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Immune perturbations in patients along the perioperative period: Alterations in cell surface markers and leukocyte subtypes before and after surgery

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ABSTRACT

Background: Surgery renders patients susceptible to life-threatening complications, including infections, multiple organ failure, and presumably cancer metastases. Surgery-induced immune perturbations were suggested to contribute to such deleterious effects, but also to facilitate post-injury healing. Preoperative psychological and physiological stress responses may contribute to these immune perturbations, and could thus jeopardize patients even before surgery. The current study assessed the effects of various operations on an array of immune indices during the perioperative period. To qualify immune changes before surgery, patients' immune status was also compared to that of healthy controls.
Methods: A total of 81 subjects (operated patients and healthy controls) provided up to five daily blood samples during the perioperative period, for assessment of leukocyte subtypes (granulocytes, monocytes, Tc, Th, NK, NKT, $CD4^+CD25^+$, $CD8^{bright}CD4^{dim}$, and B cells) and their surface markers (HLA-DR and LFA-1).
Results: Even before surgery patients displayed immune perturbations, including reduced lymphocyte HLA-DR expression and increased monocyte LFA-1 expression. Following surgery, we recorded a reduction in lymphocyte numbers that was subtype specific, increased granulocyte numbers, and reduced expression of HLA-DR by lymphocytes and monocytes. Finally, no significant associations were found between alteration in leukocyte numbers and cell surface markers (although these indices showed high correlations with other variables), implying differential mediating mechanisms.
Conclusion: Several immune alterations are manifested prior to surgery, and contribute to the marked postoperative changes, which are commonly interpreted as immune suppression. We discuss the possible adaptive and maladaptive nature of these perturbations in the context of natural injury, stress, and surgery.

1. Introduction

Following surgery and trauma, changes in a myriad of immune indices are believed to impair host defense mechanisms, leaving the body susceptible to infections and dormant diseases (Angele and Faist, 2002). For instance, postoperative leukocyte expression levels of HLA-DR have been found to negatively correlate with sepsis (Ditschkowski et al., 1999; Haveman et al., 1999; Hershman et al., 1990; Schinkel et al., 1998). Additionally, an exaggerated and prolonged pro-inflammatory cytokine response was reported to contribute to multiple organ failure (MOF), (Hietbrink et al., 2006; Lin et al., 2000). Finally, the long-term appearance of cancer metastases has been linked to reduced cellular immunity in the postoperative period (Ben-Eliyahu, 2003).

Studies have reported several post-surgical alterations in numbers of circulating leukocyte subsets and in their activity: neutrophils, a first line of defense against invading organisms, increase in number after surgery, and release an oxidative burst (Smith et al., 2006). Lymphocyte numbers decrease (excepting B cells) (Franke et al., 2006), and their in vitro proliferation and cytokine secretion abilities are impaired (Angele and Faist, 2002; Hensler et al., 1997). Circulating monocytes express low MHC class II levels (Ayala et al., 1996; Zieren et al., 2000), possibly indicating reduced antigen presentation ((Flohe et al., 2004) but see (Hensler et al., 1997)). The Th1/Th2 cytokine balance has been reported to shift toward Th2 dominance, which limits Th1 pro-inflammatory reactions, and induces a relative paralysis of cellular immunity vis-à-vis of its targets (Hietbrink et al., 2006). It is now believed that the initial postoperative response is pro-inflammatory, contributing to immune activation at the site of injury. However, this pro-inflammatory response induces a systemic anti-inflammatory response that in turn causes suppression of cellular immunity.

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The anti-inflammatory response is thought to be adaptive in restricting inflammation to the site of injury, preventing inflammatory damage to tissue and organs and limiting undesirable systemic immune reactions toward newly exposed host determinants (Munford and Pugin, 2001).

It is unclear why the body promotes seemingly detrimental changes following surgery, including systemic immune suppression. Are they undesirable side-effects of the reaction to trauma, or do they have an adaptive value? We believe that although the above postoperative responses have evolved to promote survival or other adaptive processes following natural injury, some of them are maladaptive in the clinical setting of the operating room. Moreover, the unnatural setting of lesions induced during surgical procedures (sterile as opposed to natural infected injury) may contribute to impaired immune function after surgery. Last, unlike natural injury, patients awaiting surgery may already exhibit altered immune profiles as a result of the underlying disease, medication, and psychological stress (Lutgendorf et al., 2005, 2008). All of these factors were shown to alter metabolic and endocrine processes, and to cause a shift toward Th2 dominance and suppression of cellular immunity, which further contribute to the exaggerated postoperative immune suppression (Shakhar and Ben-Eliyahu, 2003; Ni Choileain and Redmond, 2006).

The aim of the current study was to provide a comprehensive view of immune responses, both before and following surgery, focusing on established and new indices relevant to postoperative immune suppression, and identifying preoperative perturbations that may contribute to postoperative effects. Our long-term goal is to promote the development of prophylactic measures against postoperative immune suppression, with minimal disturbance to beneficial postoperative responses. We therefore measured peripheral concentrations of leukocyte subtypes, as well as cell surface expression of MHC class II (HLA-DR) and the LFA-1 adhesion molecule (CD11a). In addition, we studied the cytokine network (specifically IL-10, IL-6, IL-12 and IFN-g) and natural killer (NK) cell activity. These indices were assessed each morning, before surgery and along the postoperative hospitalization period, and were compared to their levels in a non-operated control group. Various types of operations were studied, which were categorized as either 'major' or 'minor–intermediate' (minor and intermediate) surgeries. Although grouping different operations may mask or even cancel-out effects that are unique to a specific surgery, results that are manifested in multiple surgeries could be considered common and robust.

Importantly, in an attempt to assure that our measurements reflect the in-vivo status of the immune system, we analyzed fresh blood samples withdrawn no later than 5 h earlier (excluding approximately 20% of the samples that were taken in the evening and kept overnight), and conducted whole-blood assays that maintain the presence of autologous plasma factors, such as cytokines and hormones. Given the large scope of this study, and in order to allow an in depth evaluation and discussion of the results, the cytokine and NK cytotoxicity data have been published separately (Greenfeld et al., 2007). Last, the study was planned to accommodate technical and administrative constraints on blood sampling and analysis (see Section 2.3). These constraints are the result of using fresh samples and employing patients that were hospitalized for different surgeries and different durations (from 1 to 4 days). Thus, whereas all patients provided blood samples on the morning before surgery, and the great majority of them provided blood the morning after surgery, only 50% or less of the subjects provided blood samples on the evenings before and after surgery, and on the mornings of days 2, 3 and 4 after surgery. Our statistical approaches and deductions are therefore adapted to these constraints (see Section 2.6).

2. Materials and methods

2.1. Patients and controls

Fifty-nine patients (age 53, SD 15) that provided multiple blood samples (all of which gave blood on the morning before surgery) were included in the study. Patients underwent various operations under general anesthesia, and were recruited from July to September 2005 in "Hasharon" and "Soroka" Hospitals, in Israel. Patients were recruited during the visit to the preoperative clinic by one of the attending anesthesiologists that participated in the study. Only patients of American Society of Anesthesiologists' (ASA) physical status classification I–III were recruited for this study (see Table 1). Additional exclusion criteria were alcoholism, drug abuse, and consumption of psychotropic drugs or antidepressants.

Three surgeons and two anesthesiologists independently ranked the severity of the surgical procedure to major vs. minor–intermediate, based on the duration, invasiveness, and amount of tissue damage during surgery (Table 1). Ranking corresponded well between these physicians, and the *a priori* distinction between the two categories was almost unanimous.

A control group consisted of 22 healthy age-matched subjects (age 50, SD 9), each providing a single morning blood sample. Controls were recruited by an advertisement posted at the Tel Aviv University campus, indicating restrictions on age and health status, and offering financial compensation for one blood sample. Only healthy control subjects were recruited. Additional exclusion criteria were acute sickness during the last two weeks, alcoholism, drug abuse, and consumption of psychotropic drugs or antidepressants. Females constituted 59.8% of all subjects and their ratio was similar in all groups (χ^2 test did not indicate significant difference in the male/female ratio between the groups). As expected, patients received premedication prior to surgery, while control subjects (non-operated) did not. The study was approved by the Institutional Review Board (Helsinki committee) of both hospitals, and all participants gave written informed consent before participation.

Table 1
Classifications of surgery types (Surgery types, their categorization, and patient numbers).

Category	Type of surgery	*n*
Control	Non	22
Minor and intermediate surgery	Hernia	1
	Laparoscopic hernia repair	1
	bladder tumor resection	1
	Laparotomy cholecyctectomy	9
	Pyeloplasty	1
	laparotomy gastric banding	11
	laparotomy revision gastric Banding	1
	laminectomy	1
	laparotomy adrenalectomy	1
	Prostatetectomy	1
Major surgery	laparotomy sygmoidectomy	1
	Thoracotomy lobectomy	1
	radical hysterectomy	1
	TKR	6
	Right hemi colectomy	1
	laparotomy hemycolectomy	3
	Gastrectomy	1
	Laparotomy & colon resection	1
	spinal fusion	2
	pancreas whipple resection	1
	Right nephrectomy	1
	Open nephrectomy	1
	Inguinal Hernia	3
	THR	7
	TAH BSO	1

2.2. Blood withdrawal and the immediate assessment of immunological indices

Venous blood was collected from patients and control subjects between 7:30 and 9:00 A.M. into heparinized vacuum tubes (30 U of preservative-free heparin per ml blood). Blood sample from control subjects were collected throughout the study period, one morning sample per subject simultaneously with patients' samples. In operated subjects, day 0 represents the morning of surgery (before preparation of the patient for surgery and before premedication), and the following mornings are numerically ordered (1, 2, and 3–4). Blood samples were immediately delivered to our laboratory at Tel Aviv University, where FACS analyses were preformed on the fresh samples starting at 10:30–11:00 A.M. and ending at 12:30–1:00 P.M. (time of antibody staining for the FACS analysis).

2.3. Repeated blood sampling and evening samples

Whereas all patients provided blood samples on days 0, only the great majority of them provided blood on day 1, and most patients provided samples until their release from hospital (Fig. 1). As planned, blood samples were not collected on Saturday, during which the laboratory did not operate, a fact which accounts for most of the absent morning samples in some patients. Minor–intermediate surgery patients provided blood samples up to day 1, and major surgery patients up to day 4, although days 3 and 4 were addressed as one category ("3–4"), given the relatively small sample size at these time points. Additionally, blood was withdrawn from approximately 50% of the patients on the evening (6:30–9:00 P.M.) before surgery (day −0.5) and the evening following surgery (day 0.5), depending on patients' accessibility. These samples were refrigerated overnight and delivered to the lab on the following morning, thus analyzed approximately 17 h after withdrawal. In a pilot study we found that some indices are altered following whole blood storage, but refrigeration slowed down this process. Thus, it is expected that at least some indices will be affected by overnight storage, and we have therefore treated the interpretation of these results accordingly.

2.4. Flow cytometry

Fluorescence-activated cell sorter (FACScan, Becton Dickinson) analysis was used to assess the white blood cell numbers. Fifty microliters of whole blood were incubated for 15 min at room temperature with the following three sets of mouse anti-human antibodies: (1) RPE-Cy5-conjugated anti-CD3, FITC-conjugated anti-CD16 and PE-conjugated anti-CD56 (Dako, Glostrup, Denmark). (2) RPE-conjugated anti-CD8, FITC-conjugated anti-CD25 and RPE-Cy5-conjugated anti-CD4. (3) RPE-conjugated anti-CD14, FITC-conjugated anti-CD11a and PE-Cy5-conjugated anti-HLA-DR. Erythrocytes were then lysed using Lysing Solution (Becton Dickinson, San Jose, CA) and washed twice in PBS containing 2% fetal calf serum and 2% NA_2N_3. A total of 20,000 events were acquired.

2.4.1. Cell identification

Fluorescent cell labeling was analyzed by three-color flow cytometry. Total lymphocyte numbers were identified according to side and forward scatter. Lymphocyte subsets were identified within the lymphocyte population as follows: T helper cells (Th): $CD4^+$, CTLs (Tc): $CD8^{+bright}$, $CD4^+CD25^+$, Natural killer cells (NK): $CD3^-$ and either $CD16^+$ or $CD56^+$. Natural killer T cells (NKT): $CD3^+$ and either $CD16^+$ or $CD56^+$. B cells: Total lymphocytes—(Th + Tc + NK + NKT). Monocytes were identified as $CD14^+$, and granulocytes were identified by side and forward scatter, minus $CD14^+$ cells.

To assess the absolute number of cells per μl of blood, a fixed number of polystyrene microbeads (20 μm; Duke Scientific, Palo Alto, CA) were added to the blood samples before preparation for cytometric analysis.

2.4.2. Expression levels

LFA-1 and MHC class II expression levels were assessed by quantifying expression levels of CD11a and HLA-DR, respectively, on different cell types (lymphocytes, monocytes, granulocytes) that were positive for these markers.

2.5. Assessment of cortisol levels

Total plasma cortisol levels where evaluated using ELISA (R&D Systems, Minneapolis) based on manufacture instructions. The manufacture reported sensitivity of the assay was 0.071 ng/mL with an average intra-assay coefficients of variances of 8.6%.

2.6. Statistical analysis

As detailed above, the study was planned to accommodate technical and administrative constraints on blood sampling. Thus, most patients did not provide blood samples at all time points, and only 50% or less of the subjects provided samples on the −0.5, 0.5, 3 and 4 time points (Fig. 1). Therefore, a within-subject statistical analysis would be inappropriate, and we always conducted a between-subject factorial ANOVA. It should be noted that substituting a within-subject analysis with a between-subject analysis yields more conservative results (specifically when within-subjects correlations are positive, as in our study).

Specifically, days were used as the independent variable in analyses that aimed at comparing day differences within each surgery group (e.g., within the major surgery group) as is specified in Tables 2 and 3. Groups were used as an independent variable when comparing between the groups on day 0 (i.e., comparing control to major surgery to minor–intermediate surgery, on day 0) as is specified in Table 4. To compare between the two surgery groups along the perioperative period, a 2×4 between-subject ANOVA was used (major surgery vs. minor–intermediate surgery, and the −0.5, 0, 0.5 and 1 time points). To evaluate specific pair-wise differences, and given that ANOVA indicated significant differences (e.g., day 0 vs. day 1), Fisher protected least significant difference (PLSD) post hoc analyses were conducted. When only two groups

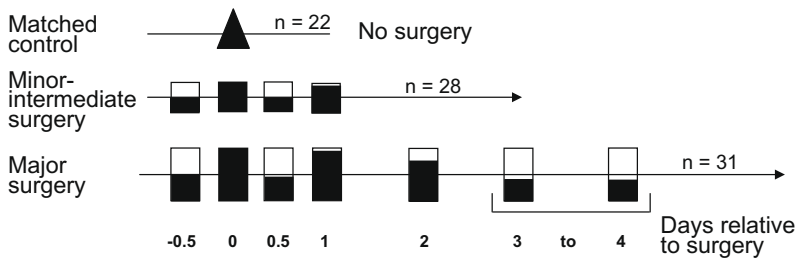

Fig. 1. A schematic presentation of the study's design and blood sampling along the perioperative period. All subject in each group (number indicated by "*n* =") provided blood samples on day 0 (morning before surgery). The proportion of patients providing blood samples in each of the other time point is reflected by the level of black filing.

Table 2
Leukocyte subtypes and statistical analysis. For each leukocyte subpopulations, mean number per μl (SEM) is given for days 0 and 0.5 (Figs. 2 and 3 provide the other 4 time points), followed by ANOVA outcome for day differences and significance levels ($^{*}p < 0.05$, $^{**}p < 0.01$). Significant post hoc PLSD comparison between day 0 and the other days, and between day −0.5 versus day 0.5 are indicated.

Index	Day 0	Day 0.5	*F* value	PLSD $p < 0.05$
	Mean (SEM)	Mean (SEM)		
Major surgery				
Total Lymphocytes	2369(191)	1051(154.7)	$F(5,149) = 9.3^{**}$	Day 0 vs. day 0.5,1,2
Tc ($CD8^+$)	461(42)	172(26)	$F(5,145) = 7.4^{**}$	Day 0 vs. 0.5,1,2; −0.5 vs. −0.5
Th ($CD4^+$)	1063(107)	343.3(53)	$F(5,147) = 9.8^{**}$	Day 0 vs. 0.5,1,2; −0.5 vs. 0.5
NKT	118(17.5)	67.2(16.4)	$F(5,145) = 2.5^{*}$	Day 0 vs. 3–4, −0.5
NK	251(36)	153(22.6)	$F(5,146) = 2.9^{*}$	Day 0 vs. 0.5, 2; −0.5 vs. 0.5
$CD8^{bri}CD4^{dim}$	53(12.4)	21.8(4.9)	$F(5,146) = 1.8$	—
$CD4^+CD25^+$	66(10.4)	14.5(2.5)	$F(5,145) = 5.6^{**}$	Day 0 vs. 0.5
$CD4^+CD25^+$/Th	6.2(0.6)	4.7(0.6)	$F(5,148) = 4.8^{**}$	Day 0 vs. 2
$HLA\text{-}DR^+$ lymph	395(52.7)	219(39.7)	$F(5,149) = 2.2$ (p = 0.06)	
Granulocytes	3860(369)	7723(1084)	$F(5,148) = 6.7^{**}$	Day 0 vs. 0.5,1,2; 0.5 vs. −0.5
Monocytes	309(43)	423(89)	$F(5,148) = 1.5$	—
B cells	470(107)	316(66.6)	$F(5,147) = 1.4$	—
Minor surgery				
Total Lymphocytes	2803(199)	2057(211)	$F(3,90) = 3.6^{**}$	Day 0 vs.. 0.5, day −0.5 vs. 0.5
Tc ($CD8^+$)	557(56)	358(56)	$F(3,91) = 3.3^{*}$	Day 0 vs. 0.5, and 0.5 vs. −0.5
Th ($CD4^+$)	1262.5(120)	813(100)	$F(3,91) = 6.1^{**}$	Day 0 vs. 0.5; −0.5 vs. 0.5,1.
NKT	145(15.3)	124(22.4)	$F(3,91) = 0.3$	—
NK	306.5(38)	247(41)	$F(3,90) = 1.4$	—
$CD8^{bri}CD4^{dim}$	44.6(9.7)	48.5(13.3)	$F(3,91) = 1$	—
$CD4^+CD25^+$	75.8(9.6)	35.8(6.3)	$F(3,91) = 4.9^{**}$	Day 0 vs. 0.5
$CD4^+CD25^+$/Th	5.8(0.4)	4.3(0.4)	$F(3,91) = 9.1^{**}$	Day 0 vs. 0.5, −0.5
$HLA\text{-}DR^+$ lymph	389(37.4)	322.5(47)	$F(3,91) = 0.9$	—
Granulocytes	3883(432)	6440(851)	$F(3,91) = 7.7^{**}$	Day 0 vs. 0.5,1
Monocytes	308(40)	358.4(59)	$F(3,91) = 1.8$	—
B cells	532(132)	515(79)	$F(3,90) = 0.2$	—

* $p < 0.05$.
** $p < 0.01$.

Table 3
Expression levels of CD11a and HLA-DR on lymphocytes, monocytes, and granulocytes. For each of the three subpopulation, mean expression (SEM) is given for days 0 and 0.5 (Figs. 5 and 6 contain the other time points studied), followed by ANOVA outcome for day differences and its significance levels ($^{*}p < 0.05$, $^{**}p < 0.01$). Significant post hoc PLSD comparison between day 0 and the other days, and day −0.5 versus day 0.5 are presented. HLA-DR expression levels on granulocytes are not presented, as granulocytes are negative for this marker.

	Day 0	Day 0.5	*F* value	PLSD $p < 0.05$
	Mean (SEM)	Mean (SEM)		
Major surgery				
Monocytes $CD11a^+$	236(7.2)	207(6.9)	$F(5,148) = 7.8^{**}$	Day 0 vs. 1,2, 3–4; 0.5 vs. −0.5
Granulocytes $CD11a^+$	58(1.8)	60.4(1.8)	$F(5,148) = 2.7^{*}$	Day 0 vs. 2
Lymphocytes $CD11a^+$	128(4.9)	122(8)	$F(5,148) = 0.4$	—
Lymphocyte $HLA\text{-}DR^+$	176(10.3)	167(11.7)	$F(5,148) = 2.6^{*}$	Day 0 vs. 1
Monocyte $HLA\text{-}DR^+$	107(5.3)	88.4(8.1)	$F(5,148) = 4.4^{**}$	Day 0 vs. 1,2; 0.5 vs. −0.5
Minor surgery				
Monocytes $CD11a^+$	246(9.1)	224(7.4)	$F(3,91) = 5^{**}$	Day 0 vs. 1
Granulocytes $CD11a^+$	57(1.5)	60.4(1.9)	$F(3,91) = 1.3$	—
Lymphocytes $CD11a^+$	128(3.7)	122(6.3)	$F(3,91) = 0.6$	—
Lymphocyte $HLA\text{-}DR^+$	174(11.8)	176(13.3)	$F(3,91) = 0.9$	—
Monocyte $HLA\text{-}DR^+$	112(6.5)	100(5.6)	$F(3,91) = 2.5$, $p = 0.066$	

* $p < 0.05$.
** $p < 0.01$.

Table 4
Differences in expression levels of CD11a and HLA-DR between patients awaiting surgery (day 0) and control subjects.

	Day 0 mean (SEM)				
	Control	Major	Minor–intermediate	*F* value	PLSD $p < 0.05$
Monocytes $CD11a^+$	204(10.3)	236(7.2)	246(9.1)	$F(2,79) = 5.8^{**}$	Control vs. major and minor–intermediate
Granulocytes $CD11a^+$	63.3(2.5)	58(1.8)	57(1.5)	$F(2,78) = 3.3^{*}$	Control vs. major and minor–intermediate
Lymphocytes $CD11a^+$	124(4.7)	128(4.9)	128(3.7)	$F(2,79) = 0.3$	—
Lymphocyte $HLA\text{-}DR^+$	204(10.8)	176(10.3)	174(11.8)	$F(2,77) = 2.9$ $p = 0.059$	
Monocyte $HLA\text{-}DR^+$	118(5.9)	107(5.3)	112(6.5)	$F(2,79) = 0.8$	—

* $p < 0.05$.
** $p < 0.01$.

were compared to each other on a specific day, unpaired *t* test was conducted. Pearson correlation was used to evaluate correspondence within variables (e.g., correlation between numbers of $CD4^+CD25^+$ and the number of $CD4^+$ lymphocytes on a specific day). Pearson correlation was the only within-subject analysis conducted in the study, and it was conducted either within a specific day (correlation between # of $CD4^+$ and # of $CD8^+$ cells on day 2) or across all days (irrespective of day), as specified for each analysis. α level was set to 0.05 for all ANOVA, PLSD and *t* tests, and to 0.01 for all Pearson correlation (to compensate for multiple tests—not more than five for each index).

3. Results

3.1. Outliers and sample exclusion

In each of the indices tested, few outlier results (approximately 1–2% of the samples, ranking higher than 2 SD above the mean) were excluded from the analysis. These outliers are most likely technical measurement errors, as highly correlated indices within a subject were not exceptional. Because of a technical obstacle (plasma samples lost due to a freezer shutdown), plasma cortisol levels were measured only in 17 operated subjects and three controls (see Fig 1). Within these 17 patients, 9 underwent major surgery and 8 minor–intermediate surgery, all of whom had cortisol levels on day 0, and 5–8 from each group had cortisol levels on the other time points. Given the small number of cortisol samples left, the 2 patients groups were joined for a surgery group in all time points.

3.2. Effects of surgery on circulating leukocyte numbers

3.2.1. Lymphocytes and their subtypes

See Table 2 for detailed statistical analysis. In accordance with the available literature, the overall number of circulating lymphocytes was significantly lower following major surgery ($F_{(5,149)} = 9.3$, $p < 0.01$) , while numbers of B cells were unaffected. This lower numbers compared to baseline levels was most prominent on postoperative day 0.5 (55% lower compared to day 0, PLSD $p < 0.01$), and gradually diminished on days 1 and 2 (PLSD = 0.024; PLSD = 0.049, respectively), becoming marginally significant (PLSD = 0.08) on postoperative days 3–4 (Fig. 2). The same pattern, but with smaller alterations, was evident in patients undergoing minor–intermediate surgeries (for detailed statistical analyses see Table 2). Most subsets of non-B lymphocytes displayed a similar pattern, although certain variations seem apparent. Specifically, following major surgery T cells were the major contributors to the lower numbers following surgery. On day 0.5, the greatest effect (79% less cells) was observed in $CD4^+CD25^+$ cells, less so in $CD4^+$ Th cells (67% less) and $CD8^+$ Tc cells (63% less), still lower in NKT cells (42% less), and least in NK cells (24% less), in which the difference reaches statistical significance only at the 0.5 time point (Fig. 2, and Table 2). The statistically significant difference in $CD4^+CD25^+$ numbers with respect to baseline levels was abolished rapidly after surgery (day 1), while Th cell numbers were still different from baseline until day 3–4. this underlies the changes observed in the $CD4^+CD25^+$/Th ratio in major surgeries, which was significantly above baseline levels on postoperative day 2 ($F_{(5,148)} = 4.8$, $p < 0.01$, *PLSD* < 0.05 day 0 vs. 2) (Fig. 3).

3.2.2. Granulocytes and monocytes

See Table 2 for detailed statistical analysis. As existing literature indicates, granulocyte numbers were significantly higher following surgery. Granulocyte numbers were highest on postoperative days 0.5 and 1, and gradually lower on days 2 and 3–4 (For major surgery, $F_{(5,148)} = 6.7$, $p < 0.01$. *PLSD* found $p < 0.05$ for day 0 vs. 0.5, 1, 2; −0.5 vs. 0.5). Monocyte numbers ($CD14^+$) did not show a significant change during the entire period in both major and minor–intermediate groups (Fig. 4).

3.2.3. Differences between operated patients before surgery (on day 0) and control subjects

The number $CD8^{bri}CD4^{dim}$ lymphocytes were significantly different between the three groups on the morning before surgery ($F_{(2,77)} = 4.77$, $p < 0.05$), and PLSD revealed that each of the surgery groups was significantly lower than the control group (PLSD < 0.05 for control vs. major and for control vs. minor–intermediate) (Fig. 2).

3.2.4. Differences between the two surgery groups along the perioperative period

The two surgery groups were compared to each other on days −0.5, 0, 0.5, and 1 using a 2×4 ANOVA (surgery type by time points). There was a significant main effect for surgery type in total lymphocytes, Th, Tc and NKT cells ($F_{(1,192)} = 11.6$, $F_{(1,192)} = 12.8$, $F_{(1,192)} = 13.92$, $F_{(1,192)} = 5.8$, respectively), indicating an overall lower number of these subpopulations in major surgery compared to minor–intermediate surgery along this time period. No significant interaction between the effects of surgery and time points was revealed.

3.3. Expression levels of cell surface markers

Tables 3 and 4 provide details on statistical comparison of the findings that are presented in Fig. 5 and 6. Expression levels of the adhesion molecule LFA-1 ($CD11a^+$) and of MHC class II ($HLA\text{-}DR^+$) were measured on a per-cell level (irrespective of cell numbers) in lymphocytes, granulocytes, and monocytes that were positive for the marker. These cell types markedly differ in expression levels from each other, and surgery-related changes were relatively small (up to 30% difference from baseline levels) yet highly significant and in accordance with effect size reported by other laboratories (Ditschkowski et al., 1999a; Kawasaki et al., 2001; Schneider et al., 2004).

3.3.1. CD11a (LFA-1) expression levels

CD11a expression levels on monocytes were significantly higher before surgery (by 20%) than in the control subjects (Fig. 5a) ($F_{(2,79)} = 5.8$, $p < 0.01$; PLSD = $p < 0.05$ for control vs. major and control vs. minor–intermediate) and were significantly lower after surgery than on day 0, in both the major surgery and the minor–intermediate surgery group (e.g., major surgery $F_{(5,148)} = 7.8$ $p < 0.01$), PLSD = $p < 0.05$ for day 0 vs. day 1, 2 and 3–4 and for day 0.5 vs. −0.5). Granulocytes showed an opposite pattern (Fig. 5b). Their expression levels on day 0 were significantly lower before surgery than in the control subjects ($F_{(2,78)} = 3.3$, $p < 0.05$; PLSD = $p < 0.05$ for control vs. major and control vs. minor–intermediate), and were higher significantly after surgery, reaching control levels (e.g., for major surgery $F_{(5,148)} = 2.7$, $p < 0.05$; PLSD < 0.05 for day 0 vs. day 2). Lymphocyte CD11a expression levels were very similar to control levels and remained unchanged along the perioperative period (Fig. 5c).

3.3.2. HLA-DR expression levels

Expression levels of HLA-DR on lymphocytes and monocytes showed a similar pattern (Fig. 6b and c). After both major and minor–intermediate surgery, expression levels were significantly lower compared to preoperative levels, (e.g., in major surgery monocytes $F_{(5,148)} = 4.4$, $p < 0.01$; PLSD < 0.05 for day 0 vs. day 1 and 2, and day 0.5 vs. day −0.5). Relative to control levels, lymphocytes HLA-DR expression on day 0 was significantly lower (e.g., major surgery vs. control $t_{(51)} = 2.13$ $p = 0.038$). Last, in addition to a significantly lower expression levels, the number of lymphocytes that were positive for HLA-DR after major surgery was 50% lower before surgery

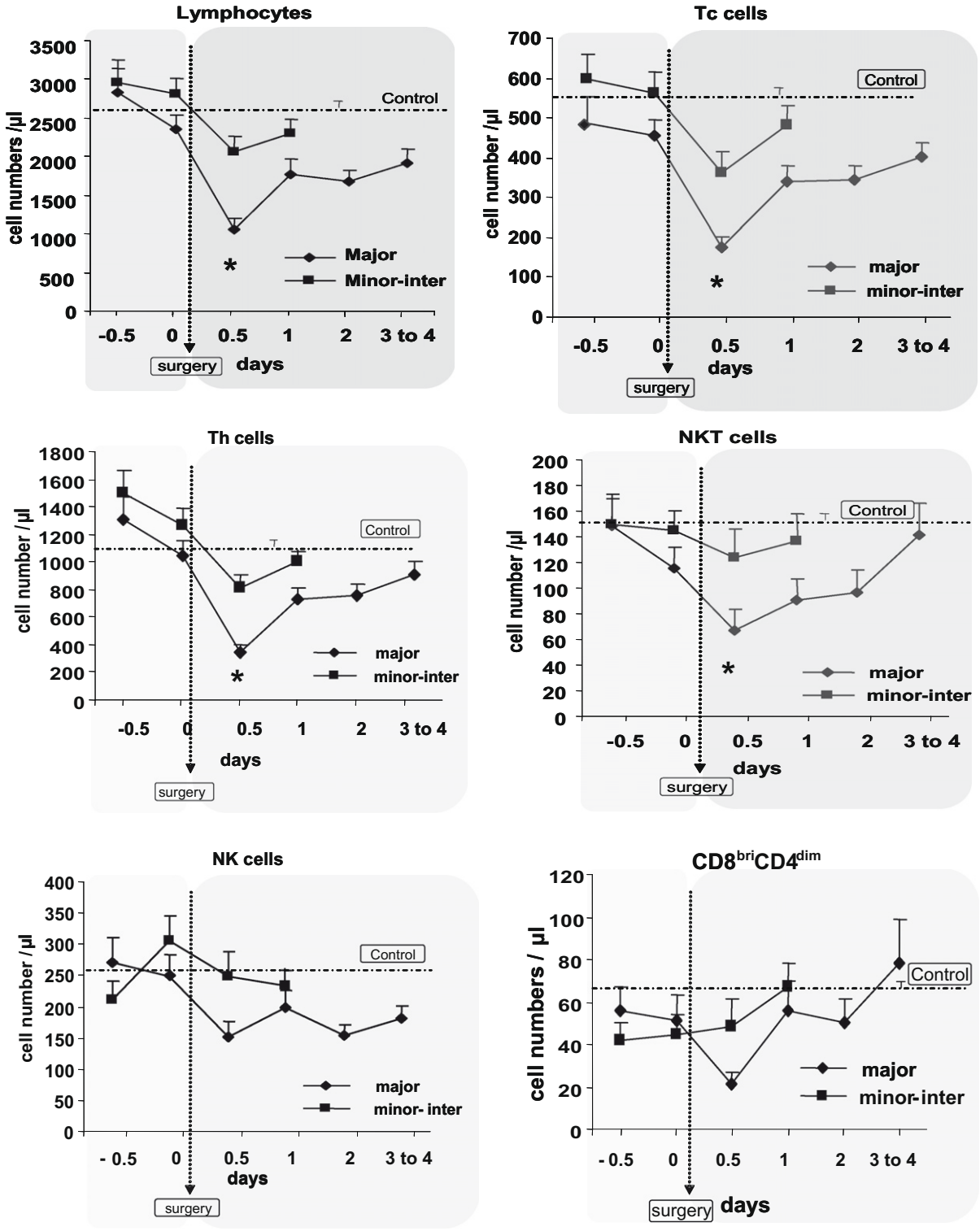

Fig. 2. Mean (+SEM) of lymphocyte subtype cell numbers in major and minor–intermediate (minor–inter) surgeries throughout the perioperative period. The dashed horizontal lines indicate mean levels (+SEM) in the control group. In most lymphocyte subsets we observed a similar pattern (which was more pronounced in major surgery, as marked by asterisk, $p < 0.05$): immediately following surgery cell numbers are significantly lower, and this difference is slowly abolished in the days following surgery. NK cells and $CD8^{bright}CD4^{dim}$ showed different patterns.

($F_{(5,149)} = 2.2$ ($p = 0.06$) for ANOVA, days as independent; $t_{(44)} = 2.58$, $p < 0.05$ for day 0.5 vs. day −0.5)) (Fig 6a).

3.4. Cortisol levels

Cortisol levels in plasma were always assessed in samples taken between 7:30 and 9 A.M., except on time points 0.5 and −0.5, during which blood samples were taken in the evening (6:30–9:00 P.M.). Since levels were assessed only in a relatively small number of subjects (17 patients and 3 controls—plasma samples lost due to a freezer shutdown), for statistical evaluation major and minor–intermediate surgeries (which demonstrated a similar pattern) were grouped to form one operated category. Prior to surgery (day 0), cortisol levels tended to be higher in patients awaiting surgery

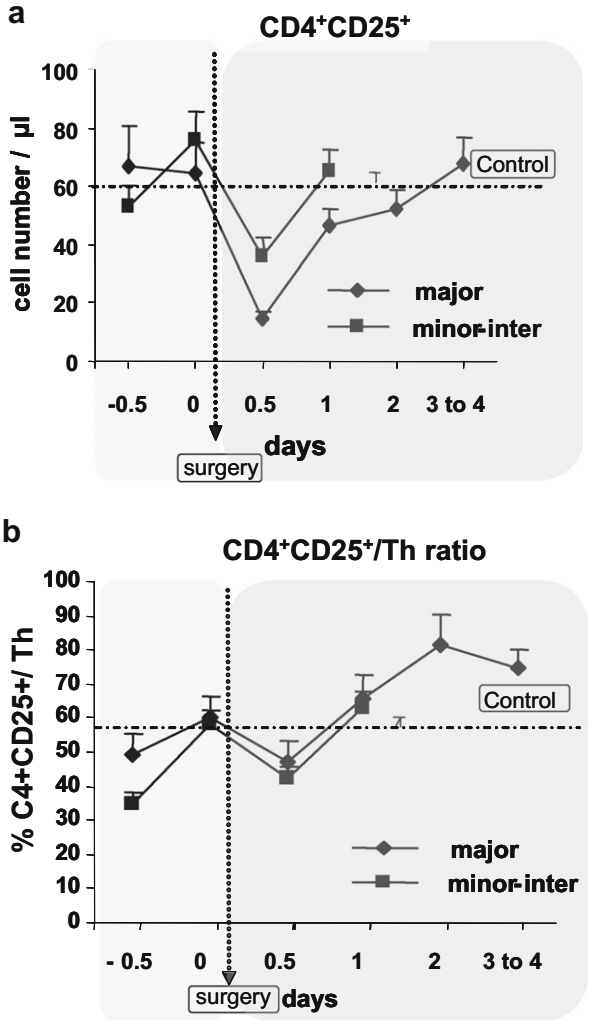

Fig. 3. Mean (+SEM) of (a) $CD4^+CD25^+$ cell numbers in major and minor–intermediate (minor–inter) surgeries. (b) The ratio of $CD4^+CD25^+$ to Th cells, which is significantly higher in the postoperative period. The dashed horizontal lines indicate mean levels (+SEM) in the control group.

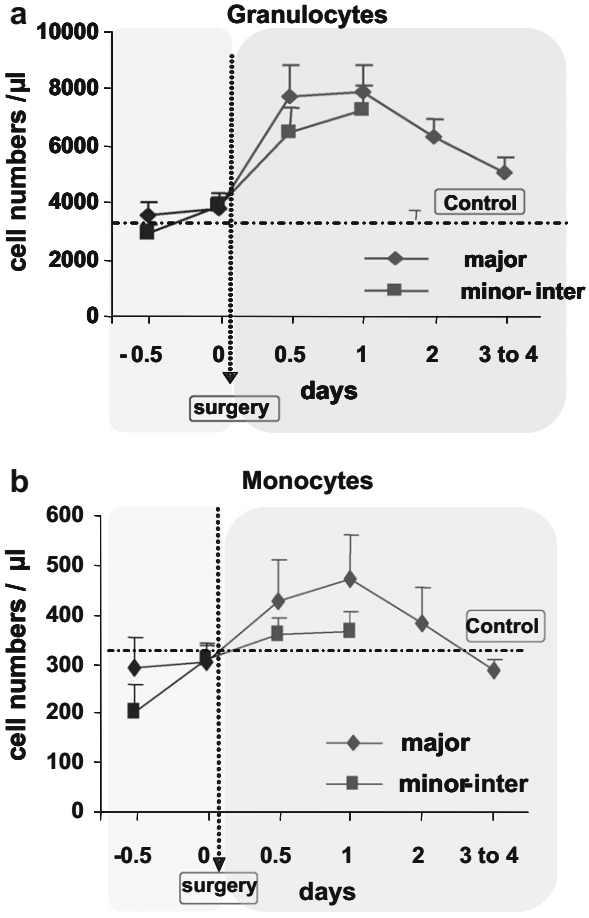

Fig. 4. Mean (+SEM) cell numbers of granulocytes (a) and monocytes (b) in major and minor–intermediate (minor–inter) surgeries along the perioperative period. The dashed horizontal lines indicate mean levels (+SEM) in the control group. Granulocyte numbers are significantly higher postoperatively, but monocyte numbers did not alter significantly along the perioperative period.

(34.42 mcg/dl, SEM = 5.36) than in controls (16.42 mcg/dl, SEM = 4.27) ($t_{(16)} = 2.07$, $p = 0.055$). Notably, in the normal population values of morning cortisol levels are around 20 mcg/dl (Guyton and Hall, 1996). On the evening before surgery (day −0.5) patients' cortisol levels (18.24 mcg/dl, SEM = 5) were higher than standard levels (around 5 mcg/dl), and postoperative evening levels (46.19 mcg/dl, SEM = 8.18, day 0.5) were significantly higher relative to the preoperative evening levels ($t_{(26)} = 3.77$, $p < 0.01$) (Fig. 7).

3.5. Results in subpopulations of patients

Because our patient population was heterogenic, we assessed whether the above findings can be ascribed to definable specific subcategories of patients. Four categories were identified: cancer-bearing patients ($n = 26$), overweight patients ($n = 12$), orthopedic patients ($n = 13$), and gender (26 males). This categorization was added as an independent variable in each of the ANOVAs conducted for each dependent variable, testing for interaction with other factors. No significant interaction between this categorization and any of the above results was revealed, and the patterns of effects were, most commonly, similar in all subgroups of patients. Thus, we cannot ascribe any of the findings to one of the subgroups of patients.

3.6. Correlations

This is the only section in the Results in which we conduct a within-subject analysis (Pearson correlation). As expected, we found very high correlations between different lymphocyte subsets ($r > 0.8$) as described below, as well as moderate correlations between granulocytes and monocytes ($r > 0.6$). On the other hand, we observed negligible and non-significant correlations between expression of surface markers and cell numbers, indicating that separate mechanisms underlie the changes observed in cell numbers and in surface markers that were studied here.

3.6.1. Correlation between indices

A significant correlation was found between $CD4^+$ and $CD8^{bri}$ lymphocyte numbers across all days ($r = 0.76$, $r = 0.83$, for all groups and for major surgery alone, respectively, $p < 0.0001$). Results were similar for $CD4^+$ and total lymphocytes ($r = 0.78$, $r = 0.83$, all groups and major surgery group, respectively, $p<0.0001$), as well as $CD8^{bri}$ and total lymphocyte number ($r = 0.82$, $r = 0.83$ for all surgery groups and major surgery alone, respectively, $p < 0.0001$). $CD4^+CD25^+$ correlated well with $CD4^+$ and $CD8^{bri}$ across all days except day 2 ($r = 0.71$ $p < 0.0001$, day 2 $r = 0.25$ $p > 0.05$ in major surgery).

3.6.2. Correlations with indices described in Greenfeld et al. (2007)

Partition of the data collected in this study into the different manuscripts was not random, but rather based on identifying clus-

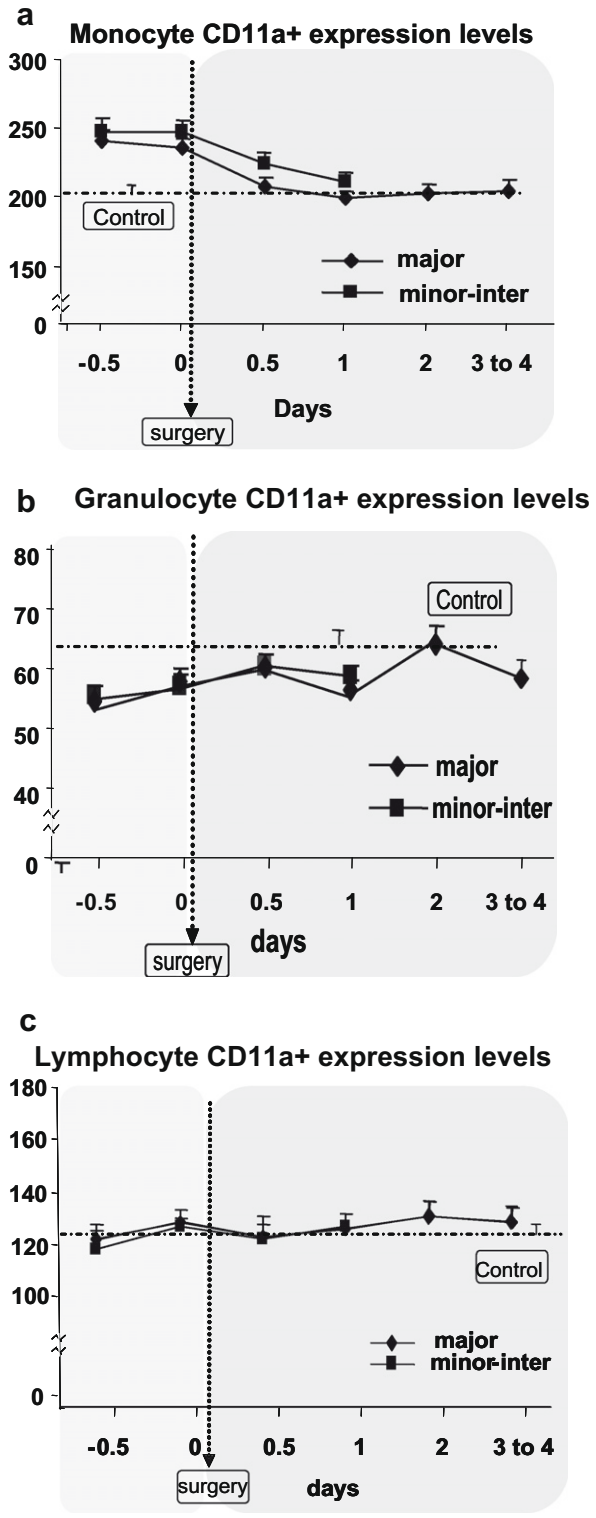

Fig. 5. Mean (+SEM) expression levels of CD11a (LFA-1) in major and minor–intermediate (minor–inter) surgeries along the perioperative period. Levels were markedly higher before surgery on monocytes (a), markedly lower on Granulocytes (b), and remained unaltered in lymphocytes (c). The dashed horizontal lines indicate mean levels (+SEM) in the control group.

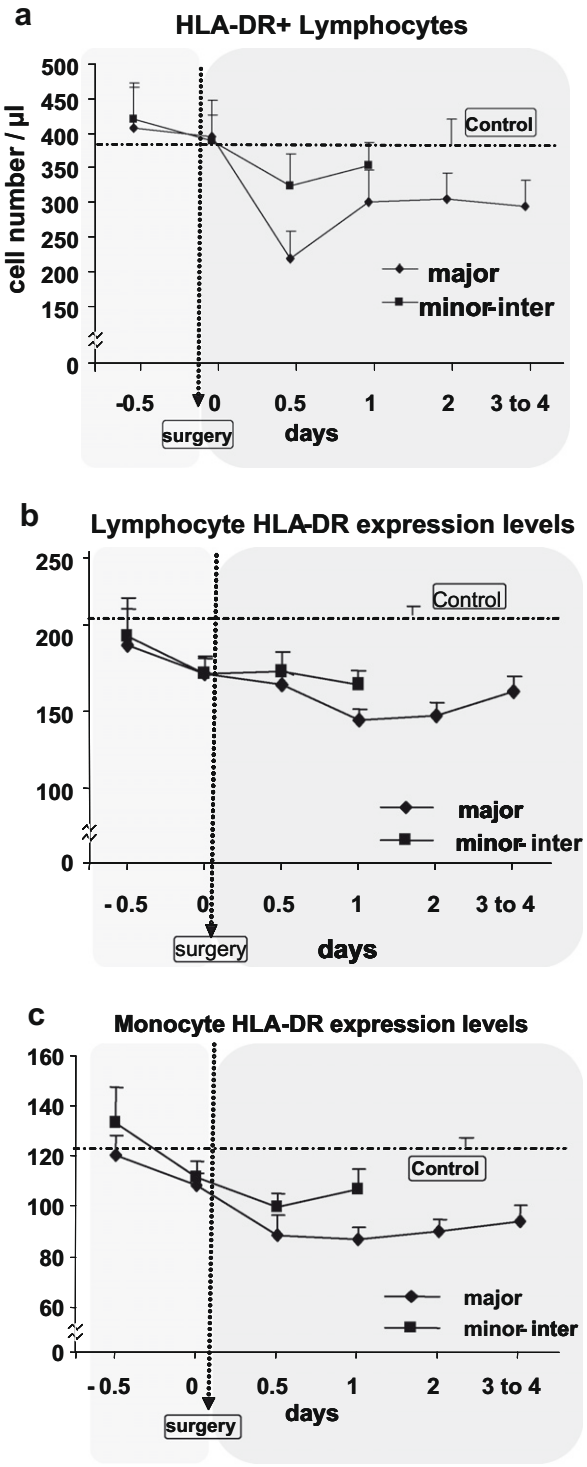

Fig. 6. (a) Number of circulating HLA-DR (MHC II) positive cells/µl blood (Mean + SEM) is significantly lower following major surgery. (b and c) Expression levels of HLA-DR in major and minor–intermediate (minor–inter) surgeries along the perioperative period in HLA-DR positive cells. In lymphocytes (b), levels were significantly lower than control levels before surgery and even more so postoperatively. In monocytes (c) significantly lower numbers were evident in the postoperative period. The dashed horizontal lines indicate mean levels (+SEM) in the control group.

ters of variables that correlated with each other (within a manuscript), while not correlating (or weakly relating) to variables of the other manuscript. Variables in each manuscript did not show substantial correlations with variables of the other manuscript ($R^2 < 0.15$), whereas within each manuscript R^2 was often >0.4.

When indices were correlated separately for each day in the perioperative period of the major surgery group, a few moderate, yet significant correlations were found. IL-6 induced levels moderately correlated with total lymphocyte number on day 2, and on

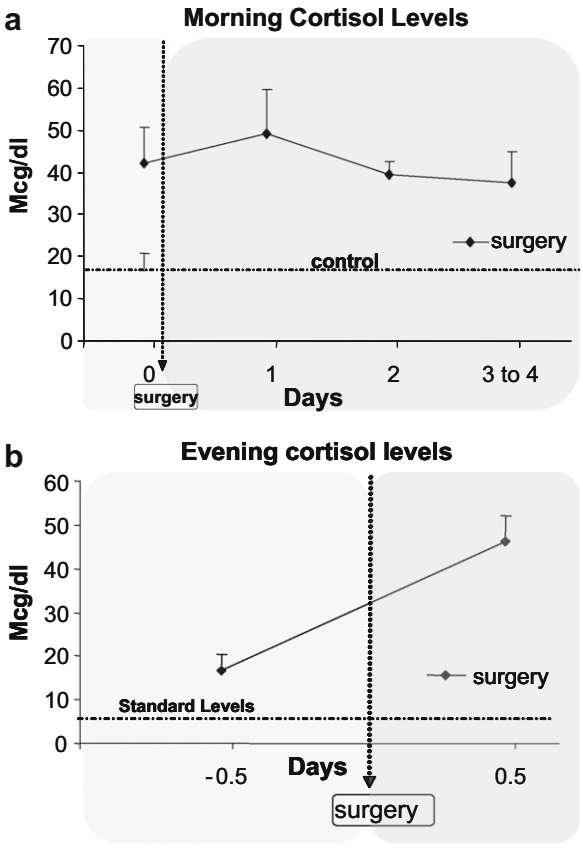

Fig. 7. Mean (+SEM) levels of cortisol. (a) Significantly higher levels of morning cortisol levels were evident in patients awaiting surgery, and remained high along the postoperative period. (b) Evening levels were markedly higher than standard levels both before and following surgery. The dashed horizontal lines indicate mean levels (+SEM) in the control group/standard population levels.

day 3–4 (day 2, $R^2 = 0.18$, day 3–4 $R^2 = 0.28$ $p < 0.05$). This pattern was repeated for some lymphocyte subsets: $CD8^{bri}$ (day2 $R^2 = 0.13$, day 3–4 $R^2 = 0.26$ $p < 0.05$) $CD4^+$ (day2 $R^2 = 0.17$, day 3–4 $R^2 = 0.21$ $p < 0.05$), $CD4^+CD25^+$ (day2 $R^2 = 0.19$, day 3–4 $R^2 = 0.15$ $p < 0.05$). Another medium correlation was found between IFNγ plasma levels and some lymphocyte subsets, but only for the preoperative period. INFγ correlated with $CD8^{bri}$ (day −0.5, $R^2 = 0.2$, day 0 $R^2 = 0.25$ $p < 0.05$), with $CD4^+CD25^+$ (day −0.5, $R^2 = 0.26$, day 0 $R^2 = 0.17$ $p < 0.05$). The lack of correlation on the postoperatively operative days in this index could be due to a floor effect of postoperative IFNγ levels.

4. Discussion

Our study aimed at depicting the immune profile of patients along the perioperative period, across various operation types. A specific objective was to characterize potential differences in preoperative immune status between patients prior to surgery and healthy controls. As detailed below, we found several surgery-related alterations in leukocyte subtype concentrations, a decrease in HLA-DR (MHC II) expression on lymphocytes and monocytes, and alterations in CD11a (LFA-1) expression, which were leukocyte-subtype specific. As expected, patients exhibited high levels of cortisol before and in the days following surgery. Lastly, patients prior to surgery exhibited alterations in several immune indices, some of which were exacerbated by the surgical procedure.

Attempts to ascribe clinical significance to these and similar results should be made with caution. The processes underlying changes in peripheral immune indices are often unknown, and it is unclear whether the changes themselves have detrimental or adaptive value. For instance, the herein observed postoperative drop in monocyte CD11a expression levels could have resulted from a decrease in CD11a expression level of individual monocytes, or from elevated ratio of un-primed naïve monocytes that express low levels of CD11a. The original "primed" monocytes expressing high levels of CD11a may have extravagated into injured tissue or may have died. With respect to circulating T cells, the seemingly detrimental postoperative reduction in their concentration, which was evident in this and other studies (Franke et al., 2006; Kolsen-Petersen et al., 2004; Leaver et al., 2000), is believed to be the result of cell necrosis and cell migration (Makarenkova et al., 2006; Smith et al., 2006). This reduction, however, could actually reflect adaptive processes. T cell necrosis could protect the organism against targeting of newly exposed self determinants. Likewise, T cell migration into organs and injured tissue could promote tissue healing, and facilitate interactions with invading organisms found in the skin and lymphatic system (Viswanathan and Dhabhar, 2005). Thus, caution should be exercised when interpreting fluctuations in immune indices in the circulation.

4.1. Immune modulation prior to surgery

In the current study patients prior to surgery were found to differ from healthy controls in several of the immune indices measured. These patients exhibited lower lymphocyte expression of HLA-DR, specific cellular alterations in CD11a expression, and higher plasma cortisol levels. In addition, as indicated in our other publication describing this study (Greenfeld et al., 2007), these patients also demonstrated reduced NK activity and a suppressed Th1 profile (reduced IL-12 production and IFNγ plasma levels).

These differences may be attributed to several factors, including the patients' pre-existing illnesses, the use of medications, and the exposure to psychological stress, all of which have been shown to influence immune competence e.g., (Ader, 2007; Bauer et al., 2001). It is our hypothesis, however, that preoperative chronic or acute stress is a significant contributor to the above preoperative immune perturbations. Supporting this hypothesis is the fact that different subgroups of our patient population (e.g., cancer patients, $n = 26$; overweight patients, $n = 12$; orthopedic patients, $n = 13$), which suffered from different diseases and were differentially medicated, all showed the same pattern of preoperative immune alterations. Additionally, patients awaiting surgery display significantly higher plasma cortisol levels compared to controls, as evident in the current study and in previous studies (Castejon-Casado et al., 2001; Lutgendorf et al., 2008). Corticosteroids and catecholamines, the major stress hormones, are known to promote a shift toward Th2 dominance in the Th1/Th2 balance (Elenkov et al., 1999), and dexamethasone has been shown to induce T cell apoptosis while delaying neutrophil apoptosis (Liles et al., 1995), all of which were evident in the current study before surgery.

Stress effects on immunity have been suggested by some researchers to have an adaptive value, preparing the organism for an optimal response toward upcoming injury that involves infections in the skin compartment. Dhabhar et al. demonstrated that acute stress has adaptive effects as expressed by increased skin delayed type hypersensitivity reaction. They also demonstrated that this effect is mediated by leukocyte trafficking to the skin, and leads to enhanced resistance to local infections (Dhabhar, 2002). Fleshner et al. demonstrated that acute physical or psychological stress causes an increase in eHsp70, which binds to the surface of macrophages and increased pro-inflammatory cytokine secretion, thus enhancing host defense mechanisms in the subcutaneous compartment (Fleshner and Laudenslager, 2004). Specifically related to our findings, the priming of cellular innate immunity could ensure a faster, more potent reaction to invading infectious agents. Increased CD11a expression on monocytes, as

evident in our patients awaiting surgery, is considered indicative of monocyte priming (Torsteinsdottir et al., 1999). CD11a facilitates extravasation and presentation of foreign determinants to lymphocytes. As cortisol was reported to increase CD11a expression on monocytes (Torsteinsdottir et al., 1999), its high preoperative levels in our patients could underlie the observed increase in CD11a.

Another potential benefit of pre-injury stress responses is the prevention of self recognition that might be induced by extracts from damaged tissue following injury. The herein evident decrease in number of HLA-DR positive lymphocytes (from 70% to 40%), and the two-fold decrease in the expression levels of HLA-DR in positive cell (previously also reported by others (Kawasaki et al., 2001)), can reduce self recognition by limiting the presentation of MHC II:self-molecule complexes to T cells (Cozzo et al., 2003). On the other hand, it is important to note that decreased HLA-DR expression is usually considered detrimental, as it represses recognition of foreign microorganisms.

Finally, the $CD8^{bri}CD4^{dim}$ cell population was significantly lower in patients awaiting surgery relative to healthy controls. This cell population has been referred to in literature as activated Tc cells (Kenny et al., 2000) that demonstrate high antigen specificity. It is likely that these cells have a role in controlling prevalent dormant infections such as HSV-1 or HSV-2. A reduced prevalence of this population would potentially lead to increased susceptibility to such infections, and could be the result of the preoperative stress. Indeed, psychological stress has been reported to increase the reactivation of HSV-1 or HSV-2 (Glaser, 2005).

4.2. Immune reaction to trauma

Following surgery, we observed significant alterations to most immune indices measured. Some of these alterations were already evident before surgery. Specifically, a further decrease in HLA-DR expression on lymphocytes and monocytes was evident postoperatively. These exacerbations may be accounted for by the physiological stress responses generated by the surgical procedure, as a decrease in monocyte HLA-DR expression has been vastly documented in the literature following various physiological traumas, both in humans and animals (e.g. (Wakefield et al., 1993)). Interestingly, CD11a expression on monocytes and granulocytes, which were also abnormal prior to surgery, normalized during the postoperative period. These normalizations could be the result of eliminating the cause for the operation, or may reflect other postoperative processes.

Other indices were normal in patients awaiting surgery, but markedly altered following surgery. Most of these results are in accord with existing literature. The rise in circulating neutrophil numbers (as indicated herein by increased granulocyte number), is considered a first line of CMI response, and may be a reaction to danger signals emanating from the injured tissue (Matzinger, 2002). However, following injury neutrophils have also been shown to contribute to immune suppression via release of an oxidative burst and inhibitory factors (e.g., PGE_2), which in turn negatively affect macrophages and lymphocytes (Smith et al., 2006).

The literature is inconsistent concerning alteration in monocyte numbers following surgery. Some researchers reported a decrease in monocyte numbers (Galle et al., 2000; Walsh et al., 2005), while others, as was also evident in the current study, did not observe such a decrease (Frantz et al., 2005). However, it is generally agreed that the functions of monocytes are suppressed by surgery. The reduction we and others observed in monocyte HLA-DR expression supports this notion, and may bring about a postoperative disruption of monocyte-T cell interactions (Schinkel et al., 1998). Following injury, monocyte suppression is thought to result from multiple factors, including increased levels of prostaglandins, nitric oxide, and anti-inflammatory cytokines, as well as decreased metabolic activity (Smith et al., 2006). As monocytes are believed to be a crucial source for pro-inflammatory cytokines (Collado-Hidalgo et al., 2006), their suppression could contribute to the dominant systemic anti-inflammatory reaction in the later stages of the postoperative period.

The herein observed reduction in lymphocyte numbers, first recorded in our study several hours after surgery, has also been vastly documented (e.g., (Lante et al., 2005; Leaver et al., 2000). In the current study we also quantified different subset of lymphocytes (including Tc, Th, NK, NKT, $CD4^+CD25^+$, $CD8^{bri}CD4^{dim}$) before and after surgery, and found that most of them exhibited a similar decrease, excluding B cells. It is noteworthy that lymphocyte numbers, particularly NK cells, increase during surgery and following an initial rise in catecholamine levels (Sullivan et al., 1998), prior to their later disappearance from circulation (Franke et al., 2006).

It is noteworthy that a naturally inflicted abrasion is inevitably contaminated by microorganisms, which activate a strong immune reaction, including a Th1 response. The above immune suppression observed after trauma could have evolved as a regulatory action against this expected Th1 response. However, following a sterile surgical procedure, this regulatory response is unleashed upon a relatively inactive immune system, leading to exaggerated immune suppression.

4.3. Regulatory cells

T regulatory (Treg) cells limit the activity of T cells, and inhibit tumor-primed CD4 cells (Casares et al., 2003). Treg cells are generated in the thymus, as well as in the periphery, where they proliferate following exposure to self-MHC II complexes, in an IL-2 dependant mechanism. Thus, MHC II levels influence Treg homeostasis (Cozzo et al., 2003). Here we studied $CD4^+CD25^+$ cells that were considered T regulatory cells until the discovery of FOXP3. Today it is known that $CD4^+CD25^+$ cells contain recently activated T cells in addition to the regulatory population. Our results indicate that correlations between $CD4^+CD25^+$ and other lymphocytes are disrupted at day 2 postoperatively, at which point there is a significant increase in the ratio of $CD4^+CD25^+$:T lymphocytes. These findings concord with previous literature (Sietses, 1999). We tentatively suggest that this increase in ratio could be a result of exposure to many self-MHC II particles that appear following tissue injury and cell damage. As a result, T lymphocyte action may be highly inhibited following surgery. As Treg cells inhibit tumor-primed $CD4^+$ cells, it may contribute to increased vulnerability to metastases suggested to occur during the postoperative period (Shakhar and Ben-Eliyahu, 2003).

4.4. Limitations and Summary

Several limitations of the study are noteworthy. The diversity of the surgical procedures is a source of both limitations and advantages. Different surgeries may have opposite effects on specific immune indices, and the current study cannot have depicted such surgery-specific perturbations. On the other hand, significant results revealed herein may have broad generalizability. Individual differences stemming from variables such as risk score, existing illness, chronic medication, and subjective anxiety surely influence the reaction to surgery, and need to be taken into account. The current sample size and the diversity of surgery types preclude this study from addressing these issues. Lastly, as no psychological indices were studied, our hypothesis regarding the potential impact of preoperative stress on immune measures is merely based on the high cortisol levels found preoperatively in the current and previous studies (Castejon-Casado et al., 2001; Lutgendorf et al., 2008). However, without interventions to reduce preoperative stress levels or to antagonize stress hormones, no causative relations can be drawn.

In conclusion, postoperative alterations in immune indices are complex, and underlying mechanisms are only partly understood. Here we showed that some of these alterations are already evident preoperatively, may be related to psychological distress, and may contribute to postoperative immune perturbation. It is unclear whether specific postoperative alterations in immune indices have an adaptive value, and to what degree specific non-naturalistic aspects of surgery (e.g., anesthetic agents and lack of bioactive infections) contribute to the detrimental impacts of surgery.

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