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INTERLEUKIN 12 AND INTERFERON-γ SYNTHETIC DEFICIENCY IS ASSOCIATED WITH DENDRITIC CELL CYTOPENIA AFTER CARDIAC SURGERY

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ABSTRACT—Traumatic or inflammatory injury associates with deactivation of monocytes and impaired synthesis of proinflammatory cytokines. We conducted a prospective, observational study to test whether cardiac surgery additionally impaired dendritic and natural killer (NK) cell functions responsible for innate immune production of interleukin (IL)-12-dependent interferon (IFN)-γ in response to bacteria or toll-like receptor agonists. Blood samples were taken just before induction of anesthesia and 24 h postoperatively. LPS- and fixed *Staphylococcus aureus*-inducible IFNγ synthesis in whole blood culture after surgery was reduced to 5% of preoperative values ($P < 0.001$). Production of IL-12 p70, a critical inducer of IFNγ in the innate immune response, was reduced to 30% of that produced by preoperative samples ($P = 0.013$). Circulating $CD11c^+$, DR^+ myeloid dendritic cells (DC) that are known sources of IL-12 p70 in normal blood, declined to approximately 25% of presurgical numbers ($P = 0.004$). Experimental depletion of $CD11c^+$, but not $CD14^+$, cells from normal peripheral blood mononuclear cell (PBMC) similarly disabled *Staphylococcus aureus* Cowan 1 (SAC)-induced production of IL-12 p70 and IFNγ. Consistent with SAC-induced IFNγ expression in $CD56^+$ NK and NK-T cells, CD56 depletion ablated IFNγ production in normal whole blood. However, repletion of IL-12 p70, IL-18, IL-15, and IL-23 in postoperative blood failed to restore presurgical levels of IFNγ synthesis ($P < 0.05$). We conclude that DC cytopenia after major surgery is sufficient to explain postoperative IL-12 p70 and IFNγ synthetic deficiency. In addition, postoperative blood became hyporesponsive to IFNγ-inducing cytokines as a further contribution to IFNγ insufficiency. The novel finding of DC cytopenia after major surgery may portend a lack of other immunologic functions provided by this potent accessory cell population.

KEYWORDS—Interferon-γ, interleukin-12, endotoxin, dendritic cell, monocyte deactivation

INTRODUCTION

The systemic inflammation associated with cardiac surgery with cardiopulmonary bypass is very similar to the systemic response seen in other injured populations (1), including trauma, burns, and sepsis. It is well recognized that systemic inflammation in all of these settings is frequently followed by depressed immune activation, as characterized by reduced delayed type hypersensitivity (DTH) reactivity and decreased monocyte expression of the MHC II molecule, HLA-DR (2–4). This monocyte-deactivation phenotype also associates with decreased proinflammatory cytokine synthesis and may be linked to increased susceptibility to nosocomial infection (5–7). Trauma and sepsis can also impair IFNγ production triggered by mitogen or anti-CD3 antibody, suggesting that critical defects in T lymphocyte function also occur (4). By suppressing production of proinflammatory cytokines, these immune defects may significantly contribute to the infectious complications of trauma and other critical illnesses (8).

IFNγ can also be generated in an antigen-independent fashion by natural killer (NK) cells and NK-T cells responding to IL-12 produced as part of the innate cellular immune response to infection (9). Dendritic cells are a critical source of bioactive IL-12 p35/p40 heterodimer (p70) in this setting (10), and this cytokine acts synergistically with IL-18 to induce innate IFNγ synthesis by NK and NK-T cells (11, 12). IFNγ subsequently mediates significant first-line defense against localized infection (13–18). IFNγ and IL-12 generated during the innate response may also critically promote Th1-type T cell differentiation, further contributing to the resolution of bacterial infection (19). Therefore, the status of innate cellular immune function in injured patients may be highly relevant to understanding the clinical relationship between trauma-induced immune suppression and increased susceptibility to nosocomial bacterial infection. For instance, LPS-induced IL-12 synthesis by monocytes is reduced in patients with sepsis and after major visceral surgery and predicts an increased risk of infection (20, 21).

Although routine cardiothoracic surgery similarly disrupts monocyte expression of HLA-DR and mitogen-induced

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production of IFNγ by peripheral T lymphocytes (22), the integrity of NK cell-dependent IFNγ produced via innate cellular immune pathways has not been characterized. We hypothesized that cardiac surgery with cardiopulmonary bypass would similarly reduce bioactive IL-12 p70- and IL-12-mediated IFNγ production in response to nonviable microbial challenges represented by lipopolysaccharide- and formalin-fixed *Staphylococcus aureus*. Our findings confirm the presence of a profound cytokine deficiency in postsurgical patients and newly identify reduced numbers of $CD11c^+$ dendritic cells in the peripheral blood that are sufficient to explain postoperative decreases in IL-12 and IFNγ synthesis.

MATERIALS AND METHODS

Patient selection

Participants in this study were consenting patients scheduled for elective cardiac surgery. Fifty-six patients were enrolled in pre- and postoperative studies.

An additional 11 volunteers provided blood for *in vitro* studies. Fifteen milliliters of whole blood was collected in a sterile tube containing 100 U/mL sodium heparin (BD Vacutainer no. 366489; Becton Dickinson, Franklin Lakes, NJ) just before surgery and again 24 h later. Individuals with immunosuppressive disorders, renal insufficiency (creatinine clearance <30 mL/min), or human immunodeficiency virus infection were excluded from the study. Corticosteroids were not administered at the time of surgery. The human subjects protocol and consent forms were approved by the Institutional Review Boards of the University Hospitals of Cleveland and the Louis Stokes Cleveland Veterans Affairs Medical Center.

Flow cytometry for monocyte HLA-DR expression and numbers of leukocyte subsets

Blood anticoagulated with 5 mM EDTA (BD Vacutainer no. 366452) was processed for cell subset analysis by the clinical flow cytometry laboratory of University Hospitals of Cleveland. Peripheral blood mononuclear cells (PBMC) were stained with fluorescent antibodies specific for CD3, CD19, and CD16/CD56 (SimulSet; Becton Dickinson, Palo Alto, CA) to measure T, B, and NK lymphocyte numbers using a FACSCalibur flow cytometer (Becton Dickinson). Sample gates were selected for specific analysis of cells with the forward- and side-scattering characteristics of lymphocytes. PBMC were also stained with CD14- and pan-allelic HLA-DR-specific antibodies and were analyzed for mean fluorescence intensity of HLA-DR on $CD14^+$ cells with the forward- and side-scatter characteristics of monocytes. Dendritic cells were analyzed using the Becton Dickinson Dendritic Cell Staining kit. Myeloid and plasmacytoid dendritic cells were defined as lineage 1^-, $HLA\text{-}DR^+$, and $CD11c^+$ or $CD123^+$, respectively. Total white blood counts were concurrently obtained by automated flow cytometry in the clinical laboratories of University Hospitals of Cleveland.

Whole blood cultures

The technique of cytokine generation by whole blood culture has been previously characterized (23). One-milliliter fractions of heparinized blood were incubated with 10 μL of saline alone or saline containing twice-washed *Staphylococcus aureus* Cowan Strain I (SAC, Pansorbin; Boehringer Mannheim, Indianapolis, IN) for a final concentration in culture of 0.01% (w/v) or *Salmonella enteritidis* lipopolysaccharide (Sigma Chemical, St. Louis, MO) for a final concentration of 10 ng/mL. These concentrations of SAC and LPS were determined in preliminary studies to be optimal for stimulating IFNγ synthesis. These reagents were partially characterized for activity. Endotoxin activity, assayed by limulus lysate assay (Sigma Chemical) was undetectable in the SAC reagent at the concentrations used in these cultures. Conversely, the LPS reagent failed to induce cytokine production in spleen cultures from Toll-like receptor (TLR) 4-deficient C3H/HeJ and TLR4KO C57BL/6 mice. After 18 h of culture at 37°C in 5% CO_2, the samples were centrifuged at 200*g* for 10 min, and the conditioned plasma was removed for enzyme-linked immunoabsorbant assay (ELISA) analysis. In replicate cultures, 20 μg/mL neutralizing anti-IL-10 MAb (JES3-19F1; BD PharMingen, San Diego, CA) was added to test the inhibitory effects of IL-10 on IFNγ production during *in vitro* stimulation. It was determined that 12.9 ng/mL antibody neutralized 90% of the activity of 5 ng/mL IL-10. Other samples were coincubated with recombinant human IL-12 (R&D Systems, Minneapolis, MN, specific activity 1.2×10^7 U/mg), recombinant human IL-18 (BioSource International, Camarillo, CA), and recombinant IL-23 (BioSource International).

Intracellular staining for IFNγ

To determine cellular sources of IFNγ, whole blood from healthy volunteers (n = 5) was incubated with 0.01% SAC in the presence of a 1:100 dilution of monensin (GolgiStop; BD PharMingen) for 4 h. Red cells were lysed using PharMLyse (BD PharMingen) and nucleated cells were labeled with fluorescein isothiocyanate anti-CD3 and PE anti-CD56. The cells were then fixed, permeabilized, and stained intracellularly with APC anti-HuIFNγ.

PBMC cultures

PBMC were separated from heparinized blood by density gradient centrifugation over Ficoll-Hypaque (1.077; Sigma Chemical). Interface cells were washed three times in normal saline and were suspended to a concentration of 10^7 cells/mL culture media (RPMI/10% fetal bovine serum [FBS]) consisting of RPMI 1640 supplemented with 20 mM Hepes (7.4), 100 μg/mL each streptomycin and penicillin G, 2 mM L-glutamine, and 10% FBS (HyClone Laboratories, Logan, UT). In preliminary studies, fixed *S. aureus*, but not LPS, induced as much IFNγ as was observed in whole blood cultures. One hundred microliters of the cell suspension was incubated with 100 μL of culture media, 0.01% SAC, 0.01% SAC plus 1 ng/mL rhIL-12 p70, or 0.01% SAC plus plasma at 37°C in 5% CO_2 for 18 h. The conditioned supernatant was removed and stored at −70°C until assayed by ELISA.

Depletion studies

Aliquots of PBMC (10^7 total cells each) from healthy volunteers (n = 6) were incubated separately with magnetic beads specific for human CD14, CD11c, or CD56 (Miltenyi Biotech, Auburn, CA) and applied to magnetized columns. Unlabeled cells were eluted using 5 mL of wash buffer (phosphate-buffered saline, 0.5% bovine serum albumin, and 5 μM EDTA at pH 7.4), and labeled cells were recovered by washing of columns after removal from the magnetic field. The extent of depletion and the composition of the magnetically selected cells were confirmed by FACS analysis. Preselection and postdepletion cell populations were separately suspended at 1×10^6 cells/mL in RPMI/10% FBS culture media with or without 0.01% SAC and were cultured for 24 h. Supernatants were assayed for IL-12 p70, IL-12 p40, and IFNγ.

ELISA

Human IFNγ, IL-12 p40, IL-10, and IL-18 levels were measured using ELISA kits and recombinant cytokine standards from BD PharMingen. Human IL-12 p70 was measured using medium-sensitivity (limit of detection, 15.4 pg/mL; Endogen, Woburn, MA) or high-sensitivity (limit of detection, 0.781 pg/mL; R&D Systems, Minneapolis, MN) commercial ELISA kits.

Statistical analysis

Data were tested for normality, and appropriate tests were selected. Non-normal distributions were compared using the Wilcoxon signed-rank test, and normally distributed data were compared using paired Student's *t* test. Spearman's rank correlation was used to test correlations. For the purpose of calculation, undetectable levels of cytokine were assigned values corresponding to the lower limit of detection for each assay (7.8 pg/mL for IFNγ).

RESULTS

Baseline data

Clinical characteristics of the study population are summarized in Table 1. The presence of monocyte deactivation in this population was confirmed by flow cytometry on $CD14^+$ cells in 37 subjects. There was a uniform, 41.1% median decrease in HLA-DR mean fluorescence intensity (Fig. 1), from a median of 517.70 preoperatively (IQR of 405.36-588.85) to a median of 342.44, 24 h postoperatively (IQR of 214.12-413.71;

TABLE 1. **Demographic data and clinical characteristics**

Characteristic	Value
Total subjects (male)	47 (30)
Median age (interquartile range; IQR)	66 (59–74)
Diabetes (%)	23 (49)
Congestive heart failure (%)	11 (23)
Previous cardiothoracic surgery (%)	2 (4)
Median bypass minutes (IQR)	84 (61–113)
Median cross-clamp min (IQR)	62 (52–86)

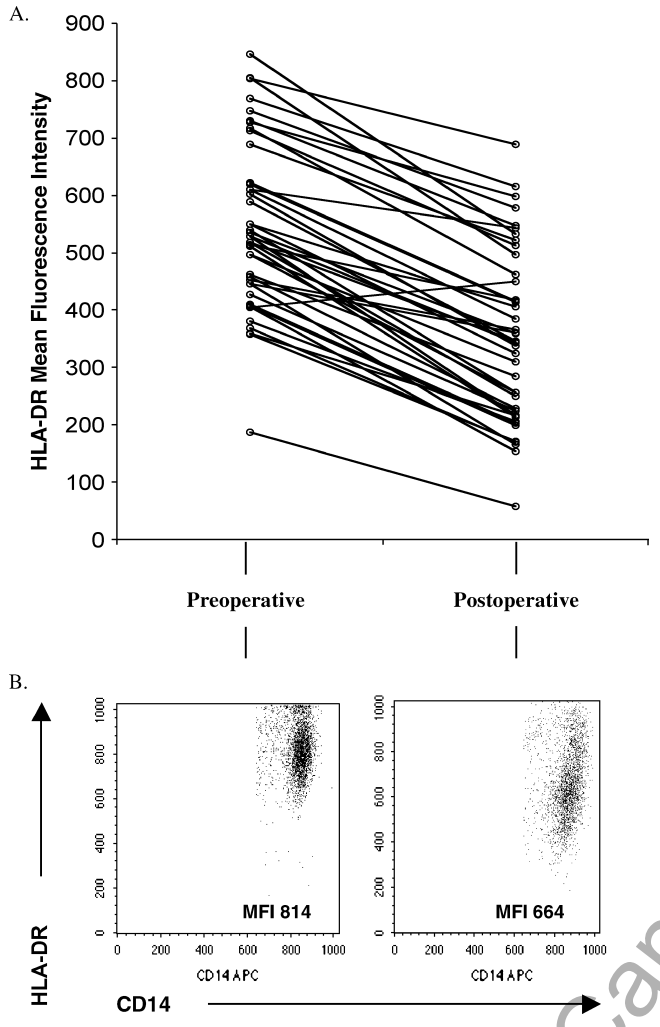

FIG. 1. **Reduced monocyte expression of HLA-DR after cardiothoracic surgery**. Data represent the mean fluorescence intensity of HLA-DR on $CD14^+$ monocytes 24 h before cardiac surgery (preoperative) and 24 h afterward (postoperative). Each pair of data points (A) indicates findings from each of 37 subjects. (B) A representative dot-plot analysis of HLA-DR expression on monocytes for one individual, including mean fluorescence intensity values. Analytical gates were chosen to select for $CD14^+$ cells with side- and forward-scattering values characteristic of monocytes.

$P < 0.001$). Flow cytometric analysis showed that T cell and NK cell numbers per square millimeter declined after surgery to about 40% of preoperative numbers, whereas monocyte numbers were unchanged (Table 2).

Innate IFNγ production is significantly reduced after cardiac surgery

Having established the presence of monocyte deactivation 1 day after cardiac surgery, we then examined production of cytokines critical to the integrity of innate cellular immunity. Peripheral blood was incubated with fixed SAC or LPS, both of which activate toll-like receptor functions that lead to production of IL-12 and IL-12-dependent IFNγ synthesis by NK cells in culture (9). Comparison of pre- and postoperative IFNγ levels in conditioned plasma demonstrated that IFNγ synthetic capacity 1 day after cardiac surgery was only 5% of that observed before surgery (Fig. 2). SAC was a greater stimulus for IFNγ production than was LPS, but both revealed significant decreases in production ($P < 0.01$; Wilcoxon signed-ranks test) in more than 95% of postoperative subjects. This synthetic defect could not be attributed wholly to the lesser decrease in NK cell or T lymphocyte populations in postoperative blood (40% of preoperative values), as there remained an additional 10-fold difference in IFNγ production. IFNγ deficiency in SAC-stimulated whole blood culture was persistent, remaining at 5% of preoperative values for at least 4 days after surgery (n = 6 subjects). Because SAC was the more potent stimulus for IFNγ production, this reagent was used in subsequent studies aimed at determining the mechanism of IFNγ underproduction after surgery.

TABLE 2. **Changes in circulating leukocyte subsets after cardiothoracic surgery**

Subset	Cell Numbers (per mm^3)		$P^{\|}$
	Preoperative	Postoperative	
Total leukocyte count	8100 (4500–13000)	8500 (3400–14500)	0.033
T cells*	952 (101–2181)	399 (55–1409)	<0.001
NK cells†	161 (35–469)	69 (8–176)	<0.001
B cells‡	187 (28–301)	163 (9–486)	0.12
Monocytes§	496 (101–1020)	586 (101–1378)	0.064
Neutrophils¶	5759 (260–10287)	7375 (2924–10,428)	0.005

Total leukocyte count was measured by a Coulter cytometer. Subset numbers were determined by concurrent FACS analysis (FACSCalibur). Counts expressed as median (range); n = 29 subjects.
*Numbers of $CD3^+$ cells/mm^3 (percentage of $CD3^+$ cells in lymphocyte gate × total leukocyte count).
†Numbers of $CD16^+/CD56^+$ cells/mm^3 (percentage of $CD16^+/CD56^+$ cells in lymphocyte gate × total leukocyte count).
‡Numbers of $CD19^+$ cells/mm^3.
§Numbers of $CD14^+$ cells/mm^3 (percentage of $CD14^+$ cells in monocyte gate × total leukocyte count).
¶Numbers of cells identified as neutrophils by a Coulter cytometer.
‖Wilcoxon signed-ranks test preoperative versus postoperative.

Cardiac surgery decreases the synthetic capacity of IL-12 p70, but not IL-12 p40

In the innate cellular immune pathway, previous synthesis of bioactive heterodimeric IL-12 (IL-12 p70) is critically required for IFNγ production (10). To determine if postoperative IL-12 p70 deficiency might account for IFNγ underproduction, IL-12 p70 levels in 24 individuals were assayed using a commercial ELISA with a lower limit of detection of 15.4 pg/mL. Although only 10 samples contained detectable preoperative concentrations, the postoperative decline in IL-12 p70 synthetic capacity to 35% of preoperative values was significant in this subset ($P = 0.004$) and remained significant when all samples were included in the analysis ($P = 0.01$). A comparable decrease (to 30%; $P = 0.013$) in IL-12 p70 was subsequently confirmed in another group of nine subjects using a high-sensitivity ELISA that detected IL-12 p70 in all stimulated samples above the detection limit of 0.78 pg/mL (Fig. 2C). Consistent with a concentration-dependent induction of IFNγ by IL-12 p70, supernatant concentrations of IL-12 p70 and IFNγ in individual preoperative cultures were significantly and positively correlated ($r = 0.554$, $P < 0.001$, n = 17; Spearman rank correlation). IL-12 p70 was not detected in unstimulated cultures before or after surgery. Concentrations of IL-12 p40 levels in SAC-stimulated culture increased significantly, from 1.13 ± 0.25 ng/mL preoperatively to 1.76 ± 0.46 ng/mL

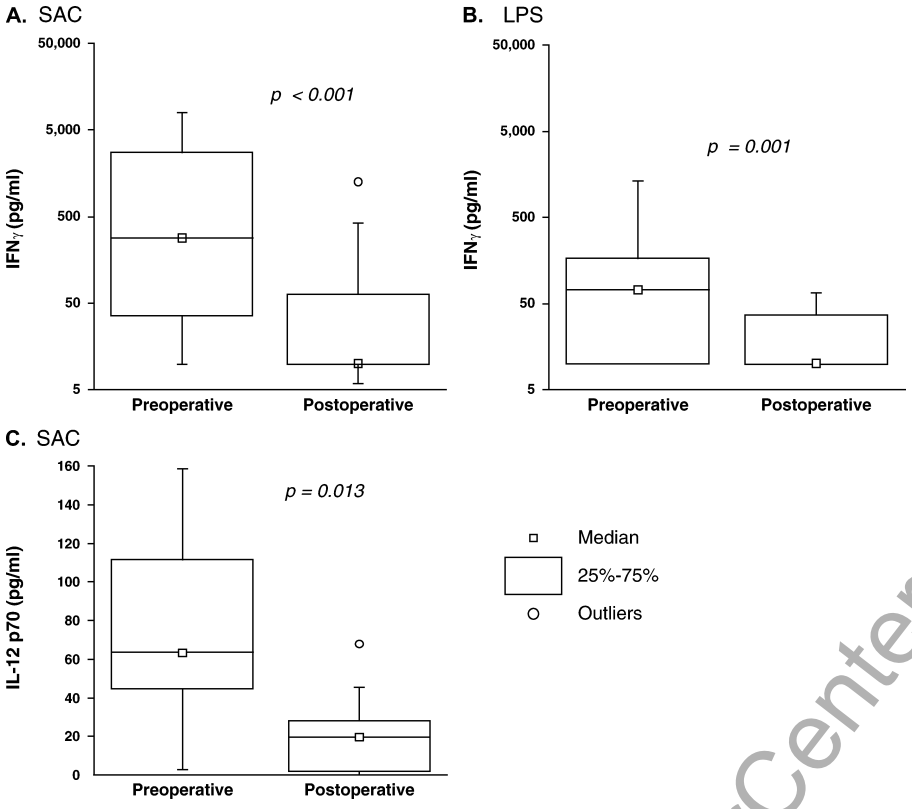

FIG. 2. **Production of IFNγ and IL-12 p70 in response to formalin-fixed SAC and LPS is reduced after cardiothoracic surgery**. Whole blood culture collected just before (preoperative) or 24 h after (postoperative) cardiac surgery was cultured for 18 h with (A) SAC or (B) LPS. These data represent conditioned plasma IFNγ (picograms per milliliter) as measured by ELISA. Large boxes represent interquartile ranges, small boxes represent medians, circles represent outlier values, and whiskers represent the total range for each pre- or postoperative set of data. The decline in the postoperative levels is statistically significant for SAC (n = 47; $P < 0.001$) and LPS (n = 25; $P = 0.001$). (C) SAC-stimulated production of IL-12 p70 in whole blood is reduced after cardiothoracic surgery ($P = 0.013$; n = 9) using an ELISA with a sensitivity of 0.781 pg/mL. The decline in the postoperative levels of IL-12p70 was also statistically significant using standard sensitivity ELISA (n = 10, $P = 0.004$; sensitivity 15.4 pg/mL).

postoperatively ($P = 0.02$, n = 32). Unstimulated whole blood produced less than 0.2 ng of IL-12 p40 in both groups.

Cardiac surgery decreases numbers of circulating myeloid dendritic cells

Although blood monocytes are capable of IL-12 p40 synthesis, isolated peripheral blood dendritic cell populations are demonstrably more potent producers of IL-12 p70 (10, 24), with myeloid dendritic cells ($CD11c^+/CD123^-/HLA\text{-}DR^+$) reportedly generating more than plasmacytoid dendritic cells ($CD11c^-/CD123^+/HLA\text{-}DR^+$) (25). We found that both subsets of circulating dendritic cells were markedly reduced in the postoperative period. In the 11 subjects studied, peripheral blood myeloid dendritic cells declined from a median of 20/mm^3 preoperatively (range of 6–31) to 5/mm^3 postoperatively (range of 1–18; $P = 0.004$; Fig. 3). Plasmacytoid dendritic cells also reduced from a median of 6/mm^3 preoperatively (range of 2–16) to 1/mm^3 postoperatively (range of 0–8; $P = 0.003$). This novel finding suggested that postoperative dendritic cell deficiency in the peripheral blood might be responsible for reduced IL-12 p70 production in response to bacterial stimuli, and that this would consequently prevent IFNγ synthesis.

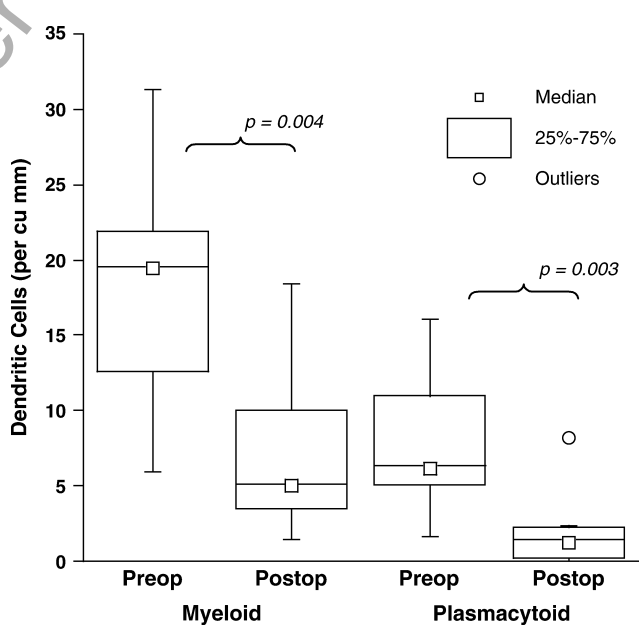

FIG. 3. **Numbers of circulating myeloid and plasmacytoid dendritic cells decline 1 day after surgery**. Peripheral blood cells were stained using a commercial kit (B&D Immunocytometry Systems, Mountain View, CA) wherein lin$^-$ PBMC cells were analyzed for expression of CD123, HLA-DR, and CD11c. Shown are the absolute numbers of total circulating cells with the myeloid or plasmacytoid phenotype before and after surgery for a cohort of 11 subjects. Median, interquartile ranges, and total range are indicated as before. Pre- to postoperative values for myeloid and plasmacytoid DC numbers were significantly decreased ($P = 0.004$ and 0.003, respectively).

Experimental depletion of CD11c$^+$ cells disrupts SAC-induced IFNγ and IL-12 p70 synthesis in normal volunteer PBMC

To test this hypothesis, we obtained PBMC from healthy volunteers and examined the effects of specific cell depletions on SAC-induced cytokines (Fig. 4). Removal of $CD11c^+$ cells, which were shown by flow cytometry to consist of $CD11c^+/DR^+$ myeloid dendritic cells and $CD11c^+/CD14^+$ monocytes, significantly reduced SAC-stimulated IFNγ secretion to 1% of control values ($P = 0.04$) and IL-12 p70% to 33% ($P = 0.04$). In contrast, specific depletion of $CD14^+$ monocytes had no significant effect on IFNγ or IL-12 p70 production. Magnetic removal of $CD56^+$ cells, consisting of NK and NK-T

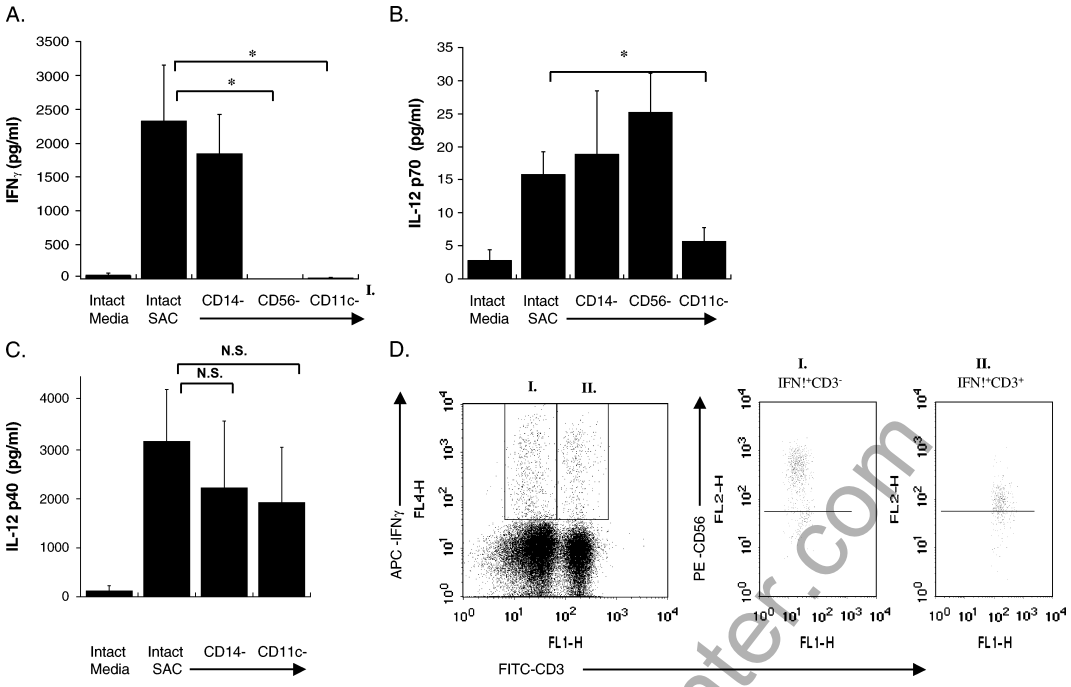

FIG. 4. **Peripheral blood dendritic cells and NK cells are necessary for SAC-induced cytokine production in blood from healthy volunteers**. Subsets of normal PBMC were depleted using CD14-, CD56-, or CD11c-specific magnetic beads. Shown are the mean ± SEM concentrations of (A) IL-12 p70 and (B) IFNγ and (C) IL-12 p40 after overnight culture of control and depleted populations (n = 6 subjects) with media or 0.01% fixed SAC. Asterisks denote statistical significance for differences indicated by brackets ($P < 0.05$; paired *t* test, two-tailed). Flow cytometry confirmed that $CD14^+$ cells were depleted to 2%, $CD56^+$ cells to 40%, and $CD11c^+$ cells to 19% relative to predepletion values. CD11c-specific beads depleted monocytes ($CD14^+/DR^+$) and dendritic cells $CD14^{lo}/DR^+$, but CD14- and CD56-targeted deletions were specific for monocytes and NK/NKT cells, respectively. (D) NK and NK-T cells express intracellular IFNγ after culture of whole blood with SAC. Shown is a FACS histogram for cells from a representative normal donor stained with anti-CD3 antibody (FL1 *x* axis) and then stained for intracellular APC-anti-IFNγ (FL4 *y* axis). The indicated analytical gates were used to determine the frequency of CD56 expression on cells that were (I) $IFN\gamma^{+/}CD3^-$ or (II) $IFN\gamma^{+/}CD3^+$. These values were 95% ± 7% and 50% ± 6% $CD56^+$, respectively, for these cells (mean and SEM; n = 5 subjects).

cells by flow analysis of bead-bound cells, reduced SAC-induced IFNγ production to undetectable levels, but did not affect IL-12 p70 production. IL-12 p40 was equally reduced by CD14 or CD11c depletion. Finally, we found that $CD3^-$ and $CD3^+$ cells produced intracellular IFNγ in SAC-stimulated whole blood (Fig. 4). The majority (95% ± 5.6%) of IFNγ-expressing $CD3^-$ cells coexpressed the NK cell marker CD56, as did 50.1% ± 5.6% of the $IFN\gamma^+/CD3^+$ cells, consistent with NK and NK-T cell sources of IFNγ. An average of 13.9% ± 3.8% of $CD56^+/CD3^-$ NK cells and 1.5% ± 0.6% of all $CD3^+$ T cells stained positive for intracellular IFNγ. Taken together, these results show that a $CD11c^+$ nonmonocyte cell population is necessary to support SAC-induced IL-12 p70 in normal PBMC and that $CD56^+$ NK and NK-T cells are the source of IFNγ synthesis.

Recombinant IL-12 p70, IL-18, and IL-23 do not restore normal IFNγ production in postoperative whole blood culture

If postoperative IFNγ deficiency was due solely to removal of IL-12 p70, the addition of recombinant IL-12 p70 to culture should restore near-normal levels of IFNγ synthesis in response to SAC. In preoperative blood, SAC supplemented with 1.0 ng/mL IL-12 p70 significantly ($P < 0.001$; n = 21 subjects) increased absolute IFNγ levels compared with culture with SAC alone (Fig. 5). Intracellular staining confirmed cytokine-assisted IFNγ production was still NK and NK-T cell based (data not shown). Although recombinant IL-12 also increased absolute

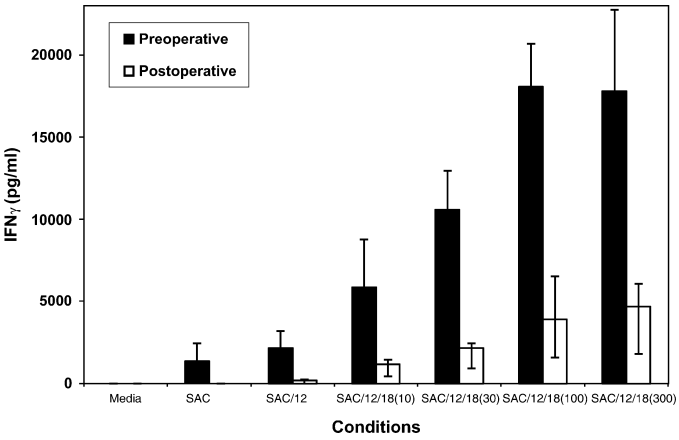

FIG. 5. **Supplementation with recombinant IL-12 p70 and IL-18 fails to restore IFNγ production in postoperative whole blood culture**. Blood collected just before (preoperative) or 24 h after (postoperative) cardiac surgery was cultured for 18 h with addition of media only, 0.01% SAC, SAC supplemented with 1.0 ng/mL recombinant human IL-12 p70, or SAC with IL-12 and increasing concentrations of recombinant human IL-18. The concentrations of IL-18 used are indicated in the parentheses in nanograms per milliliter. Postoperative declines in IFNγ production were statistically significant for all the conditions except media control ($P < 0.05$ by Wilcoxon signed-ranks test, n = 6 subjects).

postoperative IFNγ levels significantly ($P = 0.001$), IL-12 replacement did not restore normal levels of IFNγ production after surgery. The 6% residual production of IFNγ induced by SAC in postoperative blood only increased to approximately 10% of preoperative values after the addition of IL-12 (pre- and postoperative medians of 1015.5 and 105.4 pg/mL, $P = 0.002$).

Increasing IL-12 concentrations up to 5 ng/mL produced only minor incremental increases in IFNγ levels without reducing the defect in postoperative IFNγ synthesis relative to preoperative function (data not shown).

We next tested whether postoperative IFNγ deficiency might be because of loss of synergistic or alternative IFNγ-inducing signals provided by IL-18, IL-15, and IL-23 (11, 26). Although SAC-inducible IL-18 was detectable in preoperative and postoperative blood cultures in concentrations ranging from 0.2 to 1.0 ng/mL, these differences were not significant with regard to operative status (data not shown). The addition of up to 300 ng/mL recombinant human IL-18 to SAC- and rIL-12-stimulated whole blood culture increased preoperative IFNγ production 9-fold compared with culture without IL-18 (Fig. 5), but the maximal response of IL-12/IL-18-stimulated IFNγ in postoperative blood was still only 20% relative to preoperative cultures ($P = 0.028$). Similarly, the addition of 3 ng/mL rIL-15 in combination with IL-12 or with 3 ng/mL rIL-23, which induces IFNγ through a receptor distinct from IL-12R, failed to additionally increase postoperative IFNγ production even when all four cytokines were used in combination (data not shown). These findings indicate that IFNγ-inducing cytokines that were highly active in preoperative subjects were markedly less able to induce postoperative production of IFNγ in whole blood culture.

Deficient IFNγ production after cardiac surgery is not restored by neutralization of IL-10

We confirmed that IL-10 concentrations increased significantly in response to SAC in pre- and postoperative whole blood cultures, respectively, although the maximal induced responses were not significantly different before and after surgery (Table 3). We tested the physiologic significance of *in vitro* IL-10 production by measuring IFNγ responses to SAC in the absence or presence of 20 μg/mL neutralizing anti-IL-10 antibody. Although IL-10 neutralization did increase IFNγ production in preoperative and postoperative samples, postoperative SAC-inducible IFNγ levels remained only 11% relative to those in preoperative cultures.

Down-regulation of IFNγ production is not dependent on the presence of plasma in PBMC culture

The postoperative decreases in IFNγ and IL-12 production in whole blood culture might have reflected suppressive activities mediated by circulating factors other than IL-10 present at the time of sampling. Therefore, we measured IFNγ levels generated by Ficoll-Hypaque-purified PBMC in defined media (Fig. 6). After stimulation with 0.01% SAC, median IFNγ levels were 59.8 pg/mL preoperatively (range of 7.8–2359.3 pg/mL) and 7.8 pg/mL postoperatively ($P = 0.005$; n = 11 subjects). Cells cultured in media alone did not produce detectable IFNγ (<7.8 pg/mL). The addition of autologous plasma obtained from the same blood draw supported a modest increase in preoperative levels of IFNγ to 101.2 pg/mL (range of 8.0–1472.0 pg/mL) in response to SAC, but postoperative levels remained at or below the limit of detection ($P = 0.028$). Finally, although IL-12 supplementation increased IFNγ production overall, postoperative levels were still reduced to 17% of preoperative values compared with 13% in the absence of IL-12.

TABLE 3. **Neutralization of IL-10 produced in whole blood culture fails to normalize SAC-stimulated IFNγ**

	IL-10 Concentration (ng/mL)*		
	Preoperative	Postoperative	$P^{\dagger}$
Media	0.12 (0.10–5.69)	0.10 (0.05–4.15)	0.074
SAC	1.96 (0.10–6.02)	2.04 (0.36–5.63)	0.078
$P^{\ddagger}$	0.004	0.001	
	SAC-Induced IFNγ (pg/mL)§		
	Preoperative	Postoperative	$P^{\dagger}$
Control IgG	51 (8–4333)	8 (6–298)	0.005
Anti-IL-10 MAb (20 μg/mL)	91 (8–4333)	11 (8–458)	0.005
$P^{\P}$	0.110	0.086	

*n = 16 subjects; data expressed as median (range).
†Wilcoxon signed-ranks test: preoperative versus postoperative values.
‡Wilcoxon signed-ranks test: media control versus SAC.
§n = 11 subjects; data expressed as median (range).
¶Wilcoxon signed-ranks test: control IgG versus anti-IL-10 IgG.

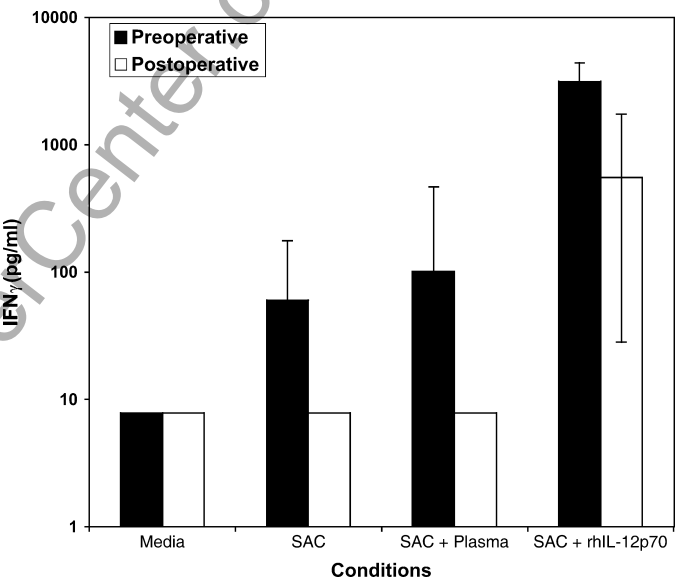

FIG. 6. **IFNγ production by PBMC is decreased after cardiac surgery**. Ficoll-purified mononuclear cells were cultured in media (RPMI and 10% fetal bovine serum), 0.01% fixed SAC, 0.01% SAC, 0.01% SAC, and autologous plasma, and 1.0 ng/mL recombinant human IL-12 p70 (rhIL-12p70). Plasma was obtained from same blood draw as PBMC. Postoperative declines in IFNγ levels were statistically significant in the SAC ($P = 0.005$), SAC + plasma ($P = 0.028$), and SAC + rhIL-12p70 ($P = 0.004$) groups compared with their respective preoperative values. Data represent median values for 11 subjects.

DISCUSSION

Because cardiothoracic surgery is a scheduled event, this population of subjects provides a readily accessible and self-controlled model for the study of mechanisms contributing to immune dysfunction in critically ill or traumatized humans. Previously, post-traumatic or sepsis-related immune deficiency was defined by decreased monocyte HLA-DR expression and proinflammatory monokine production (4, 5, 20, 28). We confirm that the controlled trauma of cardiothoracic surgery similarly reduces HLA-DR expression on monocytes and we show that TLR-mediated production of IL-12 and IFNγ is

markedly impaired in this specific setting. This finding complements and extends similar observations of IL-12 deficiency after major visceral surgery that was ascribed to macrophage dysfunction (7). In contrast, the central and novel observation of this report is that dendritic cell numbers in postoperative blood are significantly reduced after surgery. Furthermore, the experimental depletion of $CD11c^+$ dendritic cells in normal PBMC culture markedly impairs SAC-induced IL-12 and IFNγ production, whereas depletion of $CD14^+$ monocytes did not. This provides an alternative and dendritic cell-based mechanism for impaired innate immune production of IL-12 p70 and IFNγ after surgery that is distinct from macrophage deactivation. We further demonstrate that deficient postoperative IFNγ production by whole blood is not recovered by treatment with IFNγ-inducing cytokines such as IL-12, IL-18, IL-15, and IL-23. A similar IL-12 unresponsiveness occurs in humans with sepsis (20). Therefore, postoperative immune deficiency appears to be mediated by multiple mechanisms. In addition to effects mediated by macrophage deactivation and/or production of immunosuppressants (29, 30), we now conclude that dendritic cell cytopenia distinctly contributes to IL-12 p70 underproduction and that IL-12 deficiency, possibly in combination with a decreased IL-12 responsiveness by NK/NK-T cells, results in a marked loss of IFNγ synthetic capacity in postsurgical whole blood.

These studies initially focused on cytokine synthesis after activation of innate cellular immunity by TLR-active reagents, whereas many previous reports of postsurgical IFNγ deficiency used mitogens to test T cell synthetic competency. Formalin-fixed *S. aureus* proved to be a potent IFNγ-inducing agent in whole blood culture or PBMC and is known to stimulate cytokines through innate immune mechanisms that require Myd88, TLR2, and other toll receptors (18). Because dendritic cells express TLR2 and respond to SAC with IL-12 production (31), the significant decrease in circulating dendritic cells after surgery provides a plausible mechanism for IL-12 p70 deficiency. In support of this, CD11c-depleted PBMC underproduced IL-12 p70 and IFNγ in response to SAC. Although $CD11c^+$ depletion also removed monocytes, CD14-depleted PBMC showed little or no impairment in SAC-induced IL-12 p70 or IFNγ release. A previous report similarly described a nonessential role for $CD14^+$ monocytes in peripheral blood production of SAC-induced IL-12 p70 (24). IL-12 p40 synthesis, which normally parallels p70 production, paradoxically increased after surgery. In contrast to effects on IL-12 p70, depletion of $CD14^+$ or $CD11c^+$ cells each reduced IL-12 p40 production by one-half, suggesting redundant sources of IL-12 p40 in normal blood that are distinct from myeloid dendritic cells and monocytes and that may account for preserved synthesis in postoperative subjects.

The mechanisms responsible for dendritic cell cytopenia after surgery are not identified in this study. Cytopenia could be because of redistribution of cells from peripheral blood into other tissues or to surgery-induced apoptotic depletion of these cells. Lymphocyte and macrophage apoptosis is described in experimental or clinical models of trauma and sepsis (29), and apoptotic dendritic cell depletion occurs in mice systemically exposed to LPS or *Toxoplasma gondii* (32–34). More recently, depletion of follicular dendritic cells in the spleens of patients with sepsis has also been described, although these cells represent a lineage functionally and biologically distinct from circulating dendritic cells (35). Because DC are potent antigen-presenting cells and mediate critical costimulatory and regulatory functions in adaptive and innate immunity, we speculate that dendritic cell depletion may have adverse consequences on immune function extending beyond specific cytokine deficits. For instance, the loss of delayed type hypersensitivity (DTH) responsiveness after cardiac surgery (36) may be a consequence of dendritic cell depletion in tissue and blood.

The initial endpoint used in these studies was production of IFNγ by SAC- or LPS-stimulated innate immunity. SAC-induced IFNγ is IL-12 dependent and mediated by NK cells in PBMC (37, 38). Using intracellular staining techniques, we confirmed that NK and NK-T cells contribute to SAC-induced IFNγ production in whole blood. Although the numbers of NK cells declined 50% in whole blood after surgery in our study, this decrement did not account for the 95% reduction in IFNγ levels after surgery. Although loss of IL-12 p70 synthesis is sufficient to explain this defect, we also show that postoperative NK cells may become less responsive to IFNγ-stimulating cytokines. At best, using supraphysiologic amounts of IL-12 and IL-18, the postoperative deficiency in SAC-induced IFNγ synthesis was improved only from 6% to 10% of preoperative IFNγ levels. Postsurgical hyporesponsiveness also extended to IL-15 and IL-23. These data do not exclude the possibility that the partial reduction of T and NK cells after surgery was specific for a subset of cells capable of producing IFNγ. Although inhibitors of IFNγ production, such as IL-10, may be present in plasma (27, 29, 30), SAC-induced IFNγ levels in plasma-free cultures of PBMC culture were still markedly reduced relative to preoperative levels and anti-IL-10 antibody failed to restore IFNγ production in whole blood culture. Further studies are needed to determine if multiple NK cell cytokine receptors or common signal transduction pathways are disrupted as a result of surgery or if transient and previous exposure to IL-10 or other suppressant molecules persistently disables cellular function (39).

In summary, cardiothoracic surgery induces multiple defects in the innate cellular immune system that normally supports IFNγ synthesis in response to acute gram-negative and gram-positive microbial stimuli. Specifically, we show that myeloid dendritic cells are necessary for production of SAC-induced IL-12 p70 and IFNγ in PBMC and, therefore, that surgery-induced dendritic cell cytopenia is sufficient to explain postoperative IFNγ deficiency. Dendritic cell dysfunction adds to other mechanisms of postsurgical immune dysfunction due to macrophage desensitization or circulating suppressive factors (29, 30). We note similarities between postsurgical and endotoxin-induced immune defects, both of which result in macrophage deactivation, dendritic cell depletion, and combined IL-12 and IFNγ deficiency (34, 40). Because injection with TNFα reproduces these cytokine defects in mice, surgically induced production of TNFα from myocardium or other tissues (41) may mediate some of immune defects observed after cardiothoracic surgery. Separate from these mechanistic questions, the consequences of trauma- or sepsis-induced immune deficiency for the

host also need to be better understood. Although an attenuated inflammatory response may protect against death from overwhelming systemic inflammation, these same defects in toll-dependent cytokine production may increase risk of infection (7, 17, 18). Further comparisons between animal and post-surgical models of immune deficiency may identify a rationale and a therapeutic approach for preventing dendritic cell depletion *in vivo*, an intervention that may restore appropriate production of important antimicrobial cytokines in injured patients.

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