Heparin-induced thrombocytopenia: further evidence for a unique immune response

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Short title: Unique kinetics of the anti-PF4/H IgG response

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Abstract

Cardiopulmonary bypass surgery (CPB) is associated with a high incidence of immunoglobulin G (IgG) antibodies against platelet factor 4/heparin (PF4/H) complexes by day 6 after surgery. These antibodies are associated with an immune-mediated adverse drug reaction, heparin-induced thrombocytopenia. While the early onset of anti-PF4/H IgG response is compatible with a secondary immune response, the rapid decline of antibody titers thereafter is not. To shed light on the origin of these antibodies, we prospectively compared their kinetics to that of antibodies against two recall antigens and to that of autoantibodies in 166 CPB patients over four months. Surgery induced strong inflammation as shown by an increase of the mean C-reactive protein levels. Consistent with previous studies, anti-PF4/H-IgG OD transiently increased between baseline and day 10 (p<0.001; not associated with CRP levels), followed by a decrease over the next months. In contrast, concentrations of anti-diphtheria toxin IgG and anti-tetanus toxin IgG constantly increased over the four months after surgery by 25-30%. IgG autoantibodies did not change. Thus the transient kinetics of the anti-PF4/H-IgG response resembled neither that of recall antibodies nor that of IgG autoantibodies, but rather shows a unique profile.
Introduction

Platelet factor 4 (PF4), a chemokine stored in large amounts in platelet alpha granules, is rapidly released upon platelet activation. The positively charged molecule forms tetramers, which readily associate with polyanions such as heparin forming large PF4/heparin complexes (PF4/H). PF4 complexed to heparin exposes neoepitopes, which can be targeted by antibodies. The resulting immune complexes can give rise to heparin-induced thrombocytopenia (HIT), the most common adverse drug reaction affecting blood cells. HIT is clinically relevant because it results in a paradoxical prothrombotic state.

The immunobiology of the anti-PF4/H antibody response is not well understood. It exhibits unusual kinetics, which neither fit a primary immune response, nor a typical secondary immune response: in two studies of orthopedic surgery patients’ antibodies of all classes, IgM, IgG, and IgA, but especially IgG appeared from day 4 after beginning of heparin exposure, even in heparin naïve patients. This has also been shown for trauma surgery and cardiac surgery patients. However, in the latter patient groups anti-PF4/H antibodies were usually only determined starting from day 6. This early onset of IgG production strongly indicates that patients have been pre-immunized against PF4/polyanion complexes and we have recently shown that this probably occurs via PF4-coated bacteria. In fact in-vitro data and a mouse model suggest that PF4 might have a role in bacterial host defense mechanisms. The risk of developing anti-PF4/H antibodies is influenced by patient related factors, e.g. it is strongly increased when heparin is given within the context of trauma or inflammation. Trauma and inflammation might provide additional “danger signals” facilitating activation of the involved B cells.

While HIT patients regularly possess anti-PF4/H-IgG, only a minority of individuals with an anti-PF4/H response develop overt HIT. Interestingly, during the acute phase of HIT, some anti-PF4/H antibodies show characteristics of autoantibodies, i.e., they bind to PF4 on platelets even in the absence of heparin. This autoimmune feature of HIT is most pronounced in patients with “delayed onset HIT”, in whom HIT symptoms typically occur several days after cessation of heparin.
HIT shows features of a secondary immune response, of an autoimmune response, and it might be part of a general activation of a host defense system triggered by inflammation. On the other hand, anti-PF4/H antibody titers typically decrease rapidly,\textsuperscript{3,14} even though a secondary immune response should lead to persisting high titers of IgG antibodies.

Anti-PF4/H IgG antibodies are generated in about 50\% of patients undergoing cardiopulmonary bypass surgery (CPB). Besides necessitating the administration of heparin for anticoagulation during extracorporeal circulation, CPB poses a substantial challenge to the immune system due to mechanical tissue damage, anesthesia and other medication, extensive contact with foreign surfaces and ischemia-reperfusion injury to tissues. Together, these induce strong inflammation with rapid increase of serum cytokines, such as IL6, IL8, TNF\(\alpha\), and IL10.\textsuperscript{15-18}

This situation provided us with the opportunity to investigate whether the anti-PF4/H IgG response after CPB is part of (i) a general disinhibition of autoreactive B cells (general loss of tolerance) and/or (ii) associated with polyclonal activation of memory B cells against recall antigens.\textsuperscript{19-22} We followed CPB patients prospectively for more than four months after surgery in order to compare the kinetics of the anti-PF4/H IgG response with that of antibodies targeting other autoantigens as well as antibodies specific for the recall antigens tetanus toxoid (TT) and diphtheria toxin (DT). Our results clearly show that the kinetics of the anti-PF4/H antibody response has a unique profile: Its rapid but transient increase was distinct from that of the antibody response to the recall antigens TT and DT, which gradually increased over the whole observation period without showing the decrease in titers typically observed for anti-PF4/H antibodies. Neither was it part of a general break in tolerance since an increase in the titer of antibodies directed against other typical autoantigens was not observed.
Patients and Methods

Patients had been enrolled in a previously reported, prospective study in adult patients requiring CPB surgery.\textsuperscript{23-25} From these patients serum samples had been collected before surgery and on days three and ten after surgery. At discharge from hospital, patients were invited to participate in this long term study and to provide an additional blood sample at least four months after surgery. This time period corresponds to around six half-lives of serum IgG. After this time period more than 98% of the antibodies generated perioperatively should have been degraded. This allows assessment of the long-term effects of CPB surgery on the B cell response.

The study was approved by the Ethics Committee of the Ernst-Moritz-Arndt University in Greifswald, Germany.

\textit{Antibody assays and CRP-measurement}

Anti-PF4/H-IgG antibodies were assessed by enzyme immunoassay (EIA)\textsuperscript{26} and reactivities are given as optical densities (ODs). As several patients showed an OD > 0.5 in the baseline sample, we defined as responders patients experiencing an increase of the OD at day 10 of $\geq 0.5$ compared to the baseline OD values. Patients with a lower increase or no increase at all were classified as nonresponders.

Concentrations of anti-DT and anti-TT recall antibodies were measured using the SERION ELISA classic kit according to the manufacturer’s instructions (Virion\Serion, Würzburg, Germany). The optical densities were compared with an approved standard and are given in international serum units (IU). For the determination of IgM or IgG total serum concentrations, Nunc MaxiSorp\textsuperscript{®} 96 well plates (NUNC, Langenselbold, Germany) were coated overnight at 4°C with goat anti-human Ig (Southern Biotech, Birmingham, USA). After washing, free protein binding sites were blocked with 2% goat normal serum in PBS/Tween. Serum was diluted with the blocking buffer, added to the sample wells, and incubated for one hour. Bound antibodies were detected with goat anti-human IgM-HRPOD (Dianova, Hamburg, Germany) or goat anti-human IgG-HRPOD (Dianova, Hamburg, Germany). The plates were developed with BD OptEIA™ TMB Substrate Reagent, stopped with 2 N H\textsubscript{2}SO\textsubscript{4} and read at 450 nm. Concentrations were determined with an appropriate standard curve.
To screen for autoantibodies, we used binding to the human epidermoid carcinoma cell line HEp2 as a read-out, which allows the detection of antibody binding to a large spectrum of nuclear, cytoplasmic and cytoskeletal autoantigens. Human sera were diluted 1:100 in 20% FCS/PBS and incubated on HEp2-ANA slides (INOVA Diagnostics, San Diego, USA) overnight at 4 °C. After washing with PBS, bound antibodies were visualized with one drop of fluorescein-labeled anti-human IgG conjugate with Evan's Blue - ready to use (INOVA Diagnostics, San Diego, USA). Slides were exposed for an equal length of time and pictures taken with a ZEISS-Axio Imager A.1 microscope and Spot Advanced software.

C-reactive protein was measured by a standard high sensitivity assay (CRPL3 and Cobas Integra 800, Roche, Grenzach, Germany) as part of the standard post-surgical monitoring protocol.

**Statistical analysis**

We used Friedman’s test with Dunn’s post test, Mann-Whitney U test or Wilcoxon matched pairs test where indicated using GraphPad Prism 5 software.
Results

In total, 166 patients were enrolled in this long-term study. As expected, CPB surgery induced a strong inflammatory response. The patients’ maximal CRP serum concentrations three days after surgery averaged 167 mg/dl (range: 3 mg/dl – 350 mg/dl; reference value for healthy controls < 1 mg/dl) (Figure 1). The CRP concentrations on day three (maximum) did not correlate with the increases of total or antigen-specific IgG concentrations described below (Figure S1).

Kinetics of the anti-PF4/H response

Anti-PF4/H IgG increased by at least 0.5 OD by day 10 after surgery in 72 patients (responders; Figure 2A). When these patients were re-assayed at > 4 months, anti-PF4/H ODs had decreased and responders became indistinguishable from nonresponders (Figure 2B) fitting earlier observations of a transient nature of the anti-PF4/H antibody response. Ninety-four patients were nonresponders (ΔOD < 0.5; Figure 2A).

Kinetics of autoreactive antibodies

In contrast to the rapid transient increase in anti-PF4/H IgG, serum antibody binding to typical autoantigens did not change after CPB surgery. When we screened the sera for anti-cellular antibodies using binding to HEp2 cells as a readout, the majority of patients, 124 out of 166 (74.7%), did not show any sign of autoreactivity during the entire study period (Figure 3A); 22 patients had weakly binding IgG autoantibodies (not shown), and 20 patients exhibited strong IgG binding with typical patterns of autoreactivity (Figure 3B, C). In the patients with autoreactive antibodies, these antibodies were already present in the baseline sample taken before surgery. Neither the pattern nor the binding intensities of these IgG autoantibodies changed during the postsurgical period. This renders it very unlikely that the anti-PF4/H antibody response is part of a general activation of autoreactive B cells.
**Polyclonal B cell activation**

Total IgG serum concentration increased by a mean of 30% between baseline and day 10 after surgery (p<0.001; Table 1) and was even higher in the sample taken more than 4 months after surgery (p<0.001; Figure 4 A). This increase was observed in the anti-PF4/H responders and the nonresponders (Figure 4 B and C) with no difference in total IgG levels reached at day 10 and at > 4 months (p<0.25 and p<0.16, Mann-Whitney-U test). At baseline, anti-PF4/H responders had significantly lower IgG concentrations than nonresponders (p = 0.0047, Figure 4 B and C and Figure S2). This difference was due to those patients, whose baseline IgG concentration exceeded 15 mg/ml, which was the case in 21 responders and 5 nonresponders. The 26 patients maintained or even increased their very high IgG serum concentrations over the whole study period (Figure S3). Yet neither the baseline IgG concentrations nor the changes in total IgG serum concentration correlated with the increase of anti-PF4/H ODs (Figures S4 A and B). Furthermore, the 26 patients with high baseline IgG concentrations did not differ from the others with regard to their general inflammatory response as determined by serum CRP concentrations at day 3 after surgery (Figure S5).

The kinetic of total serum IgM concentrations was very similar to the changes in total serum IgG concentrations (Figure S6).

We then focused on established B cell memory for the classical recall antigens TT and DT and measured a small but significant increase of IgG binding to these antigens over time (p<0.001 in both cases; Figure 5A), with no difference between anti-PF4/H responders and nonresponders (TT: p<0.49; DT: p<0.59, Mann-Whitney-U test; Table 2 and Figure 5 B and C). Also on an individual basis, the changes in IgG binding to the recall antigens TT and DT on the one hand and to PF4/H on the other did not correlate (Figure S7 A and B). Thus the long-lasting effects of CPB surgery on total as well as antigen-specific Ig serum concentrations differed markedly from the transient kinetics of IgG binding to PF4/H.
Discussion

This study provides further evidence that the immune response to PF4/H after major surgery has distinct features that cannot be explained by either a general response of autoreactive B cells nor a moderate polyclonal activation of memory B cells to recall antigens following CPB surgery.

The immune response to PF4/H is of great interest because it mediates the most frequent adverse drug reaction involving blood cells, HIT. To the best of our knowledge, there is no other iatrogenic immune reaction that occurs so frequently in adults. After CPB surgery, about 50% of the patients are expected to develop an anti-PF4/H immune response. Because the immune response to PF4/H always occurs in the second week of heparin treatment, clinical studies are feasible. In this study, we systematically assessed the acute and long-term B cell response against PF4/H in comparison with immune responses to other antigens in humans under “real world” conditions. Since IgG has a serum half-life of around 21 days, we followed patients for more than four months. After this time, more than 98% of the serum IgG, present before surgery or produced perioperatively, has decayed. Thus any changes in the plasma cell pool, in particular generation of new long-lived plasma cells or loss of pre-existing plasma cells, were detectable by changes of serum antibody titers.

CPB surgery elicited a pronounced increase of IgG binding to PF4/H that was preceded by a strong inflammatory response. However, there was no correlation between the serum concentrations of CRP three days after surgery and the subsequent increase in anti-PF4/H-IgG binding. We cannot differentiate, whether the involved B cells require a more specific signal rather than broad activation of inflammation, or whether the inflammatory stimulus during cardiac surgery is so strong that maximal activation of the involved B cells may already be reached even in patients with a relatively low increase in CRP. This could be studied by correlation of the CRP levels and anti-PF4/H antibody response in patients undergoing less invasive procedures.

It remains uncertain whether the anti-PF4/H immune response should be classified as an autoimmune response or as an alloimmune response to a neoeptope. Although PF4 presents repetitive epitopes when complexed with heparin, it remains an endogenous protein. In addition, there is
increasing evidence that anti-PF4/H antibodies can cross-react with PF4 bound to platelets without the need of additional heparin. In some patients, HIT occurs even several days after heparin is no longer administered (delayed-onset HIT), that is, in the absence of circulating pharmaceutical heparin. This would be compatible with an in vivo autoimmune response. However, this feature may be restricted to a subset of platelet-activating anti-PF4/H-IgG antibodies, occurring in patients with symptomatic HIT. Very rarely, HIT occurs spontaneously. These patients either had an underlying autoimmune disorder such as systemic lupus erythematosus or had a recent bacterial infection.

We reasoned that the increase of anti-PF4/H antibodies after CPB surgery might be part of a general activation of (natural) autoreactive B cells derived, e.g., from marginal zone B cells or from B1 cells. It has already been shown in a mouse model that such B cells are triggered by tissue injury and ischemia-reperfusion. In addition, Tiller and co-workers have discovered an abundance of autoreactive specificities among class-switched memory B cells derived from B2 cells. During homeostasis, these cells do not give rise to antibody-secreting plasma cells, implying stringent control. This balance, however, could be disturbed by major surgery and inflammation. We performed the same screening assay for the detection of autoreactive antibodies as the group of Tiller (IgG binding to HEp2 cells) and found that after CPB surgery the control of these autoreactive B cell population was clearly maintained. Serum IgG binding to other autoantigens increased neither in prevalence nor in intensity and there was no correlation between the presence of autoantibodies and generation of anti-PF4/H antibodies. Our findings rule out that an entire class of “silent” IgG+ memory B cells with autoreactive potential is unleashed by CPB surgery.

Recently we observed the appearance of anti-PF4/H antibodies during bacterial sepsis in mice. Moreover, the natural immune response to PF4/H in the general population, that is, in individuals not treated with heparin, is strongly associated with periodontal disease. Anti-PF4/H antibodies might therefore be part of a bacterial host defense mechanism. In this case, PF4/H complexes could represent a recall antigen against which a secondary antibody response is triggered by heparin treatment in conjunction with PF4 release induced by trauma and/or inflammation. This
hypothesis is in line with the observation that anti-PF4/H IgG antibodies occurred in about half of the patients in the present study until day 6.\textsuperscript{23,24} and HIT typically occurs between days 5 and 10 after surgery.\textsuperscript{24,36,37} These observations are difficult to reconcile with the notion of a primary immune response.

The immune response pattern after cardiac surgery has been studied previously. Two groups found a decrease of total IgG concentrations following CPB, which in the study of Lante and co-workers amounted to 30% on the first day after surgery.\textsuperscript{38,39} Considering the serum half-life of IgG, which is around 20 days, this rapid effect must be due to hemodilution and/or sequestration of IgG,\textsuperscript{39} since changes in IgG production can account for a decrease of maximally 3% within one day.

Furthermore, memory B cells are highly responsive to polyclonal stimuli such as microbial products, CD40-ligation and common \(\gamma\)-chain cytokines.\textsuperscript{19} It has been proposed that polyclonal activation regularly boosts humoral memory and contributes to its maintenance in the absence of cognate antigen.\textsuperscript{19-22} We therefore assessed whether CPB surgery and the associated inflammatory response led to an antigen-independent activation of B cells. For this we measured the total serum IgG concentrations as well as the response to two prototypic recall antigens, TT and DT. These antigens were selected because most individuals are vaccinated against these proteins and possess specific memory B cells as well as long-lived plasma cells, which maintain the constant serum antibody concentrations.\textsuperscript{40} Obviously, there will be no antigen-driven reactivation of TT- or DT-specific memory B cells during CPB surgery.

IgG serum concentrations moderately increased over baseline by day 10 after surgery and continued to rise over the next months. This strongly indicates that cardiac surgery has long-term effects on antibody-producing plasma cells. Focussing on antigen-specific antibodies, Lante et al. observed that the concentration of TT-binding IgG had moderately increased by day 5 after CPB surgery.\textsuperscript{38} Our data corroborate and extend their observations showing that the increase was stable over several months and that anti-DT antibodies exhibit a similar behavior. Thus stimulation of antibody production to recall antigens appears to be a more general feature of CPB intervention, indicating that memory B cells are indeed polyclonally activated in major surgery patients.
If the antibody titers to DT and TT had shown a transient increase, similar to the kinetics of the PF4/H response, the latter could have been attributed to polyclonal activation of memory B cells, too. However, this was not the case, and thus the polyclonal activation of memory B cells cannot fully explain the anti-PF4/H IgG response to CPB surgery. Firstly, a continuous moderate increase characterized IgG binding to the “classical” recall antigens, whereas in many individuals, the anti-PF4/H response was strong, even dramatic initially. This strong initial increase can easily explained by antigen exposure. However, secondly, the PF4/H IgG titers declined and after 4 months there was no longer any difference between responders and nonresponders detectable. This is consistent with our previous findings that IgG+ memory B cells with specificity for TT but not for PF4/H are found in the peripheral blood of CPB patients prior to surgery. Furthermore, from the transience of its kinetics it is obvious that long-lived plasma cells do not readily develop during an anti-PF4/H response. Finally, there is evidence that an anti-PF4/H immune response can be elicited in MyD88-knock-out mice, who are deficient in their response to many polyclonal B cell stimuli. Taken together, the anti-PF4/H response shows a very different pattern compared with the polyclonal response to classic recall antigens. This suggests that the anti-PF4/H response is antigen-driven, which is likely since the immune system was challenged with heparin during and after surgery but not with DT and TT.

Besides polyclonal activation of B cells, for example via toll-like receptors, also a shift in the T cell subset composition could contribute to alterations in the antibody response after major surgery. Following an initial systemic inflammatory response (SIRS), major surgery (including cardiac surgery) leads to an anti-inflammatory state characterized by transient lymphocytopenia with impairment of T cell activation and downregulation of CD14 on monocytes. When stimulated ex vivo, PBMCs and T lymphocytes obtained from such patients released less interferon-γ, shifting the balance from a T helper-1 towards a T helper-2 profile. This alteration can be (partially) reversed by the addition of IL-12. While there was no net increase in the number of IL4-secreting cells nor in the amount of released IL4, DiPiro et al. have measured increases of IgE concentration and eosinophil count in the blood following CPB surgery or sepsis. This is intriguing in view of recent reports that eosinophils are constituents of the niche for long-lived plasma cells. Megakaryocytes are also part of this niche and it is very well known, that a transient increase in platelet counts is typical after major
surgery. Thus activation of the B cells producing anti-PF4/H antibodies might be in part caused by
downregulation of the adaptive immune system. We already hypothesized that the anti-PF4/H immune
response is an evolutionary ancient defense mechanism.7 Perhaps transient lymphocytopenia provides
a favorable environment for these B cells, which are silenced again when the T-cell population
recovers. This may in part explain the transience of anti-PF4/H antibody production. The recent
finding that IL-10 promoter polymorphisms are associated with the risk for anti-PF4/H immune
response50 would fit to such a regulatory feedback loop.

In this regard, the observation that individuals with very high IgG plasma levels at baseline
had a much lower risk for responding to PF4/H is of interest. Assuming a causal relationship, one may
speculate that the high baseline IgG levels are due to a stimulated adaptive immune system, which
may have a negative feedback on those B cells which produce PF4/H antibodies. Since these patients
did not have measurable anti-PF4/H antibodies before surgery, neutralization of PF4/H complexes is
not a likely explanation. Furthermore, with regard to their general inflammatory response, as measured
by CRP levels, the patients with high baseline IgG concentrations did not differ from others, ruling out
a general dampening effect of the IgG on inflammation, such as it is observed after therapeutic
application of high dose intravenous IgG59-61. Potentially, a high serum IgG serum concentration
reflects an ongoing (secondary) immune response. A vigorous plasma cell response could interfere
with anti-PF4/H-plasma cell proliferation, differentiation and survival, because the spleen has a finite
capacity to sustain plasma cells.62 However, most nonresponders had baseline IgG serum
concentrations well below 15 mg/ml and those patients with high IgG levels at baseline did not
experience a decrease of their IgG concentrations over time as it would be expected from a fading
secondary antibody response (Figure S3). Thus at this stage there is no final answer, as to how very
high IgG concentrations may prevent the development of anti-PF4/H antibodies.

Our findings have also implications of clinical relevance: Clearly, CPB did not interfere with
long-term immune memory. It caused no loss of anti-DT and anti-TT protection but, on the contrary,
moderately boosted the memory response.51-53 On its own, such a small boost may be of little
biological significance, but when repeated, this mechanism could stabilize memory. Obviously, the
bone marrow niches for antigen-specific long-lived plasma cells remained intact after CPB surgery. Either the polyclonally activated B cells were unable to displace resident long-lived plasma cells or they replaced them with newly generated plasma cells of the same antigen specificity.40,54-57 These findings are in good agreement with the results of a recent long-term study addressing humoral memory to vaccination antigens in which extremely long serum half-lives of protective serum antibody responses that were in the range of decades had been demonstrated.58

In summary, our data implicate that the increase in anti-PF4/H IgG after CPB surgery is an antigen-driven B cell response that can be explained neither by secondary or memory response nor by general nonspecific activation of memory B cells. Furthermore, the autoimmune-like features of some anti-PF4/H IgG antibodies cannot be explained by a general activation of B cells with autoreactive potential. This provides further evidence for our hypothesis that the anti-PF4/H antibody response shows a rather unique profile and may belong to a class of ancient humoral defense mechanisms at the interface between the innate and adaptive immune system. In the case of heparin administration, this response mechanism is misdirected to the platelet surface.
Acknowledgements

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Authorship

All authors contributed to the study design and the data evaluation, CP and SS performed experiments, and CP, BMB and AG wrote the manuscript.

Disclosure statement

The authors declare no conflict of interest.
References


Tables

Table 1: IgG serum concentrations in the whole study group (A), nonresponders (B) and responders (C).

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<td>IgG (mg/ml)</td>
<td>d0</td>
<td>d10</td>
<td>&gt; 4 mo</td>
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<td>9.46±6.02</td>
<td>12.92±7.9</td>
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Table 2: Serum concentrations of IgG specific for DT and TT in the whole study group (A), nonresponders (B) and responders (C).

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<td>DT (IU/ml)</td>
<td>d0</td>
<td>d10</td>
<td>&gt; 4 mo</td>
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<td>0.28±0.3</td>
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<td>TT (IU/ml)</td>
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Figure legends

Figure 1: Kinetics of the CRP concentrations in CPB patients.

C-reactive protein was measured pre- and postoperatively. The figure illustrates that the patients developed transient strong inflammation after CPB surgery (normal < 1mg/dL). The box plots indicate the median, upper and lower quartiles and the range.

Figure 2: Following CPB surgery, patients experienced a transient increase of IgG binding to PF4/H.

One hundred sixty-six patients undergoing CPB surgery were recruited into the study. Anti-PF4/H IgG binding was determined preoperatively, 10 days later, and re-analyzed around four months postoperatively. The differences between the OD values on day 10 or > 4 months and baseline OD values are shown. The means are shown by solid lines.

(A) Patients were classified into responders or nonresponders according to the increase in their anti-PF4/H IgG serum binding from day 0 to day 10 with a threshold of ∆OD = 0.5 (hatched line).

(B) Around four months after CPB anti-PF4/H antibodies had decreased and responders became indistinguishable from nonresponders. Very few patients still remained above the threshold for responders (hatched line).

Figure 3: CPB surgery did not induce autoreactive antibodies.

Human sera before (d0) and after CPB (d10, > 4 mo) were diluted in 20% FCS/PBS and incubated on commercially available HEp2-ANA slides. Bound antibodies were visualized with FITC-conjugated anti-human IgG. The staining did not reveal any increase of autoreactive antibodies postoperatively. Patient A is representative of the majority of patients (124 out of 166) who showed no autoreactive
antibodies at any time. The two patients, B and C, already had antibodies against nuclear or cytoplasmic antigens before surgery. These did not change in quality or quantity after surgery. Twenty out of the 166 patients showed a similar picture.

**Figure 4: Serum concentrations of total IgG moderately increased over the observation period.**

Serum IgG concentrations were determined by ELISA before (d0), 10 days (d10) and more than four months (> 4 mo) after CPB. Friedman’s test with Dunn’s post test revealed significant increases of IgG over time in all patients (A), with no major difference in the pattern between nonresponder (B) and responder (C) groups. However, the mean value for IgG in nonresponders to PF4/H at baseline was lower than in responders. This was due to a larger proportion of individuals with total IgG levels >15 mg/mL (see also Figure S2).

n = 166, ***: p < 0.001

**Figure 5: IgG binding to the recall antigens DT and TT moderately increased following CPB surgery.**

Serum concentrations of antibodies binding to DT and TT were determined by ELISA before (d0), 10 days (d10) and more than four months (> 4 mo) after CPB. Friedman’s test with Dunn’s post test revealed a significant increase of the concentrations over time in the patient group as a whole (A). The increase was similar in nonresponders (B) and responders (C). Means are shown (solid lines).

n = 166; *: p < 0.05, **: p < 0.01, ***: p < 0.001
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