Chapter 1

Plasma Cytokine and Growth Factor Profiling during Free Flap Transplantation

Juliane C. Finke, Jingzhi Yang, Marius Bredell, Uwe von Fritschen and Michael O. Glocker

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.70054

Abstract

Ischemia and reperfusion (I/R) is an unavoidable condition during free flap transplantation. Restoration of blood flow is usually associated with a profound inflammatory response. Cytokines and growth factors are the functional proteins which exert their specific influence on injury or repair during the healing period. Plasma concentrations of 18 cytokines and growth factor proteins (IL6, IL8, IP10, TNFα, MCP1, Fractalkine, GRO, bFGF, GMCSF, IFNg, MIP1a, VEGF, sCD40L, IL10, TGFα, IL1β, IL12P40, and TNFβ) have been analyzed with respect to I/R status during microsurgery tissue transplantation in both, artery and vein, from patients by multiplexed immunoassay. Both technical feasibility and biostatistics data analysis approaches were thoroughly assessed. It has been found that, from all investigated proteins, the venous plasma levels of IL6 significantly increased during the ischemia period and mostly sustained their high levels during reperfusion, while venous plasma levels of IL8 showed in general a significant increase in the ischemia period followed by a rapid decrease in the reperfusion period. In conclusion, these findings direct toward an active involvement of tissue-resting leukocytes which may become therapeutic targets for concomitant medication in flap surgery to improve wound healing.

Keywords: IL6, IL8, multiplex analysis, microsurgery, free flap surgery, ischemia-reperfusion injury, reconstructive surgery, protein profiling

1. Introduction

Reconstructive microsurgery represents the most efficient approach to close large or complex tissue defects of the human body [1]. Microsurgical tissue transplantation, a standardized

technique in plastic and reconstructive surgery, unavoidably involves a time of ischemia in which the transplanted tissue can be harmed [2]. Also inevitable is the ischemia-reperfusion injury (I/R injury) [3, 4], limiting tissue survival in any microsurgical tissue transplantation [5]. Minutes after the onset of ischemia, reversible changes appear in tissues [6]. Irreversible injury develops after 20–40 min of sustained ischemia in muscle cells [7]. Intracellular contents of necrotic cells initiate an inflammatory response and activate immune mechanisms. The main pathomechanisms of I/R injury involve the pathologic leukocyte-endothelium interaction [8, 9], production of reactive oxygen species [10, 11], and activation of the complement system [12] which can cause tissue damage [13] but also results in healing. The healing process starts immediately after injury and consists of three phases: inflammation, proliferation, and tissue remodeling [14]. Knowledge about pathophysiology of I/R injury is mostly based on animal models [5, 15]. There are just a few reports focusing on free human muscle tissue transfer [2, 16], and up to now data about molecular processes that occur during free flap tissue transfers of human skin or bone tissue are missing.

Since different tissues react in different ways to ischemia because of their specific metabolisms, I/R injury was studied in subgroups: the microvascular transfer of muscle flaps, fascio-cutaneous flaps, and osteo-cutaneous flaps. Assuming an involvement of tissue-resting leukocytes, intraoperative blood samples from 21 patients from artery and vein were collected at three different time points. To provide data on the protein concentration dynamics of cytokines and growth factors (CGFs), a multiplex bead array assay was applied because of the assay's high sensitivity, high throughput capability, and little sample consumption (only 25 μl plasma/serum) for analyzing numerous analytes in parallel [17, 18].

2. Results

2.1. Assay assessment, full analysis set, and per protocol set

A study with 21 patients encompassed 10 female and 11 male adults with various flap transplants (**Table 1**). The osteo-cutaneous flaps (n = 9) were free fibula flaps for head and neck reconstructions in tumor diseases. Their ischemia times ranged from 90 to 220 min, in average 133 min. The muscle flaps (n = 6) were mostly latissimus dorsi muscle flaps (n = 4), one gracilis muscle flap, and one serratus anterior muscle flap. All muscle flaps were needed for lower limb reconstruction. Their ischemia times varied from 60 to 120 min, in average 78 min. All fascio-cutaneous flaps (n = 6) were radialis flaps. They were used to cover defects in the head and neck areas in tumor diseases. Their ischemia times ranged from 60 to 150 min, in average 78 min.

Mean weights of the transplanted flaps for the osteo-cutaneous group, the muscle group, and the cutaneous group were 92.67 ± 25.63, 249.25 ± 38.26, and 36.25 ± 39.92 g, respectively.

From each of the patients, three blood samples were taken during surgery. Artery (n = 21) represented the blood protein composition at a starting point, for comparison. At time point Vein 1, 18 samples, and at time point Vein 2, 19 samples were taken (see Appendix). Proteins whose concentrations were to be analyzed (i) were selected according to their main functions inflammation, angiogenesis, and apoptosis upon study of literature with respect to ischemia

Free flap type	Patient ID	Gender	Age (y)	Skin island (cm^2)	Bone (cm)	Transplant tissue	Weight (g)	Ischemia (min)
Osteo-cutaneous	101	Male	62	9 × 6	5.5	Fibula	80	90
	102	Male	54	10 × 5	21	Fibula	n.d.[a]	120
	103	Male	55	n.d.[a]	20	Fibula	n.d.[a]	220
	104	Female	66	6.5 × 5	10.5	Fibula	110	95
	105	Male	43	5 × 3	12	Fibula	72	150
	106	Male	58	7 × 4	15	Fibula	n.d.[a]	120
	107	Female	57	6.5 × 5	8	Fibula	98	120
	108	Female	67	6 × 4	12	Fibula	132	100
	109	Female	70	7 × 5	13	Fibula	64	180
Muscle	201	Male	60	–	–	Latissimus dorsi	272	65
	202	Female	66	–	–	Latissimus dorsi	198	60
	203	Male	56	–	–	Latissimus dorsi	284	75
	204	Male	40	–	–	Serratus anterior	n.d.[a]	80
	205	Male	62	–	–	Gracilis	n.d.[a]	70
	206	Female	49	–	–	Latissimus dorsi	243	120
Fascio-cutaneous	301	Female	26	5 × 4	–	Radialis	14	80
	302	Female	51	13 × 11	–	Radialis	96	150
	303	Female	64	6 × 5	–	Radialis	20	80
	304	Male	69	6 × 5	–	Radialis	15	100
	305	Female	79	9 × 5	–	Radialis	n.d.[a]	60
	306	Female	37	14 × 12	–	Radialis	n.d.[a]	60

[a]n.d.: not determined.

Table 1. Patient information and clinical parameters.

and I/R injury and (ii) were matched with a commercially available bead-based immunoassay. From each of the patient samples, plasma concentrations of IL6, IL8, IP10, TNFα, MCP1, Fractalkine, GRO, bFGF, GMCSF, IFNg, MIP1a, VEGF, sCD40L, IL10, TGFα, IL1β, IL12P40, and TNFβ were simultaneously measured in duplicate. The raw data set of all 18 cytokines and growth factors of all samples encompassed 2088 data points which, after averaging and curation, were merged to the full analysis set (FAS) with 1044 protein concentration values (data not shown).

Data from the FAS were inspected for completeness and categorized into three groups based on the detection rates. Group I contained those CGFs for which plasma protein concentrations could be determined in over 80% of all samples. Group II included those CGFs for which plasma protein concentrations could be determined in over 60% but less than 80% of all samples. Group III contained proteins whose concentrations were determined in less than 60% of all samples and sCD40L which did not pass the QC test. Group I proteins were IL8, IP10, MCP1, TNFα, GRO, IL6, Fractalkine, bFGF, and GMCSF. Group II contained four proteins: IFNg, MIP1α, VEGF, and IL10. Five proteins were placed in group III: TGFα, IL1β, IL12P40, TNFβ, and sCD40L (**Table 2**). Group III proteins were not subjected to further data analysis. Hence, the per protocol set (PPS) consisted of just group I and II proteins (13 proteins in total) and contained a total of 754 data points.

Some of the determined average plasma protein concentrations in the analyzed samples matched well with reported reference concentrations (e.g., TNFα and bFGF), whereas others did not. Differences between the "reference concentrations" and the concentrations determined in the here described study could be caused by (i) using different assays, (ii) different specimen (serum instead of plasma), (iii) different laboratory conditions, and (iv) different health conditions. Irrespective of such discrepancies, all the proteins of the PPS fell into the assay's detection range and fulfilled the quality requirements, enabling further data analysis.

The intra-assay precision (CV%) of the determined protein concentrations were calculated for two assay plates of all group I and group II proteins to determine technical reproducibility (**Table 2**). The lowest CV% value of 5.97 was obtained for IP10, and the highest (16.99) for GMCSF. These values were slightly higher than those stated by the assay provider, most likely due to the fact that in the investigated study protein concentration levels varied from sample to sample because of the biological heterogeneities of the donors. Nevertheless, the CV% values were below 20 for all of the 13 cytokines and growth factors of the PPS, which is considered satisfactory [25–27]. Inter-patient CV% values between the averaged samples ranged from 352.63 for IL6 (in artery) to 40.76 for GRO (in artery).

2.2. Determination of data homogeneity

For testing whether or not Vein 1 samples of patients followed a trend with respect to ischemia time, linear regression analyses were performed to correlate ischemia time with mean concentrations of the PPS, i.e., group I and group II proteins (**Figure 1**). Both coefficients of determination (R^2 values) and associated p values showed that there was no significant correlation between the two features for any of the tested proteins. This result indicated that sample values were rather randomly distributed with a fair homogenous distribution and only a few outliers.

In addition, hierarchical cluster analysis was conducted with group I proteins to characterize distribution of protein concentrations between patients. Plasma sampling time points (artery, Vein 1, and Vein 2) were analyzed independently from each other. The dendrograms and heat maps (**Figure 2**) revealed that in none of the sampling time points, the transplant types clustered together. Instead, within the blood sampling time points, all three transplant types seemed randomly distributed.

PPS	Protein name	Uniprot acc. no.	Ref. value (pg/mL)[3]	Artery[c]		Vein 1[d]		Vein 2[e]	
				Mean ± SD (pg/mL)	CV%	Mean ± SD (pg/mL)	CV%	Mean ± SD (pg/mL)	CV%
Group I	IL6	P05231	22.80 ± 7.00[1]	433.37 ± 1528.12	352.62	749.14 ± 916.28	122.31	692.80 ± 905.71	130.73
	IL8	P10145	9.56 ± 0.40[1]	123.55 ± 161.52	130.73	138.29 ± 117.54	85.00	119.45 ± 105.28	88.13
	IP10	P02778	248.00 ± 96.50[2]	759.29 ± 485.42	63.93	860.02 ± 645.74	75.08	815.83 ± 581.65	71.30
	MCP1	P13500	173.20 ± 15.40[1]	2160.00 ± 2626.43	121.59	2654.86 ± 2462.78	92.76	2520.15 ± 2208	87.61
	TNFα	P01375	34.22 ± 11.46[1]	18.78 ± 10.70	56.98	20.50 ± 10.83	52.82	18.67 ± 9.49	50.83
	Fractalkine	P78423	423.00 ± 25.00[3]	137.17 ± 110.49	80.55	126.62 ± 119.15	94.10	151.21 ± 124.79	82.53
	GRO	P09341	212.20 ± 21.80[4]	1780.41 ± 725.66	40.76	2301.17 ± 1287.76	55.96	2423.88 ± 1253.26	51.70
	bFGF	P09038	76.60 ± 17.53[2]	90.76 ± 82.81	91.25	117.07 ± 73.80	63.04	87.27 ± 56.81	65.10
	GMCSF	P04141	2.43 ± 0.08[5]	8.59 ± 6.33	73.70	10.80 ± 10.81	100.11	8.47 ± 6.50	76.73
Group II	IFNg	P01579	18.30 ± 9.15[2]	42.18 ± 92.71	219.80	28.70 ± 39.87	138.89	51.41 ± 100.51	195.51
	MIP1[a]	P10147	88.10 ± 14.31[1]	22.14 ± 33.49	151.27	14.62 ± 16.86	115.34	14.22 ± 16.51	116.13
	VEGF	P15692	32.20 ± 21.80[6]	283.19 ± 356.83	126.00	310.49 ± 385.78	124.25	305.3 ± 349.13	114.36
	IL10	P22301	7.63 ± 5.95[2]	66.79 ± 129.79	194.31	63.28 ± 162.14	256.23	79.89 ± 170.61	213.54

[a]Total number of samples: $N = 58$ from 21 transplantation patients.

[b]Reference concentrations from healthy donors:

[1]Mean ± SE; multiplex bead assay: Yurkovetsky et al. [19].

[2]Median ± quartile difference; multiplex bead assay: Geyer et al. [20].

[3]Mean ± SE; EIA: Damas et al. [21].

[4]Mean ± SD; multiplex bead assay: Hang et al. [22].

[5]Mean ± SE; ELISA: Lee et al. [23].

[6]Mean ± SD; ELISA: Larsson et al. [24].

[c]Artery blood was collected before anastomosis ($N = 21$).

[d]Vein 1 blood was collected directly after anastomosis ($N = 18$).

[e]Vein 2 blood was collected 2 min after Vein 1 blood ($N = 19$).

Table 2. Averaged plasma concentrations of the per protocol set cytokines/growth factors in artery and vein samples[a].

http://dx.doi.org/10.5772/intechopen.70054

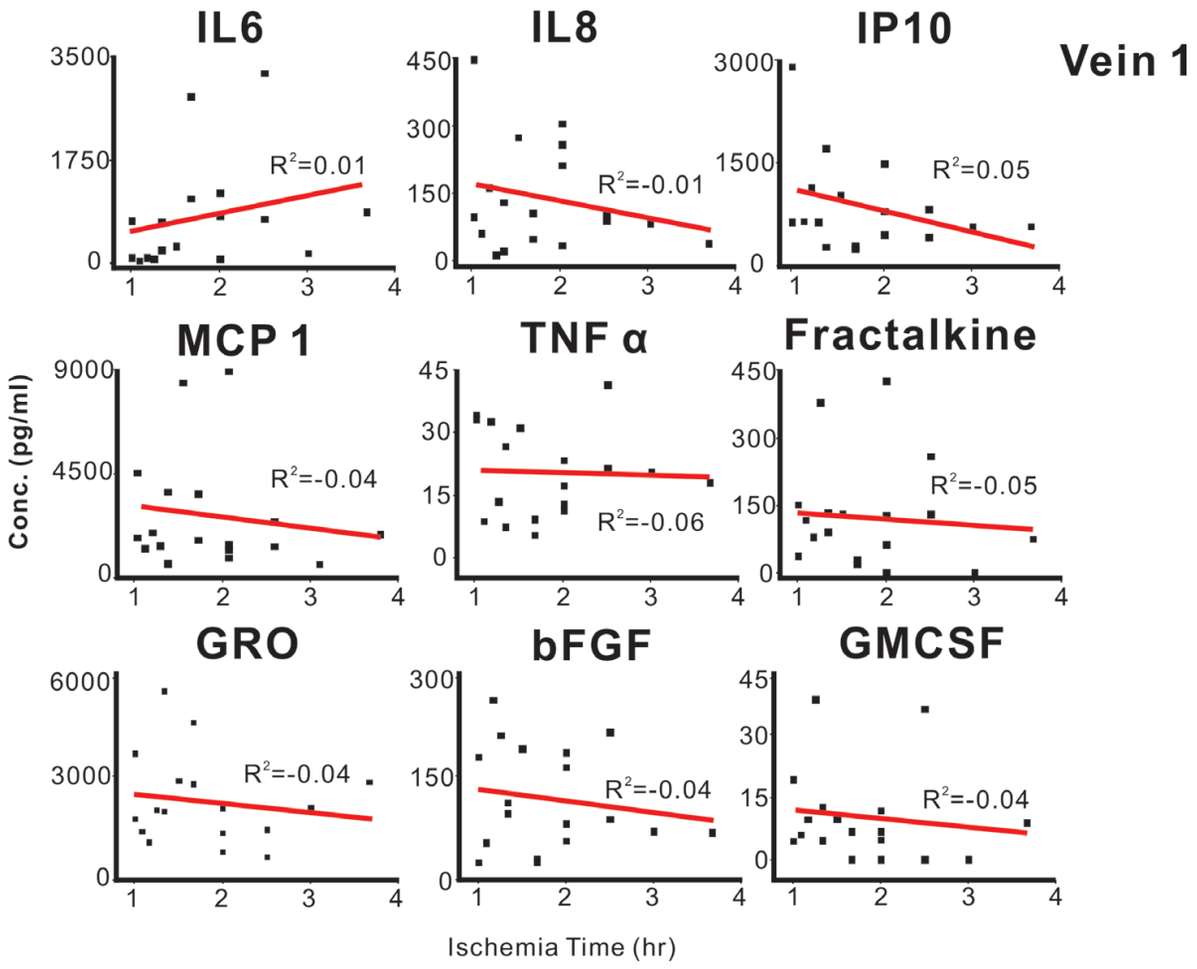

Figure 1. Linear regression plots for group I proteins between ischemia time and protein concentrations of all patients' Vein 1 samples. Trend lines are shown. R^2 values are given.

GMCSF, TNFα, Fractalkine, bFGF, and IL8 were found in low concentrations (below 500 pg/mL; colored black in **Figure 3**), whereas IP10 was found with intermediate concentration (500 pg/mL < protein concentration < 1000 pg/mL; colored dark grey) in all three sampling time points (artery, Vein 1, and Vein 2). MCP1 and GRO were present in highest concentrations (above 1000 pg/mL; colored bright grey) in most samples of all three sampling time points. Interestingly, IL6 was found to change in concentration from low in artery to intermediate in both, Vein 1 and Vein 2 samples, and prompted to analyze protein concentration differences between sampling time points.

2.3. Analysis of individual protein concentration dynamics

To investigate expression differences between the sampling time points for each patient, ratios between the respective protein concentrations were calculated. Quotient I (artery/artery), quotient II (Vein 1/artery), and quotient III (Vein 2/artery) showed individual values for all three time points in a normalized fashion. Combining these quotients, values with straight lines visualized "up" and "down" and/or "no" changes, respectively. From all investigated protein concentrations, a "dynamic" expression was observed for IL6 and IL8 in individual patients.

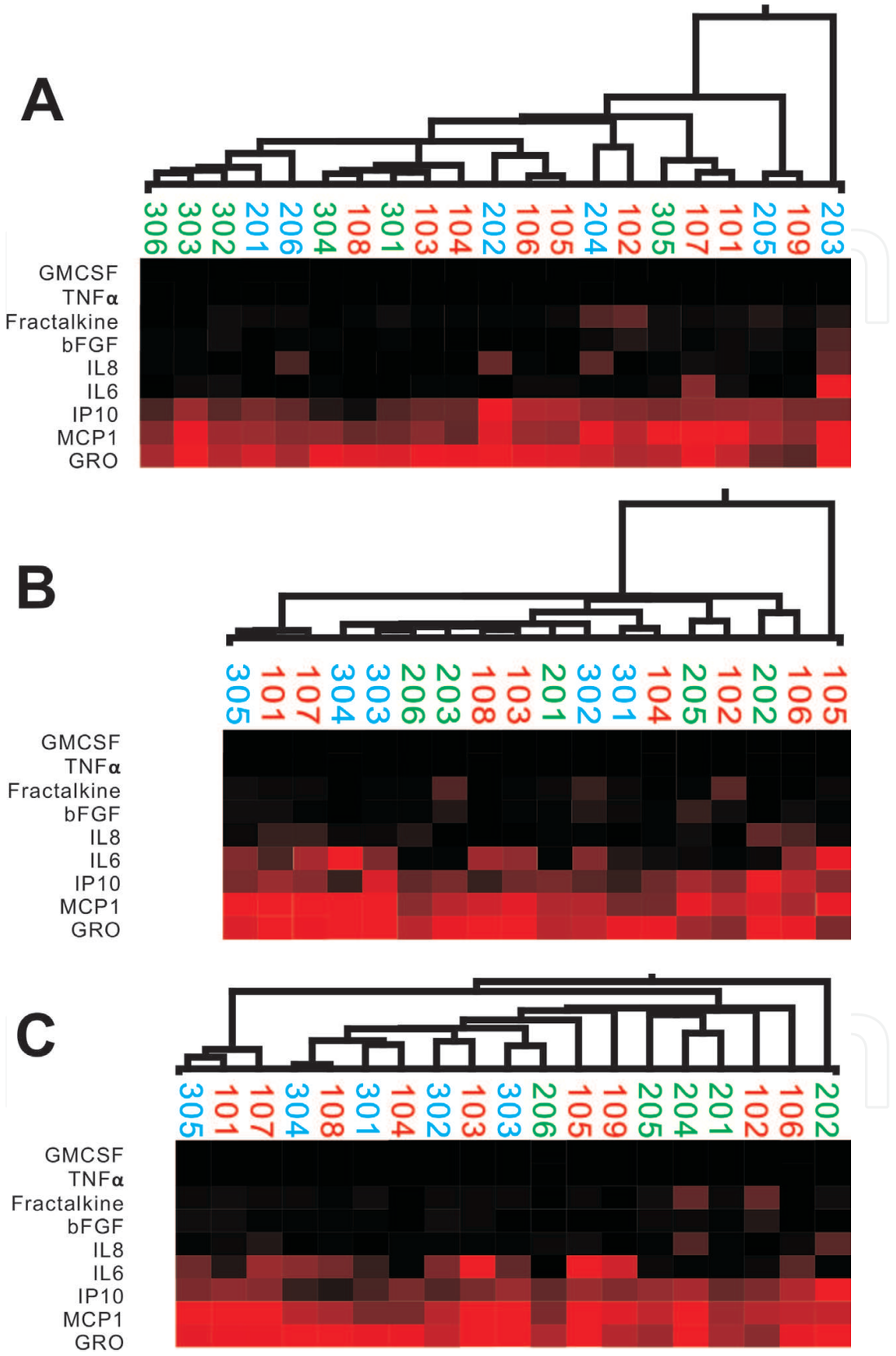

Figure 2. Hierarchical clustering analysis of protein concentrations from artery (A), Vein 1 (B), and Vein 2 (C) plasma samples of flap transplant patients. Proteins are indicated on the left. Patient IDs (**Table 1**) are given between the dendrogram and the heat map illustration. Grey scales indicate protein concentration: black—low, dark grey—medium, bright grey—high.

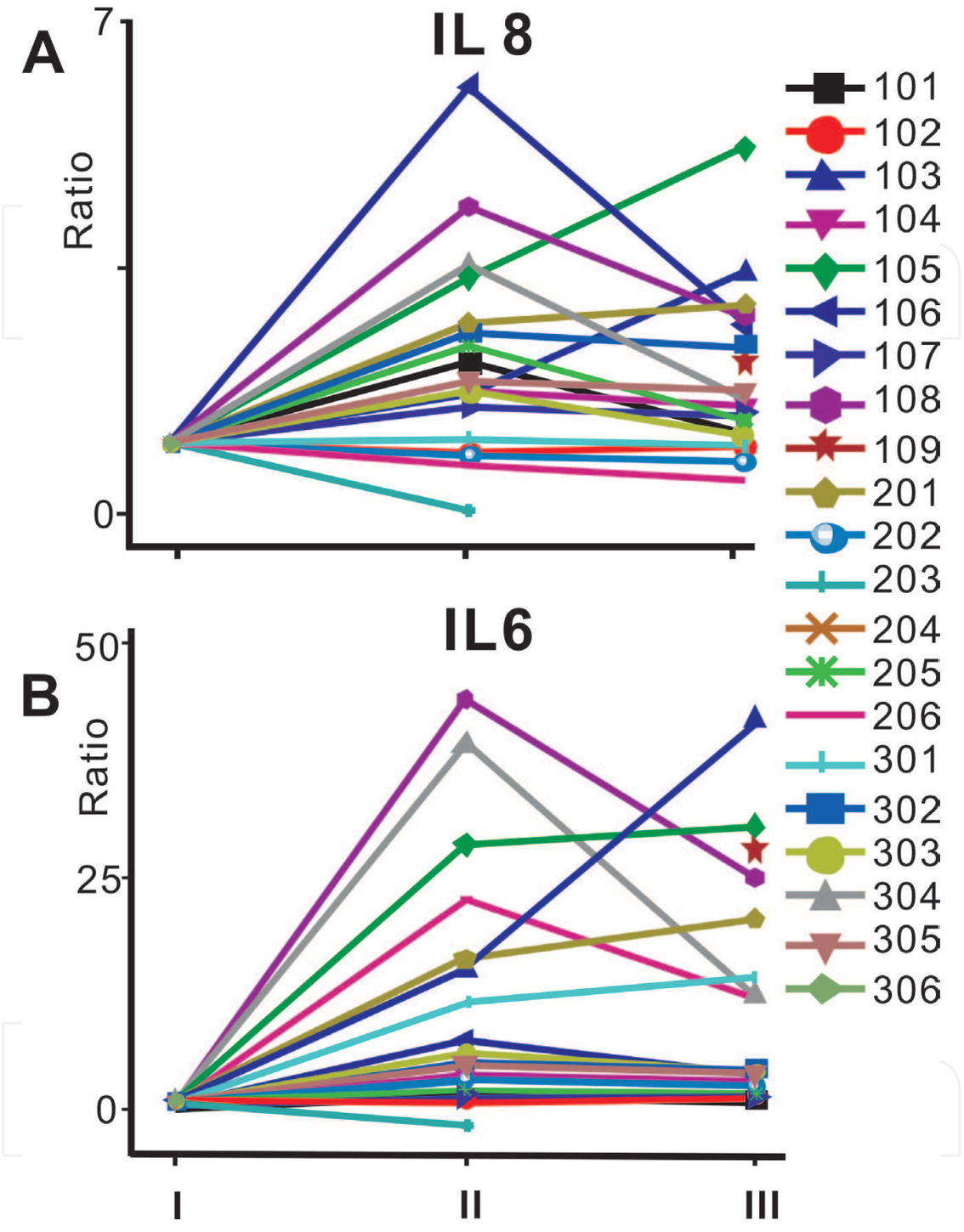

Figure 3. Line graphs of concentration ratios from 21 patients (58 plasma samples) for IL8 (A) and IL6 (B). Individual ratios were calculated from protein concentrations: I: artery/artery, II: Vein 1/artery, and III: Vein 2/artery. Patient IDs (**Table 1**) and respective symbols are shown at the right.

Nearly all line graphs for IL8 in all patients showed progression lines that were quite similar to each other (**Figure 3A**). The dominant protein concentration change profile of IL8 followed a rapid "up-down" trend. A few patients in the IL8 group (104, 107, 201, 302, and 305) represented a "slow dynamic," i.e., an "up-no" trend. Interestingly, line graphs for IL6 of most

patients followed a related progression (**Figure 3B**) as well. The dominant protein profile for IL6 followed an "up-no" trend. Noteworthy, with a few patients (104, 106, 108, 206, and 304) from the IL6 group, a "fast dynamics" of protein concentration changes ("up-down" trend) was observed.

2.4. Subgroup analysis of different free flap tissue groups

Subgroup analysis of protein profiles with respect to different transplant types was performed to check whether the trend of the general protein concentration profiles of either IL6 or IL8 was found consistently in the clinical subgroups. In each of the three transplant groups (**Figure 4**), the "up-no" trend of IL6 was dominant (10 cases), followed by the "up-down" trend (5 cases).

Notably, the PPS data in the osteo-cutaneous group showed very large differences of IL6 concentrations between the pre-ischemia time point (artery) and the second post-ischemia time point (Vein 2) in patients 103, 105, and 109 ("up-no" trend). In patient 103, the difference was 2398.4 pg/mL; in patient 105, it was 3320.68 pg/mL; and in patient 109, 1549.19 pg/mL. These three patients experienced the longest ischemia times during operation (**Table 1**). Patient 109 developed a venous thrombosis after venous anastomosis, so a revision of the anastomosis with thrombectomy and an interposition of a vein graft became necessary.

Of note, in the skin flap group, the pre-ischemia value (artery) of IL6 was higher in patients with clinical conspicuities compared to the other patients. In patient 305, the pre-ischemia IL6 concentration was 144.98 pg/mL, and in patient 302, it was 147.34 pg/mL. In the other patients of this group, the pre-ischemia IL6 concentrations were much lower and the mean was 52.15 pg/mL. Patient 305 developed an intraoperative thrombosis of the artery after venous anastomosis. In patient 302, ischemia time was the longest in the whole group with 150 min.

In case of IL8, the "up-down" trend was dominant (five cases) in both, the osteo-cutaneous group (**Figure 5A**) and the cutaneous group (**Figure 5C**), followed by the "up-no" trend (four cases). Only the muscle group seemed to behave different (**Figure 5B**). Here, no dominant trend could be defined, but "up-down" cases were present.

Again, when looking at the PPS data also in the muscle group, the two patients (patients 202 and 203) with clinical conspicuities were standing out by looking at the concentrations of IL6 and additionally of IL8. In these patients, the pre-ischemia values (artery) of both, IL6 and IL8, were significantly higher than those of the other patients. The IL6 concentration of patient 202 was 30.47 pg/mL and that of patient 203 was 7064.18 pg/mL. Although both values were very different, they were much higher than the mean concentration of the other patients which was 12.81 pg/mL. Similarly, the IL8 concentration of patient 202 was 530.33 pg/mL and that of patient 203 was 477.09 pg/mL. These concentrations were again much higher than the mean (202.16 pg/mL) of all other patients in this group. Patient 202 suffered from partial flap necrosis, whereas patient 203 developed an intraoperative venous thrombosis after venous anastomosis, so a revision of the anastomosis with thrombectomy was necessary.

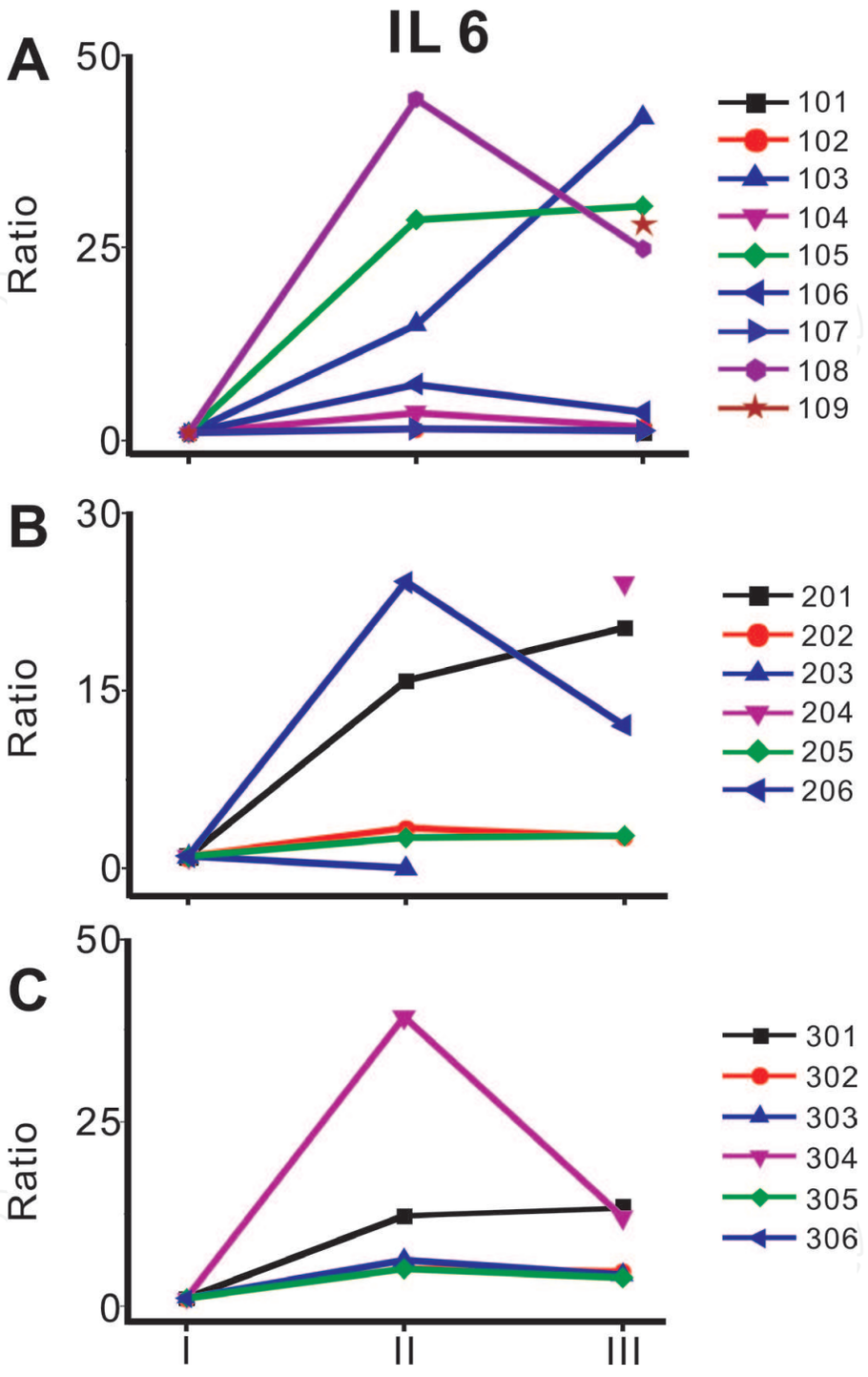

Figure 4. Line graphs of concentration ratios for IL6 from osteo-cutaneous flaps (A), muscle flaps (B), and skin flaps (C). Individual ratios were calculated from protein concentrations: I: artery/artery, II: Vein 1/artery, and III: Vein 2/artery. Patient IDs (**Table 1**) and respective symbols are shown at the right.

In sum, subgroup analysis confirmed that the dynamics of protein concentration differences of IL6 and IL8 correlated with I/R and seemed capable to characterize I/R-related processes on a molecular level for both the patients that showed clinical conspicuities or complications and those that did not. These concentration differences point toward a (transient) activation of leukocytes.

http://dx.doi.org/10.5772/intechopen.70054

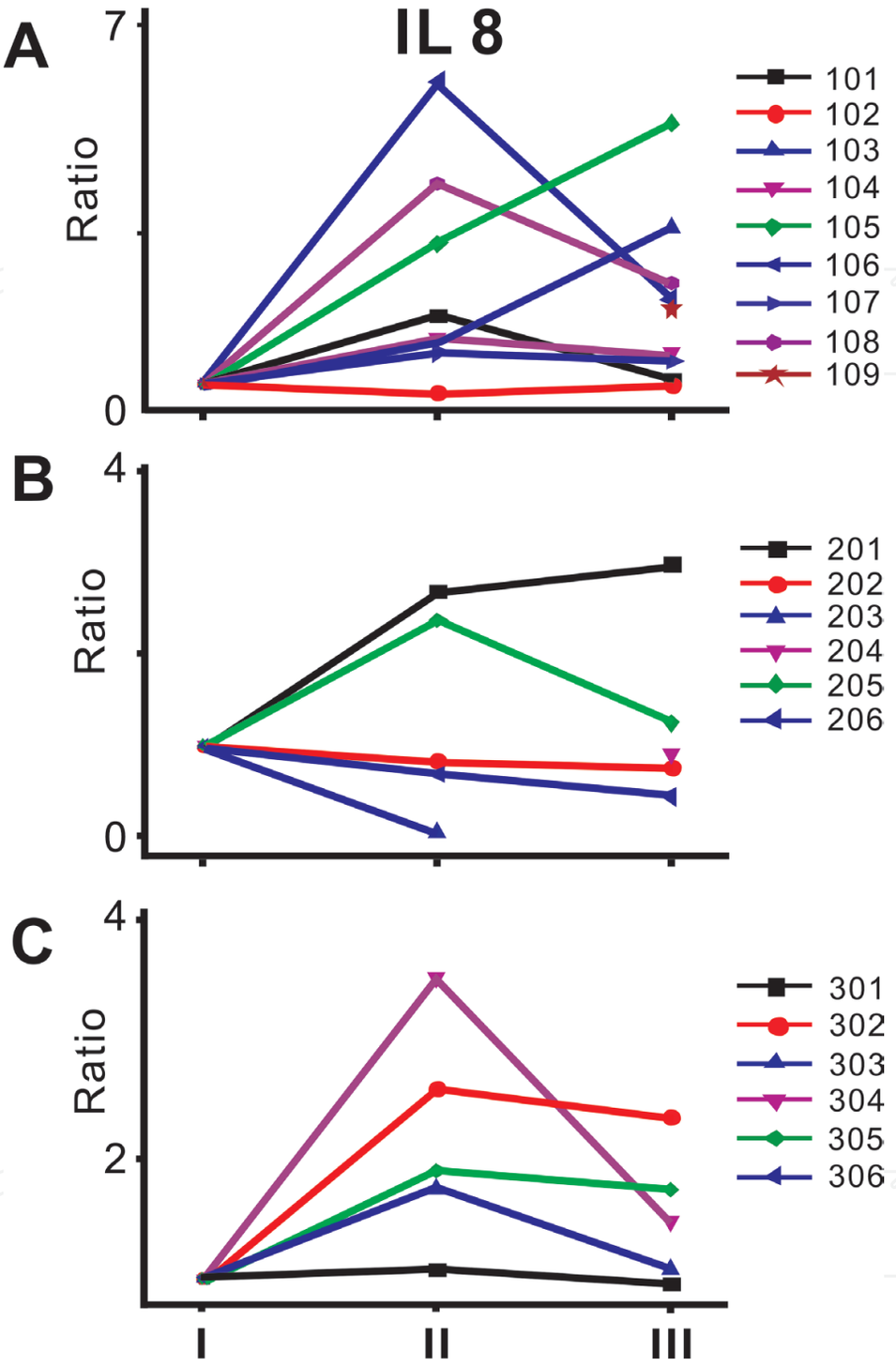

Figure 5. Line graphs of concentration ratios for IL8 from osteo-cutaneous flaps (A), muscle flaps (B), and skin flaps (C). Individual ratios were calculated from protein concentrations: I: artery/artery, II: Vein 1/artery, and III: Vein 2/artery. Patient IDs (**Table 1**) and respective symbols are shown at the right.

2.5. Conclusion

The dynamic nature of the circulating blood system and its constituents reflects diverse physiological or pathological states and, together with the ease with which blood can be sampled,

makes it a logical choice for biomarker investigations [28]. Based on the inspection of the individual protein profiles of 18 cytokines and growth factors in the investigated study cohort (**Table 3**), IL8 and IL6 showed dynamic changes within the measured time frame.

IL6 together with TNFα and IL1β belongs to the so-called pro-inflammatory cytokines. IL6 is supposed to activate the coagulation system in experimental models, due to cross-links

Classification[a]	**Cytokine/growth factor**	**Protein family**	**Receptor**[b,c]	**Cytokine/growth factor target cell types**[c,d]
Dynamic change[e]	IL8	Cytokine/chemokine	CXCR1, E482	Neutrophils, basophils, CD8 T cells, epithelial and endothelial cells
	IL6	Cytokine/interleukin	CD126, CD130	T cells, B cells
No dynamic change[f]	MCP1	Cytokine/chemokine	CCR2	T cell monocytes, NK cells, B cells, endothelial cells
	bFGF	Growth factor (fibroblast)	FGFR	Epithelial cells
	GRO	Cytokine/chemokine	CXCR2	Neutrophils, fibroblasts, melanoma cells
	Fractalkine	Cytokine/chemokine	CX3CR1	Activated T cells, neutrophils, NK cells
	GMCSF	Cytokine/interleukin	CD116, βc	Bone marrow progenitors
	IP 10	Cytokine/chemokine	CXCR3A, CXCR3B	Activated T cells, NK cells, B cells, endothelial cells
	TNF α	Cytokine (TNF)	CD120a, CD120b	T cells, B cells, endothelial cells
	MIP 1α	Cytokine/chemokine	CCR1, CCR5	Immune cells, smooth muscle cells, endothelial cells
	IL10	Cytokine/interleukin	IL-10Rα, IL-10Rβc	Macrophages, T cells
	VEGF	Growth factor (vascular endothelial)	VEGFR	Vascular endothelial cells, hematopoietic stem cells, megakaryocytes
	IFNγ	Cytokine/interleukin	CD119, IFNGR2	Monocytes, endothelial cells, macrophages

Classification[a]	Cytokine/growth factor	Protein family	Receptor[b,c]	Cytokine/growth factor target cell types[c,d]
Undetermined	sCD40L	Cytokine (TNF)	CD40	Dendritic cells, B cells, macrophages
	TGF α	Growth factor (EGF-like domain)	EGFR	Epithelial cells
	IL1β	Cytokine/interleukin	CD121a, CD121b	T cells, macrophages
	IL12P40	Cytokine/interleukin	IL-12β1c + IL-12β2	NK cells, T cells
	TNFβ	Cytokine (TNF)	CD120a, CD120b	T cells, macrophages

[a]Comparisons of means of protein concentrations between sampling time points artery, Vein 1, and Vein 2.
[b]Baggiolini et al. [29].
[c]Janeway et al. [30].
[d]Huret et al. [31].
[e]Means are different between sampling time points artery, Vein 1, and Vein 2.
[f]Means are similar between sampling time points artery, Vein 1, and Vein 2.

Table 3. Classification of proteins according to their averaged plasma concentrations.

between inflammation and coagulation [32, 33]. Activated coagulation can result in microvascular thrombosis that possibly increases I/R injury [34]. Myocardial ischemia studies assume that changes in coagulation affect the resolution of ischemia during reperfusion due to changes in no-flow regions [35, 36]. In mice, IL6 deficiency reduced myocardial infarct size at 3 h reperfusion from which it was concluded that IL6 contributed to the development of infarct size in the early phase of reperfusion [37]. These results correspond to significantly elevated concentrations of IL6 in the venous blood samples of patients that experienced long ischemia periods.

IL8 is a prototypical member of the CXC chemokine family. Chemokines control innate immune cell trafficking between the bone marrow, blood, and peripheral tissues during inflammation. IL8 is a potent chemoattractant for neutrophils in vitro [38]. It has been reported to be a chemoattractant for a subset of T-lymphocytes [39]. IL8 is also called neutrophil-activating protein 1 (NAP-1) because it stimulates release of neutrophil granules. Like many other chemoattractants, IL8 induces re-arrangement of the cytoskeleton, changes in intracellular Ca^{2+} levels, activation of integrins, exocytosis of granule proteins, and respiratory burst [40]. IL8 concentration dynamics results in the here described study are consistent with published data, demonstrating the potential of IL8 as a marker protein of I/R injury in transplantation surgery. Interestingly, in cases of clinical conspicuities or complications, IL6 and IL8 react differently as compared to inconspicuous cases.

The activated forms of both macrophages and keratinocytes can release a number of inflammatory and cytotoxic active molecules that play essential roles in wound repair and/or tissue damage [41–43]. Macrophages, by secreting IL6, were suggested to interact with keratinocytes

which are associated with epithelialization [44]. IL8 has a profound effect on the migration of keratinocytes which is again critical to wound epithelialization [45]. In vitro experiments on the effect of recombinant human IL8 on keratinocyte proliferation revealed a rise in cell numbers, whereas in vivo topically applied IL8 on human skin grafts in a chimeric mouse model enhanced re-epithelialization due to elevated numbers of mitotic keratinocytes [46]. The dynamic occurrence of chemokines IL8, GROα, MCP-1, IP-10, and Mig in the different phases of wound healing was described in a skin repair model in adult humans [47].

3. Outlook

The concept of targeting receptor cells that bind to IL6 or IL8 might become of importance for clinical interventions with the aim to accelerate wound healing as well as to attenuate fibrosis in response to individually determined cytokine/growth factor concentrations (**Figure 6**). Sequential function of endogenous IL8 and IL6 in all phases of human wound healing suggests to administering appropriate medication [48] in case of overexpression.

For instance, the anti-interleukin-6 receptor monoclonal antibody, tocilizumab, which was approved for the treatment of inflammatory diseases [49], might be tested in an off-label clinical study for its effect in modulating immune responses during free flap healing. A similar therapeutic concept may make use of HuMab 10FB, a fully human mAb against IL8, which

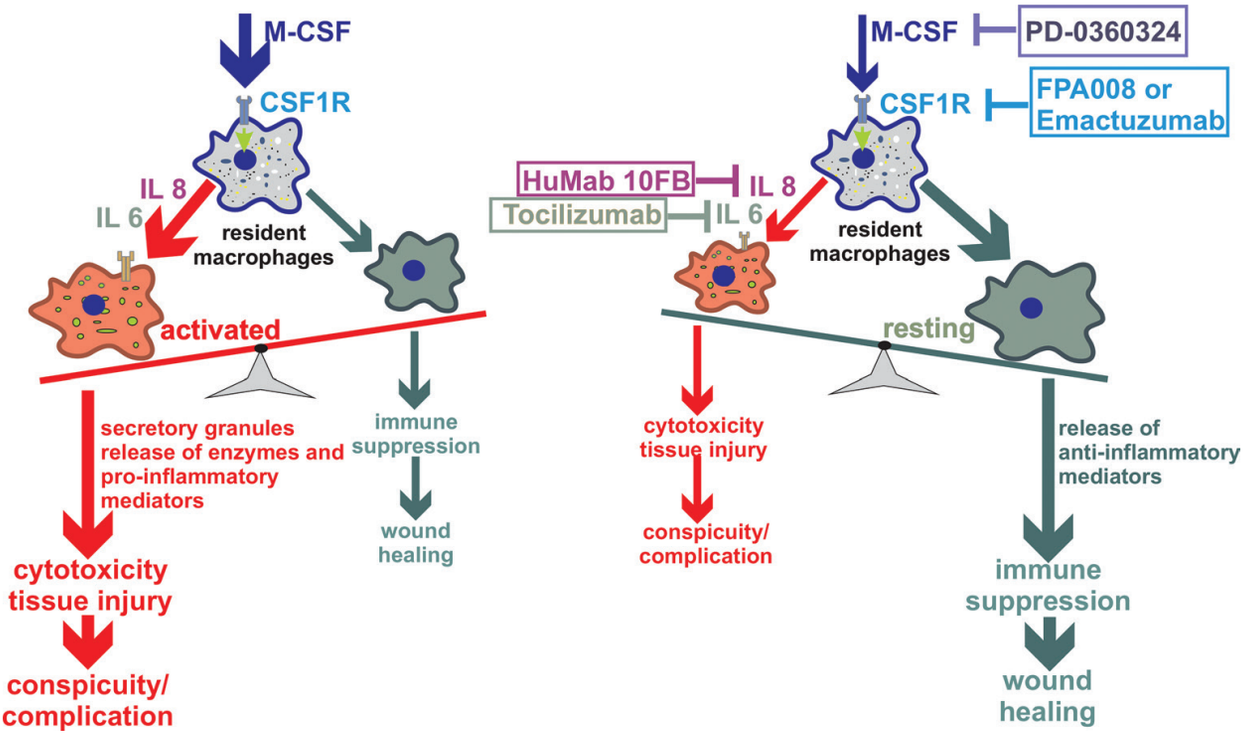

Figure 6. Implications of leucocytes (macrophages) in wound healing processes and clinical management without (left) and with (right) neutralizing antibody-based medication. Overproduced cytotoxic mediators or their receptors may be targeted. Pro-inflammatory mediators and enzymes may be clinically managed, whereas anti-inflammatory mediators would not be affected.

binds a discontinuous epitope on IL8 overlapping the receptor binding site, and it is capable to interrupt IL8 activity in vivo [50]. Both therapeutic antibodies would neutralize increased levels of interleukins and directly interfere in leukocyte signaling with potentially positive effects on tissue and wound healing processes.

These results indicate the sequential function of endogenous MCSF [51], IL8, and IL6 in wound healing which in case of overexpression may be clinically managed by administering appropriate medication, i.e., management of an individuals' systemic inflammatory status to reduce or even prevent conspicuities and complications during perioperative and postoperative periods. Concomitant medication may become an important if not indispensable part of state-of-the-art flap surgery in the future.

4. Appendix

4.1. Clinical specimen collection

Presented and discussed data are from a study that was approved by the Institutional Review Board of the University Hospital Zurich, Switzerland (StV 8-2009). Written informed consent was obtained from all participating patients. Three groups of free flap transfers were defined: (i) muscle flaps, e.g., gracilis flap, latissimus dorsi flap, serratus anterior flap, (ii) fascio-cutaneous flaps, e.g., radialis flap, anterolateral thigh flap, and (iii) osteo-cutaneous flaps, i.e., fibula flaps. Clinical measurements include the ischemia time, flap weight, length of bone (in fibula flaps), and the dimension of the skin island. Patients qualified for the study had to be with normal weight (BMI 20–25), non-diabetics, in no manifest infection situation, and of good health with no essential diseases besides the main diagnosis (**Table 1**). Blood samples were taken intraoperatively prior to arterial anastomosis from the arterial inflow and after arterial anastomosis from the venous flap outflow. Vein 1 samples were taken directly after anastomosis and Vein 2 samples 2 min after collecting Vein 1 samples in 1.5 mL portions using S-Monovette® Lithium Heparin syringes (Monovette®, Sarstedt, Germany). Blood samples were immediately subjected to sedimentation of blood cells by centrifugation at 2000 g at room temperature for 15 min. Plasma was aspirated and sterile-filtered (0.2 μm pore size) [52]. Five samples had to be excluded from the study because of too less material after centrifugation and sterile filtration: patient 109 (Vein 1), patient 203 (Vein 2), patient 204 (Vein 1), and patient 306 (Vein 1 and Vein 2). In total, 58 plasma samples had been collected. Aliquots of 100 μL per portion were stored at –80°C prior to further analysis.

4.2. Multiplexed bead-based immunoassay

The Human Cytokine/Chemokine Magnetic Bead Panel from Milliplex® (Map kit HCYTOMAG-60K, Billerica, MA, USA) contained antibodies against 18 proteins that were delivered immobilized onto color-coded beads. All kit reagents were brought to 25°C. Then, the two quality control samples were reconstituted with 250 μL deionized water, each. Serum matrix was reconstituted with 1.0 mL deionized water. The human cytokine standard mixture was reconstituted with 250 μL deionized water to give a 10,000 pg/mL concentration for each of the standards. This

solution was serially diluted by a factor of 5 and yielded in diluted human cytokine standards with the following concentrations: 10,000, 2000, 400, 80, 16, and 3.2 pg/mL. Next, each antibody-bead containing vial was sonicated for 30 s and then vortexed for 1 min. Sixty microliters of antibody-bead slurry from each of the 18 vials was added to the mixing bottle, and 1.92 mL "bead diluent" was added to achieve a final volume of 3.0 mL. Two 96-well plates were pre-wetted with 200 μL wash buffer, each. After sealing, the plates were fixed on the shaker (Heidolph® Promax 2020, Schwabach, Germany) and gently agitated for 10 min at room temperature. The wash buffer was decanted, and the residual amount was removed by inverting the plates and gently tapping onto absorbent towels for several times. Twenty-five microliters, each, of all six diluted human cytokine standards and the two quality control samples were added into their dedicated wells. Twenty-five microliters, each, of assay buffer were added to two "background" wells and to the wells that were dedicated to patient samples. Second, 25 μL of serum matrix was added into each of the diluted human cytokine standard wells, the background wells, and the quality control sample wells. Twenty-five microliters of each patient plasma was added into one of the patient sample wells. Twenty-five microliters, each, of antibody-bead slurry from the mixing bottle was added to all the wells. Afterwards, plates were sealed and incubated in the dark for 18 h at 4°C on the plate shaker. Solvents from each well were removed, avoiding loss of beads, and beads were washed twice with wash buffer (200 μL, for each well, 1 min incubation). After removal of wash buffer, 25 μL, each, of detection antibody solution was added to all wells. After 1 h incubation at 25°C, 25 μL of phycoerythrin-loaded streptavidin containing solution was added to all wells. The plates were sealed again, covered with aluminum foil, and then fixed on the plate shaker for 30 min at 25°C. Subsequently, beads were washed twice with wash buffer (200 μL, for each well, 1 min incubation). After removal of wash buffer, 150 μL of sheath fluid (Bio-Rad Laboratories, Hercules, CA, USA) was added to all wells and beads were resuspended on the plate shaker for 5 min. Plates were placed into the Bio-Plex suspension array 200 System (Bio-Rad Laboratories, Hercules, CA, USA), which had been calibrated with Bio-Plex® 200 calibration kit and validated with Bio-Plex® validation kit 4.0 (Bio-Rad Laboratories, Hercules, CA, USA). Measurement settings were as follows: data acquisition, 50 beads per region; sample size, 100 μL; and doublet discriminator gate, 5000–25,000 (low photomultiplier tube). The Bio-Plex suspension array 200 reader system contains a red laser for identification of the bead which is analyzed and a green laser for quantification of fluorescence intensity of phycoerythrin-loaded streptavidin. All standards, controls, background, and plasma samples were prepared in duplicate and measured once. From the 2088 independent measurements, the Bio-Plex® manager 6.1 software (Bio-Rad Laboratories, Hercules, CA, USA) calculated median fluorescence intensity (MFI) and standard deviations of each duplicate recording. Fluorescence values of human cytokine standards were plotted as standard curves which were used for determining plasma concentrations (pg/mL) based on their fluorescence intensities of all proteins and all time points. In total, 1044 data points (raw data set) were stored as Excel files.

4.3. Full analysis set and per protocol set

Data from the raw data set were inspected for completeness and categorized into three groups based on the detection rates. Group I contained those cytokines and growth factors (CGFs) for which plasma protein concentrations could be determined in over 80% of all samples. Group

II included those CGFs for which plasma protein concentrations could be determined in over 60% but less than 80% of all samples. Group III contained proteins whose concentrations were determined in less than 60% of all samples and sCD40L which did not pass the QC test. In both, group I and group II proteins, the missing values were imputed using the lower limit of quantitation (LLOQ) except for MCP1 for which upper limit of quantitation (ULOQ) was imputed. After imputing, the "full analysis set (FAS)" contained a total of 1044 curated data points (data not shown). The "per protocol set (PPS)" was generated out of the "full analysis set (FAS)" by including only group I and group II CGFs, resulting in 754 data points.

4.4. Biostatistical analysis

Statistical analyses were performed using the PPS with the IBM statistics software SPSS (version 20.0, SPSS Inc., Chicago, USA). Linear fit analysis between ischemia time and protein concentration was performed using the Origin statistics software (version. 8.1 G; OriginLab Corporation, Northampton, MA, USA). Linear regression was performed to calculate R^2 values, and ANOVA tests were performed to calculate p values to estimate whether protein concentration was related to the ischemia time [53]. Hierarchical cluster analysis and dendrogram presentation were performed on the Knowledge Discovery Environment (KDE) platform (InforSense Ltd., London, UK). Parameter settings were single linkage and Euclidean distance. CV% for each analyte was calculated as the ratio of the standard deviation to the plasma mean concentration [25, 26].

Author details

Juliane C. Finke[1,2#], Jingzhi Yang[1#], Marius Bredell[3], Uwe von Fritschen[2] and Michael O. Glocker[1*]

*Address all correspondence to: michael.glocker@uni-rostock.de

1 Proteome Center Rostock, University Medicine and Natural Science Faculty, University of Rostock, Rostock, Germany

2 Division of Plastic Surgery and Hand Surgery, HELIOS Clinic Emil von Behring, Berlin, Germany

3 Department of Cranio-Maxillofacial and Oral Surgery, University Hospital of Zürich, Zürich, Switzerland

# These authors contributed equally.

References

[1] Bui DT, Cordeiro PG, Hu QY, Disa JJ, Pusic A, Mehrara BJ. Free flap reexploration: Indications, treatment, and outcomes in 1193 free flaps. Plastic and Reconstructive Surgery. 2007;**119**(7):2092-2100. DOI: 10.1097/01.prs.0000260598.24376.e1

[2] Eisenhardt SU, Schmidt Y, Karaxha G, Iblher N, Penna V, Torio-Padron N, Stark GB, Bannasch H. Monitoring molecular changes induced by ischemia/reperfusion in human free muscle flap tissue samples. Annals of Plastic Surgery. 2012;**68**(2):202-208. DOI: 10.1097/SAP.0b013e3181f77ba5

[3] Cerra FB, Lajos TZ, Montes M, Siegel JH. Hemorrhagic infarction: A reperfusion injury following prolonged myocardial ischemic anoxia. Surgery. 1975;**78**(1):95-104

[4] Khalil AA, Aziz FA, Hall JC. Reperfusion injury. Plastic and Reconstructive Surgery. 2006;**117**(3):1024-1033. DOI: 10.1097/01.prs.0000204766.17127.54

[5] [5] Baumeister S, Ofer N, Kleist C, Terne P, Opelz G, Gebhard MM, Germann G, Heitmann C. Reduction of skeletal muscle injury in composite tissue allotransplantation by heat stress preconditioning. Plastic and Reconstructive Surgery. 2004;**114**(7):1832-1841

[6] [6] Eming SA, Hammerschmidt M, Krieg T, Roers A. Interrelation of immunity and tissue repair or regeneration. Seminars in Cell and Developmental Biology. 2009;**20**(5):517-527. DOI: 10.1016/j.semcdb.2009.04.009

[7] Jennings RB, Murry CE, Steenbergen C Jr, Reimer KA. Development of cell injury in sustained acute ischemia. Circulation. 1990;**82**(3 Suppl):II2-12

[8] Nolte D, Lehr HA, Messmer K. Adenosine inhibits postischemic leukocyte-endothelium interaction in postcapillary venules of the hamster. American Journal of Physiology. 1991;**261**(3 Pt 2):H651-655

[9] Farhood A, McGuire GM, Manning AM, Miyasaka M, Smith CW, Jaeschke H. Intercellular adhesion molecule 1 (ICAM-1) expression and its role in neutrophil-induced ischemia-reperfusion injury in rat liver. Journal of Leukocyte Biology. 1995;**57**(3):368-374

[10] Rashid MA, William-Olsson G. Are leukocytosis and lipid peroxidation involved in ischemic or reperfusion injury in cardiac surgery? Thoracic and Cardiovascular Surgeon. 1991;**39**(4):193-195. DOI: 10.1055/s-2007-1022707

[11] Zimmerman BJ, Granger DN. Mechanisms of reperfusion injury. American Journal of the Medical Sciences. 1994;**307**(4):284-292

[12] Lazarus B, Messina A, Barker JE, Hurley JV, Romeo R, Morrison WA, Knight KR. The role of mast cells in ischaemia-reperfusion injury in murine skeletal muscle. Journal of Pathology. 2000;**191**(4):443-448. DOI: 10.1002/1096-9896(2000)9999:9999<::AID-PATH666>3.0.CO;2-L

[13] Schmidt Y, Bannasch H, Eisenhardt SU. Ischemia-reperfusion injury leads to significant tissue damage in free flap surgery. Plastic and Reconstructive Surgery. 2012;**129**(1):174e-175e;author reply 175e-176e. DOI: 10.1097/PRS.0b013e3182362e53

[14] Wang Y, Yamamoto Y, Kuninaka Y, Kondo T, Furukawa F. Forensic potential of MMPs and CC chemokines for wound age determination. Journal of the Forensic Science. 2015;**60**(6):1511-1515. DOI: 10.1111/1556-4029.12831

[15] Chen ZG, Persons B, Lin L, Lineaweaver WC, Zhang F. Vascular endothelial growth factor upregulates inducible nitric oxide synthase expression in the muscle flap ischemia model

in the rat. Journal of Reconstructive Microsurgery. 2009;**25**(4):219-225. DOI: 10.1055/s-0028-1104550

[16] Dragu A, Schnurer S, Surmann-Schmitt C, von der Mark K, Sturzl M, Unglaub F, Wolf MB, Leffler M, Beier JP, Kneser U, Horch RE. Gene expression analysis of ischaemia and reperfusion in human microsurgical free muscle tissue transfer. Journal of Cellular and Molecular Medicine. 2011;**15**(4):983-993. DOI: 10.1111/j.1582-4934.2010.01061.x

[17] Leng SX, McElhaney JE, Walston JD, Xie D, Fedarko NS, Kuchel GA. ELISA and multiplex technologies for cytokine measurement in inflammation and aging research. The Journals of Gerontology. Series A: Biological Sciences and Medical Sciences. The Journals of Gerontology. Series A: Biological Sciences and Medical Sciences. 2008;**63**(8):879-884

[18] Vaucher J, Marques-Vidal P, Waeber G, Vollenweider P. Cytokines and hs-CRP levels in individuals treated with low-dose aspirin for cardiovascular prevention: A population-based study (CoLaus Study). Cytokine. 2014;**66**(2):95-100. DOI: 10.1016/j.cyto.2014.01.003

[19] Yurkovetsky ZR, Kirkwood JM, Edington HD, Marrangoni AM, Velikokhatnaya L, Winans MT, Gorelik E, Lokshin AE. Multiplex analysis of serum cytokines in melanoma patients treated with interferon-alpha2b. Clinical Cancer Research. 2007;**13**(8):2422-2428. DOI: 10.1158/1078-0432.CCR-06-1805

[20] Geyer AI, Kraus T, Roberts T, Wisnivesky J, Eber CD, Hiensch R, Moran TM. Plasma level of interferon gamma induced protein 10 is a marker of sarcoidosis disease activity. Cytokine. 2013;**64**(1):152-157. DOI: 10.1016/j.cyto.2013.07.010

[21] Damas JK, Boullier A, Waehre T, Smith C, Sandberg WJ, Green S, Aukrust P, Quehenberger O. Expression of fractalkine (CX3CL1) and its receptor, CX3CR1, is elevated in coronary artery disease and is reduced during statin therapy. Arteriosclerosis Thrombosis and Vascular Biology. 2005;**25**(12):2567-2572. DOI: 10.1161/01.ATV.0000190672.36490.7b

[22] Hang H, Yuan S, Yang Q, Yuan D, Liu Q. Multiplex bead array assay of plasma cytokines in type 2 diabetes mellitus with diabetic retinopathy. Molecular Vision. 2014;**20**:1137-1145

[23] Lee J, Kim Y, Lim J, Kim M, Han K. G-CSF and GM-CSF concentrations and receptor expression in peripheral blood leukemic cells from patients with chronic myelogenous leukemia. Annals of Clinical and Laboratory Science. 2008;**38**(4):331-337

[24] Larsson A, Skoldenberg E, Ericson H. Serum and plasma levels of FGF-2 and VEGF in healthy blood donors. Angiogenesis. 2002;**5**(1-2):107-110

[25] O'Connell MA, Belanger BA, Haaland PD. Calibration and assay development using the four-parameter logistic model. Chemometrics and Intelligent Laboratory Systems. 1993;**20**:97-114

[26] Quinn CP, Semenova VA, Elie CM, Romero-Steiner S, Greene C, Li H, Stamey K, Steward-Clark E, Schmidt DS, Mothershed E, Pruckler J, Schwartz S, Benson RF, Helsel LO, Holder PF, Johnson SE, Kellum M, Messmer T, Thacker WL, Besser L, Plikaytis BD, Taylor TH Jr, Freeman AE, Wallace KJ, Dull P, Sejvar J, Bruce E, Moreno R, Schuchat A, Lingappa JR, Martin SK, Walls J, Bronsdon M, Carlone GM, Bajani-Ari M, Ashford DA,

Stephens DS, Perkins BA. Specific, sensitive, and quantitative enzyme-linked immunosorbent assay for human immunoglobulin G antibodies to anthrax toxin protective antigen. Emerging Infectious Diseases. 2002;**8**(10):1103-1110. DOI: 10.3201/eid0810.020380

[27] Staples E, Ingram RJ, Atherton JC, Robinson K. Optimising the quantification of cytokines present at low concentrations in small human mucosal tissue samples using Luminex assays. Journal of Immunological Methods. 2013;**394**(1-2):1-9. DOI: 10.1016/j.jim.2013.04.009

[28] Hanash SM, Pitteri SJ, Faca VM. Mining the plasma proteome for cancer biomarkers. Nature. 2008;**452**(7187):571-579. DOI:10.1038/nature06916

[29] Baggiolini M, Loetscher P. Chemokines in inflammation and immunity. Immunology Today. 2000;**21**(9):418-420

[30] Janeway C, Paul Travers J, Walport M, Shlomchik M. Immunobiology: The Immune System in Health and Disease. New York: Garland Science; 2001

[31] Huret JL, Ahmad M, Arsaban M, Bernheim A, Cigna J, Desangles F, Guignard JC, Jacquemot-Perbal MC, Labarussias M, Leberre V, Malo A, Morel-Pair C, Mossafa H, Potier JC, Texier G, Viguie F, Yau Chun Wan-Senon S, Zasadzinski A, Dessen P. Atlas of genetics and cytogenetics in oncology and haematology in 2013. Nucleic Acids Research. 2013;**41**(Database issue):D920-924. DOI: 10.1093/nar/gks1082

[32] van der Poll T, Levi M, Hack CE, ten Cate H, van Deventer SJ, Eerenberg AJ, de Groot ER, Jansen J, Gallati H, Buller HR, et al. Elimination of interleukin 6 attenuates coagulation activation in experimental endotoxemia in chimpanzees. Journal of Experimental Medicine. 1994;**179**(4):1253-1259

[33] Stouthard JM, Levi M, Hack CE, Veenhof CH, Romijn HA, Sauerwein HP, van der Poll T. Interleukin-6 stimulates coagulation, not fibrinolysis, in humans. Thrombosis and Haemostasis. 1996;**76**(5):738-742

[34] Erlich JH, Boyle EM, Labriola J, Kovacich JC, Santucci RA, Fearns C, Morgan EN, Yun W, Luther T, Kojikawa O, Martin TR, Pohlman TH, Verrier ED, Mackman N. Inhibition of the tissue factor-thrombin pathway limits infarct size after myocardial ischemia-reperfusion injury by reducing inflammation. American Journal of Pathology. 2000;**157**(6):1849-1862. DOI: 10.1016/S0002-9440(10)64824-9

[35] Li RA, Leppo M, Miki T, Seino S, Marban E. Molecular basis of electrocardiographic ST-segment elevation. Circulation Research. 2000;**87**(10):837-839

[36] Gehrmann J, Frantz S, Maguire CT, Vargas M, Ducharme A, Wakimoto H, Lee RT, Berul CI. Electrophysiological characterization of murine myocardial ischemia and infarction. Basic Research in Cardiology. 2001;**96**(3):237-250

[37] Jong WM, ten Cate H, Linnenbank AC, de Boer OJ, Reitsma PH, de Winter RJ, Zuurbier CJ. Reduced acute myocardial ischemia-reperfusion injury in IL-6-deficient mice employing a closed-chest model. Inflammation Research. 2016. DOI: 10.1007/s00011-016-0931-4

[38] Palomino DC, Marti LC. Chemokines and immunity. Einstein (Sao Paulo). 2015;**13**(3):469-473. DOI: 10.1590/S1679-45082015RB3438

[39] Huber AR, Kunkel SL, Todd RF 3rd, Weiss SJ. Regulation of transendothelial neutrophil migration by endogenous interleukin-8. Science. 1991;**254**(5028):99-102

[40] Paccaud JP, Schifferli JA, Baggiolini M. NAP-1/IL-8 induces up-regulation of CR1 receptors in human neutrophil leukocytes. Biochemical and Biophysical Research Communications. 1990;**166**(1):187-192

[41] Raja, Sivamani K, Garcia MS, Isseroff RR. Wound re-epithelialization: Modulating keratinocyte migration in wound healing. Frontiers in Bioscience. 2007;**12**:2849-2868

[42] Laskin DL, Sunil VR, Gardner CR, Laskin JD. Macrophages and tissue injury: Agents of defense or destruction? Annual Review of Pharmacology and Toxicology. 2011;**51**:267-288. DOI: 10.1146/annurev.pharmtox.010909.105812

[43] Koh TJ, DiPietro LA. Inflammation and wound healing: the role of the macrophage. Expert Reviews in Molecular Medicine. 2011;**13**:e23. DOI: 10.1017/S1462399411001943

[44] Rodero MP, Khosrotehrani K. Skin wound healing modulation by macrophages. International Journal of Clinical and Experimental Pathology. 2010;**3**(7):643-653

[45] Jiang WG, Sanders AJ, Ruge F, Harding KG. Influence of interleukin-8 (IL-8) and IL-8 receptors on the migration of human keratinocytes, the role of PLC-gamma and potential clinical implications. Experimental and Therapeutic Medicine. 2012;**3**(2):231-236. DOI: 10.3892/etm.2011.402

[46] Rennekampff HO, Hansbrough JF, Kiessig V, Dore C, Sticherling M, Schroder JM. Bioactive interleukin-8 is expressed in wounds and enhances wound healing. Journal of Surgical Research. 2000;**93**(1):41-54. DOI: 10.1006/jsre.2000.5892

[47] Engelhardt E, Toksoy A, Goebeler M, Debus S, Brocker EB, Gillitzer R. Chemokines IL-8, GROalpha, MCP-1, IP-10, and Mig are sequentially and differentially expressed during phase-specific infiltration of leukocyte subsets in human wound healing. American Journal of Pathology. 1998;**153**(6):1849-1860

[48] Maude SL, Teachey DT, Porter DL, Grupp SA. CD19-targeted chimeric antigen receptor T-cell therapy for acute lymphoblastic leukemia. Blood. 2015;**125**(26):4017-4023. DOI: 10.1182/blood-2014-12-580068

[49] Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochimica et Biophysica Acta. 2011;**1813**(5):878-888. DOI: 10.1016/j.bbamcr.2011.01.034

[50] Skov L, Beurskens FJ, Zachariae CO, Reitamo S, Teeling J, Satijn D, Knudsen KM, Boot EP, Hudson D, Baadsgaard O, Parren PW, van de Winkel JG. IL-8 as antibody therapeutic target in inflammatory diseases: Reduction of clinical activity in palmoplantar pustulosis. Journal of Immunology. 2008;**181**(1):669-679

[51] Yang J, Finke JC, Yang J, Percy AJ, von Fritschen U, Borchers CH, Glocker MO. Early risk prognosis of free-flap transplant failure by quantitation of the macrophage colony-stimulating factor in patient plasma using 2-dimensional liquid-chromatography multiple reaction monitoring-mass spectrometry. Medicine. 2016;**95**(39):e4808. DOI: 10.1097/MD.0000000000004808

[52] Koy C, Heitner JC, Woisch R, Kreutzer M, Serrano-Fernandez P, Gohlke R, Reimer T, Glocker MO. Cryodetector mass spectrometry profiling of plasma samples for HELLP diagnosis: An exploratory study. Proteomics. 2005;**5**(12):3079-3087. DOI: 10.1002/pmic.200402098

[53] Montgomery DC. In: Anderson W, editor. Design and Analysis of Experiments. United States of America: John Wiley & Sons; 2001. pp. 65-75