

# The Effects of Glucocorticoid Therapy on Inflammatory Responses to Coronary Artery Bypass Graft Surgery

Vishnu Rumalla, MD; Steve E. Calvano, PhD; Alan J. Spotnitz, MD; Tyrone J. Krause, MD; Edward Lin, DO; Stephen F. Lowry, MD

**Hypothesis:** Delayed or reduced polymorphonuclear leukocyte (PMN) apoptosis may contribute to prolongation of systemic inflammation after cardiopulmonary bypass.

**Background/Objective:** Preoperative administration of glucocorticoids has been used ostensibly to attenuate the systemic inflammation associated with cardiopulmonary bypass. Therefore, this study evaluated, in patients undergoing cardiopulmonary bypass, the efficacy of glucocorticoids in restoring peripheral blood PMN apoptosis and modulating PMN surface receptors (CD95, tumor necrosis factor receptor [TNFR]) known to be involved in proapoptotic or antiapoptotic signal transduction.

**Design:** Randomized control study.

**Setting:** Medical school and affiliated tertiary care hospital.

**Patients:** Thirteen patients undergoing coronary artery bypass grafting.

**Intervention:** Patients were randomly assigned to the control group (n=7) or to receive 1 g of methylprednisolone sodium succinate on anesthetic induction (n=6).

**Main Outcome Measures:** Blood samples were drawn before induction, 20 minutes after sternotomy and by-

pass, immediately postoperatively, and on postoperative day 1. Isolated PMNs were incubated for 6 hours with or without the CD95 agonist CH 11. Polymorphonuclear leukocyte apoptosis was measured using propidium iodide–RNAase staining and flow cytometry. Levels of PMN cell-associated receptors (TNFR and CD95), cytokines (TNF- $\alpha$ , interleukin 6 [IL-6], IL-8, and IL-10), and soluble receptors (sTNFR1 and sTNFR2) were measured.

**Results:** In all 13 patients, spontaneous and Fas-mediated PMN apoptosis decreased more than 80% from baseline ( $P<.001$ ) by postoperative day 1. Polymorphonuclear leukocyte CD95 increased ( $P<.003$ ) by postoperative day 1 compared with baseline, whereas PMN TNFR was unchanged. Methylprednisolone administration did not modulate PMN apoptosis or immunocyte receptor expression; however, such treatment did decrease postoperative IL-6 secretion ( $P<.001$ ) and increase postoperative IL-10 secretion ( $P<.001$ ).

**Conclusions:** The complications of major surgery include persistent inflammation, which can lead to multi-system organ failure. Polymorphonuclear leukocyte resistance to apoptosis may contribute to this process. A single preoperative dose of glucocorticoids did not effect PMN apoptosis or receptor phenotype.

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From the Divisions of Surgical Sciences (Drs Rumalla, Calvano, Lin, and Lowry) and Thoracic Surgery (Drs Spotnitz and Krause), Department of Surgery, University of Medicine & Dentistry of New Jersey–Robert Wood Johnson Medical School, New Brunswick, NJ.

**C**ARDIOPULMONARY bypass (CPB) initiates a cascade of humoral and cell-mediated inflammatory responses that leads to the development of the systemic inflammatory response syndrome.<sup>1-3</sup> Specifically, CPB has been demonstrated to increase both proinflammatory cytokine production and cytotoxic immune effector cell function.<sup>4,5</sup> Because CPB is a defined intervention performed in a controlled hospital environment, the systemic inflammation associated with this procedure is generally short-lived and usually re-

solves without deleterious consequences for the patient. However, in a small subset of patients with preexisting comorbidities or with other as yet to be defined variables for susceptibility, systemic inflammatory response syndrome is amplified and prolonged. Such patients are at increased risk for the development of multi-system organ failure and death.

Endogenous polymorphonuclear leukocytes (PMNs) recruited to sites of injury and/or infection play a major role in inflammation-induced injury.<sup>6</sup> Recent attention has focused on regulation of PMN turnover as an important mecha-

## PATIENTS AND METHODS

### PATIENTS

Thirteen patients undergoing coronary artery bypass graft surgery were prospectively studied during a 6-month period at a university medical center under a protocol approved by the Robert Wood Johnson Medical School (New Brunswick, NJ) institutional review board. All patient participation involved written consent before enrollment in the study. Patients with known immunodeficiency conditions and those undergoing short- or long-term steroid therapy were excluded from study. Patients meeting criteria for the study were randomly assigned by the investigators to the control group ( $n=7$ ) or to receive 1 g of methylprednisolone sodium succinate on anesthetic induction ( $n=6$ ) (Table).

Blood samples were drawn before anesthetic induction, 20 minutes after sternotomy, 20 minutes after bypass, immediately postoperatively in the recovery room, and on postoperative day (POD) 1. The specimens from each time point were analyzed for spontaneous and CD95-inducible PMN apoptosis, TNFR and CD95 cell-associated receptor expression, and plasma cytokine levels as described herein.

### PREPARATION OF PMNs

Whole blood was collected and anticoagulated with EDTA. After centrifugation for 10 minutes at 250g and removal of platelet-rich plasma, the cells were diluted 1:4 with Hanks balanced salts solution without calcium, magnesium, or phenol red (HBSS; BioWhittaker, Walkersville, Md). Ten milliliters of the diluted blood cells was then layered over 4 mL of Histopaque (Sigma-Aldrich Corp, St Louis, Mo) and centrifuged for 35 minutes at 450g. The cell pellet containing erythrocytes and PMNs was resuspended in 40 mL of HBSS containing 3% dextran (Sigma-Aldrich Corp, molecular weight=500000), and the aggregated erythrocytes were sedimented at 1g for 20 minutes. The PMNs were recovered by centrifugation, and 10 mL of 0.2% hypotonic saline was added dropwise to lyse the remaining erythrocytes. Tonicity was restored by addition of 10 mL of 1.6% saline.

### ASSESSMENT OF PMN APOPTOSIS

Purified (>95%) PMNs were washed 1 time with HBSS and diluted to  $1 \times 10^6$ /mL in complete medium (RPMI-1640; BioWhittaker, plus 10% fetal bovine serum) and cultured for 6 hours at 37°C with or without an agonist antibody to CD95 (clone CH 11; Upstate Biotechnology, Lake Placid, NY) to induce apoptosis. After culturing, the cells were washed with HBSS, fixed in cold 70% ethanol, and stained with a solution of propidium iodide (50  $\mu$ g/mL, Sigma-Aldrich Corp) and RNase (1.5  $\mu$ g/mL; Sigma-Aldrich Corp).

After storage overnight in the dark, PMN apoptosis was determined flow cytometrically as the percentage of PMNs expressing hypodiploid (<2n) DNA.

### CELL-ASSOCIATED TNFR AND CD95

Surface TNFR expression was determined by saturation binding of TNF- $\alpha$  as previously described.<sup>21</sup> Briefly, erythrocytes from 0.4 mL of peripheral blood were lysed with bicarbonate-buffered 0.826% ammonium chloride solution (pH 7.2). The PMNs were recovered by centrifugation and washed with cold phosphate-buffered saline containing 1% bovine serum albumin (PBS-BSA). Specific staining was with 1  $\mu$ g/mL of biotinylated human TNF- $\alpha$  followed by incubation for 15 minutes at 4°C. The cells were then washed with cold PBS-BSA and further stained with 0.5  $\mu$ g/mL of streptavidin conjugated with phycoerythrin for 15 minutes at 4°C. Nonspecific staining for background fluorescence was by the addition only of 0.5  $\mu$ g/mL of streptavidin-phycoerythrin. We have previously demonstrated that this background is identical to that observed with the addition of 1  $\mu$ g/mL of biotinylated TNF- $\alpha$  and 0.5  $\mu$ g/mL of streptavidin-phycoerythrin plus 100-fold excess nonbiotinylated TNF- $\alpha$ . The cells were then washed and resuspended for flow cytometric analysis. Analysis was performed on a flow cytometer (Profile; Coulter, Miami, Fla). The photomultiplier voltage was standardized using phycoerythrin-conjugated beads (Coulter). Mean channel fluorescence at 560 to 590 nm of forward- and side-angle scatter-gated PMNs was assessed. After lysis of erythrocytes, PMN surface CD95 expression was determined by staining with 1  $\mu$ g/mL of monoclonal mouse anti-human phycoerythrin-conjugated CD95 (Becton-Dickinson, San Jose, Calif) using phycoerythrin-conjugated mouse IgG1 (Becton-Dickinson) as the isotype control for background staining as previously described.<sup>21</sup> Incubation, washing, and flow cytometric analyses were performed in a similar manner to that described for TNFR staining.

### CYTOKINE AND SOLUBLE RECEPTOR LEVELS

Plasma levels of TNF- $\alpha$ , soluble TNFR1 (sTNFR1), sTNFR2, IL-6, IL-8, and IL-10 were quantified using standard, commercially available enzyme-linked immunosorbent assay kits (Pharmigen, San Diego, Calif, and R&D Systems, Minneapolis, Minn) as previously described.<sup>21</sup> Whole blood from each patient was collected in heparin sodium tubes kept on ice until they were centrifuged at 800g to separate plasma. The plasma was then stored at -70° before being assayed.

### STATISTICAL ANALYSIS

Two-way analysis of variance with repeated measures was performed on all data over time points. Post hoc analysis by the Newman-Keuls test was applied.  $P<.05$  was considered statistically significant.<sup>33</sup>

nism for modulating the inflammatory response.<sup>7,8</sup> Influx of PMNs from the vascular compartment into the tissues is up-regulated by chemotactic and inflammatory mediators, which induce expression of adhesion molecules on both the vascular endothelium and leukocytes.<sup>9,10</sup> This leads to firm adhesion of PMNs to the en-

dothelium followed by diapedesis into the underlying tissue. Efflux of PMNs that have extravasated into tissues from the vasculature does not occur to an appreciable extent. Rather, these cells are eliminated from inflammatory foci either by necrotic or apoptotic cell death.<sup>11,12</sup>

### Demographic and Functional Characteristics of Study Patients

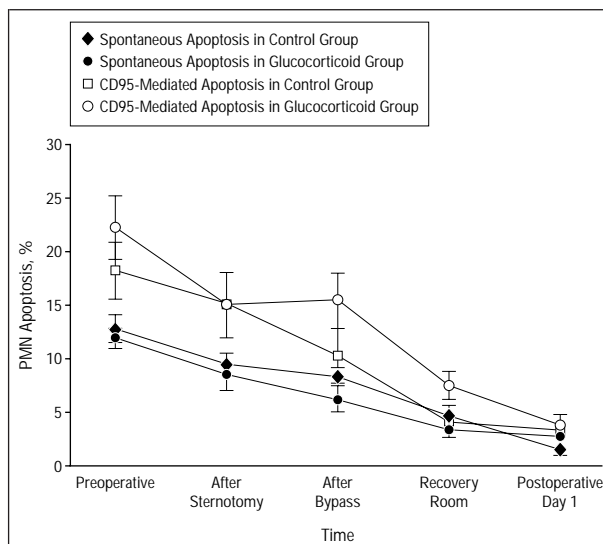
Characteristic	Control Group (n = 7)	Glucocorticoid Group (n = 6)
Age, mean, y	61	63
Ejection fraction, mean	>60%	>60%
Hypertension, No.	4	3
Diabetes mellitus, No.	1	4
No. of grafts, mean	3	3
Bypass time, min	75	100
Clamp time, min	45	60

Cardiopulmonary bypass causes an increase in PMN adhesion receptor expression and margination, leading to enhanced extravasation of these cells into the heart, where direct and bystander tissue damage can occur, especially on reperfusion.<sup>4,13-15</sup> Fortunately, PMNs have a constitutive apoptotic program that limits their life span.<sup>16,17</sup> However, it has been shown that this constitutive program for cell death can be down-regulated by inflammatory mediators, including tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin 6 (IL-6).<sup>18,19</sup> Such a delay of PMN apoptosis would be important at sites of injury or infection, where premature termination of granulocyte antimicrobial behaviors would have deleterious consequences for the host. On the other hand, if this delay in PMN apoptosis is excessive or extends beyond sites of local inflammation, this could be an important mechanism by which systemic inflammation is sustained and amplified. Indeed, peripheral blood PMN apoptosis is reduced in a variety of inflammatory conditions, including those associated with trauma, burns, sepsis, and major surgery.<sup>18-20</sup>

Modulation of cell surface receptors that are capable of transducing proapoptotic or antiapoptotic signals is one mechanism by which PMN apoptosis can be regulated. Such receptors include CD95 and tumor necrosis factor receptor (TNFR) types 1 and 2.<sup>21,22</sup> The only known function of CD95 is to transduce proapoptotic signals, whereas TNFR signaling can be either proapoptotic or antiapoptotic, depending on the status of other extracellular and intracellular regulatory variables. The relationship between peripheral blood PMN CD95 and TNFR and apoptosis in the context of patients undergoing CPB has not, to our knowledge, been previously investigated.

To abrogate the inflammation associated with CPB, several immunomodulatory strategies have been attempted.<sup>23-26</sup> Glucocorticoids inhibit inflammation through a variety of different mechanisms,<sup>27-31</sup> and several regimens of glucocorticoid therapy have been used ostensibly to attenuate the postoperative inflammatory processes associated with CPB.<sup>23-26</sup> Although preoperative glucocorticoids have been shown to decrease intraoperative and postoperative cytokine expression,<sup>23,24</sup> others have suggested that glucocorticoid administration has little or even detrimental clinical benefit.<sup>25,26</sup>

Because prior studies in our laboratory showed that glucocorticoid administration restored PMN apoptosis during a human model of experimental endotoxemia,<sup>32</sup>



**Figure 1.** Percentage of apoptosis of polymorphonuclear leukocytes (PMNs) before and at various times during and after cardiopulmonary bypass in patients who did not (control) or did (glucocorticoid) receive 1 g of methylprednisolone sodium succinate on anesthetic induction. Spontaneous and CD95-mediated apoptosis indicate PMNs cultured with medium alone or with a CD95 agonist antibody, respectively, for 6 hours. For both spontaneous and CD95-mediated apoptosis time effects,  $P < .001$ . For spontaneous apoptosis vs CD95-mediated apoptosis,  $P < .02$  at baseline; there were no significant differences for control vs glucocorticoid groups at any of the time points. Data represent the mean  $\pm$  SE.

it was thought that therapy with these agents in the context of CPB might reverse the reduced PMN apoptosis hypothesized to contribute to postoperative systemic inflammation. To this end, the present study evaluated the effect of treatment with glucocorticoids on peripheral blood PMN apoptosis, CD95 and TNFR modulation, and cytokine production in patients undergoing CPB.

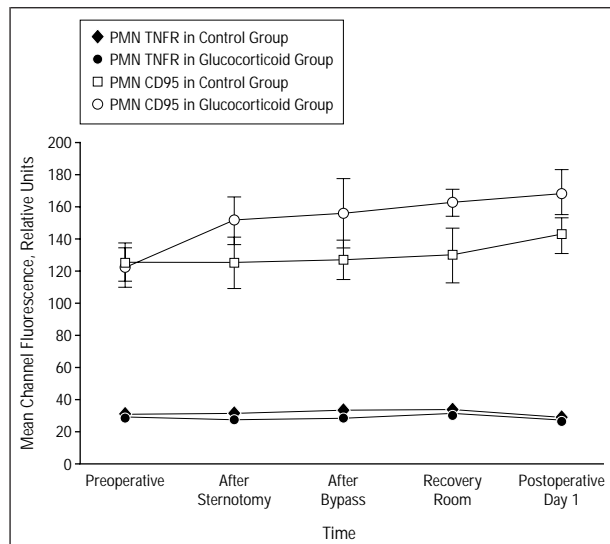
## RESULTS

### PMN APOPTOSIS

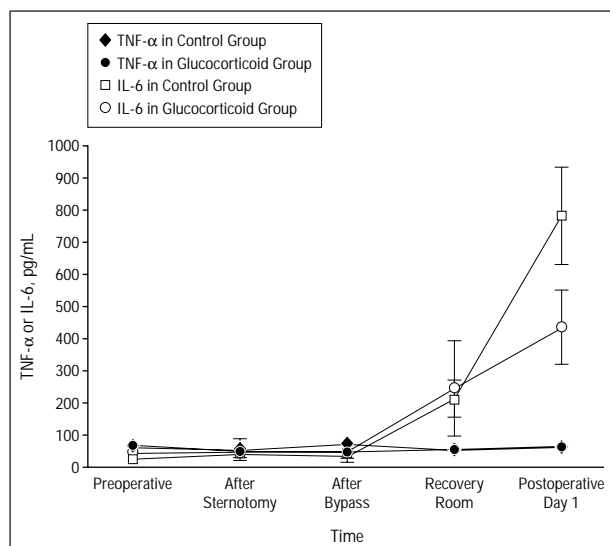
At baseline there was a statistically significant difference between percentage of spontaneous vs CD95-mediated PMN apoptosis (12.1% vs 22.2%;  $P < .02$ ) (**Figure 1**). In all 13 patients, both spontaneous and CD95-mediated PMN apoptosis decreased significantly from the respective baselines within 20 minutes after initiating CPB. The percentage of PMN apoptosis continued to decrease and reached a nadir of less than 20% of baseline on POD 1 for all patients ( $P < .001$ ). Glucocorticoid administration at the time of anesthetic induction did not affect spontaneous or CD95-mediated apoptosis at any of the subsequent time points.

### PMN RECEPTOR EXPRESSION

Compared with baseline, PMN cell-surface CD95 receptor expression rose significantly in both groups by the recovery room time point and continued to rise through POD 1 ( $P < .02$  and  $P < .003$ , respectively), whereas PMN TNFR was statistically unchanged (**Figure 2**). Methylprednisolone administration did not alter either PMN CD95 or TNFR expression.



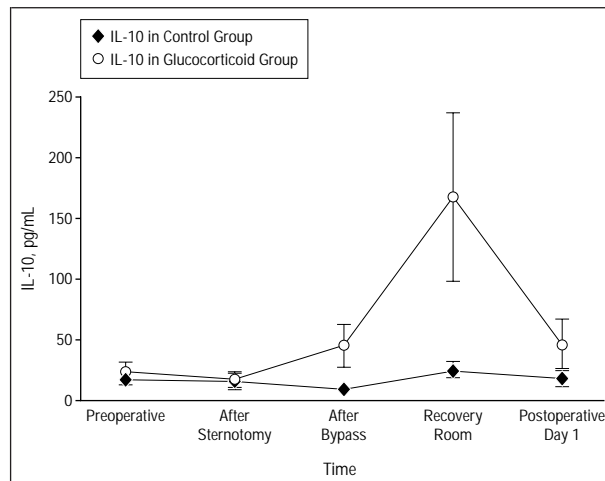
**Figure 2.** Cell surface polymorphonuclear leukocyte (PMN) CD95 and tumor necrosis factor receptor (TNFR) expression in control and glucocorticoid groups expressed as mean channel fluorescence over time. For the PMN CD95 time effect,  $P < .02$  at recovery room time point vs preoperative time point and  $P < .003$  on postoperative day 1 vs preoperative time point; there were no significant differences for glucocorticoid effect, or for both PMN TNFR time effect and glucocorticoid effect. Data represent the mean  $\pm$  SE.



**Figure 3.** Soluble tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin 6 (IL-6) concentrations over time in the control and glucocorticoid groups. For TNF- $\alpha$ , there were no significant time or glucocorticoid effects. For IL-6, time effect,  $P < .001$ ; time  $\times$  glucocorticoid interaction effect,  $P < .001$ . Data represent the mean  $\pm$  SE.

### SOLUBLE CYTOKINE AND RECEPTOR RESULTS

Tumor necrosis factor  $\alpha$  was detectable in 6 patients preoperatively, 3 in each group, and IL-8 was detectable in 3 patients preoperatively, 2 in the control and 1 in the glucocorticoid group. Mean TNF- $\alpha$  and IL-8 levels did not statistically change over any time points for either the control or steroid groups (IL-8 data not shown). Similarly, sTNFR1 and sTNFR2 levels did not change over any time points for either group (data not shown). The IL-6 levels, on the other hand, rose severalfold postopera-



**Figure 4.** Soluble anti-inflammatory cytokine interleukin 10 (IL-10) concentrations over time in the control and glucocorticoid groups. For time  $\times$  glucocorticoid interaction effect,  $P < .001$ . Data represent the mean  $\pm$  SE.

tively compared with preoperative baseline in both groups ( $P < .001$ ). However, this rise was significantly less in the glucocorticoid group by POD 1 compared with the control group ( $\Delta = 691.7$  vs  $393.4$  pg/mL, respectively;  $P < .001$ ) (Figure 3). In contrast, IL-10 levels rose significantly in the immediate postoperative period in patients receiving methylprednisolone compared with controls ( $\Delta = 168$  vs  $6.8$  pg/mL, respectively;  $P < .001$ ) (Figure 4).

### CLINICAL OUTCOMES

There were no deaths in the study. One patient in the control group had a stroke on POD 3, and 1 patient in the steroid group required reexploration within several postoperative hours for anastomotic bleeding. No infections occurred during hospital stay in any patient.

### COMMENT

Programmed cell death of immune cells may direct the nature and perhaps magnitude of the postinjury response.<sup>16,17,34</sup> An orderly and efficient apoptosis process represents the systematic deletion of senescent and dysfunctional cells following initial activation and/or proliferative response to injury. This process is believed to occur without the release of proinflammatory cytokines or potent chemical mediators, including toxic granule components and reactive oxygen metabolites.<sup>7,8,16</sup> However, at sites of inflammation, the altered mediator environment may disrupt this process. Several proinflammatory cytokines, including TNF- $\alpha$ , IL-1, IL-6, IL-8, and endotoxin, have been shown to reduce or delay immunocyte apoptosis in vitro.<sup>17,22,35</sup> As a consequence, these cells may predominantly undergo necrosis with the further release of toxic metabolites into the local environment.<sup>18-20</sup> Alternatively, delayed or reduced apoptosis may increase immunocyte half-life and prolong an acute or chronic inflammatory response.<sup>12,36</sup> In the present study, both spontaneous apoptosis and CD95-mediated apop-



tosis were decreased in response to CPB. This is in agreement with a previous report by Sakai and Watanabe,<sup>37</sup> who observed a reduction in PMN apoptosis in patients undergoing CPB. However, in the present study, no effect on PMN apoptosis was noted in patients who were administered a substantial dose of glucocorticoid before surgery. These patients showed an identical reduction in the rate of PMN apoptosis compared with patients not receiving glucocorticoids. This observation did not support our working hypothesis nor did it coincide with previous work where glucocorticoid administration restored delayed PMN apoptosis in a human model of endotoxemia.<sup>32</sup>

Current understanding is that steroids have differential effects on immune cell populations. Glucocorticoids have been shown to increase lymphocyte and monocyte apoptosis *in vitro* in both mice and humans.<sup>38-43</sup> On the other hand, it has also been demonstrated that glucocorticoids can be potent inhibitors of neutrophil apoptosis in murine and human models.<sup>44,45</sup> Kato et al<sup>45</sup> found this suppressive effect to be dose dependent in both spontaneous and TNF-mediated neutrophil apoptosis. Similarly, in concurrent studies we found that continuous glucocorticoid administration alone to healthy volunteers caused a decrease in PMN apoptosis, whereas glucocorticoid administration in the context of endotoxin-induced systemic inflammation significantly restored apoptosis to normal levels.<sup>32</sup> Such an effect was not discernible or may have been masked in the present study secondary to the overwhelming inflammatory mediator and neuroendocrine response elicited by CPB. Thus, glucocorticoid administration neither increased nor decreased PMN apoptosis, both of which have been previously reported.<sup>32,44,45</sup>

Polymorphonuclear leukocyte CD95 receptor significantly increased postoperatively, whereas TNFR was unchanged in both control and glucocorticoid groups. Methylprednisolone administration did not modulate this response. To date, no studies have characterized the effect of CPB on PMN TNFR and CD95 expression. Both TNFR species (types 1 and 2) and CD95 are members of the superfamily of TNFR complexes that are present on virtually all cells.<sup>46</sup> Specifically, the only known function of CD95 activation is to induce programmed cell death. CD95 induces apoptosis through the activation of intracellular death domains, leading to the recruitment of signaling proteins that collectively form the death-inducing signaling complex.<sup>47</sup>

Expression of PMN cell-associated CD95 receptor exhibits dynamic modulation during states of inflammation.<sup>21</sup> Previous work in this laboratory demonstrated an initial decline followed by a steady rise in PMN CD95, which becomes statistically significant after 48 hours compared with baseline receptor expression after endotoxin administration to healthy volunteers.<sup>21</sup> In the present study, this rise in PMN CD95 was statistically significant after CPB by the recovery room time point and continued through POD 1. Methylprednisolone administration did not significantly modulate this response. Although PMN CD95 receptor expression increases over time, the rate of CD95-mediated apoptosis steadily declined. This observation suggests that the inflammatory challenge elic-

ited by major surgery and/or CPB leads to a decline in PMN apoptosis despite proapoptotic PMN CD95 receptor upregulation. Whether this increase in CD95 receptor expression is a compensatory response to overcome the decrease or delay in PMN apoptosis is unknown. At the minimum, dynamic PMN CD95 receptor modulation further defines the inflammatory response seen during and after CPB.

Preoperative glucocorticoid administration significantly decreased IL-6 concentrations by POD 1 in the study. This is consistent with prior studies that demonstrated a decrease in proinflammatory cytokine production elicited by CPB in patients receiving preoperative and intraoperative glucocorticoids.<sup>23,24</sup> Glucocorticoids blunt the production of IL-6 at both the transcriptional and translational level.<sup>29</sup> Interleukin 6 is a cytokine that promotes stem cell growth, B- and T-lymphocyte activation, and the regulation of the hepatic acute-phase protein response.<sup>9</sup> Elevated circulating levels of IL-6 are often detected in conditions associated with inflammation, including elective surgery.<sup>48-50</sup> In fact, some studies suggest that IL-6 levels correlate with outcomes in that higher levels are noted in nonsurvivors vs survivors of burns, pancreatitis, and sepsis.<sup>51</sup>

In contrast to IL-6, preoperative glucocorticoid administration dramatically increased intraoperative and postoperative IL-10 levels in the study. This finding is consistent with previous observations of glucocorticoid administration in human models of inflammation.<sup>32</sup> Interleukin 10 is a potent anti-inflammatory cytokine that partially mediates its effects through the inhibition of gene expression and synthesis of several major proinflammatory cytokines, including TNF- $\alpha$ , IL-1, and IL-6, and may explain the decrease in IL-6 levels seen in patients receiving glucocorticoids.<sup>52,53</sup> In experimental sepsis models, exogenously administered IL-10 or gene transfer of the IL-10 gene decreased mortality in endotoxin-challenged mice.<sup>54,55</sup> Others have suggested that IL-10 may partially reverse the antiapoptotic effect of endotoxin and TNF- $\alpha$  in activated human neutrophils.<sup>34</sup>

There were no deaths in this study, and there were no demonstrable differences in clinical outcomes between the group that received glucocorticoids and the group that did not. In addition, no patients exhibited prolonged systemic inflammatory response syndrome or late complications that could be attributed to decreased PMN apoptosis. No modulation in PMN receptor phenotype or function related to exogenous glucocorticoids was demonstrable in the present study. Nevertheless, we cannot rule out an independent effect of endogenous steroid release or a more complex interaction with other neuroendocrine responses. A discernible trend toward reduced PMN apoptosis is already under way before CPB is initiated. Whether this effect would persist in the absence of superimposed CPB is a matter of conjecture. Our previous studies suggest that the endogenous release of glucocorticoids may be sufficient to alter PMN apoptosis in a manner observed in these studies. The delay in spontaneous and CD95-mediated PMN apoptosis and immunocyte receptor modulation represent novel findings and further define the inflammatory response induced by CPB. Increased comprehension of the

complicated immune response elicited by CPB may allow us to someday intervene and modulate the process.

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Corresponding author and reprints: Stephen F. Lowry, MD, Department of Surgery, Robert Wood Johnson Medical School CN 19, New Brunswick, NJ 08903-0019.

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