counts in patients undergoing minor surgery suggesting that, as with the HLA-DR phenotype on monocytes, the effects of surgery on T cells is related to the degree of tissue damage. Since roughly half of all circulating T cells were lost within 24 hours it would be important to know what happens to them. They may simply be hidden by sequestration in secondary immune sites or destroyed by apoptosis.

Though we cannot quantitatively distinguish the contribution of these factors we did ask whether apoptosis might play a significant role in reducing circulating T cell numbers. Apoptosis is an evolutionarily conserved and highly regulated program of cell death, which plays an important role in both normal physiological processes and in disease states. (88, 155-157). Apoptosis is characterized by chromatin condensation, a reduction in cell volume, and endonuclease cleavage of DNA into nucleosomal size fragments followed by association with membrane blebbing (158, 159)161). One early feature of apoptosis is the loss of membrane phospholipid asymmetry which results in the exposure of phosphatidylserine, normally restricted to the inner leaflet of the membrane, on the outer surface of the cell. Surface

expression of phosphatidylserine (PS) thus serves to label cells for removal by macrophages (160).

The presence of phosphotidylserine on the outer surface of intact cells can be detected using lebelled Annexin V. We used this approach to determine the fraction of apoptotic T cells present in the circulation of patients before and after surgery. A rapid increase in the fraction of apoptotic peripheral T lymphocytes was observed in post surgical patients. This demonstrates that the apoptotic processes in the peripheral T cell pool are disturbed in these patients. Whether this disturbance lies at the level of increased apoptosis induction or a decrease in the efficiency of removal of apoptotic cells from the circulation remains to be determined. Since we do not know the half life of these $PS^+$cells in the circulation, it is not possible to calculate the contribution which apoptosis makes to the loss of circulating T cells.

It would be important to know what might induce this increase in induction of apoptosis in the T-cells. It is well estabilished that $CD4^+$ $CD8^+$ double positive thymocytes are exquisitely sensitive to glucocorticoid induced apopotosis (161) and serum cortisol levels do rise after surgery (162). However circulating T cells are, in contrast to thymocytes, quite resistant to cortisol (161). To gain more insight into the changes taking place within the T cell population, we have carried out microarray analysis of T cell gene expression profiles.

Microarray analysis experiments typically require 5-20 µg of total RNA per slide for sample labeling and hybridization. This makes microarray-based gene expression analysis of the patient samples which can be realistically recovered in a clinical situation difficult due to the very low amounts of total RNA available. The blood volume available in each sample was restricted to 10 ml which yielded on average $5\text{-}6x10^6$ $CD3^+$ T cells which in turn yielded 2-3 µg of total RNA. Because of this it was necessary to amplify the RNA prior to array analysis. Amplification can be carried out by random primed PCR after cDNA systhesis. However, the procedure is subject to sequence preferences of the polymerase which results in a skewing of the sequence representation. The quantitative relationships between the expressed genes, (163) a parameter critical for expression profiling are therefore not maintained. An alternative approach involves linear amplification by *in vitro* transcription of the cDNA template from a phage T7 promoter (164) using the T7 polymerase. This amplification has been shown to retain the relative frequencies of transcripts with reasonable fidelity over a wide amplification range (165-167). For these reasons we chose to use T7 RNA polymerase based linear amplification and the amplified RNA template was then labelled for transcriptional profiling (168).

## 4.6 Gene expression changes in T cells of surgical patients

Since arrays are expensive, an analysis of gene expression of all patients at all times points would be prohibitively costly. Because of this, array analysis of clinical samples requires that sensible compromises be made as to the choice of the patient material to be used. If the arrays are interrogated with probes from single patients, this may yield distorted signals specific to the individual. On the other hand pooling samples from many individuals risks losing the contribution of genes regulated at low levels. Therefore, to concentrate on commonalities between different subjects, pooling of two patients samples was choosen for microarray examination. The differential expression of genes was then confirmed by RT-PCR. This also permitted the number of patients examined to be expanded.

For both array analysis and RT-PCR the results are dependent on the assessment of the signal strength in the post operative sample relative to that in the pre-operative control. For the arrays one would expect that a gene whose expression is unaltered would show unaltered signal intensity for the Cy3 and Cy5 dyes. However, there are differences in the labelling efficiency of the different probes (sample to sample variation) and difference in labelling efficiency of the two dyes (Cy3 versus Cy5). Because of this normalisation of the data is necessary to define the ratio of Cy3:Cy5 expected from a gene whose expression does not change. For this we have used a so-called global normalisation procedure. Global normalisation is based on the assumption that only a small fraction of all genes will change their expression ratio in the compared samples. Relative expression data from all genes are pooled and averaged. Regulated genes are identified as those whose expression ratio differs significantly from this value. Genes common to the septic and non septic patients, which differed from the global normalization mean with p values $<0.005$, were considered to be regulated. In this comparison four data sets were available (two inflammatory complication and two uneventful recovery). Genes regulated only in the inflammatory patients were selected as having median ratio intensities on the duplicate slides within 12.5% of each other and ratios of medians which differed from the normalized value by a factor of at least 3. In this case p values could not be calculated as we only had two data sets available to us. We found 589 genes were regulated in the post operation period both in patients with inflammatory complications and in those with an uneventful recovery. Of these genes 554 were down regulated and 35 were up regulated (Appendix Table III).

To confirm the array results and to be able to expand them to more patient samples we chose to employ RT-PCR. Since many genes were potentially of interest we chose to use the

SYBR green procedure rather than TaqMan probes. This approach determines relative differences in gene expression instead of absolute copy numbers, eliminating the need for many precisely quantified templates as standards. The advantages of using SYBR-Green I quantification over a 5'-nuclease assay with TaqMan probes are the relative simplicity and the reduced cost of the SYBR-Green I procedure (169). The disadvantage is that SYBR Green will bind to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products. For this reason all RT-PCR results were checked by doing a melting curve on the product. A single amplified product yields a sharp melting curve. Non specific amplification yields a broad complex of melting curves. As with array analysis, RT-PCR is crucially dependent on the availability of a suitable normalisation control which is frequently a house keeping gene. The 'ideal' housekeeping gene should be constantly transcribed in all cell types and tissues and remain stable between samples taken from different time points and under different experimental conditions. *ACTB*, *GAPDH* and 18S rRNA are examples of commonly used housekeeping genes. However for each of them studies have provided evidence that their transcription levels vary considerably between different individuals, different cell types, different developmental stages and under different experimental conditions (170, 171). 18S rRNA as a component of ribosomes is relatively stable unless the cell type under investigation changes its protein systehsis capacity radically as may happen during the development of blast cells. Since circulating T cells do not form blasts we chose to use 18S rRNA as the normalisation standared as others have previously suggested (172).

Both inflammatory complications and non-septic surgical patients showed down regulated expression of many genes with few up-regulated genes. These changes were more marked in T cells from patients who subsequently suffered from severe inflammatory complications. However, the up-regulation of a few genes indicates that T lymphocytes are not simply completely paralysed in post surgical patients. To get more insight into the pattern of the down regulated genes, the different genes were put into functional groups. To generate and rank functional networks of genes the Ingenuity Pathways Analysis Software (IPAS) was used. For this each gene was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base, a data base consisting of millions of individually modelled presumptive relationships between proteins, genes, cells and tissues for the identification of key functions and pathways. However, though we found numerous genes showing differential expression in our study are multiply associated with cellular pathways connected to cellular

signalling mechanisms, these pathways are not centered on any particular function or molecule. Nevertheless though the network approach turned out to be disappointing in this case many genes associated with important T-cell functions are affected in all of the post surgical patients examined.

The T cell proteins IL-21, CD44, CCR5 and CD3ε which are involved in various aspects of migration and activation were down-regulated in post surgical patients. IL-21 is a product of activated $CD4^+$ T cells that stimulates NK and T cells to secrete IFN-γ (173). It enhances the proliferation and cytotoxicity of NK cells and of $CD8^+$ T cells (174).

CD44 is the major receptor for hyaluronan (HA) (175), and also binds to fibronectin and collagen (176). As HA is captured *via* CD44 on endothelial cells, CD44 is important for leukocyte rolling (177, 178) and firm adhesion to vessel endothelium. It is also involved in cell motility, proliferation and apoptosis resistance (179-181). The cytoplasmic tail of CD44 associates with ERM (Ezrin, Radixin, Moesin) proteins which cross-link transmembrane. proteins to the actin cytoskeleton (182), that supports lymphocyte polarization (183). CD44 is constitutively associated with lck, lyn and fyn (184), Src family protein tyrosine kinases (PTK) important in leukocyte activation.

CCR5 is a receptor for MIP-1α, MIP-1β and RANTES expressed on some memory T-cells in the circulation. Its expression has been shown to be important for selective leucocyte migration (32) in response to chemotactic stimuli. Furthermore, CCR5 receptor stimulation induces an intracellular cascade of events including G-protein activation and increased production and release of interleukin (IL-2) and interferon-γ from T lymphocytes (185), providing an alternative indirect mechanism potentially involved in the T cell migration and activation.

CD3ε is part of the TCR complex. After TCR cross-linking, CD3ε and ζ are the predominant tyrosine-phosphorylated TCR subunits. Thus down regulation of CD3ε may affect TCR signalling events (186). In addition to these, many genes involved in transcriptional regulation, intermediary metabolism, transporter functions and signal transduction were down-regulated. Taken together, the changes in gene expression of post-surgical patients suggest global down- regulation of the transcriptome of T lymphocytes with a modest subset of genes upregulated. These results support the previously reported T cell dysfunction characterised by decreased T cell proliferation, production of cytokines (IL-2, IFN-γ) and decreased expression of the TCR due to loss of the ζ-chain peptide (72, 187).

Interpretation of the array data is made difficult by the fact that arrays do not provide data on a single cell level. A modest change in expression of a gene may be due to a large change in a small population or alternatively, to a small change in a large fraction of all cells. An analysis of this point for individual genes would require single cell PCR or some other approach yielding data at the single cell level. However as initial approach we have asked whether there are obvious large differences in the regulation of individual genes in the $CD4^+$ & $CD8^+$ populations. We have excluded the possibility that the change in the gene expression profile is restricted to the $CD4^+$ or to the $CD8^+$ population.

The perioperative analysis of monocyte and T cell status suggests that there is a generalised transitory immune dysfunction which affects both innate and adaptive immune system. The persistence of this immune dysfunction in surgical patients may predispose patients to the development of post surgical infections. Thus, the identification of critical factors associated with the induction of T cell and monocyte dysfunction will be of great importance as a prerequisite in the development of therapeutic strategies to control post operative immune suppression.

# SUMMARY

Major surgery causes alterations in immune function which results in immune suppression in post surgical patients. Deactivation of monocytes in these patients is characterised by the reduced ability of these cells to produce pro-inflammatory cytokines on stimulation with LPS *in vitro* and by markedly reduced HLA-DR expression. Immune suppression in patients with systemic inflammation has also been associated with a high level of apoptosis in both the circulating T and B cell populations. In addition post surgical T cells have a reduced capacity to proliferate ex vivo in response to co-ligation of the T cell receptor and CD-28. Considering these impairments of immune system, this study aimed to define the extent of immune modulation in both innate and adaptive system in a cohort of surgical patients.

Measurment of the level of HLA-DR expression of monocytes in these patients showed a considerable change in monocyte phenotype in the immediate post operative period. In line with previous work, all patients showed a considerable reduction in monocytic surface HLA-DR expression which persisted for many hours and those who had post surgical septic complications showed the most severe reduction. Importantly, patients with minor surgical intervention also exhibited decreased HLA-DR expression. Gene expression analysis of monocyte in these patients showed the up-regulated transcripts of genes involved in extravasation and realignment of the cytoskeleton. Analysis of periperal T cell demonstrated a significant reduction in their number in the circulation and a sharp raise in the number of apoptotic T –cells in the immediate post surgical period. Microarray analysis of T cells from patients who developed sepsis and patients with an uneventful recovery within the post-operative period (3 days) showed a substantial reduction in the transcriptional activity of many genes in both groups. However, this down regulation of T cell transcriptional activity appears to be a rather broad and non specific effect since it is not restricted to particular functional pathways. Real time PCR analysis of both the $CD4^+$ and $CD8^+$ populations using selected down-regulated genes showed that the change in transcriptional profile is equally evident both in $CD4^+$ and $CD8^+$ T-cells.

The cause of this transient immune depression following surgery remains to be established and it may represent an important enabling factor which contributes to the development of post surgical infections and inflammatory complications.

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# APPENDIX

**Tale I. List of patients recruited in this study**

| Patient (No.) | Age (Yr) | Gender (M/F) | Type of surgical procedure | Major/Minor surgery | Post surgical septic complications |
|---|---|---|---|---|---|
| 1 | 65 | F | stomach operation | Major Surgery | SS |
| 2 | 68 | F | rectum resection | Major Surgery | No |
| 3 | 44 | M | esophageal surgery | Major Surgery | No |
| 4 | 32 | M | rectum resection | Major Surgery | No |
| 5 | 69 | M | colon resection | Major Surgery | No |
| 6 | 72 | M | lung surgery | Major Surgery | No |
| 7 | 32 | M | stomach operation | Major Surgery | SS |
| 8 | 74 | M | pancreatic surgery | Major Surgery | No |
| 9 | 49 | M | liver resection | Major Surgery | No |
| 10 | 46 | F | rectum resection | Major Surgery | No |
| 11 | 52 | M | stomach operation | Major Surgery | S |
| 12 | 74 | M | stomach operation | Major Surgery | No |
| 13 | 65 | M | esophageal surgery | Major Surgery | No |
| 14 | 62 | F | pancreatic surgery | Major Surgery | No |
| 15 | 81 | F | pancreatic surgery | Major Surgery | S |
| 16 | 71 | M | lung surgery | Major Surgery | No |
| 17 | 67 | M | pancreatic surgery | Major Surgery | SS |
| 18 | 71 | M | lung surgery | Major Surgery | No |
| 19 | 41 | M | lung surgery | Major Surgery | No |
| 20 | 47 | M | pancreatic surgery | Major Surgery | No |
| 21 | 60 | M | liver resection | Major Surgery | No |
| 22 | 75 | F | stomach operation | Major Surgery | SS |
| 23 | 80 | M | lung surgery | Major Surgery | No |
| 24 | 55 | M | esophageal surgery | Major Surgery | No |
| 25 | 78 | F | stomach operation | Major Surgery | S |
| 26 | 74 | M | colon resection | Major Surgery | No |
| 27 | 26 | M | liver resection | Major Surgery | No |
| 28 | 49 | F | stomach operation | Major Surgery | SS |
| 29 | 61 | M | rectum resection | Major Surgery | No |
| 30 | 68 | F | esophageal surgery | Major Surgery | SS |
| 31 | 71 | M | colon resection | Major Surgery | No |
| 32 | 67 | M | lung surgery | Major Surgery | No |
| 33 | 68 | M | lung surgery | Major Surgery | No |
| 34 | 69 | M | rectum resection | Major Surgery | No |
| 35 | 43 | M | lung surgery | Major Surgery | No |
| 36 | 66 | F | pancreatic surgery | Major Surgery | No |

| Patient (No.) | Age (Yr) | Gender (M/F) | Type of surgical procedure | Major/Minor surgery | Post surgical septic complications |
|---|---|---|---|---|---|
| 37 | 69 | F | lung surgery | Major Surgery | No |
| 38 | 44 | M | colon resection | Major Surgery | No |
| 39 | 61 | F | pancreatic surgery | Major Surgery | No |
| 40 | 40 | M | stomach operation | Major Surgery | SS |
| 41 | 35 | F | liver resection | Major Surgery | S |
| 42 | 66 | F | pancreatic surgery | Major Surgery | No |
| 43 | 34 | M | lung surgery | Major Surgery | No |
| 44 | 80 | M | stomach operation | Major Surgery | No |
| 45 | 73 | M | colon resection | Major Surgery | No |
| 46 | 60 | M | stomach operation | Major Surgery | No |
| 47 | 61 | F | colon resection | Major Surgery | No |
| 48 | 52 | M | esophageal surgery | Major Surgery | No |
| 49 | 70 | F | colon resection | Major Surgery | No |
| 50 | 81 | M | rectum resection | Major Surgery | No |
| 51 | 65 | M | rectum resection | Major Surgery | No |
| 52 | 81 | M | rectum resection | Major Surgery | No |
| 53 | 74 | F | stomach operation | Major Surgery | No |
| 54 | 64 | F | stomach operation | Major Surgery | SS |
| 55 | 75 | F | rectum resection | Major Surgery | No |
| 56 | 77 | F | rectum resection | Major Surgery | No |
| 57 | 53 | F | stomach operation | Major Surgery | No |
| 58 | 71 | M | lung surgery | Major Surgery | No |
| 59 | 69 | M | rectum resection | Major Surgery | No |
| 60 | 38 | F | stomach operation | Major Surgery | SS |
| 61 | 62 | M | rectum resection | Major Surgery | No |
| 62 | 50 | M | pancreatic surgery | Major Surgery | No |
| 63 | 69 | F | pancreatic surgery | Major Surgery | No |
| 64 | 57 | M | pancreatic surgery | Major Surgery | No |
| 65 | 68 | M | stomach operation | Major Surgery | No |
| 66 | 70 | F | stomach operation | Major Surgery | No |
| 67 | 78 | M | lung surgery | Major Surgery | No |
| 68 | 74 | F | rectum resection | Major Surgery | No |
| 69 | 84 | M | stomach operation | Major Surgery | No |
| 70 | 80 | M | stomach operation | Major Surgery | No |
| 71 | 65 | M | colon resection | Major Surgery | No |
| 72 | 72 | F | stomach operation | Major Surgery | SS |
| 73 | 67 | M | stomach operation | Major Surgery | S |
| 74 | 69 | M | esophageal surgery | Major Surgery | SS |
| 75 | 50 | M | pancreatic surgery | Major Surgery | No |

| Patient (No.) | Age (Yr) | Gender (M/F) | Type of surgical procedure | Major/Minor surgery | Post surgical septic complications |
|---|---|---|---|---|---|
| 76 | 59 | F | pancreatic surgery | Major Surgery | SS |
| 77 | 65 | F | pancreatic surgery | Major Surgery | No |
| 78 | 70 | M | stomach operation | Major Surgery | No |
| 79 | 79 | F | stomach operation | Major Surgery | No |
| 80 | 59 | F | stomach operation | Major Surgery | No |
| 81 | 65 | F | colon resection | Major Surgery | No |
| 82 | 55 | M | colon resection | Major Surgery | No |
| 83 | 83 | M | colon resection | Major Surgery | No |
| 84 | 66 | M | rectum resection | Major Surgery | No |
| 85 | 67 | M | colon resection | Major Surgery | S |
| 86 | 64 | M | colon resection | Major Surgery | No |
| 87 | 55 | M | colon resection | Major Surgery | No |
| 88 | 64 | F | rectum resection | Major Surgery | No |
| 89 | 65 | F | lung surgery | Major Surgery | No |
| 90 | 41 | F | colon resection | Major Surgery | No |
| 91 | 46 | M | pancreatic surgery | Major Surgery | No |
| 92 | 51 | M | pancreatic surgery | Major Surgery | No |
| 93 | 89 | F | colon resection | Major Surgery | No |
| 94 | 65 | M | colon resection | Major Surgery | S |
| 95 | 65 | M | colon resection | Major Surgery | No |
| 96 | 69 | M | colon resection | Major Surgery | No |
| 97 | 62 | M | liver resection | Major Surgery | No |
| 98 | 64 | F | colon resection | Major Surgery | No |
| 99 | 74 | M | rectum resection | Major Surgery | No |
| 100 | 65 | M | colon resection | Major Surgery | No |
| 101 | 46 | F | liver resection | Major Surgery | No |
| 102 | 46 | M | herniar epair | Minor Surgery | No |
| 103 | 42 | F | thyreoid resection | Minor Surgery | No |
| 104 | 62 | M | hernia repair | Minor Surgery | No |
| 105 | 25 | M | hernia repair | Minor Surgery | No |
| 106 | 63 | F | thyreoid resection | Minor Surgery | No |
| 107 | 52 | F | thyreoid resection | Minor Surgery | No |
| 108 | 74 | F | thyreoid resection | Minor Surgery | No |
| 109 | 48 | F | hernia repair | Minor Surgery | No |
| 110 | 88 | M | gallblader surgery | Minor Surgery | No |
| 111 | 82 | M | hernia repair | Minor Surgery | No |
| 112 | 47 | M | gallblader surgery | Minor Surgery | No |
| 113 | 62 | F | gallblader surgery | Minor Surgery | No |
| 114 | 41 | M | hernia repair | Minor Surgery | No |
| 115 | 62 | M | gallblader surgery | Minor Surgery | No |

| Patient (No.) | Age (Yr) | Gender (M/F) | Type of surgical procedure | Major/Minor surgery | Post surgical septic complications |
|---|---|---|---|---|---|
| 116 | 80 | F | gallblader surgery | Minor Surgery | No |
| 117 | 65 | M | gallsblader surgery | Minor Surgery | No |
| 118 | 62 | M | hernia repair | Minor Surgery | No |

**S**-Sepsis;
**SS**-Septic Shock

**Table II. Functions of differentially regulated genes in monocytes**

| Gene | Gene Bank Accession No | Functions |
|---|---|---|
| Beta actin | BC014861 | Involved in cell motility, structure and integrity. |
| Calgranulin A12 (S100A12) | NM_005621 | A ligand for RAGE. Engagement of RAGE by S100A12 activates nuclear factor-κB and induces adhesion molecules such as VCAM-1 and ICAM-1 in endothelial cells. It also mediates the migration and activation of monocytes. |
| Calgranulin A8 (S100A8) | NM_002964 | Involved in the modulation of the tubulin dependent cytoskeleton during migration of phagocytes and it is a potent leukocyte chemoattractant. |
| CAP 1 | NM_006367 | Directly regulates filament dynamics and has been implicated in a number of complex developmental and morphological processes, including mRNA localization and the establishment of cell polarity. |
| CD163 | Y18391 | This acute phase-regulated receptor involved in clearance and endocytosis of hemoglobin/haptoglobin complexes. |
| CD31 | AF281294 | CD31 is associated with cell motility and the transmigration of leukocytes across endothelial cell junctions. |
| FLAP | M63262 | Required for the activation of 5-lipoxygenase activating protein which is involved in the biosynthesis of leukotrienes. |
| Gamma actin | NM_001614 | Functions as a component of the cytoskeleton and mediator of cell motility. |
| Leuk A4 hydrolase | BC032528 | Hydrolyzes an epoxide moiety of leukotriene A4 (LTA-4) to form leukotriene B4 (LTB-4). |
| L-Selectin | BC020758 | A cell surface adhesion protein. which mediates the adherence of leucocytes to endothelial cells. |

| Gene | Gene Bank Accession No | Function |
|---|---|---|
| L-Plastin | NM_002298 | Regulates cell movement by interacting with actin, a major cellular protein that directly controls cell movement. |
| NOD2 | AJ303140 | Plays a role in the immune response to intracellular bacterial lipopolysaccharides (LPS) by recognizing the muramyl dipeptide (MDP) derived from them and activating the NFKB protein. |
| RAC 1 | AF498964 | Members of this superfamily regulate a diverse array of cellular events, including the control of cell growth, cytoskeletal reorganization, and the activation of protein kinases. |
| TM3 | NM_153649 | Tropomyosins are actin-binding proteins; component of cytoskeletal microfilaments; tropomyosins mediate the effect of Ca2+ on the myosin-actin interaction |
| Versican | U26555 | May play a role in intercellular signaling and in connecting cells with the extracellular matrix. |
| EDRF | NM_003792 | This protein functions as a bridging molecule that interconnects regulatory proteins and the basal transcriptional machinery, thereby modulating the transcription of genes involved in endothelial differentiation. |
| MAPK6 | NM_002748 | MAP kinases are activated through protein phosphorylation cascades and act as integration points for multiple biochemical signals. |
| PKCß | NM_002738 | Protein kinase C (PKC) is a family of serine- and threonine-specific protein kinases that can be activated by calcium and second messenger diacylglycerol. |
| ZNF83 | XM_030944 | Involved in transcriptional regulation. |

| Gene | Gene Bank Accession No | Function |
|---|---|---|
| AASDHPPT | NM_015423 | Catalyzes the post-translational modification of target proteins by phosphopantetheine. |
| ECRP | X55989 | A cytotoxin and helminthotoxin with low-efficiency ribonuclease activity. |
| Glutaminyl cyclase | BC015460 | Responsible for the biosynthesis of pyroglutamyl peptides |
| Haptoglobin | NM_005143 | Haptoglobin combines with free plasma haemoglobin |
| Aquaporin 9 | NM_020980 | Forms a channel with a broad specificity and mediates passage of a wide variety of non-charged solutes |
| TXNIP | NM_006472 | Inhibitor of thioredoxin which is a major intracellular antioxidant |

## Table III. List of T cell genes down regulated in patients with sepsis and in those with uneventful recovery

| No | Gene Bank Accession No | Down Regulation | p-value | Gene Name |
|---|---|---|---|---|
| **Immune Response** | | | | |
| 1 | AB023135 | 0,6 | P < 0.001 | activation-inducible lymphocyte immunomediatory molecule ailim |
| 2 | AC004382 | 0,6 | P < 0.001 | cc chemokine stcp-1; a-152e5.1 |
| 3 | AF010446 | 0,7 | P < 0.005 | mhc class i related protein 1 isoform b; mr1b |
| 4 | AF088219 | 0,5 | P < 0.001 | small inducible cytokine a14 precursor; scya14 |
| 35 | AF129756 | 0,3 | P < 0.001 | HLA associated transcript B |
| 5 | AF193766 | 0,5 | P < 0.005 | cytokine-like protein c17; c17 |
| 6 | AF212016 | 0,4 | P < 0.001 | interleukin-1 receptor 9; il1r9 |
| 8 | AF430693 | 0,2 | P < 0.005 | t cell receptor beta variable region |
| 9 | AF502291 | 0,4 | P < 0.001 | toll-like receptor 2 |
| 10 | AF523361 | 0,5 | P < 0.005 | cd34 antigen; cd34 |
| 11 | AJ225109 | 0,7 | P < 0.001 | nk cell activating receptor (nkp44) |
| 12 | AY101192 | 0,6 | P < 0.001 | cd44 antigen; cd44 |
| 13 | BC015768 | 0,3 | P < 0.005 | interleukin 13 receptor, alpha 1 |
| 14 | BC020739 | 0,7 | P < 0.001 | interleukin 13 receptor, alpha 2 |
| 15 | BC036042 | 0,3 | P < 0.005 | similar to chemokine-like factor super family 3 |
| 16 | D21849 | 0,6 | P < 0.005 | t cell receptor alpha chain |
| 17 | D85245 | 0,5 | P < 0.001 | tr3beta |
| 18 | J03171 | 0,6 | P < 0.001 | ifnar |
| 19 | L34716 | 0,3 | P < 0.005 | t-cell receptor alpha; tcra |
| 20 | M13838 | 0,6 | P < 0.005 | tcrb |
| 21 | NG_0000026 | 0,3 | P < 0.001 | tgfb1p |
| 23 | NG_001332 | 0,6 | P < 0.001 | traj28 |
| 22 | NG_00133220 | 0,4 | P < 0.001 | traj7 |
| 24 | NG_001333 | 0,5 | P < 0.005 | trbv1 |
| 26 | NG_001333 | 0,4 | P < 0.001 | trbva |
| 25 | NG_0013335 | 0,4 | P < 0.001 | trbv5-2 |
| 27 | NM_000579 | 0,3 | P < 0.001 | chemokine (c-c motif) receptor 5; ccr5 |
| 28 | NM_000733 | 0,6 | P < 0.005 | cd3e antigen, epsilon polypeptide (tit3 complex); cd3e |
| 29 | S67783 | 0,5 | P < 0.001 | lymphocyte-specific protein 1; lymphocyte-specific protein 1 |
| 30 | U39105 | 0,4 | P < 0.005 | t cell receptor alpha chain |
| 31 | XM_069925 | 0,4 | P < 0.001 | similar to t-cell receptor beta chain precursor v-d-j region |
| 32 | XM_087167 | 0,3 | P < 0.005 | similar to cll-associated antigen kw-1 splice variant 1; kiaa1641 |
| 33 | XM_094993 | 0,3 | P < 0.001 | similar to t-cell receptor beta chain v region phds203 precursor |
| 34 | XM_095019 | 0,5 | P < 0.005 | similar to tcrbv6s8; loc168320 |
| **Transcriptional Regulation** | | | | |
| 1 | AB022083 | 0,5 | P < 0.001 | sox30 protein; sox30 |
| 2 | AB054067 | 0,4 | P < 0.005 | hypoxia-inducible factor-3 alpha; hif-3a |
| 3 | AF045239 | 0,6 | P < 0.005 | brain expressed ring finger protein |
| 4 | AF048721 | 0,4 | P < 0.001 | all1 responsive protein arp1b; arp1 |
| 5 | AF055470 | 0,5 | P < 0.001 | znf258 |
| 6 | AF059292 | 0,5 | P < 0.005 | transcriptional repressor e2f-6 |
| 7 | AF113226 | 0,3 | P < 0.001 | mstp046 |
| 8 | AF117236 | 0,7 | P < 0.005 | matrin 3 |
| 9 | AF155648 | 0,5 | P < 0.001 | putative zinc finger protein |
| 10 | AF198487 | 0,5 | P < 0.005 | transcription factor lbp-1b; lbp-1 |

| No | Gene Bank Accession No | Down Regulation | p-value | Gene Name |
|---|---|---|---|---|
| 11 | AF125507 | 0,4 | P < 0.005 | origin recognition complex subunit 3; orc3 |
| 12 | AF249672 | 0,5 | P < 0.005 | homeodomain protein nkx3.1 isoform v4; nkx3a |
| 13 | AF251684 | 0,5 | P < 0.005 | tbxx t-box containing protein; tbx22 |
| 14 | AF255565 | 0,3 | P < 0.005 | nuclear body protein sp100c |
| 15 | AF272897 | 0,6 | P < 0.005 | pr-domain zinc finger protein 5; prdm5 |
| 16 | AF301906 | 0,7 | P < 0.005 | forkhead transcription factor foxl2; foxl2 |
| 17 | AF373867 | 0,7 | P < 0.001 | t-box 1 transcription factor c; tbx1c |
| 18 | AF386743 | 0,7 | P < 0.005 | f-box protein; fbg4 |
| 19 | AF492004 | 0,3 | P < 0.001 | truncated zinc finger protein isoform; znf396 |
| 20 | AF539427 | 0,7 | P < 0.005 | zinc finger protein znf183l1 |
| 21 | AF548353 | 0,7 | P < 0.005 | btb/poz and zinc-finger domains factor on chromosome 1; bozf1 |
| 22 | AK000928 | 0,4 | P < 0.005 | weakly similar to cyclic-amp-dependent transcription factor atf-6 |
| 23 | AK027363 | 0,3 | P < 0.005 | cdna clone weakly similar to zinc finger protein 91; |
| 24 | AK027679 | 0,3 | P < 0.001 | cdna clone weakly similar to zinc finger protein 84; |
| 25 | AK075032 | 0,6 | P < 0.005 | cdna clone weakly similar to zinc finger protein 157; |
| 26 | AK091550 | 0,6 | P < 0.001 | highly similar to mus musculus (clone pmlz-1) zinc finger protein |
| 27 | AK093123 | 0,7 | P < 0.005 | cdna clone moderately similar to zinc finger protein 84; |
| 28 | AK093518 | 0,5 | P < 0.005 | cdna clone weakly similar to zinc finger protein 135 |
| 29 | AK096221 | 0,3 | P < 0.001 | cdna clone moderately similar to zinc finger protein 85 |
| 30 | AL009182 | 0,6 | P < 0.005 | liver-specific bhlh-zip transcription factor |
| 31 | AY029765 | 0,3 | P < 0.001 | zinc finger 1111 |
| 32 | AY049744 | 0,5 | P < 0.005 | zinc finger dna binding protein p71; zer6 |
| 33 | AY149174 | 0,6 | P < 0.005 | zinc finger protein 12; hzf12 |
| 34 | BC006322 | 0,6 | P < 0.001 | activating transcription factor 3 |
| 35 | BC007035 | 0,2 | P < 0.001 | nuclear transcription factor y, beta |
| 36 | BC014068 | 0,5 | P < 0.005 | similar to e4f transcription factor 1 |
| 37 | L39061 | 0,4 | P < 0.005 | transcription factor sl1 |
| 38 | M63896 | 0,3 | P < 0.001 | transcription enhancer factor; tef-1 |
| 39 | NM_003443 | 0,6 | P < 0.005 | zinc finger protein 151 (phz-67); znf151 |
| 40 | NM_003447 | 0,5 | P < 0.005 | zinc finger protein 165; znf165 |
| 41 | NM_006963 | 0,7 | P < 0.001 | zinc finger protein 22 (kox 15); znf22 |
| 42 | NM_031456 | 0,6 | P < 0.005 | charcot-marie-tooth duplicated region transcript 1; c17orf1a |
| 43 | NM_134433 | 0,6 | P < 0.001 | regulatory factor x2, isoform b; rfx2 |
| 44 | U71600 | 0,6 | P < 0.005 | zinc finger protein zfp31; zf31 |
| 45 | XM_017340 | 0,6 | P < 0.005 | similar to zinc finger protein 135; loc158954 |
| 46 | XM_064011 | 0,6 | P < 0.005 | similar to zinc finger protein 29 (zfp-29); loc124171 |
| 47 | XM_066289 | 0,5 | P < 0.005 | similar to ret finger protein-like 3; loc129081 |
| 48 | XM_088521 | 0,3 | P < 0.001 | similar to zinc finger protein 135; znf79 |
| 49 | XM_091893 | 0,4 | P < 0.005 | similar to endothelial zinc finger protein induced by tumor necrosis factor; |
| 50 | XM_091958 | 0,6 | P < 0.001 | similar to zinc finger protein 14 (kox 6); |
| 51 | XM_091968 | 0,6 | P < 0.005 | similar to zinc finger protein 91 (hpf7, htf10); loc163059 |
| 52 | XM_093185 | 0,5 | P < 0.005 | similar to activating transcriptionn factor 4; loc170217 |
| 53 | XM_094502 | 0,6 | P < 0.005 | similar to zinc finger protein 91 (hpf7, htf10); loc167465 |
| 54 | XM_166669 | 0,6 | P < 0.005 | similar to zinc finger protein 33b; loc219750 |
| 55 | XM_167147 | 0,5 | P < 0.005 | similar to zinc finger protein 193 (prd51); loc222696 |
| 56 | XM_167216 | 0,3 | P < 0.001 | similar to heterogeneous nuclear ribonucleoprotein l (hnrnp l) |
| 57 | XM_167224 | 0,6 | P < 0.001 | similar to transcription factor b2, mitochondrial; |
| 58 | XM_171233 | 0,5 | P < 0.001 | similar to phd finger protein 2; kiaa1111 |
| 59 | XM_171772 | 0,1 | P < 0.001 | similar to actin 3 - fruit fly (drosophila melanogaster) (fragments) |
| 60 | XM_171979 | 0,3 | P < 0.001 | similar to zinc finger protein 93 (zinc finger protein htf34); l |
| 61 | XM_172592 | 0,7 | P < 0.001 | similar to heterogeneous nuclear ribonucleoprotein a1 |

| No | Gene Bank Accession No | Down Regulation | p-value | Gene Name |
|---|---|---|---|---|
| **Transporter Functions** | | | | |
| 1 | AB011082 | 0,6 | P < 0.005 | organic-cation transporter like 4; orctl4 |
| 2 | AB011082 | 0,4 | P < 0.001 | huntingtin |
| 3 | AB011082 | 0,8 | P < 0.005 | organic anion transporter 4; hoat4 |
| 4 | AB011082 | 0,6 | P < 0.005 | voltage-gated sodium channel alpha1 subunit; scn1a |
| 5 | AB011082 | 0,6 | P < 0.005 | beta subunit of epithelial amiloride-sensitive sodium channel; a-279b10.1 |
| 6 | AB011082 | 0,2 | P < 0.001 | retinal rod na-ca+k exchanger splice variant; nckx1 |
| 7 | AB011082 | 0,5 | P < 0.005 | pallid |
| 8 | AB011082 | 0,2 | P < 0.001 | two pore domain k+ channel; task-2 |
| 9 | AB011082 | 0,5 | P < 0.001 | intracellular chloride channel p64h1 |
| 10 | AB0110820 | 0,5 | P < 0.005 | putative rna binding protein |
| 11 | AB0110821 | 0,3 | P < 0.001 | bo,+ amino acid transporter; slc7a9 |
| 12 | AB0110822 | 0,6 | P < 0.001 | orphan neurotransmitter transporter ntt5; ntt5 |
| 13 | AB0110823 | 0,8 | P < 0.005 | zinc transporter hzip2; hzip2 |
| 14 | AB0110824 | 0,6 | P < 0.005 | putative rna-binding protein 3 rnpl; rbm3 |
| 15 | AB0110825 | 0,5 | P < 0.001 | potassium large conductance calcium-activated channel beta 3b subunit |
| 16 | AB0110826 | 0,6 | P < 0.005 | exp35 |
| 17 | AB0110827 | 0,6 | P < 0.005 | kinesin superfamily protein kif1b |
| 18 | AB0110828 | 0,6 | P < 0.005 | sh3-containing protein sh3glb2 |
| 19 | AB0110829 | 0,7 | P < 0.005 | testis-specific calcium-binding protein cbp86-iii |
| 20 | AB0110820 | 0,2 | P < 0.001 | atp-binding cassette protein c11; abcc11 |
| 21 | AB0110821 | 0,6 | P < 0.001 | potassium intermediate/small conductance calcium-activated channel |
| 22 | AB0110822 | 0,3 | P < 0.001 | koyt binding protein 1 |
| 23 | AB0110823 | 0,4 | P < 0.001 | guanine nucleotide binding protein alpha oa; gnao1 |
| 24 | AB0110824 | 0,5 | P < 0.005 | cardiac potassium channel subunit (kv6.2); kv6.2 |
| 25 | AB0110825 | 0,4 | P < 0.005 | organic anion transporting polypeptide 8 (oatp8); slc21a8 |
| 26 | AB0110826 | 0,6 | P < 0.005 | putative trp cation channel; knp3 |
| 27 | AB0110827 | 0,6 | P < 0.005 | cdna clone highly similar to amiloride-sensitive sodium channel delta-subunit |
| 28 | AB0110828 | 0,5 | P < 0.005 | cdna clone moderately similar to carboxypeptidase a1 precursor (ec 3.4.17.1) |
| 29 | AB0110829 | 0,4 | P < 0.005 | dj1068e13.2 (novel protein similar to bovine scp2 (sterol carrier protein 2) |
| 30 | AB0110820 | 0,4 | P < 0.005 | small-conductance calcium-activated potassium channel sk3; kcnn3 |
| 31 | AB0110821 | 0,5 | P < 0.001 | similar to solute carrier family 1 (high affinity aspartate/glutamate transporter) |
| 32 | AB0110822 | 0,5 | P < 0.005 | similar to solute carrier family 13 |
| 33 | AB0110823 | 0,6 | P < 0.001 | dna-binding protein |
| 34 | AB0110824 | 0,4 | P < 0.001 | fatty acid binding protein |
| 35 | AB0110825 | 0,7 | P < 0.005 | potassium channel |
| 36 | AB0110826 | 0,8 | P < 0.001 | cyclic nucleotide-gated cation channel |
| 37 | AB0110827 | 0,4 | P < 0.001 | phosphoprotein |
| 38 | AB0110828 | 0,5 | P < 0.001 | methyl-cpg-binding protein; mecp-2 |
| 39 | AB0110829 | 0,7 | P < 0.005 | k+ channel beta-subunit |
| 40 | AB0110820 | 0,3 | P < 0.005 | calmodulin |
| 41 | AB0110821 | 0,5 | P < 0.005 | aspartate aminotransferase precursor (2.6.1.1) |
| 42 | AB0110822 | 0,6 | P < 0.001 | na+, k+ -atpase alpha subunit |
| 43 | AB0110823 | 0,5 | P < 0.005 | guanine nucleotide-binding regulatory protein; g-y-alpha |
| 44 | AB0110824 | 0,4 | P < 0.001 | sterol carrier protein-x/sterol carrier protein-2; scp-x/scp-2 |
| 45 | AB0110825 | 0,7 | P < 0.005 | ca2+-atpase |
| 46 | AB0110826 | 0,6 | P < 0.005 | guanine nucleotide binding protein (g protein), q polypeptide; gnaq |
| 47 | AB0110827 | 0,4 | P < 0.005 | solute carrier family 16 (monocarboxylic acid transporters), member 1; |
| 48 | AB0110828 | 0,6 | P < 0.005 | thyroglobulin; tg |
| 49 | AB0110829 | 0,7 | P < 0.001 | potassium voltage-gated channel, shaker-related subfamily |

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|---|---|---|---|---|
| 50 | AB0110820 | 0,5 | P < 0.005 | potassium inwardly-rectifying channel, subfamily j, member 4; kcnj4 |
| 51 | AB0110821 | 0,3 | P < 0.001 | angiotensin receptor-like 1; agtrl1 |
| 52 | AB0110822 | 0,5 | P < 0.005 | gata binding protein 6; gata6 |
| 53 | AB0110823 | 0,4 | P < 0.005 | regulatory solute carrier protein, family 1, member 1; rsc1a1 |
| 54 | AB0110824 | 0,7 | P < 0.001 | oxysterol-binding protein-like protein 7; osbpl7 |
| 55 | AB0110825 | 0,6 | P < 0.005 | chloride channel 6, isoform clc-6b; clcn6 |
| 56 | AB0110826 | 0,4 | P < 0.001 | mitochondrial citrate transport protein |
| 57 | AB0110827 | 0,5 | P < 0.005 | similar to mannose receptor, c type 1; loc90010 |
| 58 | AB0110828 | 0,7 | P < 0.005 | similar to l-type calcium channel alpha-1; loc121181 |
| 59 | AB0110829 | 0,5 | P < 0.005 | similar to calmodulin 1 (phosphorylase kinase, delta); loc121916 |
| 60 | AB0110820 | 0,5 | P < 0.005 | similar to calmodulin 2; loc124827 |
| 61 | AB0110821 | 0,5 | P < 0.005 | similar to sodium/iodide cotransporter (na(+)/i(-) cotransporter) |
| 62 | AB0110822 | 0,6 | P < 0.005 | g4 protein; g4 |
| 63 | AB0110823 | 0,8 | P < 0.005 | connexin25; cx25 |
| 64 | AB0110824 | 0,6 | P < 0.005 | cdna clone highly similar to c-terminal binding protein 2; |
| 65 | AB0110825 | 0,5 | P < 0.005 | ba462d18.3.1 (ribosome binding protein 1 (dog 180 kda homolog) |
| 66 | AB0110826 | 0,4 | P < 0.001 | ba353j17.1 (novel protein similar to nuclear rna export factor 1 (nxf1)) |
| 67 | AB0110827 | 0,5 | P < 0.005 | tropomodulin |
| 68 | AB0110828 | 0,5 | P < 0.001 | inhibitor of dna binding 3, dominant negative helix-loop-helix protein |
| 69 | AB0110829 | 0,4 | P < 0.005 | pancreatic lipase-related protein 2 |
| 70 | AB0110820 | 0,4 | P < 0.005 | similar to deiodinase, iodothyronine, type i |
| 71 | AB0110821 | 0,3 | P < 0.005 | coronin, actin binding protein, 2b |
| 72 | NM_134324 | 0,8 | P < 0.005 | tar rna binding protein 2, isoform b; tarbp2 |
| 73 | XM_058449 | 0,5 | P < 0.001 | similar to ysosomal amino acid transporter 1; loc120103 |
| 74 | XM_064801 | 0,5 | P < 0.005 | similar to sorting nexin 19; loc125799 |
| 75 | XM_065045 | 0,6 | P < 0.005 | similar to apoa-i binding protein; loc126358 |
| 76 | XM_066257 | 0,3 | P < 0.005 | similar to poly(a) binding protein, cytoplasmic 1; loc128943 |
| 77 | XM_066446 | 0,6 | P < 0.005 | similar to pol protein; loc139051 |
| 78 | XM_070894 | 0,5 | P < 0.005 | similar to ferritin light chain; loc138414 |
| 79 | XM_090399 | 0,5 | P < 0.005 | similar to grp1-associated scaffold protein grasp; loc160622 |
| 80 | XM_090998 | 0,5 | P < 0.005 | similar to nucleophosmin (npm) (nucleolar phosphoprotein b23) |
| 81 | XM_091124 | 0,5 | P < 0.001 | similar to microtubule-associated proteins 1a/1b light chain 3; loc161785 |
| 82 | XM_093276 | 0,3 | P < 0.005 | similar to golgin-like protein; loc170359 |
| 83 | XM_094561 | 0,5 | P < 0.005 | similar to metaxin 1; loc167562 |
| 84 | XM_095131 | 0,5 | P < 0.001 | similar to connexin 31.3; loc168486 |
| 85 | XM_095730 | 0,7 | P < 0.005 | similar to transactivator hsm-1; loc157954 |
| 86 | XM_171547 | 0,8 | P < 0.001 | similar to microtubule-associated proteins 1a/1b light chain 3; loc255731 |
| 87 | XM_172132 | 0,5 | P < 0.005 | similar to bactericidal/permeability-increasing protein-like 2; loc254240 |
| 88 | AF338732 | 0,6 | P < 0.005 | thymic stromal lymphopoietin protein tslp |
| 89 | AF498094 | 0,7 | P < 0.005 | amphiphysin i variant ct4; amph |
| 90 | AJ400877 | 0,3 | P < 0.001 | cegp1 protein; cegp1 |
| 91 | AK027871 | 0,3 | P < 0.001 | cdna clone weakly similar to putative importin beta-4 subunit |
| 92 | AK054727 | 0,4 | P < 0.001 | cdna clone weakly similar to ankyrin 2; unnamed protein product. |
| 93 | AK095193 | 0,6 | P < 0.005 | cdna clone weakly similar to ankyrin 1; unnamed protein product. |
| 94 | BC000258 | 0,4 | P < 0.005 | similar to delta-tubulin |
| 95 | BC000319 | 0,7 | P < 0.001 | similar to dynactin 3 (p22) |
| 96 | BC000712 | 0,5 | P < 0.005 | similar to kinesin family member c1 |
| 97 | BC031090 | 0,5 | P < 0.005 | similar to rna-binding protein (autoantigenic) long isoform; |
| 98 | BC009738 | 0,5 | P < 0.005 | mawd binding protein |
| | | | | |

| No | Gene Bank Accession No | Down Regulation | p-value | Gene Name |
|---|---|---|---|---|
| **Enzymes and Intermediary Metabolism** | | | | |
| 1 | AB013885 | 0,7 | P < 0.005 | beta-ureidopropionase |
| 2 | AB021981 | 0,3 | P < 0.005 | udp-n-acetylglucosamine transporter |
| 3 | AB028125 | 0,5 | P < 0.001 | regucalcin |
| 4 | AB039834 | 0,7 | P < 0.005 | h-haspin; h-haspin |
| 5 | AB040537 | 0,5 | P < 0.005 | rna helicase; ddx10 |
| 6 | AB041407 | 0,7 | P < 0.005 | beta 1,3-galactosyltransferase polypeptide 1; b3galt1 |
| 7 | AB052622 | 0,3 | P < 0.001 | hddm36 |
| 8 | AB052907 | 0,8 | P < 0.005 | alcan-beta; alcan-beta |
| 9 | AB077207 | 0,6 | P < 0.005 | thymidylate synthase; ts |
| 10 | AB080265 | 0,5 | P < 0.005 | cytochrome p450 2j2; cyp2j2 |
| 11 | AF019386 | 0,5 | P < 0.005 | heparan sulfate 3-o-sulfotransferase-1 precursor; 3ost1 |
| 12 | AF025794 | 0,3 | P < 0.001 | methionine synthase reductase; mtrr |
| 13 | AF038650 | 0,6 | P < 0.05 | core 2/core 4 beta-1,6-n-acetylglucosaminyltransferase; c2/4gnt |
| 14 | AF044127 | 0,5 | P < 0.005 | peroxisomal short-chain alcohol dehydrogenase; scad-srl |
| 15 | AF052732 | 0,5 | P < 0.001 | 10-formyltetrahydrofolate dehydrogenase |
| 16 | AF054821 | 0,6 | P < 0.005 | cytochrome p-450 |
| 17 | AF057034 | 0,4 | P < 0.001 | sterol/retinol dehydrogenase |
| 18 | AF063612 | 0,4 | P < 0.001 | 2'-5'oligoadenylate synthetase-related protein p30; oasl |
| 19 | AF067127 | 0,7 | P < 0.005 | 7-dehydrocholesterol reductase; dhcr7 |
| 20 | AF073770 | 0,4 | P < 0.005 | carnitine octanoyltransferase |
| 21 | AF081287 | 0,7 | P < 0.005 | serine phosphatase fcp1a; fcp1 |
| 22 | AF084521 | 0,3 | P < 0.005 | brefeldin a-inhibited guanine nucleotide-exchange protein 2 |
| 23 | AF084559 | 0,7 | P < 0.005 | fatty acid desaturase 2; fads2 |
| 24 | AF096290 | 0,5 | P < 0.005 | very long-chain acyl-coa synthetase |
| 25 | AF098269 | 0,7 | P < 0.005 | procollagen c-terminal proteinase enhancer protein 2; pcolce2 |
| 26 | AF135162 | 0,6 | P < 0.005 | cyclin i; cyc1 |
| 27 | AF141332 | 0,5 | P < 0.001 | apolipoprotein b48 receptor; apob48r |
| 28 | AF142099 | 0,5 | P < 0.005 | aggrecanase-2; adamts11 |
| 29 | AF152929 | 0,6 | P < 0.005 | a-kinase anchoring protein 18 gamma |
| 30 | AF153430 | 0,6 | P < 0.001 | cyclin-dependent kinase related protein; cdk10 |
| 31 | AF171933 | 0,5 | P < 0.005 | metallaproteinase-disintegrin beta; adam30 |
| 32 | AF176832 | 0,3 | P < 0.001 | low density lipoprotein receptor related protein-deleted in tumor; lrpdit |
| 33 | AF185696 | 0,5 | P < 0.001 | oxysterol-binding protein 1; osbp1 |
| 34 | AF187981 | 0,7 | P < 0.005 | delta7-sterol-c5-desaturase; s5des |
| 35 | AF207881 | 0,8 | P < 0.005 | tumor-associated hydroquinone (nadh) oxidase tnox |
| 36 | AF231121 | 0,3 | P < 0.005 | iron-regulated transporter ireg1; ireg1 |
| 37 | AF247551 | 0,5 | P < 0.005 | cyclin-dependent kinase inhibitor p27kip1 |
| 38 | AF252297 | 0,3 | P < 0.005 | cytochrome p450 retinoid metabolizing protein p450rai-2 |
| 39 | AF252538 | 0,6 | P < 0.001 | endoplasmic reticulum oxidoreductin 1-lbeta; ero1lbeta |
| 40 | AF261917 | 0,5 | P < 0.001 | rna helicase ii/gu protein |
| 41 | AF285159 | 0,2 | P < 0.001 | topoisomerase ii alpha-4; top2a |
| 42 | AF289022 | 0,6 | P < 0.005 | formiminotransferase cyclodeaminase form c; ftcd |
| 43 | AF302784 | 0,7 | P < 0.005 | trna-guanine transglycosylase; tgt |
| 44 | AF384667 | 0,3 | P < 0.001 | carboxypeptidase a5 |
| 45 | AF385926 | 0,3 | P < 0.001 | propionyl-coa carboxylase alpha subunit; pcca |
| 46 | AF390028 | 0,6 | P < 0.005 | serine/threonine protein kinase kkialre-like 1 |
| 47 | AF411076 | 0,3 | P < 0.001 | asparaginase-like protein |
| 48 | AF478685 | 0,5 | P < 0.001 | n-benzoyl-l-tyrosyl-p-amino-benzoic acid hydrolase alpha subunit; mep1a |
| 49 | AF502905 | 0,4 | P < 0.001 | hyaluronidase 1 variant 2; hyal1 |
| 50 | AF533528 | 0,7 | P < 0.005 | thyroid peroxidase isoform 5; tpo |
| 51 | AJ005577 | 0,6 | P < 0.005 | 6-phosphofructo-2-kinase; pfkfb2 |

| No | Gene Bank Accession No | Down Regulation | p-value | Gene Name |
|---|---|---|---|---|
| 52 | AJ010840 | 0,3 | P < 0.005 | atp-dependent rna helicase |
| 53 | AJ249179 | 0,3 | P < 0.001 | 17beta-hydroxysteroid dehydrogenase type 7; hsd17b7 |
| 54 | AJ250235 | 0,5 | P < 0.001 | ferrochelatase; fech |
| 55 | AJ250460 | 0,7 | P < 0.05 | neutral sphingomyelinase ii; nsmase2 |
| 56 | AJ427355 | 0,6 | P < 0.001 | succinic semialdehyde dehydrogenase; ssadh |
| 57 | AK001250 | 0,5 | P < 0.005 | similar to dna-directed rna polymerase iii 128 kd polypeptide |
| 58 | AK001320 | 0,7 | P < 0.005 | similar to homo sapiens partial mrna for beta-transducin family protein |
| 59 | AK001553 | 0,6 | P < 0.001 | highly similar to gtp:amp phosphotransferase mitochondrial |
| 60 | AK001670 | 0,3 | P < 0.005 | weakly similar to ubiquitin-activating enzyme e1; unnamed protein product. |
| 61 | AK001687 | 0,3 | P < 0.005 | weakly similar to double-stranded rna-specific editase 1 (ec 3.5.-.-) |
| 62 | AK001968 | 0,7 | P < 0.005 | similar to s-acyl fatty acid synthase thioesterase, medium chain (ec 3.1.2.14); |
| 63 | AK002018 | 0,5 | P < 0.001 | cdna clone weakly similar to atp-dependent permease mdl1 |
| 64 | AK022890 | 0,6 | P < 0.001 | similar to dna-directed rna polymerase i 135 kd polypeptide (ec 2.7.7.6) |
| 65 | AK023323 | 0,3 | P < 0.001 | similar to oxidoreductase ucpa (ec 1.-.-.-); unnamed protein product. |
| 66 | AK024318 | 0,7 | P < 0.005 | similar to probable ubiquitin carboxyl-terminal hydrolase r10e11.3 (ec 3.1.2.15) |
| 67 | AK055852 | 0,5 | P < 0.005 | cdna clone weakly similar to glucoamylase s1/s2 precursor (ec 3.2.1.3) |
| 68 | AK056644 | 0,4 | P < 0.005 | cdna clone weakly similar to phospholipase a2 inhibitor subunit b precursor; |
| 69 | AL023805 | 0,6 | P < 0.001 | plcb4 |
| 70 | AL033397 | 0,4 | P < 0.001 | gamma-glutamylcysteine synthetase |
| 71 | AL035704 | 0,3 | P < 0.005 | tyrosine kinase isosform 2 |
| 72 | AL359553 | 0,7 | P < 0.005 | novel 3-beta hydroxysteroid dehydrogenase/isomerase family member |
| 73 | AY035399 | 0,7 | P < 0.005 | galnt7 |
| 74 | BC000971 | 0,6 | P < 0.005 | similar to phosphoserine aminotransferase |
| 75 | BC003108 | 0,5 | P < 0.005 | similar to fumarate hydratase |
| 76 | BC005231 | 0,2 | P < 0.005 | similar to serine/threonine kinase 4 |
| 77 | BC006256 | 0,7 | P < 0.005 | similar to ctp synthase ii |
| 78 | BC007402 | 0,4 | P < 0.001 | apolipoprotein d |
| 79 | BC007656 | 0,6 | P < 0.005 | ubiquitin carrier protein e2-c |
| 80 | BC007659 | 0,6 | P < 0.001 | diaphorase (nadh/nadph) (cytochrome b-5 reductase) |
| 81 | BC010942 | 0,6 | P < 0.001 | similar to acetyl-coenzyme a acetyltransferase 1 |
| 82 | BC012049 | 0,5 | P < 0.001 | similar to cytochrome p450, subfamily i (dioxin-inducible) |
| 83 | BC012287 | 0,5 | P < 0.001 | similar to lipase a, lysosomal acid, cholesterol esterase (wolman disease) |
| 84 | BC014480 | 0,5 | P < 0.001 | similar to peptidylprolyl isomerase c (cyclophilin c) |
| 85 | BC014897 | 0,6 | P < 0.001 | similar to methylcrotonoyl-coenzyme a carboxylase 2 (beta) |
| 86 | BC020723 | 0,7 | P < 0.005 | apolipoprotein c-iv |
| 87 | BC025739 | 0,7 | P < 0.001 | cholesteryl ester transfer protein, plasma |
| 88 | BC029828 | 0,6 | P < 0.005 | similar to udp-n-acetyl-alpha-d-galactosamine |
| 89 | BC030054 | 0,5 | P < 0.001 | similar to methionyl aminopeptidase 1 |
| 90 | D45421 | 0,6 | P < 0.005 | phosphodiesterase i alpha |
| 91 | D50487 | 0,5 | P < 0.001 | rna helicase (hrh1) |
| 92 | D83647 | 0,4 | P < 0.005 | metalloproteinase |
| 93 | D89858 | 0,4 | P < 0.001 | d-aspartate oxidase |
| 94 | J02694 | 0,7 | P < 0.001 | myeloperoxidase; mpo |
| 95 | J05428 | 0,6 | P < 0.005 | udp-glucuronosyltransferase (ec 2.4.1.17) |
| 96 | L03357 | 0,6 | P < 0.001 | ret tyrosine kinase/camp protein kinase a subunit ri; ret/ptc2 |
| 97 | L10404 | 0,6 | P < 0.001 | surfactant protein b-binding protein |
| 98 | M27691 | 0,6 | P < 0.005 | transactivator protein |
| 99 | M93283 | 0,6 | P < 0.001 | lipase related protein 1; plrp1 |
| 100 | NM_000053 | 0,4 | P < 0.001 | atpase, cu++ transporting, beta polypeptide (wilson disease); atp7b |
| 101 | NM_000937 | 0,7 | P < 0.001 | dna directed rna polymerase ii polypeptide a; polr2a |
| 102 | NM_001174 | 0,5 | P < 0.005 | rho gtpase activating protein 6 isoform 2; arhgap6 |
| 103 | NM_001333 | 0,6 | P < 0.001 | cathepsin l2; ctsl2 |
| 104 | NM_001488 | 0,6 | P < 0.001 | transcriptional adaptor 2-like, isoform a; tada2l |

| No | Gene Bank Accession No | Down Regulation | p-value | Gene Name |
|---|---|---|---|---|
| 105 | NM_001527 | 0,3 | P < 0.001 | histone deacetylase 2; hdac2 |
| 106 | NM_001917 | 0,4 | P < 0.005 | d-amino-acid oxidase; dao |
| 107 | NM_002215 | 0,5 | P < 0.001 | inter-alpha (globulin) inhibitor, h1 polypeptide; itih1 |
| 108 | NM_002645 | 0,5 | P < 0.005 | phosphoinositide-3-kinase, class 2, alpha polypeptide; pik3c2a |
| 109 | NM_002649 | 0,5 | P < 0.005 | phosphoinositide-3-kinase, catalytic, gamma polypeptide; pik3cg |
| 110 | NM_002703 | 0,7 | P < 0.005 | phosphoribosyl pyrophosphate amidotransferase; ppat |
| 111 | NM_002744 | 0,4 | P < 0.005 | protein kinase c, zeta; prkcz |
| 112 | NM_002844 | 0,5 | P < 0.005 | protein tyrosine phosphatase, receptor type, k precursor; ptprk |
| 113 | NM_002860 | 0,6 | P < 0.001 | pyrroline-5-carboxylate synthetase |
| 114 | NM_003559 | 0,7 | P < 0.005 | phosphatidylinositol-4-phosphate 5-kinase type ii beta isoform a; pip5k2b |
| 115 | NM_003647 | 0,6 | P < 0.005 | diacylglycerol kinase epsilon; dgke |
| 116 | NM_004104 | 0,4 | P < 0.005 | fatty acid synthase; fasn |
| 117 | NM_004385 | 0,8 | P < 0.005 | chondroitin sulfate proteoglycan 2 (versican); cspg2 |
| 118 | NM_004950 | 0,5 | P < 0.005 | dermatan sulphate proteoglycan 3; dspg3 |
| 119 | NM_004959 | 0,6 | P < 0.005 | nuclear receptor subfamily 5, group a, member 1; nr5a1 |
| 120 | NM_006204 | 0,6 | P < 0.005 | phosphodiesterase 6c, cgmp-specific, cone, alpha prime; pde6c |
| 121 | NM_007014 | 0,4 | P < 0.001 | nedd-4-like ubiquitin-protein ligase; wwp2 |
| 122 | NM_007037 | 0,4 | P < 0.005 | adamts8 |
| 123 | NM_014498 | 0,7 | P < 0.005 | golgi phosphoprotein 4; golph4 |
| 124 | NM_016395 | 0,6 | P < 0.001 | butyrate-induced transcript 1; hspc121 |
| 125 | NM_016413 | 0,4 | P < 0.001 | plasma carboxypeptidase b2 isoform b; cpb2 |
| 126 | NM_016821 | 0,3 | P < 0.005 | 8-oxoguanine dna glycosylase, isoform 2a; ogg1 |
| 127 | NM_017423 | 0,5 | P < 0.001 | polypeptide n-acetylgalactosaminyltransferase 7; galnt7 |
| 128 | NM_024492 | 0,3 | P < 0.005 | apolipoprotein(a) related gene c precursor; apoargc |
| 129 | NM_031414 | 0,5 | P < 0.005 | serine/threonine kinase 31 isoform a; stk31 |
| 130 | U42604 | 0,6 | P < 0.005 | udp-glucuronosyltransferase; ugt1h |
| 131 | U49974 | 0,7 | P < 0.005 | mariner transposase |
| 132 | U50743 | 0,5 | P < 0.005 | na,k-atpase gamma subunit |
| 133 | XM_006464 | 0,6 | P < 0.001 | similar to 150 kda oxygen-regulated protein precursor (orp150); |
| 134 | XM_042105 | 0,6 | P < 0.005 | similar to type-2 phosphatidic acid phosphatase alpha-2; ppap2a |
| 135 | XM_062440 | 0,4 | P < 0.001 | similar to chondroitin 4-sulfotransferase; loc121058 |
| 136 | XM_062822 | 0,5 | P < 0.001 | similar to lysyl-trna synthetase (lysine--trna ligase) (lysrs); loc121855 |
| 137 | XM_064055 | 0,8 | P < 0.005 | similar to fibroblast growth factor receptor 3 precursor (fgfr-3) |
| 138 | XM_064218 | 0,6 | P < 0.005 | similar to nk inhibitory receptor precursor; loc124599 |
| 139 | XM_065016 | 0,4 | P < 0.005 | similar to eosinophil lysophospholipase (charcot-leyden crystal protein) |
| 140 | XM_066095 | 0,5 | P < 0.005 | similar to protein-glutamine gamma-glutamyltransferase |
| 141 | XM_066679 | 0,1 | P < 0.001 | similar to protease; reverse transcriptase; rnaseh; |
| 142 | XM_068675 | 0,7 | P < 0.005 | similar to ornithine decarboxylase; loc134077 |
| 143 | XM_086047 | 0,4 | P < 0.001 | similar to riken cdna 4933439f11; loc148109 |
| 144 | XM_086530 | 0,3 | P < 0.001 | similar to serine/threonine kinase 35; loc149420 |
| 145 | XM_089091 | 0,6 | P < 0.005 | similar to fatty acid amide hydrolase; loc163718 |
| 146 | XM_093015 | 0,4 | P < 0.005 | similar to ornithine aminotransferase, mitochondrial precursor |
| 147 | XM_105663 | 0,5 | P < 0.005 | similar to phosphoglycerate mutase 1 (phosphoglycerate mutase isozyme b) |
| 148 | XM_116626 | 0,5 | P < 0.005 | similar to cytochrome c oxidase subunit i; loc206612 |
| 149 | XM_165421 | 0,4 | P < 0.001 | similar to phospholipid scramblase 3 (pl scramblase 3) |
| 150 | XM_167100 | 0,6 | P < 0.005 | similar to glutathione s-transferase a1 (gth1) |
| 151 | XM_167456 | 0,6 | P < 0.005 | similar to glutamic-oxaloacetic transaminase 2, |
| 152 | XM_171032 | 0,4 | P < 0.005 | similar to succinate dehydrogenase [ubiquinone] flavoprotein subunit |
| 153 | XM_171774 | 0,4 | P < 0.001 | similar to mocs1a enzyme; loc255896 |
| 154 | XM_171864 | 0,7 | P < 0.001 | similar to putative translation initiation factor; loc253604 |
| 155 | XM_171878 | 0,6 | P < 0.005 | similar to e3 ubiquitin ligase smurf2; loc254901 |
| 156 | XM_172383 | 0,7 | P < 0.005 | similar to dna-directed rna polymerase; loc253633 |
| 157 | AJ243666 | 0,2 | P < 0.001 | nice-5 protein; nice-5 |
| 158 | AK096281 | 0,5 | P < 0.005 | cdna clone highly similar to glypican-2 precursor;. |
| | | | | |

| No | Gene Bank Accession No | Down Regulation | p-value | Gene Name |
|---|---|---|---|---|
| 157 | AJ243666 | 0,2 | P < 0.001 | nice-5 protein; nice-5 |
| 158 | AK096281 | 0,5 | P < 0.005 | cdna clone highly similar to glypican-2 precursor; unnamed protein product. |
| 159 | AK096669 | 0,5 | P < 0.001 | similar to mouse mrna for tetracycline transporter-like protein |
| 160 | BC000728 | 0,5 | P < 0.005 | capping protein (actin filament), gelsolin-like |
| 161 | BC033091 | 0,5 | P < 0.005 | similar to uncoupling protein 4 |
| 162 | BC038506 | 0,4 | P < 0.005 | similar to elongation of very long chain fatty acids like 4 |
| 163 | D26528 | 0,3 | P < 0.001 | rna helicase |
| 164 | D31872 | 0,7 | P < 0.005 | metalloprotease/disintegrin-like protein |
| 165 | D89618 | 0,3 | P < 0.001 | karyopherin alhph 3 |
| 166 | J03037 | 0,6 | P < 0.001 | ca2 |
| 167 | J03810 | 0,3 | P < 0.001 | glut2 |
| 168 | J05243 | 0,3 | P < 0.001 | sptan1 |
| 169 | L32140 | 0,4 | P < 0.005 | afamin |
| 170 | M63582 | 0,6 | P < 0.001 | preprothyrotropin-releasing hormone |
| 171 | NM_000299 | 0,4 | P < 0.005 | plakophilin 1; pkp1 |
| 172 | NM_001854 | 0,7 | P < 0.005 | alpha 1 type xi collagen, isoform a preproprotein; col11a1 |
| 173 | NM_002271 | 0,7 | P < 0.005 | karyopherin (importin) beta 3; kpnb3 |
| 174 | NM_002290 | 0,5 | P < 0.001 | laminin, alpha 4 precursor; lama4 |
| 175 | NM_002378 | 0,4 | P < 0.005 | megakaryocyte-associated tyrosine kinase, isoform b; matk |
| 176 | NM_004523 | 0,4 | P < 0.005 | kinesin-like 1; knsl1 |
| 177 | NM_033157 | 0,6 | P < 0.005 | caldesmon 1, isoform 2; cald1 |
| 178 | NM_133437 | 0,5 | P < 0.005 | titin, isoform novex-2; ttn |
| 179 | NM_145699 | 0,7 | P < 0.005 | phorbolin 1; apobec3a |
| 180 | S67659 | 0,6 | P < 0.005 | calnexin |
| 181 | S82496 | 0,5 | P < 0.001 | etx1 |
| 182 | U26724 | 0,6 | P < 0.001 | calpastatin; bs-17 |
| 183 | U32672 | 0,4 | P < 0.005 | gpr10 |
| 184 | U46192 | 0,5 | P < 0.005 | rage-2 orf2; one of 3 possible coding regions |
| 185 | U54612 | 0,5 | P < 0.001 | pvhl; vhl |
| 186 | U76189 | 0,6 | P < 0.005 | extl2 |
| 187 | X71491 | 0,4 | P < 0.001 | vacuolar proton atpase; p31 |
| 188 | XM_037764 | 0,4 | P < 0.005 | similar to elkl motif kinase 1 isoform a; elkl motif kinase 1; mark2 |
| 189 | XM_069733 | 0,6 | P < 0.001 | similar to cytochrome c, expressed in somatic tissues; loc136142 |
| 190 | XM_070552 | 0,4 | P < 0.005 | similar to mitochondrial aconitase (nuclear aco2 gene); loc137697 |
| 191 | XM_070916 | 0,6 | P < 0.005 | similar to dead/h (asp-glu-ala-asp/his) box polypeptide 10; dead/h box-10; |
| 192 | XM_094346 | 0,7 | P < 0.005 | similar to carbohydrate (chondroitin) synthase 1; kiaa0990 protein; |
| 193 | XM_095533 | 0,4 | P < 0.001 | similar to myo-inositol-1(or 4)-monophosphatase (impase) (imp) |
| 194 | XM_167146 | 0,4 | P < 0.005 | similar to epididymal secretory glutathione peroxidase precursor |
| 195 | XM_167228 | 0,7 | P < 0.005 | similar to cytoplasmic antiproteinase 2 (cap2) (cap-2) |
| 196 | XM_171353 | 0,6 | P < 0.005 | similar to ascites sialoglycoprotein-2 - rat (fragment); loc256738 |
| 197 | XM_171493 | 0,6 | P < 0.005 | similar to trehalase precursor (alpha,alpha-trehalase) |
| 198 | XM_171903 | 0,7 | P < 0.005 | 43-kda form skeletal muscle and kidney enriched inositol phosphatase; |
| 199 | XM_172313 | 0,7 | P < 0.005 | similar to myosin light chain kinase,mlck |
| 200 | Y18462 | 0,5 | P < 0.001 | cathepsin l |
| 201 | AF254260 | 0,7 | P < 0.005 | tuftelin 1; tuft1 |
| 202 | AB027133 | 0,5 | P < 0.005 | male enhanced antigen-2; mea-2 |
| 203 | AF114488 | 0,6 | P < 0.005 | intersectin short isoform; itsn |
| 204 | AF266283 | 0,3 | P < 0.005 | ubiquitin specific protease; usp28 |
| 205 | AF285593 | 0,7 | P < 0.005 | ubiquitin specific protease 26; usp26 |
| 206 | AF043244 | 0,5 | P < 0.001 | apoptosis repressor arc; arc |
| 207 | AF152322 | 0,6 | P < 0.001 | protocadherin gamma a2; pcdh-gamma-a2 |
| 208 | AF152331 | 0,7 | P < 0.005 | protocadherin gamma b2; pcdh-gamma-b2 |
| 209 | AF161368 | 0,5 | P < 0.001 | hspc105 |
| 210 | AF161373 | 0,4 | P < 0.001 | hspc255 |

| No | Gene Bank Accession No | Down Regulation | p-value | Gene Name |
|---|---|---|---|---|
| 211 | AF161384 | 0,6 | P < 0.001 | hspc266 |
| 212 | AF161418 | 0,3 | P < 0.005 | hspc300 |
| 213 | AF161431 | 0,3 | P < 0.001 | hspc313 |
| 214 | AF161545 | 0,3 | P < 0.001 | hspc060 |
| 215 | AF165217 | 0,4 | P < 0.001 | sk-tropomodulin; sk-tmod |
| 216 | AF169690 | 0,5 | P < 0.005 | cadherin-like protein vr20 |
| 217 | AF169691 | 0,5 | P < 0.005 | cadherin-like protein vr8 |
| 218 | AF184110 | 0,5 | P < 0.005 | cyclophilin-related protein; nktr |
| 219 | AF194172 | 0,7 | P < 0.005 | androgen-regulated protein 6; aig6 |
| 220 | AF208070 | 0,7 | P < 0.005 | kelch-like protein klhl3b; klhl3b |
| 221 | AF467441 | 0,6 | P < 0.005 | target of myb1-like protein 2; tom1l2 |
| 222 | XM_171926 | 0,6 | P < 0.001 | similar to acidic ribosomal phosphoprotein p0; loc254004 |
| 223 | XM_172075 | 0,6 | P < 0.005 | similar to rpe-spondin; loc256288 |
| 224 | AF494536 | 0,4 | P < 0.005 | cystatin 9 |
| 225 | AJ130978 | 0,4 | P < 0.005 | ariadne-2 protein (ari2); ari2 |
| 226 | AJ243706 | 0,6 | P < 0.005 | rb-binding protein; rbbp2h1a |
| 227 | AJ344352 | 0,3 | P < 0.001 | page-5 protein; page-5 |
| 228 | AY008263 | 0,4 | P < 0.001 | cyclin-dependent kinase inhibitor isoform |
| 229 | AY083210 | 0,5 | P < 0.005 | euchromatic histone methyltransferase 1 |
| 230 | BC001630 | 0,4 | P < 0.001 | replication protein a2 (32kd) |
| 231 | BC022508 | 0,7 | P < 0.001 | glycoprotein m6a |
| 232 | AF125098 | 0,6 | P < 0.005 | hspc037 protein |
| 233 | AF125950 | 0,4 | P < 0.001 | dna repair protein rad52 beta isoform; rad52 |
| 234 | AF132967 | 0,5 | P < 0.001 | cgi-33 protein |
| 235 | AF135026 | 0,6 | P < 0.005 | kallikrein-like protein 3 splice variant 1; klk9 |
| 236 | AF136381 | 0,5 | P < 0.001 | c-cbl-associated protein sh3p12; sh3p12 |
| 237 | AF138300 | 0,3 | P < 0.005 | decorin variant a |
| 238 | AJ010346 | 0,5 | P < 0.005 | ring-h2; rnf6 |
| 239 | AJ242956 | 0,4 | P < 0.005 | l1 protein; hpv45 l1 |
| 240 | AJ271736 | 0,6 | P < 0.001 | synaptobrevin-like 1 protein; sybl1 |
| 241 | XM_166944 | 0,2 | P < 0.005 | similar to gamma-heregulin; loc220373 |
| 242 | XM_167317 | 0,5 | P < 0.005 | similar to centaurin alpha 1 - human; loc223053 |
| 243 | XM_170870 | 0,7 | P < 0.005 | similar to hspc059 protein; loc253670 |
| 244 | XM_172184 | 0,3 | P < 0.001 | similar to xage-5 protein; loc255058 |
| 245 | XM_172263 | 0,4 | P < 0.005 | similar to kelch-like protein klhl6; loc256739 |
| 246 | AF267739 | 0,6 | P < 0.005 | 26s proteasome-associated uch interacting protein 1; uip1 |
| 247 | BC002987 | 0,6 | P < 0.001 | stress-induced-phosphoprotein 1 (hsp70/hsp90-organizing protein) |
| 248 | BC007319 | 0,4 | P < 0.005 | snare protein |
| 249 | BC022288 | 0,5 | P < 0.005 | calsequestrin 2 (cardiac muscle) |
| 250 | D00025 | 0,5 | P < 0.005 | proceruloplasmin |
| 251 | D14826 | 0,7 | P < 0.005 | hcrem 2beta-b protein; hcrem-2 |
| 252 | M34875 | 0,6 | P < 0.005 | amyloid-beta protein; app |
| 253 | XM_115293 | 0,6 | P < 0.005 | similar to acidic protein rich in leucines; loc197040 |
| | | | | |
| **Receptors and Signaling** | | | | |
| 1 | AB006589 | 0,7 | P < 0.005 | estrogen receptor beta cx |
| 2 | AB006590 | 0,6 | P < 0.005 | estrogen receptor beta |
| 3 | AB009356 | 0,3 | P < 0.005 | tgf-beta activated kinase 1a |
| 4 | AB009462 | 0,5 | P < 0.001 | ldl receptor related protein 105; hlrp105 |
| 5 | AB026491 | 0,6 | P < 0.001 | pick1 |
| 6 | AB037925 | 0,5 | P < 0.005 | mail |
| 7 | AB038951 | 0,5 | P < 0.001 | amyotrophic lateral sclerosis 2; als2cr3 |
| 8 | AB045116 | 0,4 | P < 0.001 | wnt-2b isoform 1; wnt-2b |
| 9 | AB052156 | 0,7 | P < 0.005 | mapk phosphatase-7; mkp-7 |
| 10 | AB056477 | 0,7 | P < 0.005 | angiopoietin-related protein 4; arp4 |
| 11 | AB062450 | 0,6 | P < 0.001 | nek7; nek7 |

| No | Gene Bank Accession No | Down Regulation | p-value | Gene Name |
|---|---|---|---|---|
| 12 | AB062766 | 0,5 | P < 0.005 | wnt7b |
| 13 | AB063300 | 0,4 | P < 0.005 | tgf-beta induced apotosis protein 2; taip-2 |
| 14 | AB065654 | 0,4 | P < 0.005 | seven transmembrane helix receptor |
| 15 | AB065708 | 0,6 | P < 0.001 | seven transmembrane helix receptor |
| 16 | AB065717 | 0,5 | P < 0.005 | seven transmembrane helix receptor |
| 17 | AB065727 | 0,5 | P < 0.001 | seven transmembrane helix receptor |
| 18 | AB065808 | 0,8 | P < 0.001 | seven transmembrane helix receptor |
| 19 | AB065815 | 0,6 | P < 0.005 | seven transmembrane helix receptor |
| 20 | AB065929 | 0,2 | P < 0.001 | seven transmembrane helix receptor |
| 21 | AB065931 | 0,6 | P < 0.005 | seven transmembrane helix receptor |
| 22 | AB065932 | 0,6 | P < 0.005 | seven transmembrane helix receptor |
| 23 | AB070933 | 0,6 | P < 0.005 | asialoglycoprotein receptor 1; asgr1 |
| 24 | AB083625 | 0,7 | P < 0.005 | putative g-protein coupled receptor; gpcr |
| 25 | AF032119 | 0,6 | P < 0.005 | hcask; cask |
| 26 | AF037439 | 0,4 | P < 0.005 | protein kinase a anchoring protein |
| 27 | AF041245 | 0,6 | P < 0.005 | orexin receptor-2 |
| 28 | AF053537 | 0,5 | P < 0.005 | p33; ing2 |
| 29 | AF054986 | 0,4 | P < 0.005 | putative transmembrane gtpase |
| 30 | AF055033 | 0,6 | P < 0.001 | insulin-like growth factor binding protein 5; igfbp5 |
| 31 | AF062077 | 0,4 | P < 0.005 | putative protein kinase regulator |
| 32 | AF068227 | 0,4 | P < 0.005 | putative transmembrane protein; cln5 |
| 33 | AF080394 | 0,7 | P < 0.001 | transient receptor potential protein 6 |
| 34 | AF080582 | 0,6 | P < 0.005 | 5-hydroxytryptamine 3 receptor b subunit precursor |
| 35 | AF091486 | 0,6 | P < 0.005 | calmodulin-dependent protein kinase ii alpha |
| 36 | AF131839 | 0,5 | P < 0.001 | human neuronal olfactomedin related er localized protein |
| 37 | AF135562 | 0,6 | P < 0.005 | p58 killer cell inhibitory receptor kir-k78; kir-k78 |
| 38 | AF208111 | 0,6 | P < 0.005 | truncated il-17 receptor homolog precursor; evi27 |
| 39 | AF208690 | 0,7 | P < 0.001 | p70 killer cell inhibitory receptor |
| 40 | AF237763 | 0,4 | P < 0.005 | orphan g protein-coupled receptor 87; gpr87 |
| 41 | AF279865 | 0,4 | P < 0.005 | kinesin-like protein gakin |
| 42 | AF282874 | 0,5 | P < 0.001 | nectin 3 |
| 43 | AF288738 | 0,7 | P < 0.005 | p110 epidermal growth factor receptor; egfr |
| 44 | AF289204 | 0,4 | P < 0.001 | odorant receptor hor3'beta5 |
| 45 | AF303889 | 0,6 | P < 0.005 | ropporin |
| 46 | AF316830 | 0,4 | P < 0.005 | cystatin and duf19 domain-containing protein 1; csdufd1 |
| 47 | AF410901 | 0,5 | P < 0.005 | neurotrophin receptor tyrosine kinase type 2 truncated isoform; ntrk2 |
| 48 | AF435588 | 0,4 | P < 0.005 | melatonin receptor mel1a; mtnr1a |
| 49 | AF458592 | 0,3 | P < 0.001 | admp |
| 50 | AF458662 | 0,5 | P < 0.005 | calcium-dependent activator protein for secretion protein |
| 51 | AF494227 | 0,3 | P < 0.005 | candidate taste receptor tas2r46 |
| 52 | AJ012186 | 0,3 | P < 0.001 | gabab receptor, subunit 1b; gabab-r1 |
| 53 | AJ236922 | 0,5 | P < 0.005 | metabotropic glutamate receptor 8c; mglur8c |
| 54 | AJ276208 | 0,8 | P < 0.005 | orphan transporter xt3a; xt3 |
| 55 | AK022859 | 0,4 | P < 0.005 | cdna clone highly similar to rattus transmembrane receptor unc5h2 |
| 56 | AY089976 | 0,4 | P < 0.005 | g protein-coupled receptor zaq |
| 57 | AY118267 | 0,4 | P < 0.001 | vanilloid receptor like 3 protein splice variant b; trpv3 |
| 58 | BC009748 | 0,3 | P < 0.001 | dopamine receptor d5 |
| 59 | BC013992 | 0,6 | P < 0.001 | similar to mitogen activated protein kinase 3 |
| 60 | BC020972 | 0,6 | P < 0.001 | casein kinase 1, gamma 2 |
| 61 | BC028242 | 0,6 | P < 0.005 | similar to programmed cell death 6 |
| 62 | BC035058 | 0,5 | P < 0.005 | protein kinase, camp-dependent, catalytic, beta |
| 63 | BC037905 | 0,5 | P < 0.005 | similar to casp2 and ripk1 domain containing adaptor with death domain |
| 64 | D38301 | 0,7 | P < 0.005 | prostaglandin e receotor ep3 subtype 3 isoform |
| 65 | L08893 | 0,4 | P < 0.001 | bombesin receptor subtype-3 |

| No | Gene Bank Accession No | Down Regulation | p-value | Gene Name |
|---|---|---|---|---|
| 66 | M21142 | 0,5 | P < 0.001 | guanine nucleotide-binding protein g-s-alpha-2 |
| 67 | M24900 | 0,7 | P < 0.005 | triiodothyronine receptor |
| 68 | M83941 | 0,4 | P < 0.001 | receptor protein kinase; hek |
| 69 | NM_000540 | 0,7 | P < 0.001 | ryanodine receptor 1 (skeletal); ryr1 |
| 70 | NM_000813 | 0,7 | P < 0.005 | gamma-aminobutyric acid (gaba) a receptor, beta 2, isoform 2; gabrb2 |
| 71 | NM_002063 | 0,4 | P < 0.001 | glycine receptor, alpha 2; glra2 |
| 72 | NM_002565 | 0,5 | P < 0.001 | pyrimidinergic receptor p2y, g-protein coupled, 4; p2ry4 |
| 73 | NM_002644 | 0,6 | P < 0.001 | polymeric immunoglobulin receptor; pigr |
| 74 | NM_003618 | 0,6 | P < 0.005 | mitogen-activated protein kinase kinase kinase kinase 3; map4k3 |
| 75 | NM_004442 | 0,6 | P < 0.005 | ephrin receptor ephb2 isoform 1; ephb2 |
| 76 | NM_005302 | 0,6 | P < 0.001 | g protein-coupled receptor 37; gpr37 |
| 77 | NM_005544 | 0,4 | P < 0.001 | insulin receptor substrate 1; irs1 |
| 78 | NM_005874 | 0,6 | P < 0.001 | leukocyte immunoglobulin-like receptor, lilrb2 |
| 79 | NM_006260 | 0,6 | P < 0.005 | protein-kinase, prkri |
| 80 | NM_012411 | 0,5 | P < 0.005 | lymphoid-specific protein tyrosine phosphatase isoform 2; ptpn22 |
| 81 | NM_014336 | 0,7 | P < 0.001 | aryl hydrocarbon receptor-interacting protein-like 1; aipl1 |
| 82 | NM_080425 | 0,3 | P < 0.001 | guanine nucleotide binding protein (g protein), |
| 83 | NM_080426 | 0,4 | P < 0.005 | guanine nucleotide binding protein (g protein), |
| 84 | NM_130391 | 0,5 | P < 0.005 | protein tyrosine phosphatase, receptor type, d, isoform 2 precursor; ptprd |
| 85 | NM_130842 | 0,7 | P < 0.005 | protein tyrosine phosphatase, receptor type, n polypeptide 2 |
| 86 | NM_144490 | 0,5 | P < 0.005 | a kinase (prka) anchor protein 11, isoform 2; akap11 |
| 87 | S76825 | 0,6 | P < 0.005 | insulin receptor |
| 88 | S86392 | 0,5 | P < 0.001 | neurokinin-3 receptor |
| 89 | U34994 | 0,6 | P < 0.005 | dna dependent protein kinase catalytic subunit; prkdc |
| 90 | U94512 | 0,3 | P < 0.001 | lymphocyte associated receptor of death 11 |
| 91 | XM_034551 | 0,7 | P < 0.001 | similar to dual-specificity tyrosine-(y)-phosphorylation regulated kinase 4 |
| 92 | XM_048844 | 0,7 | P < 0.005 | similar to thyroliberin receptor; dkfzp586c1021 |
| 93 | XM_064909 | 0,5 | P < 0.001 | similar to putative g-protein coupled receptor; loc126023 |
| 94 | XM_086409 | 0,6 | P < 0.001 | similar to olfactory receptor, family 1, subfamily k, member 1; |
| 95 | XM_114984 | 0,4 | P < 0.001 | similar to olfactory receptor mor25-1; loc203820 |
| 96 | XM_115484 | 0,6 | P < 0.005 | similar to monocarboxylate transporter 3 (mct 3); loc201232 |
| 97 | XM_166832 | 0,5 | P < 0.005 | similar to olfactory receptor mor199-1; loc219480 |
| 98 | XM_171937 | 0,6 | P < 0.005 | similar to protein-tyrosine phosphatase mu precursor (r-ptp-mu); loc256003 |
| 99 | XM_171970 | 0,4 | P < 0.005 | similar to olfactory receptor mor130-1; loc255473 |
| 100 | XM_065234 | 0,6 | P < 0.005 | similar to positive cofactor 2 glutamine/q-rich-associated protein 29433 |
| 101 | XM_065455 | 0,5 | P < 0.005 | similar to oligophrenin 1, rho-gtpase acivating protein; loc129896 |
| 102 | XM_088102 | 0,7 | P < 0.005 | similar to coatomer gamma-2 subunit (gamma-2 coat protein) |
| 103 | XM_116458 | 0,7 | P < 0.001 | similar to cdc37 homolog; cdc37 (cell division cycle 37) |
| 104 | XM_166963 | 0,4 | P < 0.001 | similar to proteasome activator complex subunit 2 |
| 105 | XM_171746 | 0,6 | P < 0.005 | similar to capping protein alpha 2~data |
| 106 | XM_172274 | 0,6 | P < 0.005 | similar to melanoma antigen, family d, 1 |
| 107 | Z97989 | 0,6 | P < 0.005 | proto-oncogene tyrosine kinase fyn (p59-fyn, syn, slk) isoform 2; fyn |
| 108 | XM_172547 | 0,6 | P < 0.005 | similar to thymosin beta - human; loc255680 |

**Table IV. List of T cell genes up regulated in patients with sepsis and in those with uneventful recovery**

| No | Gene bank accession No | Up regulation | p-value | Gene Name |
|---|---|---|---|---|
| **Transcriptional Regulation** | | | | |
| **1** | AB027710 | 1,3 | P < 0.005 | transcription factor rbp-l; rbp-l |
| **2** | AY035371 | 1,4 | P < 0.005 | t-box transcription factor tbx22 |
| **3** | BC000876 | 1,3 | P < 0.005 | similar to zinc finger protein 174 |
| **4** | XM_087349 | 4,1 | P < 0.005 | similar to eukaryotic TE factor 1 alpha 2 |
| **5** | XM72733 | 1,3 | P < 0.001 | similar to transcription factor sox-2 |
| **Receptors and Signaling** | | | | |
| **1** | AF034780 | 1,5 | P < 0.005 | lysosphingolipid receptor edg5 |
| **2** | AF115765 | 2,9 | P < 0.005 | artemin |
| **3** | AJ298334 | 1,4 | P < 0.005 | p2y11 receptor; p2y11 |
| **4** | XM_060318 | 3,8 | P < 0.005 | similar to olfactory receptor 2t1 |
| **Enzymes and metabolism** | | | | |
| 1 | AF130420 | 3,2 | P < 0.005 | serine protease-like protein isoform; nsp |
| 2 | BC001687 | 1,3 | P < 0.001 | asparaginyl-trna synthetase |
| 3 | BC001687 | 1,3 | P < 0.001 | asparaginyl-trna synthetase |
| 4 | BC007104 | 2,9 | P < 0.005 | peptidylprolyl isomerase a (cyclophilin a) |
| 5 | BC022463 | 1,3 | P < 0.001 | dual specificity phosphatase 1 |
| 6 | BC036802 | 1,3 | P < 0.005 | similar to gpi-gamma 4 |
| 7 | L10641 | 1,3 | P < 0.005 | vitamin d-binding protein; gc |
| 8 | NM_001054 | 1,2 | P < 0.005 | sulfotransferase family, sult1a2 |
| 9 | NM_005907 | 3 | P < 0.005 | mannosidase; man1a1 |
| 10 | U41668 | 1,4 | P < 0.005 | deoxyguanosine kinase |
| 11 | U41668 | 1,4 | P < 0.005 | deoxyguanosine kinase |
| 12 | U82808 | 1,2 | P < 0.005 | muscle-specific serine kinase 1; mssk1 |
| 13 | XM_064826 | 1,6 | P < 0.005 | glyceraldehyde 3-phosphate dehydrogenase |
| 14 | XM_068321 | 4,3 | P < 0.005 | similar to inhibin/activin; loc133384 |
| **Others** | | | | |
| 1 | AF009227 | 1,7 | P < 0.005 | gamma-heregulin |
| 2 | AF058319 | 1,4 | P < 0.005 | yolk sac permease-like molecule 2; yspl2 |
| 3 | AF131208 | 1,3 | P < 0.005 | snail protein |
| 4 | AF161364 | 1,7 | P < 0.005 | hspc101 |
| 5 | AF249669 | 1,3 | P < 0.005 | homeodomain protein nkx3.1 ; nkx3a |
| 6 | AF254086 | 1,5 | P < 0.005 | ews/zsg fusion protein short isoform; |
| 7 | AF325213 | 1,5 | P < 0.005 | oculospanin; ocsp |
| 8 | BC002349 | 2,4 | P < 0.005 | CD-63 antigen |
| 9 | BC002361 | 2,4 | P < 0.005 | similar to transducin (beta)-like 3 |
| 10 | BC006163 | 1,6 | P < 0.005 | similar to dynactin 1 |
| 11 | NM_003632 | 1,5 | P < 0.005 | contactin associated protein 1; cntnap1 |
| 12 | NM39135 | 2,6 | P < 0.005 | smarcf1 |

## Table V. List of T cell genes down regulated only in patients with sepsis

| No | Gene Bank Accession No | Down Regulation | Gene Name |
|---|---|---|---|
| **Immune Response** | | | |
| 1 | AF001622 | 0,3 | class-i mhc-restricted t cell associated molecule; crtam |
| 2 | AF054815 | 0,3 | eukocyte differentiation antigen cd84 isoform cd84c; cd84 |
| 3 | AF105261 | 0,3 | natural killer cell receptor 2b4 |
| 4 | AF254069 | 0,2 | interleukin 21; il21 |
| 5 | AJ405728 | 0,3 | t-cell receptor beta chain; tcr beta |
| 6 | M13838 | 0,3 | tcrb |
| 7 | NM_006068 | 0,3 | toll-like receptor 6; tlr6 |
| | | | |
| **Intermediary Metabolism** | | | |
| 1 | AB023421 | 0,3 | apg-1 |
| 2 | AB074415 | 0,3 | brain acyl-coa hydrolase; bach |
| 3 | AC002543 | 0,3 | f-actin capping protein alpha-2 subunit; wugsc:h_rg300c03.1 |
| 4 | AF025794 | 0,3 | methionine synthase reductase; mtrr |
| 5 | AF049895 | 0,3 | calbindin 1; calb1 |
| 6 | AF061737 | 0,3 | microsomal signal peptidase |
| 7 | AF110957 | 0,3 | sumo-1 activating enzyme subunit 2; sae2 |
| 8 | AF135025 | 0,2 | kallikrein-like protein 5 |
| 9 | AF144566 | 0,3 | cytochrome p450 epoxygenase; cyp2j2 |
| 10 | AF144745 | 0,3 | guanine aminohydrolase; gah |
| 11 | AF252297 | 0,3 | cytochrome p450 retinoid metabolizing protein p450rai-2 |
| 12 | AF285159 | 0,2 | topoisomerase ii alpha-4; top2a |
| 13 | AF448144 | 0,3 | trub pseudouridine synthase-like protein 1; trub1 |
| 14 | AF527632 | 0,3 | insig-2 membrane protein |
| 15 | AJ224172 | 0,3 | lipophilin b |
| 16 | AJ243666 | 0,2 | nice-5 protein; nice-5 |
| 17 | AK023323 | 0,3 | cdna clone weakly similar to oxidoreductase ucpa (ec 1.-.-.-) |
| 18 | AK074873 | 0,1 | n-acetylglucosamine-6-sulfatase precursor (ec 3.1.6.14) |
| 19 | AK092307 | 0,2 | cdna clone highly similar to kilon protein precursor |
| 20 | AK094791 | 0,3 | dihydrodipicolinate synthase (ec 4.2.1.52) |
| 21 | AK096323 | 0,2 | sphingosine-1-phosphatase (orf1) |
| 22 | BC013579 | 0,3 | similar to calpastatin |
| 23 | BC035763 | 0,3 | similar to exocyst complex 84-kda subunit |
| 24 | D26528 | 0,3 | rna helicase |
| 25 | J00098 | 0,3 | apoa1 |
| 26 | J03202 | 0,3 | lamb2 |
| 27 | M19311 | 0,2 | calmodulin |
| 28 | M24486 | 0,3 | prolyl 4-hydroxylase alpha subunit (ec 1.14.11.2) |
| 29 | NM_016413 | 0,3 | plasma carboxypeptidase b2 isoform b; cpb2 |
| 30 | U87589 | 0,3 | polymerase |
| 31 | XM_044702 | 0,3 | similar to myosin heavy chain, nonmuscle type b |
| 32 | XM_066679 | 0,1 | similar to protease; reverse transcriptase; |

| No | Gene Bank Accession No | Down Regulation | Gene Name |
|---|---|---|---|
| **Receptors and signaling** | | | |
| 1 | AJ271736 | 0,2 | synaptobrevin-like 1 protein; sybl1 |
| 2 | AB009356 | 0,3 | tgf-beta activated kinase 1a |
| 3 | AC005756 | 0,3 | fos39347_1 |
| 4 | AF125093 | 0,3 | trk-fused gene-anaplastic lymphoma kinase fusion protein; tfg/alk |
| 5 | AF385591 | 0,3 | muscarinic acetylcholine receptor M5 |
| 6 | AJ276680 | 0,3 | putative liprin related protein; l2 |
| 7 | BC005231 | 0,2 | similar to serine/threonine kinase 4 |
| 8 | BC009748 | 0,3 | dopamine receptor d5 |
| 9 | M13977 | 0,3 | protein kinase c-gamma; prkacg |
| 10 | NM_005226 | 0,3 | endothelial differentiation, sphingolipid g-protein-coupled receptor, 3 |
| 11 | NM_080425 | 0,3 | (g protein), alpha stimulating activity polypeptide 1 |
| 12 | U94512 | 0,3 | lymphocyte associated receptor of death 11 |
| | | | |
| **Transcriptional Regulation** | | | |
| 1 | AF089897 | 0,3 | topoisomerase-related function protein; trf4-2 |
| 2 | AF113226 | 0,3 | mstp046 |
| 3 | AF170025 | 0,3 | zinc finger protein zfp-95; zfp95 |
| 4 | AK027679 | 0,3 | cdna clone weakly similar to zinc finger protein 84; |
| 5 | AK075318 | 0,3 | cdna clone weakly similar to histone h1; |
| 6 | AK098175 | 0,2 | cdna clone moderately similar to zinc finger protein 184 |
| 7 | BC007035 | 0,2 | nuclear transcription factor y, beta |
| 8 | BC018059 | 0,3 | similar to pre-mrna splicing factor 17 |
| 9 | M63896 | 0,2 | transcription enhancer factor; tef-1 |
| 10 | NM_001488 | 0,2 | transcriptional adaptor 2-like, isoform a; tada2l |
| 11 | NM_005587 | 0,3 | mads box transcription enhancer factor 2 |
| 12 | U68140 | 0,3 | nuclear vcp-like protein nvlp.2; nvl.2 |
| | | | |
| **Transporter Functions** | | | |
| 1 | AF035407 | 0,2 | p27-bwr1b; bwr1b |
| 2 | AF062921 | 0,2 | retinal rod na-ca+k exchanger splice variant; nckx1 |
| 4 | AF477977 | 0,2 | k-cl cotransporter kcc3 variant isoform |
| 5 | AJ387747 | 0,3 | sialin |
| 6 | AK001517 | 0,3 | cdna clone weakly similar to nucleoporin nup57 |
| 7 | AK027871 | 0,3 | cdna clone weakly similar to putative importin beta-4 subunitt. |
| 8 | BC012444 | 0,3 | similar to chloride intracellular channel 4 |
| 9 | J03810 | 0,3 | glut2 |
| 10 | J05243 | 0,3 | sptan1 |
| 11 | L13923 | 0,2 | fibrillin |

## Table VI. List of T cell genes up-regulated only in patients with sepsis

| Gene Bank Accession No | Up Regulation | Gene Name |
|---|---|---|
| AF181091 | 7,3 | cytochrome oxidase subunit ii; coii |
| AF346966 | 7,3 | atp synthase 6 |
| AJ010341 | 6,6 | cyclin-dependent kinase; pisslre |
| NM_006693 | 7,3 | cleavage and polyadenylation specific factor 4, 30kd subunit; cpsf4 |
| XM_086164 | 7,3 | similar to elongation factor 1-alpha 1 (ef-1-alpha-1) |
| NM_080685 | 5,7 | protein tyrosine phosphatase, non-receptor type 13, isoform 4; ptpn13 |

## Table VII. List of T cell genes down regulated only in patients with normal recovery

| Gene Bank Accession No | Down Regulation | Gene Name |
|---|---|---|
| AF141289 | 0,2 | bo,+ amino acid transporter; slc7a9 |
| AF113226 | 0,3 | CNOT2 CCR4-NOT transcription complex |

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# ERKLÄRUNG

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Ernst-Moritz-Arndt-Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbständig verfasst und keine anderen als die darin angegebenen Hilfsmittel benutzt habe.

Greifswald, den 14.07.2009

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## Original Paper

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