Vascular inflammation: signaling pathways in atherosclerosis and arteriogenesis
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Interferon-beta signaling is enhanced in patients with insufficient coronary collateral artery development and inhibits arteriogenesis in mice

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ABSTRACT

Stimulation of collateral artery growth in patients has been hitherto unsuccessful, despite promising experimental approaches. Circulating monocytes are involved in the growth of collateral arteries, a process also referred to as arteriogenesis. Patients show a large heterogeneity in their natural arteriogenic response on arterial obstruction. We hypothesized that circulating cell transcriptomes would provide mechanistic insights and new therapeutic strategies to stimulate arteriogenesis. Collateral flow index was measured in 45 patients with single-vessel coronary artery disease, separating collateral responders (collateral flow index, >0.21) and nonresponders (collateral flow index, <0.21). Isolated monocytes were stimulated with lipopolysaccharide or taken into macrophage culture for 20 hours to mimic their phenotype during arteriogenesis. Genome-wide mRNA expression analysis revealed 244 differentially expressed genes (adjusted P, <0.05) in stimulated monocytes. Interferon (IFN)-β and several IFN-related genes showed increased mRNA levels in 3 of 4 cellular phenotypes from nonresponders. Macrophage gene expression correlated with stimulated monocytes, whereas resting monocytes and progenitor cells did not display differential gene regulation. In vitro, IFN-β dose-dependently inhibited smooth muscle cell proliferation. In a murine hindlimb model, perfusion measured 7 days after femoral artery ligation showed attenuated arteriogenesis in IFN-β–treated mice compared with controls (treatment versus control: 31.5±1.2% versus 41.9±1.9% perfusion restoration, P<0.01). In conclusion, patients with differing arteriogenic response as measured with collateral flow index display differential transcriptomes of stimulated monocytes. Nonresponders show increased expression of IFN-β and its downstream targets, and IFN-β attenuates proliferation of smooth muscle cells in vitro and hampers arteriogenesis in mice. Inhibition of IFN-β signaling may serve as a novel approach for the stimulation of collateral artery growth.
INTRODUCTION

Collateral artery growth, also termed arteriogenesis, is a natural escape mechanism in cases of arterial obstruction.\textsuperscript{1} It alleviates symptoms of ischemia, and the extent of myocardial infarction is diminished if a sufficient collateral network is present.\textsuperscript{2} Therefore, pharmacological stimulation of arteriogenesis is of potential benefit to a large number of patients. Despite the large body of evidence for the feasibility of pharmacological stimulation of arteriogenesis in various animal models, large randomized clinical trials have not demonstrated beneficial effects of a proarteriogenic treatment in patients.\textsuperscript{3-6} Lack of knowledge on the molecular background of arteriogenesis in humans may explain the disappointing results of the clinical trials. Interestingly, a large heterogeneity exists in patients in their arteriogenic response on coronary obstruction.\textsuperscript{7} Hence, comparative studies of patients responding with either sufficient or insufficient collateral artery growth can provide insights into arteriogenesis in humans and may reveal new therapeutic strategies.

Circulating cells, especially monocytes and macrophages\textsuperscript{8} but potentially also (endothelial) progenitor cells,\textsuperscript{9} are involved in the arteriogenic remodeling process. In a previous study, we showed that CD44 expression is functionally involved in arteriogenesis in mice and is differentially regulated on stimulated monocytes in patients with either a sufficiently or an insufficiently developed coronary collateral circulation.\textsuperscript{10} We thus hypothesized that the observed heterogeneity in arteriogenic response in patients could be attributed to differences in transcriptional activity of circulating cells. Therefore, in the present study, we determined full transcriptomes of resting monocytes, cultured macrophages, and CD34\textsuperscript{+} progenitor cells from individual patients with either a sufficiently or an insufficiently developed collateral circulation, so-called arteriogenic responders and nonresponders. In addition, monocytes were activated with lipopolysaccharide (LPS) to more closely resemble the phenotype of monocytes/macrophages during arteriogenesis. LPS is an agonist of the Toll-like receptor (TLR)4, and degradation products of the extracellular matrix produced during inflammation (such as collateral remodeling) have been shown to serve as endogenous ligands of TLR4.\textsuperscript{11} Furthermore, a lack of TLR4 significantly attenuates arteriogenesis, as has been shown recently.\textsuperscript{12}
PATIENTS AND METHODS

An expanded Methods section is available in the online data supplement at http://circres.ahajournals.org.

Patient selection
This study was approved by the institutional medical ethics committee. After giving informed consent, 45 white patients undergoing percutaneous coronary intervention for stable, high-grade, single-vessel coronary artery disease were included. Exclusion criteria were as follows: multivessel disease; previous myocardial infarction, cardiac surgery, or percutaneous coronary intervention; depressed left ventricular function; diabetes mellitus; neoplastic; or inflammatory disease.

Collateral flow index
During a 1-minute balloon inflation, wedge pressure distal to the coronary occlusion ($P_w$) and aortic pressure ($P_{ao}$) were determined. Collateral flow index (CFI) was calculated as $(P_w - CVP)/(P_{ao} - CVP)$. CVP was estimated to be 5 mm Hg. Patients were dichotomized into 2 groups, using a CFI cutoff of 0.21. This cutoff value has been validated to separate patients with ST segment elevation and angina pectoris during balloon occlusion from those without ischemia (Christian Seiler, Bern, Switzerland, personal communication).

Isolation, culture, and gene expression analysis of circulating cells
From 5 mL of arterial blood, withdrawn before percutaneous coronary intervention, CD14$^+$ monocytes were positively isolated using immunomagnetic beads. Another 55 mL of blood underwent Ficoll separation. From the resulting mononuclear cells, CD34$^+$ progenitor cells were positively isolated, whereas monocytes were negatively isolated for stimulation with LPS for 3 hours, or macrophage culture for 20 hours (Figure I in the online data supplement). Monocyte purity was ±95% by flow cytometry. mRNA from all 4 cell types of 42 patients was amplified and biotinylated. Samples were randomly hybridized to HumanRef-8 Expression bead chip arrays (Illumina), followed by scanning and feature extraction.

Validation of gene array results
Gene expression of IFN-β, IFN-β, CXCL10, CXCL11, matrix metalloproteinase (MMP)-1, MMP-10, and NAD(H):quinone oxidoreductase (NQO)1 was assessed in all 45 patients using RT-PCR. IFN-β and CXCL10 were measured in supernatants of stimulated monocytes and patient plasma using ELISA. Proliferation of human smooth muscle cells (SMCs) after treatment with recombinant human IFN-β was assessed in vitro.
Animal experiments
In a murine hindlimb ligation model, perfusion restoration after 1 week of systemic treatment with IFN-β was assessed as previously described. The IFN-β concentration used is comparable to dosages used in patients with multiple sclerosis. Furthermore, we assessed hindlimb tissue IFN-β protein concentrations using ELISA and assessed tumor necrosis factor (TNF)-α gene expression using RT-PCR analysis.

Statistical analysis
Normalization and statistical analysis of the gene array data were carried out using the Limma package and scripts in R/Bioconductor. MetaCore was used for pathway analysis. Gene set enrichment analysis was used for analysis of enrichment of transcription factor binding sites in promoters of differentially expressed genes. Microarray data have been submitted to the Gene Expression Omnibus (GEO) under accession no. GSE7547.

RESULTS

Patient characteristics of responders and nonresponders
Patients were aged 62.8±12.0 years, and CFI ranged from 0.04 to 0.57 (mean value, 0.23±0.11). Genome-wide mRNA expression analysis was performed for 42 patients. Baseline characteristics were well matched between responders (n=22, CFI=0.32±0.10) and nonresponders (n=20, CFI=0.14±0.04) (Table 1). Nonresponders showed stronger ST segment elevation as a sign of ischemia during balloon coronary occlusion (1.88±1.40 mm versus 0.50±0.99 mm, P=0.001) and had a lower modified Rentrop score (0.23±0.43 versus 0.95±0.89, P=0.001). Overall, 93% of all patients had a score of 0 or 1 on a scale of 0 to 3.

Gene expression analysis: resting monocytes and progenitor cells
Numbers of circulating monocytes did not differ between responders and nonresponders (518±116/µL versus 529±154/µL, P=0.80). Resting monocytes did not show consistent gene expression differences discriminative for the 2 patient groups after correction for multiple testing (adjusted P, >0.4 for all genes). Analysis on the pathway level, however, showed that the epidermal growth factor receptor, fibroblast growth factor receptor, and insulin signaling pathways were differentially regulated in responders versus nonresponders. Neither numbers nor gene expression of CD34+ cells differed between the 2 groups (online data supplement).
Stimulated versus resting monocytes

Resting monocytes, LPS-stimulated monocytes, and macrophages showed distinctively different gene expression when subjected to unsupervised hierarchical clustering regardless of collateralization of patients. LPS stimulation and macrophage cell culture resulted in marked regulation of genes consistent with these stimuli, with changes found most significant in TLR-mediated immune response, cytokine-mediated signaling, and cell cycle signaling.

Table 1. Baseline characteristics

| Characteristics                        | CFI0.21 (n=22)       | CFI>0.21 (n=20)      | P     |
|----------------------------------------|----------------------|----------------------|-------|
| Age, years                             | 62.9±12.0            | 62.6±12.2            | 0.93  |
| Male sex, n (%)                        | 15 (68.2)            | 14 (70)              | 1.0   |
| Body mass index, kg/m2                 | 26.54±3.20           | 26.67±2.82           | 0.89  |
| Body surface area, m2                  | 1.98±0.21            | 1.99±0.12            | 0.95  |
| Hypertension, n (%)                    | 13 (59.1)            | 12 (60)              | 1.0   |
| Hypercholesterolemia, n (%)            | 11 (50)              | 10 (50)              | 1.0   |
| Family history of CAD, n (%)           | 14 (63.6)            | 10 (50)              | 0.53  |
| Present smoker, n (%)                  | 5 (22.7)             | 4 (20)               | 1.0   |
| Previous smoker, n (%)                 | 10 (45.5)            | 10 (50)              | 1.0   |
| Weeks anginal symptoms*                | 26 [9.75; 52]        | 11 [5.25; 36.5]      | 0.16  |
| β-blockers, n (%)                      | 19 (86.4)            | 16 (80)              | 0.69  |
| Statins, n (%)                         | 20 (90.1)            | 18 (90)              | 1.0   |
| Aspirin, n (%)                         | 21 (95.5)            | 18 (90)              | 0.60  |
| Clopidogrel, n (%)                     | 11 (50)              | 15 (75)              | 0.12  |
| Calcium antagonists, n (%)             | 9 (40.9)             | 7 (35)               | 0.76  |
| Nitrates, n (%)                        | 12 (54.5)            | 11 (55)              | 1.0   |
| ACE inhibitors/ARBs, n (%)             | 7 (31.8)             | 7 (35)               | 1.0   |
| Diameter coronary stenosis (QCA), %    | 74±8                 | 76±9                 | 0.41  |
| Diuretics, n (%)                       | 3 (13.6)             | 3 (15)               | 1.0   |
| C-reactive protein,* mg/dL             | 2.6 [0.73; 7.88]     | 1.8 [0.98; 4.70]     | 0.61  |
| NT-proBNP,* µg/L                       | 87.5 [53.75; 238]    | 141.5 [56.5; 623]    | 0.32  |
| Glucose, mmol/L                        | 5.76±0.81            | 5.77±1.0             | 0.96  |
| LDL cholesterol, mg/dL                 | 2.07±0.69            | 2.03±0.81            | 0.86  |
| Lipoprotein A,* mg/dL                  | 103 [39.5; 371.5]    | 118 [35.25; 533.5]   | 0.82  |
| Peripheral blood mononuclear cells/µL  | 2524±786             | 2410±597             | 0.61  |

Responders and nonresponders did not show differences in clinical characteristics. CAD indicates coronary artery disease; ARB, angiotensin receptor blocker; ACE, angiotensin-converting enzyme; NT-proBNP, N-terminal–pro-brain natriuretic peptide; QCA, quantitative coronary angiography. *Data are expressed as median [first quartile; third quartile].
LPS-stimulated monocytes and macrophages
A comparison of LPS-stimulated monocytes from responders versus nonresponders showed 244 differentially expressed genes (adjusted $P$, <0.05). Of these, 147 genes showed a stronger LPS-mediated induction in monocytes from nonresponders. A heat map illustrating the 100 most differentially expressed genes shows stronger induction of 95% of the genes in nonresponders (Figure 1).

In the cell population that was cultured to obtain a macrophage phenotype, 3 genes were found differentially expressed between responders and nonresponders (adjusted $P$, <0.05). However, although statistical significance was not reached at the single

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**Figure 1.** Heat map showing most differentially expressed genes. Significantly differentially regulated genes (adjusted $P$, <0.05) of LPS-stimulated monocyte samples from responders (n=18) and nonresponders (n=20) were sorted according to their fold change. The 100 genes with the largest fold change are visualized in a heat map, in which patients were sorted by CFI (columns). Columns range from patients with the lowest (left) to highest (right) CFI. Red denotes genes that are relatively higher expressed; blue, those that are relatively lower expressed. Of note, of these 100 most differentially expressed genes, 95% show stronger induction in nonresponders.
gene level, expression differences were consistent with those observed in LPS-
stimulated monocytes: 82% from the 100 most differentially expressed genes in LPS-stimulated monocytes showed differential expression in the same direction in the corresponding macrophage populations (exact binominal test $P<10^{-10}$), and their moderated t statistics were significantly correlated (Spearman’s rank correlation=$0.56$, $P<10^{-15}$) (Table 2). Such agreement was not observed between the transcriptomes of stimulated versus resting monocytes.

**Classification analysis**

Classification analysis was performed on the LPS-stimulated transcriptome differences as an internal validation of the data. When using 500 splits in a training set of 20 patients and a validation set of 18 patients, patients in the validation set were classified as either responder or nonresponder with an average accuracy of 67% (95% confidence interval: 50% to 83%; mean sensitivity: 65%; mean specificity:

| Symbol | Gene | Fold Change | Adjusted $P$ | Fold Change | Adjusted $P$ |
|--------|------|-------------|--------------|-------------|--------------|
| CXCL11 | Chemokine (C-X-C motif) ligand 11 | 3.184 | $7.85\times10^{-7}$ | 1.186 | NS |
| IL27   | Interleukin 27 | 2.214 | $7.85\times10^{-7}$ | 1.146 | NS |
| NCOA7  | Nuclear receptor coactivator 7 | 2.072 | $3.27\times10^{-5}$ | 1.251 | NS |
| IFNB1  | IFN-$\beta$1 | 2.038 | $5.05\times10^{-5}$ | 1.148 | NS |
| AIM2   | Absent in melanoma 2 | 2.008 | $4.09\times10^{-4}$ | 1.386 | NS |
| IFIT3  | IFN-induced protein with tetratricopeptide repeats 3 | 1.976 | $3.13\times10^{-2}$ | 1.717 | NS |
| IFNG   | IFN-$\gamma$ | 1.956 | $4.19\times10^{-2}$ | 1.028 | NS |
| CXCL9  | Chemokine (C-X-C motif) ligand 9 | 1.801 | $6.31\times10^{-4}$ | 1.065 | NS |
| CACNA1A| Calcium channel voltage-dependent P/Q type $\alpha$ 1A subunit | 1.705 | $1.46\times10^{-3}$ | 1.110 | NS |
| IFIT5  | IFN-induced protein with tetratricopeptide repeats 5 | 1.663 | $4.67\times10^{-3}$ | 1.278 | NS |
| JAK2   | Janus kinase 2 (a protein tyrosine kinase) | 1.454 | $2.28\times10^{-2}$ | 1.152 | NS |

Among the genes showing stronger induction in nonresponders are IFN-$\beta$ and a number of IFN-related genes. This table shows a selection of IFN-related genes, all showing significantly stronger induction in stimulated monocytes from nonresponders (adjusted $P<0.05$). Gene expression in macrophages was regulated in the same direction (upregulation in nonresponders) but to a lesser extent that did not result in statistical significance.
70%), as illustrated by the unsupervised clustering heat map of these classifier genes (Figure 2).

**Increased IFN signaling in nonresponders**

Among the most strongly induced genes in stimulated monocytes from nonresponders were IFN-β and several IFN-related genes (Figure 3, Table 2). Also, several genes in the classifying set were part of the IFN pathway. Pathway analysis revealed interferon (IFN) signaling- and immune response-related pathways most significantly differentially expressed (Table 3). The 2 top ranking pathways (IFN-α/β and TIR-containing adapter molecule [TICAM]-1–specific signaling) belong to the MyD88-independent arm of the TLR signaling pathway. Closer analysis of these pathways showed stronger induction of the majority of genes in nonresponders, including IFN-α/β, STAT1/2, IFN regulatory factor 1/2, and IFN-induced factor 6.

The antiinflammatory interleukin (IL)-10 family members IL-19, IL-20, and IL-24, as well as antiinflammatory SOCS-7, an inhibitor of the IFN pathway, were found significantly enhanced in responders. Alternative pathway analysis software packages confirmed these findings (data not shown). Analysis of promoter sequences of the corresponding genes using gene set enrichment analysis corroborated the important role of IFN-β, showing 52 gene sets enriched in nonresponders (adjusted P, <0.25), 14 of which were based on IFN-related transcription factor binding motifs.

**Figure 2.** Genes classifying patients as responders or nonresponders. Subjecting the genes found as classifiers (using independent training and validation sets) to unsupervised hierarchical clustering reveals separation of responders (R) (left side; n=18) and nonresponders (N) (right side; n=20) by these genes. Green displays lowly expressed genes, whereas red denotes highly expressed genes. All but 1 classifier gene (CBS) (top row of the clustering image) show stronger induction in nonresponders.
In the macrophage population, fewer pathways were differentially expressed, but the IFN-α/β signaling pathway again showed stronger activation in nonresponders (supplemental Table XI).

**Monocytes from nonresponders display enhanced apoptosis-related gene activity**
LPS-stimulated monocytes of nonresponders displayed increased expression of cytotoxic factors like perforin (FAS) and TRAIL (TNFSF10), whereas the antiapoptotic oxidoreductase NQO1 showed stronger induction in responders. Pathway analyses pointed to enhanced apoptosis in monocytes of nonresponders, where increased FASL, FAS receptor, CD45, and CASP7 genes was found in apoptosis-relevant pathways.

**Factors upregulated in responders**
A total of 97 genes were significantly upregulated when comparing LPS-stimulated monocytes from responders and nonresponders. Among these was homocysteine-metabolizing cystathionine β-synthase (CBS), which was also the only classifier gene with higher expression in responders (Figure 2). Differential homocysteine metabolism was corroborated at the pathway level. Homocysteine tended to be lower in plasma from responders (12.9±1.7 versus 16.5±7.9 mg/dL, *P*=0.09). Also, MMP-1 and MMP-10 showed stronger induction in LPS-stimulated monocytes from responders. Of note, none of the growth factors reported thus far to be related to the degree of

![Figure 3. Differentially expressed genes in resting and stimulated monocytes. Bar graphs demonstrating 2-log average values and SDs of a selection of genes in resting monocytes and LPS-stimulated monocytes. Differences that are not present at baseline become apparent on stimulation. Among the genes showing stronger induction in nonresponders (n=20) are IFN-β and a number of IFN-related genes, whereas antioxidant IL-19 and IL-24, as well as MMP-1 and MMP-10, are more strongly induced in collateral responders (n=18).](image-url)
coronary collateralization (placental growth factor, basic fibroblast growth factor, vascular endothelial growth factor, TNF-α, monocyte chemoattractant protein-1, transforming growth factor-β, granulocyte/macrophage colony-stimulating factor)\textsuperscript{21,22} was found differentially expressed.

**Real-time RT-PCR**

Using RT-PCR, stronger induction of genes of the IFN pathway in nonresponders was confirmed for all tested targets (IFN-β, IFN-β, CXCL10, CXCL11), as was stronger induction of MMP-1, MMP-10, and NQO1 in responders (Table 4).

**Enzyme-linked immunosorbent assay**

ELISA analysis of the LPS-stimulated monocyte supernatants showed significantly less secretion of IFN-β in responders versus nonresponders (36.5±16.7 versus 60.5±32.6 pg/mL, \( P=0.0045 \)). IFN-β plasma levels were not detectable in the majority of patients from both groups (data not shown). CXCL10 as an IFN-β downstream target showed significantly lower levels in plasma of responders (59.5±30.8 versus 98.1±44.1 pg/mL, \( P<0.01 \)).

**SMC Proliferation**

Incubation with IFN-β inhibited SMC proliferation dose dependently, as indicated by decreased 5-bromodeoxyuridine incorporation (Figure 4a).

**Table 3. Differentially expressed pathways between responders and nonresponders**

| No. | Metacore Pathway Name                                          | \( P \)       |
|-----|-----------------------------------------------------------------|--------------|
| 1   | IFN-α/β signaling pathway                                       | 4.07x10-5    |
| 2   | Role of TLRs 3 and 4 in cell response: TICAM1-specific signaling pathways | 1.70x10-4    |
| 3   | Role of IAP-proteins in apoptosis                               | 3.14x10-4    |
| 4   | Cytoplasm/mitochondrial transport of proapoptotic proteins Bid, Bmf and Bim | 9.72x10-4    |
| 5   | TNFR1 signaling pathway                                         | 1.33x10-3    |
| 6   | EPO-induced MAPK pathway                                        | 1.50x10-3    |
| 7   | Methionine-cysteine-glutamate metabolism                        | 2.09x10-3    |
| 8   | Apoptotic TNF family pathways                                   | 3.21x10-3    |
| 9   | Methionine metabolism                                          | 5.41x10-3    |
| 10  | Crosstalk VEGF and angiopoietin-1 signaling                     | 5.58x10-3    |

Genes found differentially expressed in stimulated monocytes between responders and nonresponders were subjected to pathway analysis. The most significant pathways (sorted by \( P \)) are shown here. Genes related to immunity or apoptosis were found overexpressed almost exclusively in nonresponders (for all significant differential pathways, please see supplemental Table VIII). EPO indicates erythropoietin; IAP, inhibitor of apoptosis; MAPK, mitogen-activated protein kinase; TNFR, TNF receptor; VEGF, vascular endothelial growth factor.
Murine Hindlimb Model of Arteriogenesis

To investigate the role of IFN-β signaling in arteriogenesis in vivo, we performed a perfusion study in an established hindlimb model of arteriogenesis. Systemic treatment with IFN-β for 7 days following unilateral femoral artery ligation led to significantly attenuated perfusion restoration as compared with saline-treated control group.

Table 4. Confirmation of Gene Array Results With Real-Time PCR

| GenBank Accession No. | Symbol | Array Fold Change | Array P | Adjusted P | PCR Fold Change | PCR P |
|-----------------------|--------|------------------|---------|------------|----------------|-------|
| NM_002176.2           | IFN-β  | 2.04             | 3.19x10^{-8} | 5.05x10^{-5} | 3.23           | 0.02  |
| NM_000619.2           | IFN-γ  | 1.96             | 4.07x10^{-11} | 4.19x10^{-7} | 2.58           | 0.02  |
| NM_001565.1           | CXCL10 | 2.19             | 9.36x10^{-4}  | 0.07        | 3.81           | 0.01  |
| NM_005409.3           | CXCL11 | 3.18             | 1.52x10^{-10} | 7.85x10^{-7} | 4.36           | 0.02  |
| NM_000903.2           | NQO1   | -1.58            | -158      | 0.001      | -1.65          | 0.04  |
| NM_002421.2           | MMP1   | -1.75            | 0.002     | 0.10       | -3.10          | 0.01  |
| NM_002425.1           | MMP10  | -1.58            | 3.63x10^{-4} | 0.04       | -1.90          | 0.03  |

A positive sign in the fold changes denotes more strongly induced genes in nonresponders; a negative sign denotes more strongly induced genes in responders. The table shows that, when validating differentially regulated genes using PCR, for all targets tested, almost identical results are obtained as compared with the gene expression levels from the arrays.

Figure 4. Effects of IFN-β on SMC proliferation and arteriogenesis. a, Increasing concentrations of IFN-β inhibit SMC proliferation in vitro as shown by decreased 5-bromodeoxyuridine incorporation. b, Relative perfusion was assessed in mice using fluorescent microsphere infusion under maximal vasodilation 7 days after femoral artery ligation and daily systemic treatment with 10⁵ IU/kg IFN-β. Perfusion ratios are expressed as percentage ligated vs nonligated hindlimb. IFN-β treatment (n = 10) significantly attenuated perfusion restoration compared with PBS-treated control group (n = 10).
(31.5±1.2% [IFN-β] and 41.9±1.9% [control] perfusion-ligated versus nonligated hindlimb, \( P=0.001 \)) (Figure 4b). We confirmed enhanced levels of IFN-β in ligated hindlimbs of IFN-β–treated animals compared with controls (41.9±17.6 versus 11.5±1.7 ng/g total protein, \( P<0.05 \)). Systemic treatment with IFN-β increased tissue gene expression of TNF-αα, indicating increased inflammation.

**DISCUSSION**

The present study demonstrates that monocytes from patients with sufficient versus insufficient coronary collateral artery development show distinctively different gene expression profiles. Stress testing by *in vitro* stimulation of monocytes with LPS most strongly revealed these differences. IFN-β and IFN-related pathways showed a stronger induction in 3 of 4 examined cell types in nonresponders, consistent with higher secretion levels of IFN-β protein. The effect of IFN-β on arteriogenesis was verified in a murine hindlimb model showing attenuated collateral artery growth after application of IFN-β.

Clinical trials on stimulation of collateral artery growth have been hitherto unsuccessful.\(^3\)\(^–\)\(^6\) In most cases, proarteriogenic factors are identified in experimental models of collateral artery growth. However, there are several pitfalls involved in experimental explorative strategies, such as variances between species and comorbidities like dyslipidemia and diabetes that are seldom implemented in experimental models. We therefore aimed to examine the molecular mechanisms of arteriogenesis in humans, exploiting the heterogeneity in arteriogenic response for the discovery of potential proarteriogenic targets. Unlike studies comparing diseased and healthy populations, in the present study, all patients only differed in their arteriogenic response to a similar level of obstructive arterial disease. Therefore, we took great care to separate responders from nonresponders, calculating CFI using intracoronary pressure measurements\(^23\) and cautiously matching the 2 patient groups. CFI measurements confirmed the variation of collateralization in patients with similar diameters of stenosis.

**Absence of classifying differences in resting monocyte transcriptome**

One of the most surprising outcomes of our study was that we were unable to detect a consistent difference in gene expression in resting monocytes from responders versus nonresponders. No single gene or gene set was able to classify patients when using appropriate correction for multiple testing. In a recent study, a similar analysis of gene expression in circulating, unstimulated monocytes from patients with coronary
artery disease in relation to their collateral status identified differential regulation of several genes using low stringency multiple-testing correction (cutoff used for array analysis: adjusted $P_{\text{adj}}$, <0.32). Indeed, the discovered genes could only partly be validated in a separate group, possibly because of the restricted number of patients ($n=16$), the varying degree of underlying coronary artery disease among these patients, or the use of angiograms for collateral grading instead of more accurate invasive pressure measurements.

**Differences in monocytic transcriptome are revealed by cellular stress testing**

Once activated, circulating cells can acquire distinct functional characteristics. Particularly monocytes become involved in vascular (patho-)physiological processes on extravasation, stimulation and transformation into macrophages. They then turn on phenotype-specific gene expression profiles that may correlate with disease. To test the hypothesis that monocytes have to be stimulated *ex vivo* to disclose arteriogenesis-related differences in gene expression, we stimulated the cells with the TLR4 agonist LPS. Activated TLR4 was recently shown to be essential in arteriogenesis. Indeed, the differences between responders and nonresponders were revealed most clearly after stimulation with LPS. This approach of cellular stress testing may prove to be valuable also in other disease entities in which circulating cells are involved, such as atherosclerosis or metastatic cancer.

**IFN-β Inhibits Arteriogenesis**

When comparing LPS-stimulated monocyte expression profiles from the 2 patient groups, no differences were found in the induction of genes that correspond to the MyD88-dependent pathway, generally considered as the default proinflammatory TLR4 response. In contrast, substantial differences were found in the expression of the MyD88-independent, TICAM-1–regulated, IFN-induced pathway. This pathway was significantly differentially expressed in stimulated monocytes, macrophages, and progenitor cells, suggesting imprinted differences in the activation response of monocytes from the 2 patient populations. Analysis of corresponding promoter sequences of these genes for specific transcription factor binding sites corroborated the role of the IFN-β pathway. Classification analysis, using multiple independent training and validation sets within our patient population, once again resulted in IFN-β and IFN-dependent genes as classifiers to separate responders from nonresponders. Given the increased IFN-β protein production we observed on nonresponder monocyte activation, we determined the effect of IFN-β treatment on arteriogenesis in a well-established mouse model to analyze whether the association of IFN-β signaling with poor collateral artery growth is causal. Results indeed substantiated...
our findings from the patient study in an experimental setting. Local inflammation, as measured by TNF- expression, was higher in IFN-β–treated mice. Arterial SMCs represent the primary target cell type of arteriogenic therapies. Our in vitro data indicate that IFN-β inhibits SMC proliferation. Conceptually, the inhibitory effect of IFN-β on arteriogenesis seems consistent with its documented importance in capillary sprouting (angiogenesis).26 A possible therapeutic approach to stimulate arteriogenesis would therefore involve inhibition of the IFN pathway and hence modulation of the inflammatory response of circulating cells. For the first time, a possible proarteriogenic therapy would thus not be proinflammatory but rather antiinflammatory. This is advantageous, given the increased risk of promoting atherosclerosis or destabilizing existing plaques that is shown to be associated with current proarteriogenic therapies based on proinflammatory agents.27

**Increased apoptotic signaling in nonresponders**
Besides upregulation of the IFN axis, expression data of monocytes from nonresponders showed stronger induction of several apoptosis-related genes and pathways than responders on stimulation. Also, the oxidoreductase NQO120 showed stronger induction in monocytes of responders, pointing toward antiapoptotic properties. Reduced apoptosis is 1 of the mechanisms by which granulocyte/macrophage colony-stimulating factor stimulates arteriogenesis.28 Furthermore, NQO1 is part of the protective cellular response activated on exposure to oxidative stress,29 which affects collateral artery growth.30

**Improved homocysteine metabolism and matrix-degrading factors in responders**
CBS showed stronger induction in responders. CBS is known to metabolize homocysteine,31 and high levels of homocysteine have been described previously to inhibit angiogenesis in a rat model of hindlimb ischemia.32 Interestingly, plasma levels of homocysteine tended to be lower in responders in our study. Furthermore, monocytes from responders showed significant upregulation of MMPs, which are known to play an important role in vascular remodeling.33

**Study limitations**
Although the diameter stenosis was similar in both patient groups, differences in microvascular resistance that were not recorded in this study could have influenced pressure-derived CFI measurements. The fact that no significant differences in gene expression could be found in resting monocytes and progenitor cells may be attributable to the relatively small size of the study, which makes negative conclusions difficult.
Moreover, monocyte stimulation may have magnified differences already present at baseline levels but not detectable in a small study population. The use of LPS as a stimulator for the more proarteriogenic phenotype of monocytes possibly resulted in LPS-specific changes in gene expression that are not generalizable to other stimuli. No independent patient cohort was included to further verify array data. Instead, we used a well-established internal validation technique. Also, we validated the hypothesis of the attenuating effect of IFN-β on arteriogenesis in the murine model.

CONCLUSION

In the present study, cellular stress testing revealed differential monocyte gene expression profiles of patients with sufficient or insufficient coronary collateral networks. The results strongly suggest that monocytes are involved in human arteriogenesis. Surprisingly, the majority of differentially regulated genes was found to be overexpressed in collateral nonresponders, indicating that differential activity of antiarteriogenic pathways rather than proarteriogenic pathways is responsible for the heterogeneous arteriogenic response of individual patients on arterial obstruction. In a bedside-to-bench approach, we verified the functional impact of observational human data in an experimental model, providing evidence for the inhibitory effect of IFN-β on arteriogenesis in mice. *In vitro* data indicated that the antiarteriogenic effect of IFN-β may be mediated by its antiproliferative effect on SMCs. These data could lead to a shift in paradigm in the research on stimulation of arteriogenesis because they suggest that intervening with antiarteriogenic pathways may serve as a novel therapeutic approach to stimulate collateral artery growth.

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Chapter 7

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