Aus der Klinik für Anästhesiologie mit Schwerpunkt operative Intensivmedizin –
Campus Virchow-Klinikum und Campus Charité Mitte
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Einfluss der thorakalen Periduralanalgesie auf die Expression von T-Helferzell-Transkriptionsfaktoren bei lungenresezierten Patienten

Influence of thoracic epidural block on the expression of T helper cell transcription factors in patients undergoing lung surgery

Zur Erlangung des akademischen Grades Doktor medicinae (Dr. med.)
vorgelegt der Medizinischen Fakultät der Charité – Universitätsmedizin Berlin
von

Junjie Zhang
aus Shanghai, Volksrepublik China

Gutachter: 1. Prof. Dr. med. C. Spies

2. Prof. Dr. med. U. Schirmer

3. Priv.-Doz. Dr. med. J.-C. Rückert

Datum der Promotion: 14. December, 2009

One hundred years ago Paul Ehrlich and Ilya Mechnikov won the 1908 Nobel Prize in physiology and medicine for their great contribution to our understanding of the immune system. Their pioneering work has led us into a new world: the critical role of immunity in human diseases. Today we are following their steps.

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1. Introduction

1.1 Immunity

Immunity is the body's capability to repel foreign substances and cells in order to protect an organism from disease. Immunity has been divided into innate immunity (also named natural or native immunity) and adaptive immunity (also named specific immunity). Innate immunity is the first line of defence against invading micro organisms and provides clues to adaptive immunity on building memory for subsequent infections. Adaptive immunity is a secondary process mediated by T cells and B cells. Their collective and coordinated response to the introduction of foreign substances is called immune response. Response to intracellular pathogens is known as cellular immune response (cell mediated immunity) and involves sensitized T cells that attack and lyse target cells. Response to circulating pathogens (extra-cellular) is mediated by humoral response (humoral immunity), which involves B cell maturation into plasma cells and production of pathogen-binding antibodies [1]. Appropriated and balanced magnitudes of both immunities determine the direction of an immune response and eventually patients' outcomes.

1.2 T helper cells

T helper (Th) subsets originate from a common naïve precursor (Th0). During the immune response Th0 cells encounter antigens presented by antigen presenting cells (APC) and differentiate into their subsets Th1 or Th2 [2, 3]. Th1-type cytokines such as tumour necrosis factor-alpha (TNF-α) or interferon-gamma (IFN-γ) are known to mediate inflammation. Th2-type cytokines such as interleukin (IL) -4, IL-5 and IL-10 mediate anti-inflammation. There is some evidence that rather relative proportions of Th1- and Th2-responses (Th1-Th2-ratio) and not absolute cytokine activity are the major determinants of the immune response.

Recently two new Th subsets, Treg (T regulatory cells) and Th17 have been described. Their role in immunity is gradually being discovered.

Activation, differentiation and development of Th cell subsets in immune responses are modulated by many factors including quantity, duration and strength of pathogen stress, cytokines secreted by APCs as well as T cell receptor (TCR) signalling. Although these factors show a rather complex interaction within the immune response, more and more evidence

indicates that each Th subset is controlled by its own specific transcription factor [4].

Modern molecular biology revealed that transcription factors, usually located in the cytoplasm, can move to cell nuclei where they bind to specific recognition motifs leading to an up-regulation of gene transcription succeeded by formation of messenger RNA (mRNA) and adjacent protein synthesis [5]. Some transcription factors are cell specific, others ubiquitous.

Recent studies showed that Th cell subsets interact with specific transcription factors including T-bet, GATA3, Foxp3 and ROR-γt. Universal, unspecific transcription factors like SOCS1 and SOCS3 have also been described. In vitro experiments proved that such transcription factors are modulated by environmental signals and influence the differentiation of Th subsets thus impacting immune response [6]. However, so far little information is available on expression patterns and interaction of transcription factors in perioperative settings. Understanding function and regulation of transcription factors in human immune response to surgery may provide novel therapeutic strategies in perioperative medicine.

1.2.1 T-bet - a Th1 specific transcription factor

IFN-γ is the key Th1-proinflammatory cytokine in cellular immunity. Interestingly Lighvani et al. assessed that secretion of IFN-γ was always accompanied with an expression of transcription factor T-bet. T-bet enhanced production of IFN-γ and IL12 forming a potential positive feedback loop of Th1 development [7, 8]. This finding elicits the key role of T-bet in Th1 differentiation and proliferation.

T-bet (T-box expressed in T cells) is a 530-amino acid protein with a 189-amino acid T box DNA-binding domain, which is rapidly and selectively induced in Th1 cells but not in Th2 cells. T-bet, also known as Tbx21 [9], belongs to the T-box family of transcription factors and is the only known T-box gene specifically expressed in the lymphoid system. Its critical role in the development of Th1 was confirmed in T-bet$^{-/-}$ mice. The study by Szabo showed that in the absence of T-bet, CD4^{+} T cells failed to differentiate into Th1 lineage and default to Th2 fate while retroviral gene transduction of T-bet into polarized Th2 and Tc2 primary T cells redirected them into Th1 and Tc1 cells [10].

Animal models described the role of T-bet in inflammation. Mice lacking T-bet failed to mount Th1 response in vivo following either protein antigen immunization or Leishmania infection but

reacted with an increase in Th2-type cytokines [11]. T-bet deficient mice were found more susceptible to tuberculosis infection with increased systemic bacterial burden, diminished IFN-γ production in the lung [12].

Although perioperative immune reaction in humans has been described in several studies the role of T-bet in a bed side setting remains unexplored.

1.2.2 GATA3 - a Th2 specific transcription factor

IL-4 has always been thought to play a central role in polarizing naïve Th cells towards Th2 and in powerfully suppressing the appearance of IFN-γ-producing Th1 cells. Recent studies surprisingly suggest that transcription factor GATA3 and not IL-4 is responsible for this shift towards Th2. Originally this factor was cloned as a transcription factor that binds to enhancers of TCR-α and δ genes [13, 14]. Three years later, Zheng W. et al. where the first to find that GATA3 is selectively expressed in Th2 cells and that in transgenic mice elevated GATA3 in $CD4^+$ T lymphocyte caused Th2 cytokine gene expression in developing Th1 cells [15]. Later this finding was confirmed by other groups [16, 17]. After that, more positive evidence emerged: antisense-GATA3 could repress Th2-cell responses in vivo, which further implied the requirement of GATA3 for Th2-cell development [18]. Even more important is that an expression of GATA3 using retroviral constructs can up-regulate endogenous GATA3, suggesting that this transcription factor auto-regulates creating a positive feedback loop that stabilizes the Th2 polarization [19, 20]. Besides the effect on Th2 commitment, GATA3 strongly inhibits production of IFN-γ which consequently lowers differentiation of Th1 and leads to a polarization of Th2 [16, 21].

Several clinical studies suggest that a shift towards Th2 in the Th1/Th2-ratio after surgery or trauma significantly worsens patients outcomes [22], [23].

Nowadays there is no hard evidence that GATA3 leads to a Th2-shift in perioperative settings and the mechanisms of perioperative immune answers in humans remain unclear. According to the importance of the Th2-response following surgery, further studies are needed to clarify underlying molecular mechanisms of this Th2-answer and the exact role of transcription factor GATA3.

1.2.3 FOXP3 - a Treg specific transcription factor

Treg (Regulatory T cell) is a subset of T lymphocytes which is characterized by a CD4 and CD25 double positive surface marker. It was first identified by Sakaguchi and his colleagues, who found that $CD4^+CD25^+$ cells can prevent T cell-mediated organ-specific autoimmune diseases and account for approximately 7-10% of peripheral $CD4^+$ lymphocytes and less than 1% of $CD8^+$ lymphocytes in adult mice and humans [24]. Before that, Treg was usually called suppressor cell because of its counter-regulation or suppression of immune responses mediated by Th1 and Th2 [25]. Besides its autoimmune suppression, Treg was found to have the ability of limiting collateral tissue damage caused by protective immune responses against pathogens and maintaining immune homeostasis. Hori et al. found out that the transfer of Treg into Pneumocystis carini-infected recombination-activating gene-2-deficient mice did not induce lethal pneumonia and prevented development of this disease while all control mice died within 13 days [26]. Treg also seems to modulate inflammation within keratitis lesions thus limiting their severity, perhaps by secreting IL-10 [27].

Recently FOXP3 (fork head box P3, an X chromosome–encoded fork head transcription factor family member,) has been identified as the specific transcription factor of Treg. Its expression is specifically restricted to the Treg subset [27-29]. The indispensable role of FOXP3 in the development of Treg cells was illustrated by recent studies: FOXP3 deficient mice develop a rapid and fatal lymphoproliferative autoimmune syndrome at 3–4 weeks of age [30, 31] and FOXP3 deficient mice fail to generate a Treg population. Mutations in the human orthologue of FOXP3 result in a fatal autoimmune syndrome known as IPEX (Immune dysregulation, polyendocrinopathy, enteropathy and X-linked inheritance) [32, 33]. Fontenot et al. verified that $CD4^+$ $CD25^+$ regulatory T cells could prevent this lethal autoimmune syndrome in FOXP3 deficient mice and expanded preferentially when it was transferred into neonatal FOXP3 deficient mice. In addition, continued expression of FOXP3 was demonstrated to be crucial for the maintenance of Treg differentiation [34]. Although the relation between Treg and FOXP3 has been confirmed in many in vitro and in vivo experiments the role of FOXP3 in clinical and particularly perioperative settings remains unclear.

1.2.4 ROR-γ - a Th17 specific transcription factor

Th17, named after its specific pro-inflammatory cytokine IL17, has been recently identified as a new, distinct Th lineage mediating tissue inflammation. It was discovered by Infante-Duarte and colleagues [35], who found out that some Th cells in a murine model but also in humans can secrete IL17. IL17 succeeds microbial stimuli and belongs neither to Th1 nor to Th2 cytokines. Specific analysis showed that Th17 development lacks the expression of all key signalling components of Th1 and Th2 differentiation, supporting the theory of Th17 as product of a separate effector lineage [36, 37]. Consistent with these findings, Oppmann et al found a new Th17 cytokine - IL23 [38]. IL23 is described as being critical for Th17-mediated immunity and host protection by amplifying and stabilizing Th17 answer through a positive feedback loop [39, 40]

Within tissues, Th17 effector cells stimulate production of a variety of inflammatory chemokines, cytokines, and other pro-inflammatory mediators and promote or enhance inflammation [41, 42]. Neutralization of IL-17, but not genetic deletion of Th1 cells, was proved to resolve tissue pathology in autoimmune models [43, 44]. Furthermore, experimental models suggest that Th17 cells may be of importance in neutrophilic inflammation of acute airway inflammation [45-49].

On the other hand, Th17 appears to interact with Treg. Patients with acute coronary syndrome show significant increase in peripheral Th17 and its relative cytokines (IL-17, IL-6 and IL-23), as well as a decrease in Treg and its related cytokines (IL-10 and TGF-β1) [50]. This imbalance of Th17 and Treg has also been observed in the treatment of galectin-9 on collagen-induced arthritis (CIA) in a mouse model [51]. All of these findings imply that Th17 and Treg play an important role in modulating T lymphocytes in inflammation.

Similar as Th1, Th2 and Treg Th17 has its own lineage-specific transcription factor: Retinoic acid receptor-related orphan receptor gamma (ROR-γ). ROR-γ was recently identified by Ivaylo et al. [52]. ROR-γ is normally expressed in developing thymocytes ($CD4^+$, $CD8^+$) as well as in lymphoid tissue inducer (LTi) cells and LTi-like cells [53]. In ROR-γ deficient mice, Th17 number and IL17 secretion were markedly reduced, though not completely eliminated. In addition, a deficiency in ROR-γ globally impaired Th17 generation and completely protected mice against experimental autoimmune encephalomyelitis [54]. Conversely, an enforced expression of ROR-γ in naive CD4 T cells resulted in IL-17 production by approximately one-

half of cells that expressed ROR-γ. This result was in contrast to that of T-bet transduced cells, which did not show significant IL-17 expression [52].

1.2.5 SOCS1 and SOCS3 - Two negative modulators of Th cell development

It is well accepted that Th cell development has to be modulated negatively, too. Otherwise, the number of Th cells and secretion of cytokines will be out of control, once immune response is initiated. Recently, SOCS (Suppressor of cytokine signalling) is thought to play this important role. SOCS comprises a family of intracellular proteins, including eight members, CIS (cytokine-inducible SH2 protein), SOCS1, SOCS2, SOCS3, SOCS4, SOCS5, SOCS6 and SOCS7. Each of them has a common central SH2 domain, a N-terminal domain of variable length and sequence and a C-terminal 40-amino-acid module called the SOCS box [55-58]. Interestingly, a comparison of the primary amino acid sequence of SOCS subfamily members shows that pairs of SOCS proteins, SOCS1 and SOCS3, CIS and SOCS2, SOCS4 and SOCS5, SOCS6 and SOCS7, are more similar to each other than to other SOCS proteins [59]. SOCS proteins are normally present in cells at low or undetectable levels, but are rapidly induced by a board spectrum of cytokines, both in vitro and in vivo [60, 61].

Negative modulation mechanisms of SOCS1 and SOCS3 have been revealed recently (Fig. 1). Briefly, cytokines bind to their receptors on the T cell surface, leading to a dimerization of receptors and activation of so called receptor-associated Janus Kinase (JAK). Activated JAK proteins phosphorylate receptors creating binding sites for STATs (signal transducers and activators of transcription) which are as well phosphorylated by JAK. Once phosphorylated, STAT proteins can dimerize and upon transport into nuclei induce transcription of many cytokine-regulated genes. Meanwhile, activated STATs also induce transcription of SOCSs, which in turn inhibit the JAK-STATs signalling pathway that initially led to their production, forming a potent negative feedback loop [62-64]. Because the JAK-STATs pathway is the crucial component to both Th1 and Th2 signalling, like JAK-STAT6 to Th1, JAK-STAT3 toTh2, an activation of STATs will induce SOCS' negative signalling modulation.

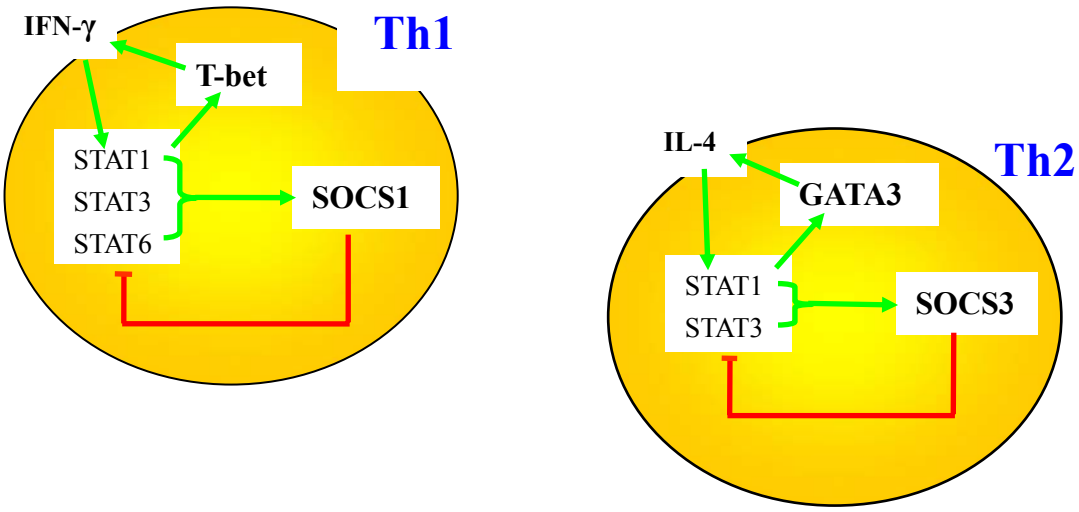

Figure 1: The role of SOCS family in the differentiation of Th cells
Green arrow indicates up-regulation, while the red arrow indicates inhibition.

STAT: Signal Transducers and Activators of Transcription; **SOCS**: Suppressor of cytokine signalling.

SOCS1 was cloned in 1997 by three independent groups and proved playing a critical role in immune response by modulating T cells activation, development and differentiation. SOCS1 deficient mice are normal at birth, later they show stunted growth and die with 3 weeks of age with a syndrome characterized by severe lymphopenia, aberrant peripheral T cells activation, fatty degeneration and necrosis of liver, as well as macrophage infiltration of major organs [65, 66]. Mice lacking of SOCS1 skew thymocyte development toward the $CD8^+$ single-positive lineage [67, 68]. SOCS1 deficient $CD4^+$ cells produced relative high amounts of IFN-γ and IL4 than do wide-type $CD4^+$ T cells in response to anti-CD3 stimulation [69] indicated that SOCS1 regulated both Th1 and Th2 polarizations. In addition, evidence from T-cell receptor-transgenic T cells suggested SOCS1 was highly expressed in Th1 cells, which implied that SOCS1 might mainly negatively regulate Th1 differentiation, toward a Th2 polarization and humoral immunity [70]. SOCS1 has also an important regulatory function in APC, such as macrophages and dendritic cells, which play key roles for inflammation, septic shock and innate immunity [71-73].

SOCS3 has a similar structure to SOCS1, but the immune functions are different. SOCS3 deficient mice exhibited embryonic lethality with death occurring between days 11 and 13 of gestation because of the defect of placental development. Leukaemia Inhibitory Factor Receptor (LIFR) deficiency rescued this defect and embryonic lethality, implying SOCS3 as an essential regulator of LIFR signalling in trophoblast giant cell differentiation [74, 75]. Like SOCS1, SOCS3 is also involved in T cell differentiation but selectively expresses in Th2 cells [70]. Seki et al demonstrated that SOCS3 transgenic mice showed increased Th2 responses and multiple pathological features characteristic of asthma in an airway hypersensitivity model system. In contrast, SOCS3 deficient mice had decreased Th2 development. These data indicate that SOCS3 has an important role in regulating the onset and maintenance of Th2 polarization [76]. SOCS3 had been shown to regulate the TLR **(**toll-like receptor) signalling, impacting the innate immune System. In Yasukawa's study, SOCS3 was strongly induced by both IL6 and IL10 in the presence of lipopolysaccharide (LPS), but selectively inhibited IL6 signalling by binding the IL6 receptor gp130 but not the IL10 receptor. Mice specifically lacking SOCS3 in macrophages and neutrophils are resistant to LPS induced septic shock [77]. Recently SOCS3 was found to be an essential negative regulator of IL-23 signalling, inhibition of which constrains the generation of Th17 differentiation [78].

1.3 Lung surgery and Anaesthesia

1.3.1 Lung surgery

Lung surgery includes a variety of procedures to diagnose and treat pulmonary diseases. Pneumonectomy, lobectomy, segmentectomy and wedge resection are standard procedures in lung surgery and are mostly performed in patients diagnosed with bronchial carcinoma or pulmonary metastases. Lung surgery is known to be associated with high tissue damage and high postoperative pain levels, which both negatively influence patient outcomes.

According to Harpole et al. thirty-day mortality of patients undergoing lobectomy and pneumonectomy is 4% and 11.5% respectively; overall thirty-day morbidity amounts to 24.1% and the most frequently occurring complication is pneumonia (11.7% for lobectomy and 9.7% for pneumonectomy) [79].

Despite significant improvements in the fields of thoracic surgery and intensive care medicine, in-hospital morbidity rates assessed in more recent studies still range between 1.95% and 4.1% (Tab.1). These findings suggest that further improvements are needed. Specific interventions aimed at reducing perioperative morbidity and mortality could save thousands of lives each year worldwide.

Table 1. **Morbidity and mortality in thoracic surgery**

Year	No. patients	No. of deaths	Setting
1999-2001 [80]	4028	103 (2.6%)	36 departments in the UK
2001-2003 [81]	3426	66 (1.9%)	27 departments in 14 countries
2004-2006 [82]	695	20 (2.3%)	3 departments in 3 countries

1.3.2 Immune response in major surgery

It is well known that surgical insult can induce a widespread alteration of immune functions such as natural killer (NK) cell activity [83], lymphocyte proliferation [84] and cytokine secretion [85]. Furthermore surgical manipulation stimulates aggregation of neutrophils and macrophages and secretion of pro-inflammatory mediators such as IL-6 and prostaglandins. NK cell activity is known to be suppressed immediately after surgery and remains decreased several days. This suppression has been shown to be more prolonged in individuals with cancer [83]. The extent of this alteration of immune function is thought to be proportional to the magnitude of surgical trauma [86].

There is some evidence that imbalances of the immune system during or after surgery significantly contribute to the development of sepsis and multi organ dysfunction syndrome (MODS). Characterizing the status of the perioperative immune function is therefore of great interest. Perioperative interventions aimed at preventing these imbalances might improve patient outcome and reduce morbidity as mortality during or after surgery.

1.3.3 Pain and immunity

The pathway from pain to immune response seems to be extremely complex. If confronted with pain the immune system firstly answers with acute inflammation. Its primary task is to detect, contain and eliminate invading microbes (bacteria, fungi, viruses) quickly, and it does so by relying on macrophages and mast cells that are stationed strategically in the tissues and circulating neutrophils to signal and engage foreign genomes. This defence becomes systemic in

major surgery and its severity is proportional to the degree of tissue injury and the length of surgery. If the acute inflammation is excessive organ damage or even death can follow. Locally surgical manipulation causes the release of immune mediators, like substance P, prostaglandins and cytokines, which can subsequently induce pain feeling and download the threshold of pain (hyperalgesia and allodynia). Meanwhile the sensory signal of this nociception is submitted to the brain via the dorsal horn of the spinal cord. Centrally, the activation of the central nervous system (CNS) and hypothalamic- pituitary- adrenal (HPA) axis pathways results the releasing of the stress hormones including the corticosteroid from the adrenal cortex. The hypothalamus also activates the sympathetic nervous system, thereby resulting in the secretion of epinephrine from the adrenal medulla and the release of norepinephrine from sympathetic terminals. All these stress hormones are considered having an inhibition on the immune response: inhibiting the antigen presentation [87], suppressing APC and Th1 production and up-regulating Th2 cytokine production [88]. In this phase, monocytes are tolerant to endotoxin and do not secrete normal amounts of pro-inflammatory cytokines [89], antigen presentation is suppressed, natural killer cell function is compromised (e.g., decreased interferon-γ(IFN-γ) production) [90], the total number of blood lymphocytes is decreased [91]. Furthermore, evidence suggests that proportion of the T cell subsets changed too [92]. The suppression of the adaptive immunity after pain stress is generally related to the increased susceptibility to sepsis, SIRS and tumour recurrence both in short and long term [93].

Therefore, an optimal pain management might provide an available method to protect the patients from not only the feeling of pain but also the following immune impairment and finally ameliorate the perioperative outcome. From this point of view, the understanding of the relationship between pain management and immune function, especially with regard T-Helper cells, seems to be of central interest.

1.3.4 Perioperative pain managements

Standard pain managements after major surgery include either intravenous infusion of opioids or epidural nerve block with local anaesthetics. Both techniques are proved to provide satisfactory results.

1.3.4.1 Intravenous analgesia

Opioids have been indispensable components of intravenous anaesthesia for over 100 years. Common opioids include Morphine and its derivates: fentanyl, remifentanil, alfentanil, sufentanil. It has been known for many years that opioids suppress hypothalamic and pituitary hormone secretion. In lower abdominal surgery, fentanyl 15 μg/kg was sufficient to inhibit cortisol and glucose responses [94]. In upper abdominal surgery, clinical doses of fentanyl have been found to be relatively ineffective in preventing the complete stress response to surgical stress [95]. In addition, Opioids are generally thought to moderately suppress the activity of natural killer (NK) cells both in animal and in human [96-99]. However, Cronin et al in recent studies demonstrated that an 8-h infusion of remifentanil did not affect NK cell activity in normal volunteers differing from all other opioids [100]. The impact of opioids on the immune system is still controversial. More evidences are needed to clear the effects of intravenous analgesia on perioperative immune response.

Remifentanil is a newer synthetic opioid which was first described in 1990 and was approved for clinical use in 1996. It acts on μ receptors systematically and is metabolized by non-specific tissue and plasma esterases, thus providing a good tolerance for patients with liver or renal failure. Remifentanil is particularly suited for Total Intravenous Anaesthesia (TIVA) according to its rapid onset and short duration of action. The potency of remifentanil is similar to fentanyl. Like fentanyl and its other derivates, remifentanil offers a remarkable hemodynamic stability. Bradycardia and hypotension can be seen only with higher dosage, which can be avoided by slowing administration and/or increasing preload. The combination of remifentanil and propofol for TIVA has been successfully used for a variety of operations, including coronary artery bypass graft (CABG) [101, 102]; major thoracic [103], abdominal [104], and orthopaedic [105] procedures in both adults and children.

1.3.4.2 Epidural analgesia

Epidural analgesia is reached by injection of drugs through a catheter placed into the epidural space. The injection can cause both a loss of sensation (anaesthesia) and a loss of pain (analgesia), by blocking the transmission of signals through nerves in or near the spinal cord.

The epidural space lies between the spinal meninges and the vertebral canal, surrounding the

dura mater (posteriorly, laterally, and anteriorly). It contains a rich venous plexus, fatty connective tissue, lymphatics and nerve roots, on which the local anaesthetic acts. Recently epidural analgesia is more frequently employed in conjunction with general anaesthesia, so called combined anaesthesia.

When epidural analgesia is performed in the thoracic region, it is called thoracic epidural anaesthesia (TEA) or thoracic epidural block (TEB). TEA is technically more difficult to accomplish than lumbar blocks because of the anatomy of vertebrae (greater angulation and marked overlapping of the spinous processes in thorax). In spite of the risks, many evidences demonstrated that TEA provided better heart-perfusion, less oxygen demand with sympathetic nerve block [106-108] and resulted in less postoperative complications [109] and lower mortality [110].

Many investigators also reported that epidural analgesia had advantageous effects on the immune response to surgical stress, including the reduction of NK cell activity suppression [111], and maintenance of Th1 cell numbers [112]. Beilin et al found that compared with intravenous analgesia, postoperative epidural analgesia provided a better pain relief, more IL-1 and IL-6 production, reduced suppression of lymphocyte proliferation and attenuated pro-inflammatory response [113]. Volk's study also revealed that postoperative epidural analgesia preserved lymphocyte rather than monocytes function compared with intravenous analgesia, which may be benefit to resist the infection [84]. Kawasaki et al. studied the production of TNF-α, IL-10, monocytes HLA-DR expression and mCD14 expression of patients with intravenous and epidural analgesia respectively, but no difference was found till the postoperative day 4 [114].

Ropivacaine is a long-acting amide local anaesthetic and is mainly metabolized in the liver. It is widely applied for epidural anaesthesia and peripheral nerve block during surgery, as well as for postoperative analgesia. The major attractive advantage of ropivacaine is that it has similar potency to Bupivacaine with regard to pain relief, but causes less CNS-toxicity, cardio-toxicity and motor block. As far, there is no evidence if the distribution of ropivacaine in circulation has a direct systematic effect on the human immune system. However, it is clear that the concentration in systemic circulation is lower than that in local epidural room after epidural anaesthesia.

1.4 Aims of the study

The most important postoperative complication in patients undergoing lung surgery is postoperative pneumonia. T1/T2 balance is well-known to be suppressed within the first three to five days after major surgery. Recent evidences revealed that the development of Th cells was controlled by its specific transcription factors. Although perioperative immune function has been widely studied by the measurement of Th cells and its cytokines, the role of Th specific transcription factors in perioperative immune response is still unclear.

Surgery induced pain is a major stressor which influences the immune response. A good pain management can be helpful for maintaining immune homeostasis and improving surgery outcomes. Clinical common used pain managements include intravenous analgesia and epidural analgesia. There is some evidence that epidural analgesia is able to maintain the perioperative immune balance better than intravenous analgesia does. But this conclusion is still not coincident. T-bet is the specific transcription factor of Th1-response and GATA3 is the regulating transcription factor of Th2-response. In vitro evidence is documented that SOCS1 negatively modulates the Th1 development and SOCS3 inhibits the Th2 development [70]. However the in vivo transcription factors' answer in a perioperative setting is unknown.

Therefore, the aim of this prospective, randomised, double blinded, controlled trial was to

1. investigate if epidural and intravenous analgesia have different impacts on the expression of Th cell transcription factors (primary outcome measure);
2. investigate if the Th1 negative regulator SOCS1 has a corresponding alteration to the Th1 positive regulator T-bet in perioperative immune response, as well as SOCS3 to GATA3 (secondary outcome measure).

2 Methods

2.1 Study design:

This study was conducted between January 2005 and June 2007 in a prospective, randomised, double-blinded controlled design at the Klinik für Anästhesiologie mit Schwerpunkt operative Intensivmedizin of the University Hospital Charité, Charité Virchow-Klinikum and Campus Charité Mitte, Charité - Universitätsmedizin Berlin. It was approved by the local ethics committee (No. EA 1/175/05), the Federal Institute for Drugs and Medical Devices (BfArM) and the Federal Opium Agency (BOPST). Quality of randomization and double-blind procedure were controlled by the Koordinierungszentrum Klinische Studien Charité (KKS Charité).

2.2 Patient selection and exclusion criteria:

One-hundred thirteen consecutive patients scheduled for elective lung surgery at the Charité University Hospital were screened for eligibility. All patients were randomized in a double-blind fashion to three groups. One of these groups was not considered in this thesis. According to the clinical trial protocol patients meeting at least one of the following exclusion criteria were not admitted to this study: 1) age under eighteen years, 2) guardianship/conservatorship, 3) refusal to participate in the study, 4) pre-existing alterations of the immune system such as infections meeting criteria of the Center for Disease Control and Prevention (CDC) or treatments and disorders with direct influence on the immune system such as immune modulating therapy or adrenal pathology, 5) pregnancy, 6) contraindications for epidural catheter insertion, 7) contraindications for ropivacaine or remifentanil application, 8) pre-existing treatment with the above-mentioned trial drugs or drugs belonging to the same pharmacological class, 9) class III or higher according to The New York Heart Association (NYHA) Functional Classification, 10) myocardial infarction in the previous eight weeks before surgery. In total, 40 patients remained in the analysis of this thesis.

Each participant gave written informed consent. Patients were randomly assigned to one of the three groups, while one arm featuring additional clonidine treatment was not considered in this thesis: 1) intravenous remifentanil analgesia during the operation and 2) thoracic epidural block

with ropivacaine during the operation (Tab. 2).

Table 2. Random group, content and pharmaceutical form of the investigational drugs

Disguised syringes	Remifentanil group	Ropivacaine group
10 ml syringe for epidural bolus application	10 ml of placebo instead of ropivacaine	10 ml of ropivacaine 0.75% (75 mg)
50 ml syringe for continuous intravenous infusion	10 mg of remifentanil diluted with 50 ml normal saline	50 ml of placebo instead of remifentanil
50 ml syringe for continuous epidural infusion	50 ml of placebo instead of ropivacaine	50 ml of ropivacaine 0.2% (2 mg/ ml)

2.3 Anaesthesia management

All anaesthetists who performed anaesthesia and/or collected blood samples were blinded to both groups. Anaesthesia protocols were based on the clinics internal standard operating procedures ("Check-up Anästhesiologie", Spies et al).

2.3.1 Premedication:

Midazolam (Dormicum®, Hoffman-La Roche AG Germany) at a dosing of 0.15 mg•kg-1 was administered orally 30 minutes prior to arrival at the anaesthesia induction room. Fasting began 6 hours before induction and clear fluids have been given up to 2 hours before anaesthesia.

2.3.2 Placement of epidural catheter

On arrival in the induction room an 18 gauge intravenous catheter was placed and an infusion of 500 ml Ringer's Lactate solution was started. Meanwhile 5 lead ECG, NIBP (non-invasive blood pressure), SpO_2 (oxygen saturation) and RR (respiration rate) were monitored continuously.

After local infiltration of 5 ml 1 % Lidocaine, thoracic epidural puncture was performed in the operating room by median approach and loss of resistance technique at a level between Th4 and Th7. After 5 cm catheter insertion into the epidural space an accidental subdural catheterization

was ruled out by injecting the standard test dose of 3 ml isobaric bupivacaine 0.5%.

2.3.3 Induction of anaesthesia

An arterial catheter was placed in the radial artery for continuous invasive blood pressure (IBP) monitoring and blood gas analysis. After preoxygenation with 100% of oxygen by means of facemask, anaesthesia was inducted. The standardized regimen of induction included Fentanyl (Fentanyl Janssen®, Janssen-Cilag, Neuss, Germany) 0.1-0.2mg, Propofol (Propofol fresenius®, Fresenius Kabi, Germany) 2mg/kg, and Cisatracurium (Nimbex®, GlaxoSmithKline, UK) 0.15mg/kg. Fentanyl was given prior to Propofol. As soon as patients lost their eyelash reflex and stopped spontaneous breathing, mask ventilation was started and cisatracurium administered. Oral tracheal intubation was performed with double-lumen endobronchial tube (Broncho-cath ™ Left, Nellcor, USA, Boulder; size: female 37 Ch, male 39 Ch). Position was verified and corrected by fiberoptic bronchoscopy if necessary**.** Finally, a 7.0 F triple-lumen central venous catheter was placed in the subclavian vein if required.

2.3.4 Maintenance of anaesthesia

Patients were pressure controlled ventilated using an Aestiva/5® anaesthesia machine (Datex-Ohmeda, GE Healthcare P.O. Box 7550 Madison, WI 53707-7550, USA). During one-lung ventilation (OLV) patients were ventilated in terms of lung protective ventilation with 5 ml/kg body weight and positive end-expiratory pressure (PEEP) of 5 cmH2O. Normocapnia was maintained by adapting respiratory rate. Initial inspiratory oxygen fraction (FiO2) of 1.0 was reduced according to blood gas analysis.

Continuous infusion of 6-8mg/kg/h Propofol was initiated immediately after induction and intraoperative analgesia was performed double-blind either with remifentanil continuously i.v. or ropivacaine continuously epidurally.

Remifentanil was infused at 0.2-0.4 μg/kg/min and ropivacaine at 6 - 10 ml/h until the end of surgery. Anaesthesia was stopped 3 minutes prior to the end of the operation and all patients received an i.v. morphine bolus of 0.1mg/kg. Extubation was conducted in the operating room.

Continuous postoperative analgesia with ropivacaine 0.2% via peridural catheter was performed on all patients. (Fig. 3).

2.4 Sample collection

Blood samples (BS) were collected on day of surgery and the first three postoperative days at morning rounds (Fig. 2). BS 2 and BS 3 were collected during lung resection and on arrival at the intensive care unit (ICU). Each time, 2.5ml blood was drawn from a central or peripheral vein using BD Vacutainer® Safety-Lok™ Blood Collection Set (Becton Dickinson GmbH, Germany, Heidelberg) and softly injected into a PAXgene Blood RNA Tube (PreAnalytiX GmbH, Germany). PAXgene Blood RNA Tubes then were gently inverted 10 times and placed upright at room temperature for at least 2 hours. After that, tubes were transferred to -20 °C for 24 hours and then stored at -80°C waiting for RNA extraction.

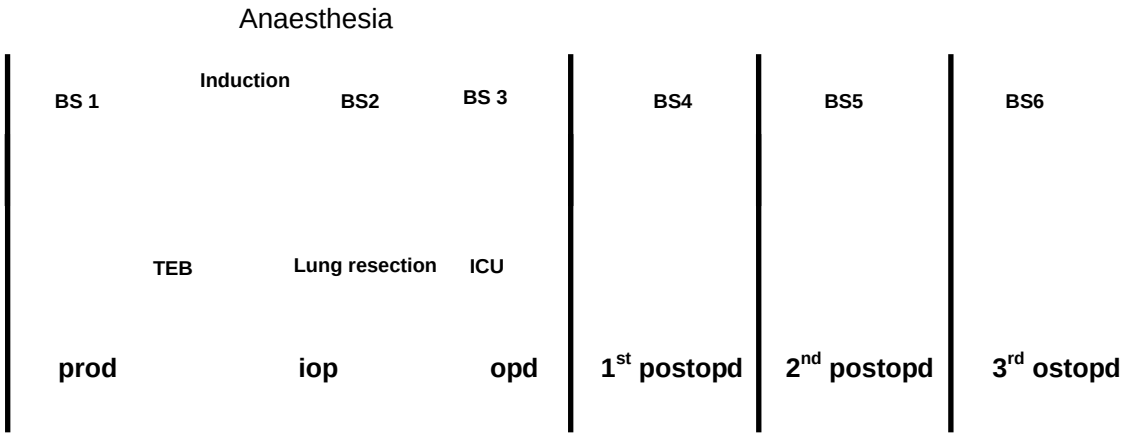

Figure 2: Schema of the study protocol

BS: blood sample; **TEB:** thoracic epidural block; **POD**: postoperative day; **ICU**: intensive care unit; prod = pre-operative day, iop = intra-operative, opd = operation day, postopd = post-operative day

2.5 Measurement of Th transcription factors mRNA by relative qRT PCR

2.5.1 Materials

2.5.1.1 Instruments

Table 3: Instruments used in the study

7500 Realtime PCR system	ABI
96 well Opital Plate	ABI
Biometra Trio-Thermoblock	Biotron
Centrifuge 5424	Eppendorf
Centrifuge 5804	Eppendorf
Heraeus Multifuge 3L-R	Thermo Scientific
Mastersycler personal	Eppendorf
MicroAmp Optical Tubes and Caps	ABI
Pipet 0.5-5000µl	Eppendorf
PCR reaction tubes 0.5ml	Eppendorf
Sterile, RNase-free pipet tips with filter	Greiner Bio One
Thermomixer Compact	Eppendorf
Vortex mixer 7-2020	Neolab

2.5.1.2 Chemicals, kits

Table 4: The chemicals and kits used in real time PCR

Ampuwa	Fresenius Kabi
DNase I	Invitrogen
M-MLV Reverse-transcriptase	Promega
Oligo (dT) Primer	Promega
Ethanol (analytical pure)	
PAXgene Blood RNA kit	PreAnalytiX
RNase Inhibitor	
TagMan universal PCR Master Mix	Roche

2.5.1.3 Primer used in relative Quantitation real time PCR

Table 5: Sequence of primers:

Symbol	Sequence
T-bet	forward primer:5′-CAA CAC AGG AGC GCA CTG G-3′
	reverse primer :5′- CCC CCT TGT TGT TTG TGA GCT-3′
GATA3	forward primer: 5′- CCT CAT TAA GCC CAA GCG AAG-3′
	reverse primer : 5′-TTG GCA TTC CTC CTC CGA AGT-3′
FOXP3	forward primer:5′-AAG TGG CCC GGA TGT GAG A-3′
	reverse primer :5′- CAT TGT GCC CTG CCC TTC T-3′
SOCS1	forward primer:5′CCG CGA CTA CCT GAG CTC CTT-3′
	reverse primer :5′AGT TAA TGC TGC GTG CAC GG-3′
SOCS3	forward primer:5′-CTT TCT GAT CCG CGA CAG CT-3′
	reverse primer :5′-TCA CAC TGG ATG CGC AGG T-3′
HPRT1	forward primer: 5′-AGT CTG GCT TAT ATC CAA CAC TTC G-3′
	reverse primer :5′- GAC TTT GCT TTC CTT GGT CAG G-3′
ROR-γ	This primer was bought from ABI, unlike others are designed by us

2.5.1.4 Fluorogenic Probe used in relative Quantitation real time PCR

Table 6: Sequence of probes:

Symbol	Sequence
T-bet	5′-CAC CTG TTG TGG TCC AAG TTT AAT CAG CAC C-3′
GATA3	5′-TCC TGT GCG AAC TGT CAG ACC ACC AC -3′
FOXP3	5′- ACT TCC TCA AGC ACT GCC AGG CGG-3′
SOCS1	5′-TTC CAG ATT TGA CCG GCA GCG C-3′
SOCS3	5′-CCA GCG CCA CTT CTT CAC GCT Cag-3′
HPRT1	5′- TTT CAC CAG CAA GCT TGC GAC CTT GA-3′
ROR-γ	This probe was bought from ABI, unlike the others designed by us

2.5.2 RNA isolation

Total RNA of each blood sample was contracted by PAXgene Blood RNA Kit according to the manufacturer's instruction. In brief, after overnight thaw, samples were centrifuged and washed

twice. Pellets of blood were then incubated with proteinase K to digest the protein. Digested solutions were transferred into the PAXgene Shredder to homogenize cell lysate and residual cell debris was removed by centrifuging again. Subsequently, cell lysate was transferred into a PAXgene RNA spin column to bind to the PAXgene silica membrane. After several efficient wash steps, total RNA was finally eluted in elution buffer and stored at -80 °C waiting for cDNA synthesis.

2.5.3 cDNA template synthesis (reverse transcription)

Total RNA was reverse-transcribed using M-MLV reverse transcriptase and oligo (dT) primer in a final volume of 40µl (Table 7). First, RNA and oligo (dT) primer were pre-incubated at 75 °C for 10 min, then for 30 min at 37 °C with prepared compound B and further for 60 min at 42 °C with compound C. RNA samples were then treated with RNase-free DNase I before reverse transcription to eliminate contaminating genomic DNA. The synthesized cDNA was stored at -20°C.

Table 7: Standard reactions and procedure of cDNA synthesis

Segment	Temperature °C	Time (min)	Compound reactions		Volume
1	75	10	A	total mRNA	21.5ul
				Oligo(dT)-Primer	2 ul
2	37	30	B	Reaction Buffer	8ul
				dNTP mix	4 ul
				RNase Inhibitor	0.5 ul
				DNase	2ul
3	75	5		---	---
4	42	60	C	Reverse-transcriptase	1 ul
				RNase Inhibitor	1 ul
5	94	5		---	---
Total		110			40 ul

2.5.4 Relative quantitation real time PCR

Gene expression of transcription factors was measured in duplicate by relative quantitative real time reverse-transcription polymerase chain reaction (relative qRT-PCR) with the use ofABI Prism 7500 Sequence Detection System (ABI 7500 SDS, Applied Biosystems, USA, Foster City). The specific primer and internal fluorogenic probe of ROR-γ was bought from ABI. Others were designed by using Primer Express 2 and synthesized by Metabion GmbH (Metabion, Germany, Martinsried) (Table 5 and 6). Reporter dye used in real time PCR was FAM (6-carboxyflurorescein) and quencher dye was TAMRA N,N,N′,N′-tetramethyl-6-carboxyrhodamine). Thermal cycling conditions were designed as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min (Table 9). Study transcription factors, T-bet, GATA3, ROR-γ, FOXP3, SOCS1 and SOCS3, were set as target genes and HPRT1 (Hypoxanthine Phosphoribosyltransferase 1) as endogenous control. Fluorescent measurements, which reflect gene expression, were recorded during each annealing step. At the end of each PCR run, fluorescent data were automatically analyzed by ABI system and plotted against time forming an amplified curve. NTC (cDNA replaced by water as blank control) was performed to check for non-pollution from reaction in each plate.

Table 8: Standard qRT-PCR reaction

Compound	Volume
cDNA	3.25ul
TagMan universal PCR Master Mix	6.25ul
primer	3ul
probe	0.5ul
Total	13ul

Table 9: Standard program of qRT-PCR

Segment	Step	Temperature °C	Duration min:sec
1	AmpErase® UNG Activation	50	2:00
2	DNA Polymerase Activation	95	10:00
3 (each of 40 Cycles)	Melt	95	0:15
	Anneal/Extend	60	1:00

2.5.5 Calculation of relative quantitation of target gene expression using $2^{-\Delta\Delta C_T}$ method

The $2^{-\Delta\Delta C_T}$ method was used to evaluate expression levels of target transcription factors at each time point [115]. Briefly, baseline and threshold value (C_T) of each sample was automatically created by SDS (Sequence Detective Software, Applied Biosystems, version 1.3.1). The level of target transcription factor was normalized to a calibrator in each case. The calibrator chosen was the sample collected before the operation. Final results were expressed as ***Rn*** (*n*-fold change) in gene expression relative to endogenous gene *HPRT1* and calibrator as follows: $\boldsymbol{Rn} = 2^{-(\triangle Ct \text{ sample-} \triangle Ct \text{ calibrator})}$, where $\triangle Ct$ values of sample and calibrator were determined by subtracting the average *Ct* value of the target gene transcript from the average *Ct* value of each sample's *HPRT1*. All measurements were made in duplicate and results were only accepted if duplicate pairs differed by less than 0.5 cycles. Final results were transferred into SPSS for further statistical analysis.

2.6 Statistical analysis

Results were expressed as arithmetic mean ± standard error of mean (SEM) [no rejection of normal distribution] or median and (25% - 75% quartiles) [non-symmetrically distributed observations], respectively. Absolute and relative frequencies have been used for categorical and dichotomous variables. In dependence on the proved distributions, Student's t-test or the non-parametric Mann-Whitney U-test were applied for inter-group comparisons. Frequencies were tested by Fisher's exact test. In case of large differences in the sample sizes and/or data with ties and/or sparse data in contingency tables, exact versions of the tests were applied (StatXact 5®, Cytel Software Corp. Cambridge, MA 02139 USA, 2001). The differences between groups (such as treatment with remifentanil or ropivacaine) with respect to various clinical endpoints were not only univariately proved but also multivariately by using the non-parametric multivariate analysis of variance for repeated measurements (Brunner, E., Domhof, S. and F. Langer (2002): Nonparametric Analysis of Longitudinal Data in Factorial Experiments. Wiley & Sons, New York). Therefore, we have compared all the time points simultaneously on the corresponding response curves. Intra-group differences (between certain time points) were tested by means of the non-parametric Wilcoxon rank-sum test for paired observations. Correlations between selected clinical parameters were estimated and tested (against H0: $\rho = 0$) by Spearman's rank

correlation coefficient. A two-tailed p-value < 0.05 was considered statistically significant. All tests should be understood as constituting exploratory data analysis, such that no adjustments for multiple testing have been made. Numerical calculations were performed with SPSS, Version 15, Copyright© SPSS, Inc., Chicago, Illinois 60606, USA, SAS, Version 9.1, Copyright© by SAS Institute, Inc., Cary, NC, USA, and StatXact 6®, CYTEL Software Corp., Cambridge, MA 02139,USA.

3. Results

3.1 Demographics and clinical characteristics

40 Patients were randomly assigned to one of the two groups as shown in figure 3.

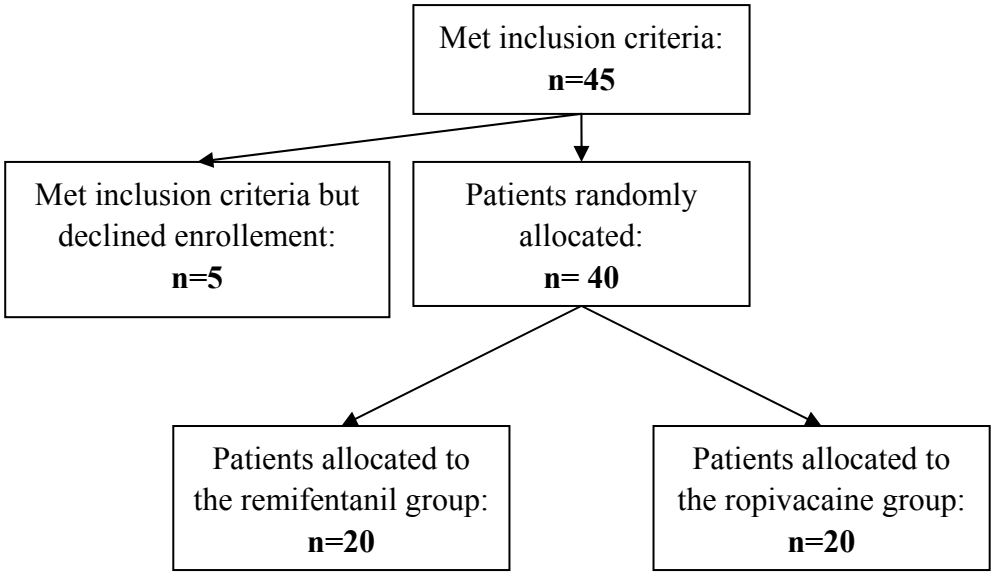

Figure 3: Flow of participants

Basic demographics and basic characteristics of both groups were equally distributed as shown in table 10.

Clinical characteristics are shown in table 11. All items were comparable between groups, no significant differences could be found. Six patients of the remifentanil group and 3 patients of the group receiving ropivacaine underwent Video Assisted Thoracic Surgery (VATS). No significant differences were found for anaesthesia duration, ICU-stay and re-surgery rate and no patient showed SIRS symptoms.

Table 10: Demographics and basic characteristics

	Remifentanil n=20	Ropivacaine n=20	p value
Age (Years)	66.5 (50.0-70.5)	65.5 (63.0-70.5)	0.429
Sex (Male/Female), No. §	13/7 (65/35 %)	16/4 (80/20 %)	0.300
BMI (kg/m^2)	26.37 (21.56-29.77)	26.56 (22.16-30.08)	0.708
ASA(I/II/III/IV), No. §	0/8/12/0 (0/40/60/0 %)	0/8/12/0 (0/40/60/0 %)	>=0.999
Pre-existing cardiovascular disease, No.§	11 (55%)	14 (70%)	0.514
Pre-existing Beta-blocker therapy, No. §	7 (35%)	8 (40%)	>=0.999
Pre-existing anti-diabetic therapy, No. §	3 (15%)	3 (15%)	>=0.999
Current Smoking, No. §	3 (15%)	7 (35%)	0.273

Data are presented as median (quartiles 25-75) or No. (frequency)

BMI: Body Mass Index; ASA: American Society of Anaesthesiologists

Two sided P values are based on the Mann-Whitney U test.

§: Two sided P values are based on the Fisher's chi-square test.

Table 11: Clinical characteristics of patients in both groups

	Remifentanil n=20	Ropivacaine n=20	p value
Duration of operation (min, mean ± SEM)	158±21	146±21	0.678
Anaesthesia duration (min, mean ± SEM)	232±20	215±17	0.521
VATS, No §	6	3	0.451
Re-surgery, No §	2	1	>=0.999
ICU stay (day, mean ± SEM)	1.85±0.67	1.70±0.74	0.841

VATS: video assisted thoracic surgery, SEM: Standard Error Mean

Two sided P values are based on the student *t* test.

§: Two sided P values are based on the Fisher's chi-square test.

3.2 T-bet expression

We detected expression of mRNA for T-bet in all samples using relative quantitative real time PCR (relative qRT-PCR). According to the $2^{-\Delta\Delta C_T}$ method we used, the final acquired value (Rn) represents the n-fold change of T-bet expression in relation to preoperative baseline values (prod) as shown in table 12.

Table 12: Cumulative (both groups) T-bet expression in quantitative real time PCR
iop = intra-operative, opd = operation day, postopd = post-operative day

	Percentile	iop	opd	1 postopd	2 postopd	3 postopd
T-BET [Rn]	25 %	0.667	0.837	0.211	0.246	0.398
	median	1.021	1.608	0.422	0.586	0.563
	75 %	1.944	3.244	0.983	1.186	0.825

T-bet expression in each group as represented in figure 4 shows no significant difference between groups in analysis of variance (p= 0.313).

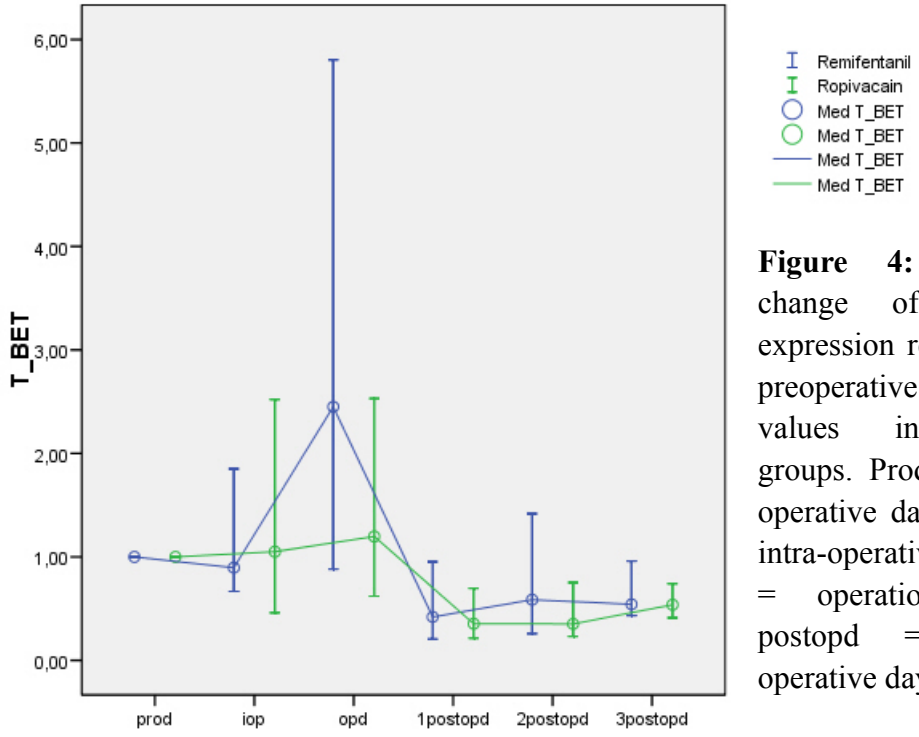

Figure 4: n-fold change of T-bet expression related to preoperative baseline values in both groups. Prod = pre-operative day, iop = intra-operative, opd = operation day, postopd = post-operative day

Cumulative alterations of T-bet expression in all patients for the time point end of surgery in the Wilcoxon rank-sum test for paired observations reached a two-sided significance (p= 0.001) for the time point opd related to preoperative baseline (prod). The remifentanil group itself showed a significant increment as well for the time point opd (p= 0.016), while the ropivacaine group showed no significance in the non-parametric Wilcoxon rank test (p= 0,058).

3.3 GATA3 expression

We detected expression of GATA3mRNA using relative qRT-PCR. According to the $2^{-\Delta\Delta C_T}$ method, the final acquired value (Rn) represents the n-fold change of GATA3 expression in relation to preoperative baseline values (prod) as shown in table 13.

Table 13: GATA3 expression in quantitative real time PCR
iop = intra-operative, opd = operation day, postopd = post-operative day

	Percentile	iop	opd	1 postopd	2 postopd	3 postopd
GATA3 [Rn]	25 %	0.687	0.544	0.247	0.285	0.373
	median	1.008	1.025	0.480	0.572	0.653
	75 %	1.266	1.576	0.775	1.021	1.043

GATA3 expression in both groups is represented in figure 5. Statistically, differences in the cumulative course are not significant (p= 0.164).

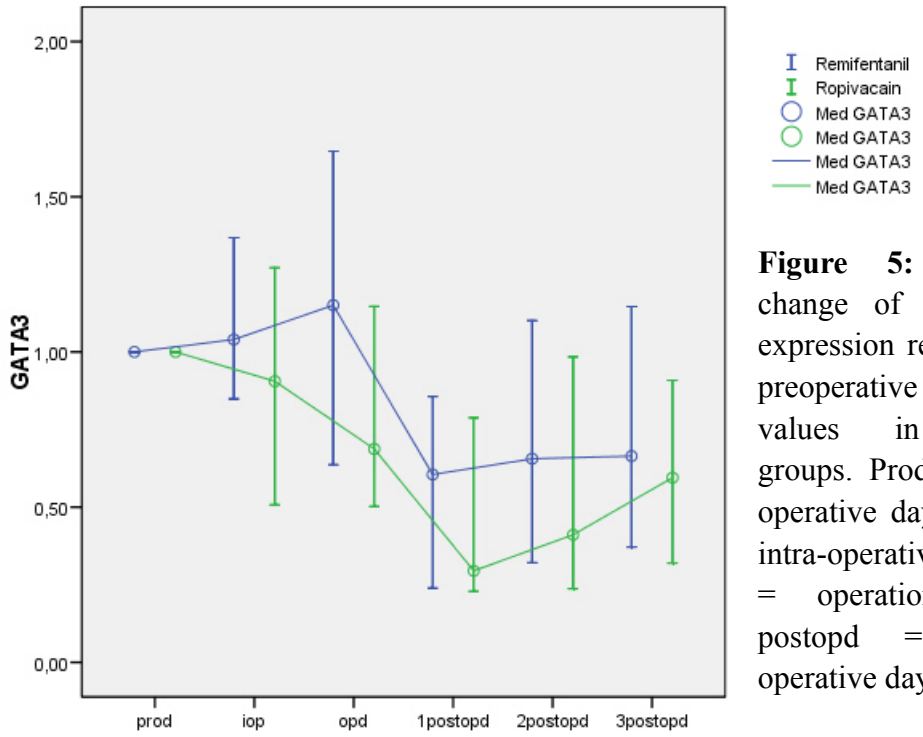

Figure 5: n-fold change of GATA3 expression related to preoperative baseline values in both groups. Prod = pre-operative day, iop = intra-operative, opd = operation day, postopd = post-operative day

Cumulative expression for all patients at the time point opd in the Wilcoxon rank-sum test was not significant for the GATA3 curve (p= 0.826). The remifentanil group itself (p= 0.215) as well as the ropivacaine group itself (p= 0.286) showed no statistical significance.

3.4 FOXP3 expression

We detected expression of FOXP3 mRNA using relative qRT-PCR and the $2^{-\Delta\Delta C_T}$method. The final acquired value (Rn) represents the n-fold change of FOXP3 expression in relation to preoperative baseline values (prod) as shown in table 14.

Table 14: FOXP3 expression in quantitative real time PCR
iop = intra-operative, opd = operation day, postopd = post-operative day

	Percentile	iop	opd	1 postopd	2 postopd	3 postopd
FOXP3 [Rn]	25 %	0.547	0.486	0.310	0.358	0.460
	median	0.940	0.813	0.690	0.665	0.673
	75 %	1.363	1.421	1.093	1.210	1.150

FOXP3 expression in both groups is represented in figure 6. The differences in the cumulative course are not of statistical significance (p= 0.425).

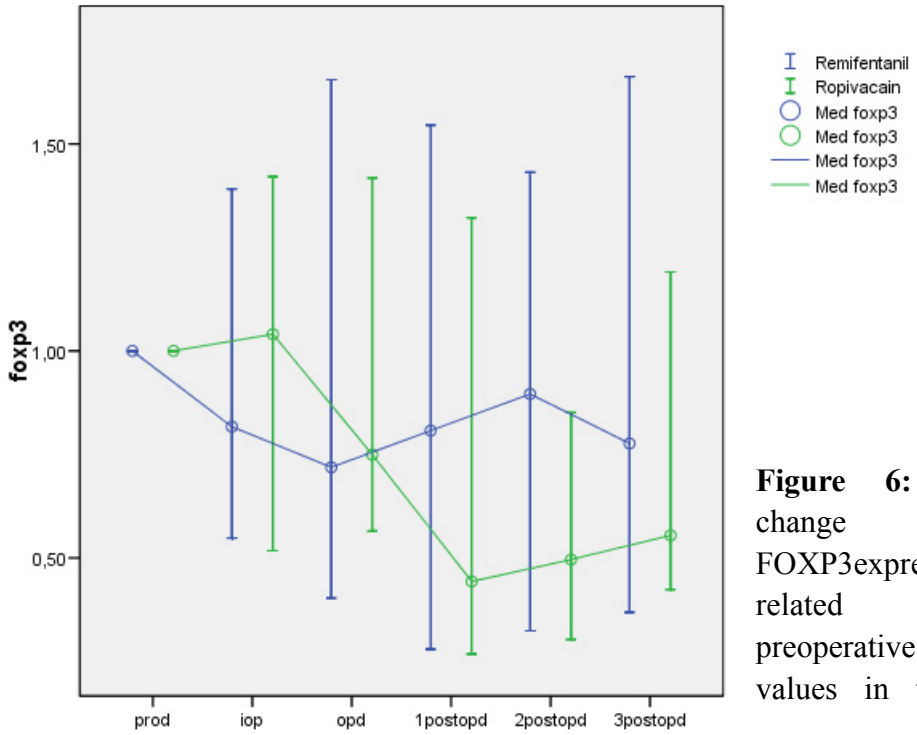

Figure 6: n-fold change of FOXP3expression related to preoperative baseline values in the two

Alteration of expression in all 40 patients at time point opd in the Wilcoxon rank-sum test for paired observations showed no two-sided asymptotic significance (p= 0.850) in relation to preoperative baseline values. The remifentanil group itself (p= 0.913) as well as the ropivacaine group itself (p= 0,647) showed no statistical significance for time point opd.

3.5 ROR-γ expression

We detected expression of mRNA for ROR-γ in all samples using relative qRT-PCR. According to the $2^{-\Delta\Delta C_T}$ method, the final acquired value (Rn) represents the n-fold change of ROR-γ expression in relation to preoperative baseline values (prod) as shown in table 15.

Table 15: GATA3 expression in quantitative real time PCR
iop = intra-operative, opd = operation day, postopd = post-operative day

	Percentile	iop	opd	1 postopd	2 postopd	3 postopd
ROR-γ [Rn]	25 %	0.654	0.508	0.324	0.327	0.388
	median	1.033	0.971	0.498	0.585	0.722
	75 %	1.482	1.622	1.058	1.105	1.087

ROR-γ expression in the two groups is represented in figure 7. The differences in the cumulative course are not of statistical significance (p= 0.651).

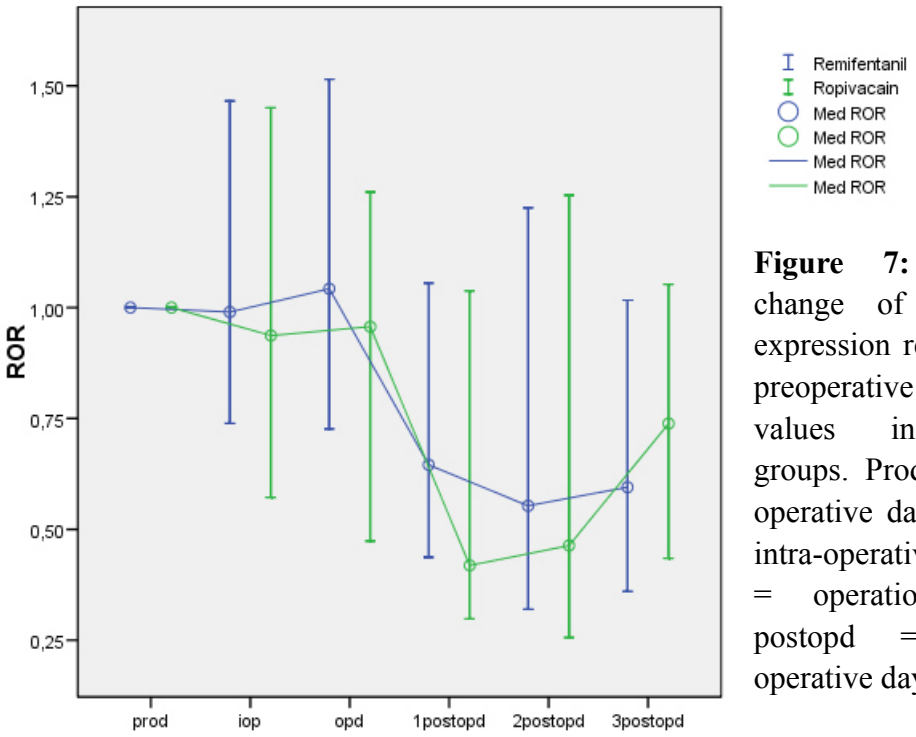

Figure 7: n-fold change of ROR-γ expression related to preoperative baseline values in both groups. Prod = pre-operative day, iop = intra-operative, opd = operation day, postopd = post-operative day

Cumulative expression of all patients at time point prod in the Wilcoxon rank-sum test was not significant for the ROR-γ curve (p= 0.675). The remifentanil group itself (p= 0.446) as well as the ropivacaine group itself (p= 0.877) showed no statistical significance.

3.6 SOCS1 expression

Expression of SOCS1 mRNA in all samples using relative qRT-PCR results in the n-fold change in relation to preoperative baseline values (prod) as shown in table 16.

Table 16: SOCS1 expression in quantitative real time PCR
iop = intra-operative, opd = operation day, postopd = post-operative day

	Percentile	iop	opd	1 postopd	2 postopd	3 postopd
SOCS1 [Rn]	25 %	0.587	0.522	0.098	0.187	0.180
	median	0.972	0.895	0.326	0.360	0.477
	75 %	1.249	2.321	0.807	0.986	1.140

SOCS1 expression in both groups is represented in figure 8. Differences in the cumulative course are not of statistical significance (p= 0.517).

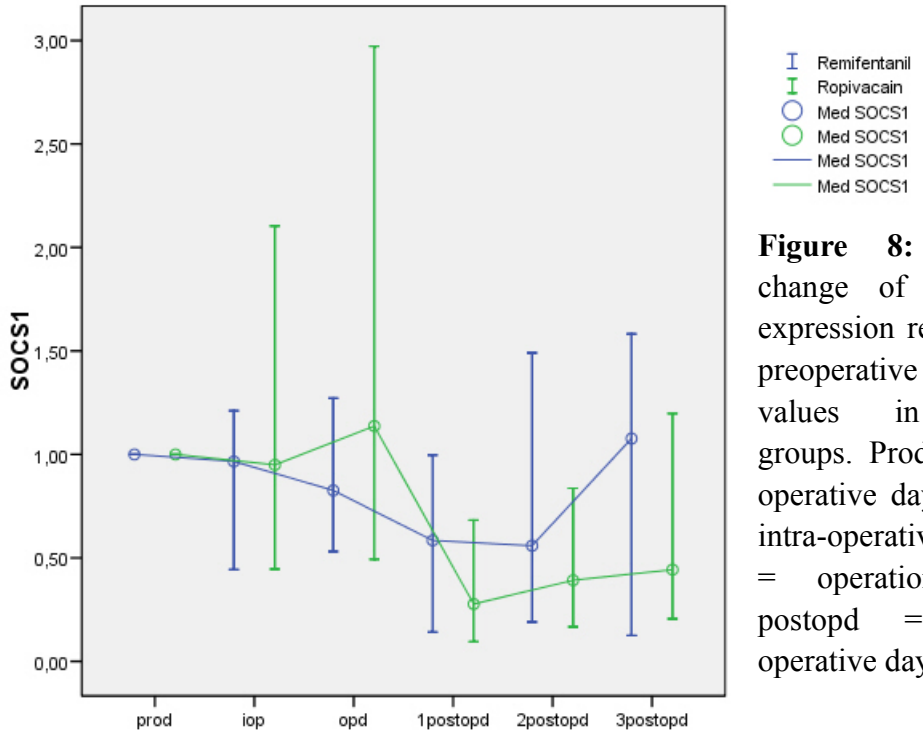

Figure 8: n-fold change of SOCS1 expression related to preoperative baseline values in both groups. Prod = pre-operative day, iop = intra-operative, opd = operation day, postopd = post-operative day.

Cumulative expression of all patients at time point prod in the Wilcoxon rank-sum test was not significant for the SOCS1 curve (p= 0.499). The remifentanil group itself (p= 0.586) as well as the ropivacaine group itself (p= 0.199) showed no statistical significance.

3.7 SOCS3 expression

The expression of mRNA for SOCS3 in all samples using relative qRT-PCR results in the n-fold changes in relation to preoperative baseline values (prod) as shown in table 17.

Table 17: SOCS3 expression in quantitative real time PCR
iop = intra-operative, opd = operation day, postopd = post-operative day

	Percentile	iop	opd	1 postopd	2 postopd	3 postopd
SOCS3 [Rn]	25 %	1.11	3.50	2.35	1.30	1.19
	median	2.51	8.59	4.63	2.63	3.03
	75 %	8.00	24.25	8.30	5.65	4.80

SOCS3 expression in the two groups is represented in figure 9. The differences in the cumulative course are not of statistical significance (p= 0.177).

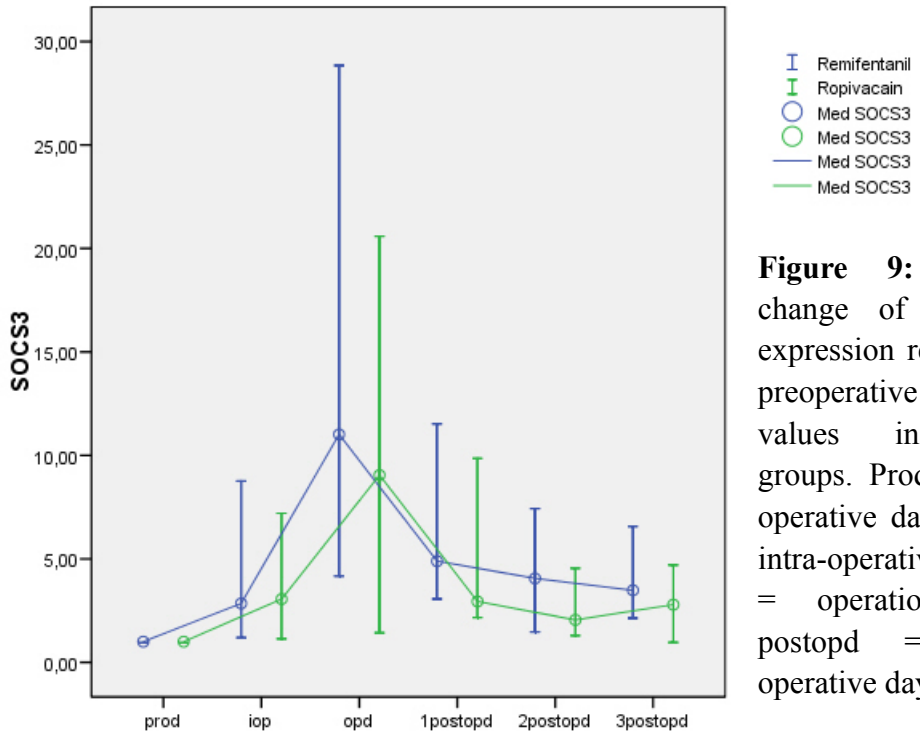

Figure 9: n-fold change of SOCS3 expression related to preoperative baseline values in both groups. Prod = pre-operative day, iop = intra-operative, opd = operation day, postopd = post-operative day.

Alteration of SOCS3 expression of all 40 patients at the end of surgery in the Wilcoxon rank-sum test for paired observations reached a two-sided high significance ($p < 0.0001$) for the time point prod related to preoperative baseline values. The remifentanil group itself ($p < 0.0001$) as well as the ropivacaine group itself ($p < 0.0001$) showed significant increments for the time point prod.

3.8 Correlation between T-bet and SOCS1

In Spearman's rank correlations (Spearman's rho), the correlation coefficient between T-bet and SOCS1 was highly significant for all time points except for 1 postopd (p= 0.050) as shown in table 18.

Table 18: Spearman's rank correlations T-bet / SOCS1

iop = intra-operative, opd = operation day, postopd = post-operative day.

Correlation between T-bet and SOCS1		
iop	Correlation coefficient	0.546
	2-sided significancy	p < 0.0001
opd	Correlation coefficient	0.514
	2-sided significancy	p= 0.001
1 postopd	Correlation coefficient	0.328
	2-sided significancy	p= 0.050
2 postopd	Correlation coefficient	0.488
	2-sided significancy	p= 0.002
3 postopd	Correlation coefficient	0.582
	2-sided significancy	p < 0.0001

3.9 Correlation between GATA3 and SOCS3

Spearman's rank correlation coefficient shows no correlation at any time point between GATA3 and SOCS3 as reported in table 19.

Table 19: Spearman's rank: correlations GATA3 / SOCS3

iop = intra-operative, opd = operation day, postopd = post-operative day.

Correlation between GATA3 and SOCS3		
iop	Correlation coefficient	-0.180
	2-sided significancy	p= 0.285
opd	Correlation coefficient	-0.278
	2-sided significancy	p= 0.101
1 postopd	Correlation coefficient	0.237
	2-sided significancy	p= 0.165
2 postopd	Correlation coefficient	0.292
	2-sided significancy	p= 0.076
3 postopd	Correlation coefficient	0.239
	2-sided significancy	p= 0.160

4 Discussion

The most important results of this study were:

1) Immediately after surgery T-bet expression is significantly increased in the remifentanil group but not in the ropivacaine group. All other transcription factors did not differ between groups.
2) The SOCS3 expression is significantly increased in both the remifentanil and the ropivacaine group. The n-fold change of T-bet expression levels significantly correlates with the n-fold change of SOCS1 expression levels at all time points except for the first postoperative day, which nevertheless shows a clear trend.

4.1 T-bet expression

In the present study we found that the expression of T-bet mRNA can be divided into 2 stages: during surgery and during the postoperative phase. T-bet expression increased during surgery and reached a significant maximum at the end of the operation.

Very little is known about T-bet expression in a perioperative setting. Additionally, as the cytokine signalling downstream of the transcription factors has not been measured in this study the interpretation of our results has to remain on a speculative level.

Following hepatobiliary pancreatic surgery, T-bet mRNA expression level was not changed after surgery but was significantly higher on postoperative day 3, 7, and 14 in patients with postoperative infection ($P < 0.05$) [116].

Besides prevention of infections, functional Th1 cells are required for surgical adhesion formation in a murine model [117].

In contrast, chronic morphine treatment resulted in a decrease in T-bet mRNA levels [118]. On one hand this might be indicative of a better maintained immune reactivity with the ropivacaine group in our setting. On the other hand, different regulations in acute surgical settings might be seen and this might result from the better maintained immune reactivity if pain is treated pre-emptively. It was shown previously in our group that remifentanil maintains immune reactivity during surgery better than fentanyl did [119].

In conclusion, the different T-bet response during surgery could either be in favor of remifentanil

to defend infections after surgical stress and to induce surgical adhesion formation, or could be an indicator of a more stressful response with remifentanil than with ropivacaine due to intraoperative increased stress and therefore postoperative inflammation.

It can be speculated that the assessed increase of T-bet is due to an alteration of IFN-γ and macrophages, both of which are important components in the early pro-inflammatory response following operation. Roumen and other researchers, by measuring macrophage secreted cytokines IL-1β and IL-6, demonstrated that macrophage activation significantly increased in major surgery [120, 121]. Macrophages present antigens to Th1 cells via MHC II and induce their proliferation. In addition macrophages produce IL-12, which binds to IL-12 receptors of Th1 cells. Both pathways have the ability to boost transcription of T-bet, which could subsequently induce IFN-γ transcript and synthesis. Furthermore, IFN-γ can activate effector-macrophages, promoting their ability to ingest and destroy microbes [122]. Thus, all of these elements together could build a positive feedback loop, increasing expression of T-bet as well as early pro-inflammatory responses. Consistent with our results, Charles and colleagues previously reported that patients undergoing colonic resection showed a significant increase of Th1 cytokines three hours past surgery [123].

Sean et al. showed that the concentration of Th1 specific cytokine IFN-γ in peripheral blood samples was suppressed in burn and trauma patients[124]. Wu and colleagues demonstrated that the percentage of Th cells decreased significantly on the first postoperative day when compared to preoperative values, but returned to preoperative levels on the second postoperative day [125]. In our study, we observed a similar phenomenon. T-bet expression dropped on the first postoperative day and was slightly lower compared to its preoperative level. This lower level continued for the next two days.

Although no statistical difference was found for pre- and postoperative T-bet levels, a decrease of T-bet on postoperative day one and relatively low T-bet levels during the following postoperative days imply that T-bet was down-regulated and that Th1 function might have been impaired, too. Observational studies after major surgery showed that after operative trauma in the early postoperative phase anti-inflammation or even immune-suppression can prevail [126, 127]. As far as we know no randomized controlled trials have been performed to investigate this central issue in a lung-surgery setting, so that it remains difficult to compare our results with

other studies. Of course, because of our study design, we have no evidence to support our hypotheses. The central limitation-factor of this study is the absence of assessment of cytokine signalling. On the other hand, none of our patients showed SIRS or other inflammation or infection symptoms. We assume that no greater, clinically relevant immune-impairment took place and speculate that the cytokine signalling described in other studies took place in our patients too.

4.2 GATA3 expression

In the present study, GATA3 expression showed fluctuations with small range tendencies: a slight increase during the operation and a decrease below preoperative levels after the operation, however not of statistical significance.

Following hepatobiliary pancreatic surgery, GATA 3 mRNA expression level was slightly increased before surgery and further increased on postoperative day 3, 7, and 14 in patients with postoperative infection ($P < 0.05$) [116].

Therefore, the controlled GATA 3 response in our study might show a better maintained immune reactivity, this is clinically coincident because no obvious infectious signs were seen postoperatively.

Generally it is thought that trauma produces a shift towards a Th2 response, the degree of which is proportional to the magnitude of trauma involved [128]. Decker et al revealed that Th subsets of patients after conventional aortic aneurysm repair showed a significant shift towards Th2 immunity, which was expressed by the ratio of IFN-γ/IL-4 producing T cells, as well as by the ratio of HLA-DR+ monocytes/CD23+ B-cells) [129]. Hensler and co workers also showed that during the postoperative period after major elective surgery, cytokine levels were decreased for IL-2 and IFN-γ, which are associated with Th1 immunity. In contrast to this, anti-inflammatory cytokine IL-10 was selectively elevated during the late postoperative period, indicating a shift of the Th1/Th2 balance towards a Th2 response [92].In our study,GATA3 expression after surgery showed only non-significant trends compared to preoperative base line. The role of Th2 immunity and its specific transcription factor GATA3 in perioperative immune responses needs further study. And, again, the central limitation-factor of this study is the absence of assessment of cytokine signalling. No specific literature showing the perioperative expression of GATA3 in a similar setting has been performed, thus making a comparison with other results merely

speculative.

4.3 FOXP3 expression

In the present study, the level of FOXP3 decreased slightly since the beginning of the operation, and kept this relative low level until the end of the investigation period. However, the extent of alterations was not significant as compared with the time point prod.

There is not any study on this T cell transcription factor in surgical settings yet.

There is some evidence that suggests a correlation between the expression level of FOXP3 and the activity of Treg. Previous in vitro experiments revealed that Treg has the ability to suppress the proliferation of Th cells primarily in a contact-dependent manner [130]. In animal experiments, Treg was revealed to suppress Th1 immune responses and promote a shift towards Th2 immunosuppressive responses in mice after burn injury [131]. More recently, Mac Conmara and colleagues observed an alteration of Treg in humans after serious injury, as well as of Th1 immunity. They showed for the first time that Treg was significantly increased in trauma patients which showed a suppressed Th1 immunity [132]. But this phenomenon was not found in our study. One possible reason is that the magnitude of injury in our study is not as hard as in theirs. Another possible explanation is the different follow up: 3 days in our study and 7 days after trauma. According to the short history of Treg and FOXP3, findings of Mac Conmara's and our study still not assess enough evidence to explain the role of Treg and FOXP3 in human immune responses after injury. Further studies including the assessment of cytokines signalling are needed to address this issue.

4.4 ROR-γ expression

Recently Pene and his group found out that chronically inflamed human tissues are being infiltrated by highly differentiated Th17 cells. IL-17 and IL-22 concentrations secreted by tissue infiltrating Th17 cells also reached high levels while Th1/Th2 associated cytokines were correlatively depressed [133]. In contrast to these findings, ROR-γ levels in our study showed only slight and transient increases during the operation and a return to preoperative levels during postoperative days. No statistical significances could have been found.

No perioperative studies investigating ROR-γ have been performed yet.

An apparent difference between the studies above and ours is that the studies above both tested samples from local inflammatory sites, while we measured systemic levels of ROR-γ. This implies that Th17 may exert its major role locally in injured tissues. Consistent with our study, Frangen and colleagues retrospectively analyzed systemic concentrations of IL-17 and IL-6 of 71 patients diagnosed with polytrauma and described that only 6% of patients showed significant elevations of IL17 [134]. Their study disagrees with systemic IL 17 as marker of immune response, too. So far, the exact function of this new T cell subset and its specific transcription factor ROR-γ remains unexplained considering human immune responses and particularly acute inflammation and needs to be investigated in larger controlled trials with regard to IL-17 cytokine signalling.

4.5 SOCS1 expression

In our study, SOCS1 levels fluctuated during surgery and declined during the postoperative period. It is known that the SOCS1 promoter contains STAT1-, STAT3- and STAT6- binding sites. If surgical induced immune mediators have the ability to bind with these sites, they would synchronously initiate the expression of SOCS1 [135]. Surgical trauma normally causes the release of various cytokines and induces differentiation of T cell subsets. IFN-γ activates JAK1, JAK2 and induces STAT1; IL-6 mainly activates STAT3 through JAK1. Th1 development is controlled by IL-12-dependent STAT4 and Th2 is so by IL-4-dependent STAT6 activation. Recently, several studies proved that SOCS proteins are involved in the negative modulation of the JAK-STAT pathway, playing an essential role for proper homeostasis of immune responses [135, 136]. An activation of the JAK-STAT pathway in an immune response may induce transcription of SOCS, which then inhibits the JAK-STAT pathway. Although our findings were not of statistical relevance and did not include the assessment of cytokine signalling, they might show a trend describing SOCS1 activation during surgery.

4.6 SOCS3 expression

An increase of SOCS3 in the perioperative period was observed in our study. Between beginning and end of surgery, levels of SOCS3 mRNA significantly rose to a maximum of up to 13-fold.

Like SOCS1, SOCS3 is also regulated by STATs and its promoter contains STAT1 and STAT3 binding sites. Studies targeting cytokine signalling demonstrated that IL-6 induced an activation of STAT1 and STAT3, while IL-10 induced the an activation of STAT3 [137, 138]. An increase of inflammatory cytokines like IL-6 and IL-10 is frequently seen after surgery or trauma. An elevation of IL-6 was reported in patients after major orthopaedic surgery [139]. In addition, both IL-6 and IL-10 were described to be significantly increased in patients with cancer after major surgery [137]. Therefore, we speculate that the release of IL-6 and IL-10 after surgical injury would be expected to activate transcription of SOCS3 as shown in our study. However no cytokine signalling assessment was performed in this study.

4.7 Correlation between T-bet and SOCS1

In our study, T-bet expression significantly increased during surgery. T-bet is the specific transcription factor of Th1 immunity. IFN-γ is the major effector cytokine of Th1 and its expression is linked to T-bet expression as shown in animal experiments with knock out mice [12]. In addition, these experiments with knockout mice indicate that IFN-γ is able to induce SOCS1 expression via STAT1and that SOCS1 is indispensable for the negative regulation of IFN-γ [140]. Mice with knocked out SOCS1 showed stunted growth and finally died within three weeks of age of a syndrome characterized by severe lymphopenia, activation of peripheral T cells, fatty liver degeneration and necrosis as well as macrophage infiltration of major organs, primarily attributed to unbridled IFN-γ signalling [140, 141]. Therefore we suppose that the increment of SOCS1 during surgery might be, at least partially, correspondent to the activation of T-bet, in order to negatively regulate Th1 cell differentiation, preventing a formation of activated Th1 cells producing excess IFN-γ.

While IFN-γ is not the only cytokine that is able to induce SOCS1, other immune mediators, like IL-6, TNF, IL-12 have also been demonstrated to have this capability[142-144]. In order to confirm that this change of SOCS1 is mainly corresponding to that of T-bet, we further analyzed their correlation. We found that levels of SOCS1 had a significant correlation with levels of T-bet on most time points, except for the time point of first postoperative, which only showed a trend (Table 18). Although we did not assess the cytokine signalling in our patients, these results could be supported by findings from Charles E. Egwuagu and colleagues. They reported that differentiation of naïve Th cells into Th1 or Th2 phenotypes is accompanied by preferential

expression of distinct SOCS proteins and that SOCS1 expression is 5-fold higher in Th1 than in Th2 cells [70]. Our study demonstrates that SOCS1 transcription levels are increased by lung surgery and significantly correlate to an activation of T-bet.

4.8 Correlation between GATA3 and SOCS3

IL-6 and IL-10 are both key cytokines of the Th2 answer. Therefore, we further analyzed the relationship between GATA3 and SOCS3, to find out whether the change of SOCS3 is mainly due to an activation of GATA3, marker of Th2 differentiation. However, analysis showed that SOCS3 did not correlate with GATA3 at any time point (Table 19). The reason for this unexpected result may be that IL6 and IL10 are also secreted from other immune cells, such as macrophages or dendritic cells. Furthermore, Th2 cytokines, including IL-6 and IL-10, are not the only cytokines induced by SOCS3 [145]. Besides, SOCS3 is not exclusively induced by STAT1 and STAT3. The slight trend ofGATA3 expression does not support this hypothesis either. In other words, while transcription of SOCS3 mRNA is extremely activated during lung surgery, GATA3 is probably not the main trigger of this SOCS3 activation. Further larger studies including cytokine signalling are needed to clear this question

4.9 Epidural versus intravenous analgesia:

Pain, anaesthesia and surgical trauma are major stress factors causing immunological alterations in perioperative settings. Different pain management concepts may affect immune responses in different ways. Supporting this idea, in patients receiving epidural anaesthesia, Holte and Kehlet demonstrated a reduction of fat metabolism, lactate and ketones, as well as reduced concentrations of endocrine and metabolic response parameters such as catecholamines, cortisol and glucose [146]. Hole also reported that lymphocyte and monocyte function was suppressed during general anaesthesia but maintained during epidural anaesthesia in patients undergoing total hip replacement [147, 148].

Many studies suggest that epidural/spinal anaesthesia has a better protective effect against perioperative stress. A meta-analysis concluded that epidural anaesthesia decreased the overall mortality by approximately one-third, reduced the odds of deep vein thrombosis by 44%, pulmonary embolism by 55%, transfusion requirements by 50%, pneumonia by 39% and respiratory depression by 59%. Incidences of myocardial infarction and renal failure were also

reduced [149]. This advantage might be explained by the mechanism of epidural anaesthesia. Confronted with surgical stress, epidural nerve blocks not only provide analgesia but also decrease neuroendocrine stress by blocking both ascending sensory pathways and descending sympathetic efferences. It is also well accepted that immune system and nervous systems mutually communicate and influence each other [150, 151].

In this study, we observed the mRNA expression of main known Th cell transcription factors in patients intraoperatively receiving either remifentanil intravenously or ropivacaine epidurally as analgesia. Our results showed that mRNA alterations of all tested transcription factors are completely comparable for both groups receiving lung surgery for every sampling time point with the exception of T-bet. T-bet expression showed a significant increment after surgery in the remifentanil group alone, while the ropivacaine group showed no significant increase after surgery but depicts a trend towards the same direction. Referring to immune function, we did not find any advantages of epidural analgesia over intravenous analgesia. Similar to our study, Kawasaki and his group investigated effects of different anaesthesia methods on surgical induced immune responses in patients undergoing upper abdominal surgery. They did not find any advantages from epidural anaesthesia over general anaesthesia [114]. A similarity between the present study and Kawasaki's is that the regions operated on include regions innervated by the phrenic nerve. It is easy to accept that a complete blockade of the afferent pathway should include the phrenic nerve. However, a complete block of the phrenic nerve can only be achieved by an epidural block as high as C3-C4, this cannot be achieved by thoracic epidural catheterisation. So, an incomplete block of the phrenic nerve in the operating field might mask benefits of epidural analgesia affecting immune function. Another possible bias may also lie in the large quantities of cytokines released in the region operated on, which might directly activate stress responses. Taken together, epidural analgesia did not show different effects on Th cell transcription factors in thoracic surgery as intravenous analgesia.

Propofol was applied for anaesthesia induction and maintenance in both groups. Some groups investigated its effects on T helper cells and immune function. Pirttikangas et al. investigated changes of T cells in patients after minor breast surgery and found that propofol can cause an increase of Th cell percentage compared to thiopental [152]. This made us consider that besides surgical stress, Propofol could have been another trigger for T-bet and GATA3 stimulation during

surgery. However, results from the M. Salo groups, who studied effects of Propofol infusion on Th1/Th2 balance by measurements of IFN-γ and IL-4 production in vitro revealed that Propofol at serum concentrations of 3.5μg/ml did not affect the IFN-γ/IL-4 ratio [153]. This dosage is higher than the blood concentration in general anaesthesia, which normally measures about 2.5 μg/ml [154]. In addition, Propofol was previously proved to inhibit the production of cortisol in a dose-related manner in vitro [155]. This profile implied that Propofol might impact immune function through the endocrine system. But van Hemelrijck et al. demonstrated that clinical doses of propofol (90-200 μg/kg/min) did not affect cortisol synthesis or the response to adrenocorticotropic hormone (ACTH) stimulation in vivo[156]. Therefore, the infusion of Propofol seems to be unlikely to affect the immune function in the perioperative setting.

4.10 Limitations

The major limitations of the present study were:

1) The cytokine signalling downstream of the transcription factors has not been measured. The perioperative reaction on protein level described in the current literature has not been verified and our conclusions must remain speculative.

2) The mRNA expression of transcription factors in whole blood provides only a surrogate parameter for evaluating the dynamic of immune responses in very complex pathways.

3) The number of patients randomised in the two groups was small; therefore, the risk of a type 2 error cannot be excluded.

4) All patients were treated with thoracic epidural block after surgery in order to provide our patients with state of the art analgesia. The study specific treatment was limited to the intraoperative period.

However, biases were kept as much reduced as possible:

- **Study design:** In order to minimise biases we planned a prospective, randomised, double-blind, controlled trial. The project was approved by the local ethics committee and the German Federal Institute for Drugs and Medical Devices (BfArM).
- **Patient selection and exclusion criteria:** Consecutive patients were screened and exclusion criteria were predefined in the study protocol. Particularly, no patients with pre-existing imbalances of the immune system were included into the study in order to minimise biases.

- **Clinical settings:** All patients were operated in the same centre by the same team following a predefined protocol in order to allow comparable general conditions for each patient. Blood withdrawals were performed on predefined time points following a standardised protocol.

- **Laboratory techniques:** The PAXgene blood RNA system used in this study provides an efficient and standardized method for blood collection. This product reduces RNA degradation and prevents it from any post-sampling stimulation, which is essential for the investigation of highly reactive genes. Indeed, some previously published studies showed that blood collection in EDTA tubes and cell purification is accompanied by profound changes in mRNA expression [157]. Despite several sources of data variation, Bustin et al. suggested that fluorescence based qRT-PCR provides more sensitivity and specificity to the detection of mRNA levels as compared with conventional PCR [158, 159]. The qRT-PCR uses fluorescent reporter dyes to combine amplification and detection steps ofthe PCR reaction in a single tube format. The assay relies on measuring an increase in fluorescent signal, which is proportional to the amount of DNA produced during each PCR cycle. The use of TaqMan in the present study is also essential for its accuracy. The main advantage of qRT-PCR with TaqMan chemistry is its selective detection of accumulating specific setting PCR products and the fact that is does not bind all double-stranded DNA which includes non-specific reaction products. To minimize the bias of mRNA level differences between patients, a $2^{-\Delta\Lambda CT}$ method was used for calculation and analysis of qRT-PCR data.

- **Demographics and clinical characteristics:** Demographic and clinical characteristics such as age, sex, BMI, ASA-classification, cardiovascular diseases, beta-blocker therapy, anti-diabetic therapy and current Smoking did not differ between both groups. In addition, anaesthesia time, surgery duration and techniques did not show any significant differences between the two groups. These are favourable premises for a reliable comparison between groups.

4.11 Conclusions

1) T-bet expression was significantly increased after surgery compared to base line. Subgroup

analysis showed that T-bet expression was significantly increased in the remifentanil group but not in the ropivacaine group, which only showed a trend towards the same direction.

2) SOCS3 expression showed no difference between groups. The n-fold changes of T-bet significantly correlate with the n-fold changes of SOCS1 at all time points except for the first postoperative day, which nevertheless shows a clear trend.

3) This study showed that maintaining intraoperative analgesia with ropivacaine via thoracic epidural catheter or remifentanil i.v. did not show significant differences in the transcription factors despite the fact that T-bet differed between groups immediately after surgery. Due to the fact that clinical outcome did not differ between groups, it can be assumed that the intraoperative analgesia considering the minimal effects on the transcriptional level can be maintained with any of these procedures. The postoperative period was not investigated as it was considered not to be ethical to allow that patients might suffer from more pain than unavoidable because they are denied to benefit from the evidence-based better maintained analgesia with the epidural catheter after surgery.

Abstract

Lung surgery causes serious alterations of human immune functions, the extent of which subsequently impacts patients' outcome. T cells, including subtypes Th1, Th2, Treg and Th17, play an important role in the immune response, involving both cell mediated and humoral immunity. There is some evidence that the differentiation and development of these Th cell subsets are controlled by their specific transcription factors T-bet, GATA3, FOXP3 and ROR-γ, and that they are being negatively regulated by SOCS1 and SOCS3. Different pain management may lead to different perioperative inflammatory reactions. In this study we investigated the perioperative alterations of these transcription factors, aiming to achieve a deeper understanding of the differences in the perioperative immune response at the genetic level between thoracic epidural block compared to i.v. analgesia.

After having obtained written informed consent, 40 patients were allocated randomly and received either intravenous remifentanil analgesia or epidural ropivacaine analgesia in a doubleblind manner. Blood samples were collected on the day before the operation as baseline, the point of lung resection, the end of operation and the first, second and third postoperative day. Total blood mRNA was extracted and synthesized into cDNA. Relative mRNA expression of the genes T-bet, FOXP3, ROR-γ, SOCS1 and SOCS3 was measured by quantitative real time PCR. Relative alterations compared to baseline levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Surgical stress rapidly induced the increase of T-bet and SOCS3 in the remifentanil group in a significant way whereas this wasn't the case in the ropivacaine group. Further significant changes were not to be found. Alterations of SOCS1 and T-bet expression levels were significantly correlated over the perioperative period. No difference between the two groups in basic patient characteristics and clinical outcome were seen.

The significant immediate postoperative elevation of the transcription factors T-bet and SOCS3 in the remifentanil group might be a hint for an accentuated inflammation due to operative stress. Nevertheless one has to take into account the fact that downstream cytokine signalling was not investigated, which is a major drawback of this thesis. A significant difference between the groups in the clinical course could not be found. However it has to be considered that for ethical reasons postoperative analgesia was maintained using the epidural catheter in both groups.

Abstract

Operative Eingriffe an der Lunge verursachen tief greifende, outcome-relevante Veränderungen des Immunsystems. T-Lymphozyten und deren Subpopulationen Th1, Th2, Treg und Th17 spielen eine Schlüsselrolle sowohl in der zellulären als auch in der humoralen Immunabwehr. Es gibt Hinweise darauf, dass die Differenzierung und Entwicklung dieser Subpopulationen durch die für sie spezifischen Transkriptionsfaktoren T-bet, GATA3, FOXP3 und ROR-γ gesteuert und durch die inhibitorischen Faktoren SOCS1 und SOCS3 gehemmt werden. Verschiedene Schmerztherapiekonzepte können zu unterschiedlichen perioperativen Inflammationsreaktionen führen. In dieser Studie wurden die entsprechenden Veränderungen dieser Transkriptionsfaktoren untersucht, um ein tieferes Verständnis der Unterschiede zwischen thorakaler Periduralanalgesie und intravenöser Opiatanalgesie in Bezug auf die perioperative Immunreaktion auf der genetischen Ebene zu gewinnen.

Vierzig Patienten wurden nach Aufklärung und Einwilligung in zwei Gruppen randomisiert und erhielten doppelblind entweder eine thorakale Periduralanalgesie oder eine kontinuierliche intravenöse Opiatanalgesie mit Remifentanil. Die Blutentnahmen erfolgten präoperativ als Baseline, intraoperativ zum Zeitpunkt der Lungenresektion, am Ende der Operation und jeweils am ersten, zweiten und dritten postoperativen Tag. Aus den Blutproben wurde mRNA extrahiert und in cDNA umgeschrieben. Mittels quantitativer RT-PCR wurde die relative Genexpression der Transkriptionsfaktoren T-bet, FOXP3, ROR-γ, SOCS1 and SOCS3 gemessen. Relative Veränderungen der Genexpression in Bezug auf die Baseline wurden mit der $2^{-\Delta\Lambda CT}$-Methode ermittelt.

Infolge des durch die Lungenresektion verursachten operativen Stresses stiegen die Transkriptionsfaktoren T-bet und SOCS3 in der Remifentanilgruppe unmittelbar postoperativ im Vergleich zur Ropivacaingruppe signifikant an. Weitere signifikante Veränderungen konnten nicht gefunden werden. Veränderungen der Genexpression des inhibierenden Faktors SOCS1 und des Th1-spezifischen Faktors T-bet zeigten eine signifikante Korrelation über die gesamte perioperative Zeit. Es haben sich zwischen den Gruppen keine Unterschiede in den demografischen und klinischen Merkmalen der Patienten sowie im klinischen Outcome gezeigt.

Die unmittelbar postoperativ signifikant erhöhten Transkriptionsfaktoren T-bet und SOCS3 in der Remifentanilgruppe könnten ein Hinweis darauf sein, dass der operative Stress zu einer stärkeren Inflammation geführt hat, obwohl in Betracht gezogen werden muss, dass die

Zytokinexpression am Ende der Kaskade nicht erfasst wurde, was die Haupteinschränkung dieser Studie bezüglich ihrer Aussagekraft darstellt. Klinisch konnte kein signifikanter Unterschied zwischen beiden Gruppen gefunden werden. Dennoch sollte beachtet werden, dass beide Gruppen aus ethischen Gründen eine postoperative Analgesie mit Ropivacain epidural erhielten.

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List of Abbreviations

ACTH	AdrenoCorticoTropic Hormone
APC	Antigen Presenting Cell
ASA	American Society of Anesthesiologist score
BMI	Body Mass Index
BS	Blood Samples
CABG	Coronary Artery Bypass Grafting
CD	Cluster of Differentiation
CDC	Centers for Desease Control and prevention
cDNA	complementary DeoxyriboNucleic Acid
Ch	Charrier
CI	Clearance Index
CIS	Cytokine-Inducable SH2 protein
CNS	Central Nervous System
CSF	CerebroSpinal Fluid
Ct	threshold cycle
ECG	ElectroCardioGramm
EDTA	EthyleneDiamineTetraacetic Acid
F	French
FiO2	inspiratory Oxygen concentration
FOXP3	FOrkhead boX P3
GATA	able to bind to the sequence G-A-T-A
H20	Water
HLA-DR	Human Leukocyte Antigen DR
HPA	Hypothalamic Pituitary Adrenal
IBP	continuous Invasive Blood Pressure
HPRT	Hypoxanthine PhosphoRibosylTransferase 1
ICU	Intensive Care Unit
IFN	Interferon
IL	Interleukin
iop	intraoperative (during lung resection)
i.v.	intravenous
JAKs	Janus kinases
LIFR	Leukaemia Inhibitory Factor Receptor
LPS	LipoPolySaccharides
LTi	Lymphoid Tissue inducer
MHC II	Major Histocompatibility Complex II
MODS	Multi Organ Dysfunction Syndrome
mRNA	messenger RiboNucleic Acid

NIBP	Non-Invasive Blood Pressure
NK cell	Natural Killer cell
NTC	Non Template Control
NYHA	New York Heart Association functional classification
OLV	One Lung Ventilation
OP	OPeration
opd	operative day (on arrival at the intensive care unit)
PEEP	Positive End-Expiratory Pressure
postopd	postoperative day
qRT-PCR	quantitative real-time Reverse Transcription Polymerase Chain
RR	Respiration Rate
SaO_2	Oxygen Saturation
SEM	Standard Error of Mean
SH2 domain	Src (structurally conserved region) Homology 2 domain
SIRS	Systemic Inflammatory Response Syndrom
SOCS	Suppressor Of Cytokine Signalling
STAT	Signal Transducers and Activators of Transcription
SV	Single Volume
T-bet	T-box expressed in T cells
Tc	T Cytotoxic lymphocyte
TCR	T Cell Receptor
TEA	Thoracic Epidural Anaesthesia
TEB	Thoracic Epidural Block
TGF	Transforming Growth Factor
Th	T helper lymphocyte
TIVA	Total IntraVenous Anaesthesia
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor

Acknowledgements

I gratefully acknowledge Professor Claudia Spies for accepting me as doctoral fellow and for providing me with the topic of this doctoral thesis. Without her continued guidance and support the completion of this thesis would not have been possible.

I am also most grateful to Edoardo Viviano for supporting me in my scientific work; working together with him is a real pleasure.

Especially I would like to thank Markus Renius for his various supports. He took care of the patients, organized the clinical sample provision and provided the clinical data necessary for the analysis of my findings.

I am very grateful to Roman Klemz for giving me always kind support and excellent advice, especially for his instructions into the theoretical background of my work and the experimental techniques.

I would also like to show my respect and thankfulness to Dr. Clarissa von Haefen for her assistance, kindness, patience, thoughtfulness, and encouragement in the study.

Also I would like to thank Jeffrey Bierbrauer for his assistance in the language revision.

I also thank Professor Wernecke for his help in the statistical work.

I thank Professor Volk, director of the Institute of Medical Immunology of the Charité for designing the primer and probes of tested genes.

Furthermore, I would like to express my gratitude to the Charité, namely the Head of the Charité and the Dean, previously Prof. Dr. D. Ganten and Prof. Dr. M. Paul, currently Prof. Dr. K. Einhäupl and Prof. Dr. Annette Grüters-Kisslich, as well as the coordinators of the program at the Charité, Prof. Dr. Dudenhausen and Dr. Arnold, currently Mrs. Voitl and the Department of Anästhesiologie mit Schwerpunkt operative Intensivmedizin, PD Dr. I. Rundshagen and PD Dr. W. Boemke, to work and to study 3 years at this institution.

Finally I give my sincere thanks to all colleagues who work in the laboratory of the Klinik für Anästhesiologie mit Schwerpunkt operative Intensivmedizin for giving me continuous help and support in my work.

Of course, I eternally appreciate my family who gave me endless love and immense mental support.

Erklärung

„Ich, Junjie Zhang, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema: „Influence of thoracic epidural block on the expression of T helper cell transcription factors in patients undergoing lung surgery“ selbst verfasst habe, keine anderen als die angegebenen Quellen und Hilfsmittel benutzt sowie ohne die (unzulässige) Hilfe Dritter verfasst habe und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

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