The Role of Monocyte-Derived Cells and Inflammation in Baboon Ductus Arteriosus Remodeling

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ABSTRACT

Inflammatory processes play a crucial role in the pathogenesis of atherosclerosis and other vascular disorders. We hypothesized that ischemia of the ductus arteriosus might initiate an active inflammatory response that could play a role in ductus remodeling and permanent closure. To test this hypothesis, we studied effects of postnatal ductus construction on inflammatory processes and remodeling in late-gestation fetal and newborn baboons, and preterm newborn baboons. After postnatal ductus constriction, the expression of several genes known to be essential for atherosclerotic remodeling [vascular cell adhesion molecule (VCAM)-1, E-selectin, IL-8, macrophage colony stimulating factor-1, CD154, interferon-γ, IL-6, and tumor necrosis factor-α] was increased in the ductus wall. We were unable to detect intercellular adhesion molecule (ICAM)-1, ICAM-2, P-selectin, monocyte chemoattractant protein-1, or IL-1 by either real-time PCR or immunohistochemistry. VCAM-1, which is newly expressed by luminal cells of the closed ductus, is an important ligand for the mononuclear cell adhesion receptor VLA4. After postnatal constriction, VLA4$^+$ monocytes/macrophages (CD68$^+$ and CD14$^+$) and, to a lesser extent, T-lymphocytes adhered to the ductus wall. Neutrophils and platelets were not observed. The extent of postnatal neointimal remodeling (both endothelial cell layering and subendothelial space thickening) was associated with the degree of mononuclear cell adhesion. Similarly, the extent of vasa vasorum ingrowth correlated with the invasion of CD68$^+$ cells, from the adventitia into the muscle media. Based on these data, we conclude that the inflammatory response following postnatal ductus constriction may be as necessary for ductus remodeling as it is for atherosclerotic remodeling. (*Pediatr Res* **57: 254–262, 2005**)

Abbreviations
IBU, ibuprofen
ICAM, intercellular adhesion molecule
IFN-γ, interferon-γ
INDO, indomethacin
L-NA, *N*-nitro-L-arginine
MCP, monocyte chemoattractant protein
MMA, *N*-monomethyl-L-arginine-monoacetate
MDH, malate dehydrogenase
TNF-α, tumor necrosis factor-α
VCAM, vascular cell adhesion molecule
VEGF, vascular endothelial growth factor

Closure of the full-term ductus arteriosus occurs in two phases. First, smooth muscle constriction obstructs the ductus' lumen. Then, anatomic remodeling permanently occludes the lumen. The initial constriction appears to be the required stimulus for anatomic closure. During constriction, loss of luminal and vasa vasorum flow produce a zone of ischemic-hypoxia in the ductus' muscle media that induces the following anatomic changes: luminal endothelial proliferation, subendothelial thickening, ingrowth of vasa vasorum, and cell death (1). The preterm newborn is capable of remodeling its ductus, just like the full-term newborn, if it can impede its luminal flow and develop the same degree of ischemic-hypoxia as found at term (2).

Although the cell death and ingrowth of vasa vasorum in the ductus wall appear to be due to ATP depletion (3,4) and VEGF induction (5), respectively, the mechanism(s) responsible for the neointimal changes are still unknown. It is now clear that inflammatory processes play a crucial role in the pathogenesis of several vascular disorders (6–9). The most studied model of

vascular inflammation is atherosclerosis, where the accumulation of monocytes/macrophages in the vessel wall plays an early role in remodeling (10). The monocytes/macrophages elaborate numerous factors that help to orchestrate the subsequent neointimal changes (8,9). Recently, several cytokines—proinflammatory [IL-6 (11,12), TNF-α (13,14), IL-1 (13,14)], chemotactic [MCP-1 (15), IL-8 (16)], immunomodulatory [M-CSF (10,17), IFN-γ (18), CD154 (19,20)]—and adhesion molecules—P-selectin (21,22), E-selectin (21), ICAM-1 (22,23), VCAM-1 (23), and VLA4 integrin (24)—have been shown to play an essential role during atherosclerotic remodeling.

We hypothesized that, after postnatal constriction and loss of luminal blood flow, an active inflammatory process might play an important role in ductus arteriosus remodeling and permanent closure. In the studies described below, we found that, after postnatal constriction, monocytes/macrophages adhere to the ductus wall, their accumulation appears to be related to the types of cytokines and adhesion molecules induced after constriction, and the extent of neointimal remodeling is directly related to the degree of mononuclear cell adhesion.

METHODS

All studies were approved by the committees on animal research at the University of California San Francisco, the University of Texas Health Science Center, and the Southwest Foundation for Biomedical Research.

We used fetal and newborn baboons (*Papio* sp., full term = 185 d gestation) to examine the effects of ductus constriction on ductus remodeling. Animal care, surgery, and necropsy were performed as previously described (1,2). Preterm [125 d gestation (68% of full term)] and mature (175 d gestation) fetal baboons were delivered by cesarean section and euthanized before breathing. Preterm newborn baboons were delivered by cesarean section at 125 d gestation and were euthanized on d 6 after delivery. Mature full-term newborn baboons were euthanized between 1 and 2 d after spontaneous full-term delivery.

Our primary goal was to examine the difference in inflammatory response between preterm newborn ductus that remain patent after birth and those that close after birth. A complete echocardiographic exam including assessment of ductal patency was performed daily using an 8-mHz transducer interfaced with a Biosound AU3 (Genoa, Italy) echocardiographic system (2). At necropsy, the ductus was dissected in 4°C PBS solution and frozen in liquid nitrogen (for RNA analysis) or embedded in Tissuetek (American Master Tech, Merced, CA) and frozen in liquid nitrogen.

Preparation of total RNA, reverse transcription, and quantitative PCR. Total RNA was isolated from the frozen ductus of eight preterm fetuses, six preterm newborns (whose ductus had closed spontaneously by 5 d after delivery), eight preterm newborns (whose ductus were still patent before necropsy on d 6), eight mature fetuses (175 d gestation), and eight full-term 1- to 2-d-old newborns, as described elsewhere (25). We used the TaqMan Universal PCR master mix of PE Applied Biosystems (Foster City, CA) to quantify the expression of molecules involved in vascular remodeling and inflammation. TaqMan probes were designed using the Primer Express program and labeled with fluorophores, 6-caboxy-fluorescein and 6 carboxy-tetramethyl-rhodamine, as reporter and quencher dyes, respectively. An ABI PRISM 7700 Sequence detection system was used to determine number of PCR cycles required for product detection [cycle threshold (CT value)]. Reactions were carried out in triplicate. The fewer the number of starting copies of a gene (mRNA), the higher the CT value required for product detection. All reactions were repeated on at least three separate days. Data were analyzed using the Sequence Detector version 1.6.3 program.

In preliminary experiments, we found that in the fetal and newborn ductus the CT value of the baboon housekeeping gene MDH was constant throughout gestation and after birth: CT (fetus 125 d) = 30.1 ± 0.4 cycles, $n = 8$; CT (fetus 175 d) = 30.0 ± 0.4, $n = 8$; CT (full term newborn) = 30.2 ± 0.3, $n = 8$.

Therefore, MDH could be used as an internal control to normalize the degree of expression of any gene under investigation. We used the method of relative gene expression, where ΔCT (MDH − gene) represents the difference in cycle threshold between MDH and the individual gene of interest. Each unit of ΔCT (MDH − gene) represents a 2-fold increase in a gene's mRNA. The more negative the ΔCT (MDH − gene), the fewer the number of starting copies of a gene.

We have previously shown that the expression of VEGF mRNA in the ductus arteriosus correlates with the degree of smooth muscle hypoxia (5). In our real-time PCR experiments, we used VEGF as a surrogate marker for hypoxia.

Immunohistochemistry: in vivo *preparation.* Our primary goal was to examine the differences between preterm newborn ductus that remain patent after birth and those that close after birth. Preterm newborn baboons frequently fail to constrict their ductus and obstruct their luminal blood flow after birth unless they are treated with inhibitors of both prostaglandin and nitric oxide production (2). Therefore, we treated preterm newborn baboons with one of five protocols to obtain a sufficient number of closed ductus for immunohistochemical analysis: group 1—INDO alone (INDO, $n = 6$); group 2—INDO plus L-NA (a nitric oxide synthase inhibitor) (INDO+L-NA, $n = 6$); group 3—INDO plus MMA (a nitric oxide synthase inhibitor) (INDO+MMA, $n = 6$); group 4—ibuprofen alone (IBU, $n = 6$); group 5—no treatment (control, $n = 10$). These treatment protocols have been previously described (2). INDO (Indocin, 0.1 mg/kg/dose) was given intravenously at 24, 48, 72, 84, 96, 108, 120, and 132 h after delivery to animals in groups 1, 2, and 3. Groups 2 and 3 received continuous infusions of either L-NA (6 mg/kg/h) or MMA (20 mg/kg/h), respectively, starting at 50 h after delivery, which continued until necropsy. Group 4 received i.v. ibuprofen (Farmacon, Westport, CT) 10 mg/kg at 24 h and 5 mg/kg at 48, 72, 96, and 120 h after delivery.

On the day of necropsy, the ductus was closed on Doppler exam in 3/6 INDO-treated, 6/6 INDO+L-NA-treated, 6/6 INDO+MMA-treated, 6/6 IBU-treated, and 3/10 control preterm newborn baboons. We have previously shown that the histologic changes that occur during ductus remodeling depend on the degree of ductus constriction and vessel wall ischemia, and not on the pharmacologic manipulations used to produce the constriction (1,2). Therefore, we sorted the ductus from the five different preterm newborn treatment groups into two groups: immature newborn ductus closed at necropsy ($n = 24$) and immature newborn ductus open at necropsy ($n = 10$).

Immunohistochemistry. Immunohistochemistry protocols were similar to those reported previously (2,26). Endothelial cells were detected with *anti-endothelial nitric oxide synthase* (Clone 3, BD Biosciences, San Jose, CA) and anti-*CD31* (9G11, R & D Systems, Minneapolis, MN). Both antibodies showed identical findings. Antibodies against the following leukocyte antigens were used to identify mononuclear cells in the ductus wall: *VLA4* [lymphocytes, dendritic cells and activated monocyte/macrophages: BU49 (Calbiochem, San Diego, CA)], *CD68* [lymphocytes, dendritic cells, activated monocytes/macrophages, and granulocytes: KP1 (DAKO, Carpinteria, CA)], *CD3* [T-lymphocytes: rabbit polyclonal (DAKO)], *CD14* [monocytes/macrophages: TUKU (DAKO)], *CD83* [mature dendritic cells: HB15a (Santa Cruz Biotechnology, Santa Cruz, CA)], *neutrophil elastase* [granulocytes: NP57 (DAKO)], and *GP1bα* [platelets: AN51 (DAKO)]. Antibodies against *VCAM-1* [1G11 (Santa Cruz Biotechnology)], *ICAM-1* [15.2 (Santa Cruz Biotechnology and Calbiochem)], *ICAM-2* [F5 (Santa Cruz Biotechnology)], *E-selectin* [1.2B6 (Santa Cruz Biotechnology)], and *P-selectin* [1E3 (Santa Cruz Biotechnology) and AK4 (BD Biosciences)]) were used to identify adhesion molecules expressed by activated endothelial cells. The number of positively stained cells lining the ductus lumen was expressed per 100 luminal-lining cells.

Histologic measurements in the newborn ductus were made at the level of minimal luminal area, which was determined from 6-μm serial cross-sections. The subendothelial zone was defined as the region between the luminal endothelial cells and the internal elastic lamina (identified by phase-contrast microscopy).

Mononuclear cells and vasa vasorum invade the ductus muscle media from the surrounding adventitia (see below). To determine the extent of muscle media invasion by mononuclear cells and vasa vasorum, we used an overlay template, containing a grid, and NIH Image software to divide the muscle media into 12 clock hours. In each clock hour section of the wall, we measured the maximal distance that the cells migrated from the adventitia into the media (expressed as a percentage of the distance from the adventitia to the lumen in that clock hour section of the wall). The maximal distances from each of the

12 sections of the ductus wall were averaged and reported as "percentage medial thickness" for that vessel.

Statistics. Results are presented as means ± standard deviations, percentages, and correlation coefficients. Intergroup differences were evaluated with either a χ^2 analysis, or unpaired *t* test. When more than one comparison was made, Bonferroni's correction was used.

RESULTS

The full-term newborn baboon ductus constricts rapidly after birth and is closed by Doppler exam within 12 h after delivery. In contrast with the mature fetal ductus, which has a single endothelial cell layer lying close to the internal elastic lamina for the majority of the luminal circumference, the full-term newborn ductus undergoes extensive neointimal expansion during the first 2 d after birth. This includes the appearance of multiple endothelial cell layers and thickening of the subendothelial zone (by migrating smooth muscle cells, matrix and edema) (Fig. 1, *A* and *B*) (1). In addition, vasa vasorum increase their penetration into the outer muscle media of the mature newborn ductus (Fig. 1*C*).

The histology of the immature fetal ductus is similar to that of the mature fetal ductus except for fewer areas of subendothelial thickening and less vasa vasorum ingrowth. Little change occurs in the immature newborn ductus that remain patent after birth (Fig. 1). In contrast, the immature newborn ductus that close after birth undergo the same changes that occur in the mature newborn ductus (Fig. 1).

We used VEGF gene expression (mRNA) as a surrogate marker for hypoxia. VEGF expression is increased in the baboon ductus after birth, but only following ductus closure; there is no change in VEGF expression after birth if the ductus
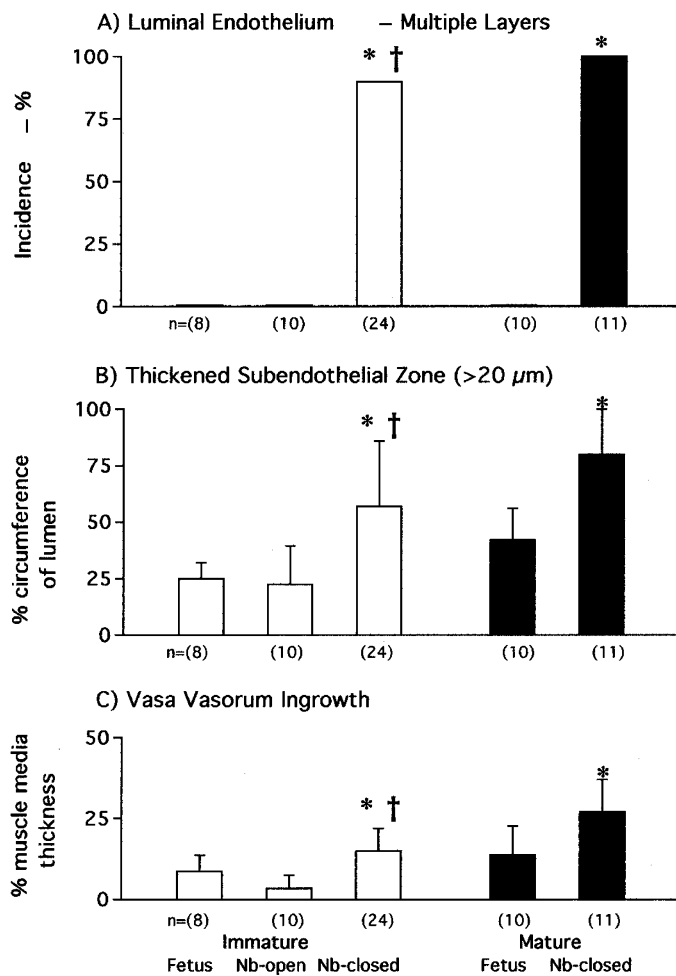

Figure 1. Relationship between the degree of ductus constriction and ductus remodeling. (*A*) Fetal and neonatal ductus from immature and mature baboons were categorized as having either areas along the lumen with multiple (three or more) layers of CD31$^+$ luminal endothelial cells (multiple layers) or a single layer of CD31$^+$ cells that completely surrounds the lumen (single layer). The vertical bars represent the number of ductus in a group (expressed as a percentage) that have areas with multiple layers of endothelium. *Nb-open*, newborn with a patent ductus at necropsy; *Nb-closed*, newborn with a closed ductus at necropsy. (*B*) Subendothelial zone thickness is distance between endothelial layer and internal elastic lamina. The percentage of the luminal circumference that is surrounded by a thickened (>20 μm) subendothelial zone is increased after postnatal constriction. (*C*) The distance that vasa vasorum migrate from the adventitia into the muscle media is expressed as the percentage medial thickness (see "Methods"). *$p < 0.05$ *vs* fetal ductus; †$p < 0.05$ *vs* newborn-open. *N*, number of ductus examined.
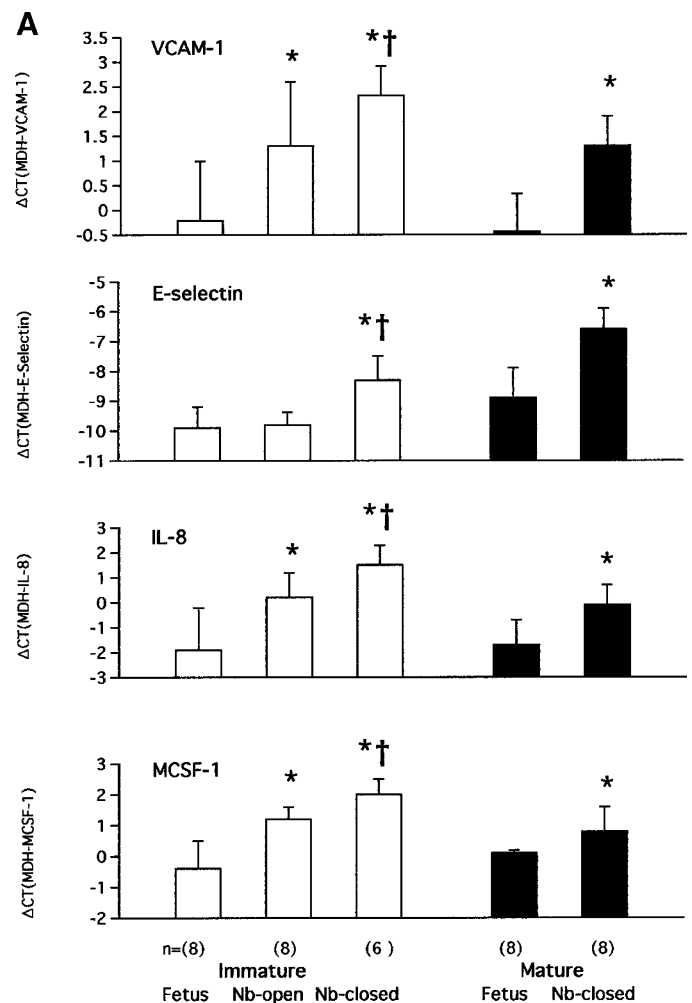

Figure 2. Real-time PCR measurements of genes known to affect vascular remodeling. Ten nanograms of cDNA from individual ductus were placed in separate wells and analyzed by TaqMan real-time PCR. ΔCT(MDH − gene) represents the difference in cycle threshold (CT) between the expression of housekeeping gene MDH and the individual gene of interest. Each unit of ΔCT(MDH − gene) represents a 2-fold increase in a gene's mRNA. The more negative the ΔCT(MDH − gene), the fewer the number of starting copies of a gene (mRNA). (*A*) Genes affecting mononuclear cell recruitment.

remains patent [ΔCT(MDH $-$ VEGF): immature (fetus $= 0.47 \pm 0.31$, newborn–open ductus $= 0.43 \pm 0.30$, newborn–closed ductus $= 2.82 \pm 0.60$; $p < 0.05$ *versus* immature fetal ductus); mature (fetus $= -0.49 \pm 0.36$, newborn–closed ductus $= 2.03 \pm 0.18$; $p < 0.05$ *versus* mature fetal ductus)].

Many of the genes found to be essential for atherosclerotic remodeling (VCAM-1, E-selectin, IL-8, macrophage colony stimulating factor-1, CD154, IFN-γ, IL-6, and TNF-α) are also expressed after postnatal ductus constriction (Fig. 2, *A* and *B*). Several of these genes are increased, even in the face of persistent postnatal patency, and, therefore, must be regulated by hypoxia and by other mechanisms.

On the other hand, several genes found to be necessary for atherogenesis are either not found or are not altered during ductus remodeling. We were unable to detect ICAM-1, ICAM-2, P-selectin, MCP-1, or IL-1 by either real-time PCR [ICAM-1, P-selectin, MCP-1, IL-1 (Fig. 2*C*)] or immunohistochemistry [ICAM-1, ICAM-2, P-selectin (data not shown)].

During atherosclerotic remodeling, circulating leukocytes use inducible ligands to roll (P- and E-selectin) and firmly adhere (ICAM-1 and VCAM-1) to the vessel's endothelium. Among these ligands, only E-selectin and VCAM-1 were found to be regulated during ductus closure and remodeling (Figs. 2*A*, 3, and 4). Cells lining the ductus lumen increased

their expression of E-selectin and VCAM-1 after postnatal closure (Figs. 3 and 4).

VCAM-1 is an important endothelial ligand for the VLA4 integrin expressed on circulating mononuclear cells (9,27). The number of VLA4$^+$ mononuclear cells adhering to the ductus lumen increased after birth (Figs. 3 and 5). The increase in VLA4$^+$ cells paralleled the increase in VCAM-1 expression and occurred only after ductus closure and loss of luminal flow (Figs. 4 and 5). The mononuclear cells that adhered to the ductus lumen were positive for monocyte and activated macrophage antigens (CD14 and CD68, respectively; Fig. 3 and 5). There was also a small but significant increase in the number of CD3$^+$ T cells that adhered to the immature ductus lumen after birth; this only occurred after ductus closure (Fig. 5). At the same time, we found a significant increase in CD14 and CD3 mRNA in the ductus after closure: ΔCT(MDH $-$ CD14): immature fetus $= -2.0 \pm 0.3$ *versus* immature newborn–closed $= 0.0 \pm 0.4$; ΔCT(MDH $-$ CD3): immature fetus $= -7.0 \pm 0.8$ *versus* immature newborn–closed $= -5.5 \pm 0.4$. No platelets (GP1bα^+) or neutrophils (neutrophil elastase-positive cells) adhered to the fetal or newborn ductus lumen (data not shown). Dendritic cells (CD83$^+$) were only occasionally found in the ductus wall (data not shown).

In both the mature and immature ductus, the adhesion of CD68$^+$, VLA4$^+$, and CD14$^+$ cells to the lumen was signifi-
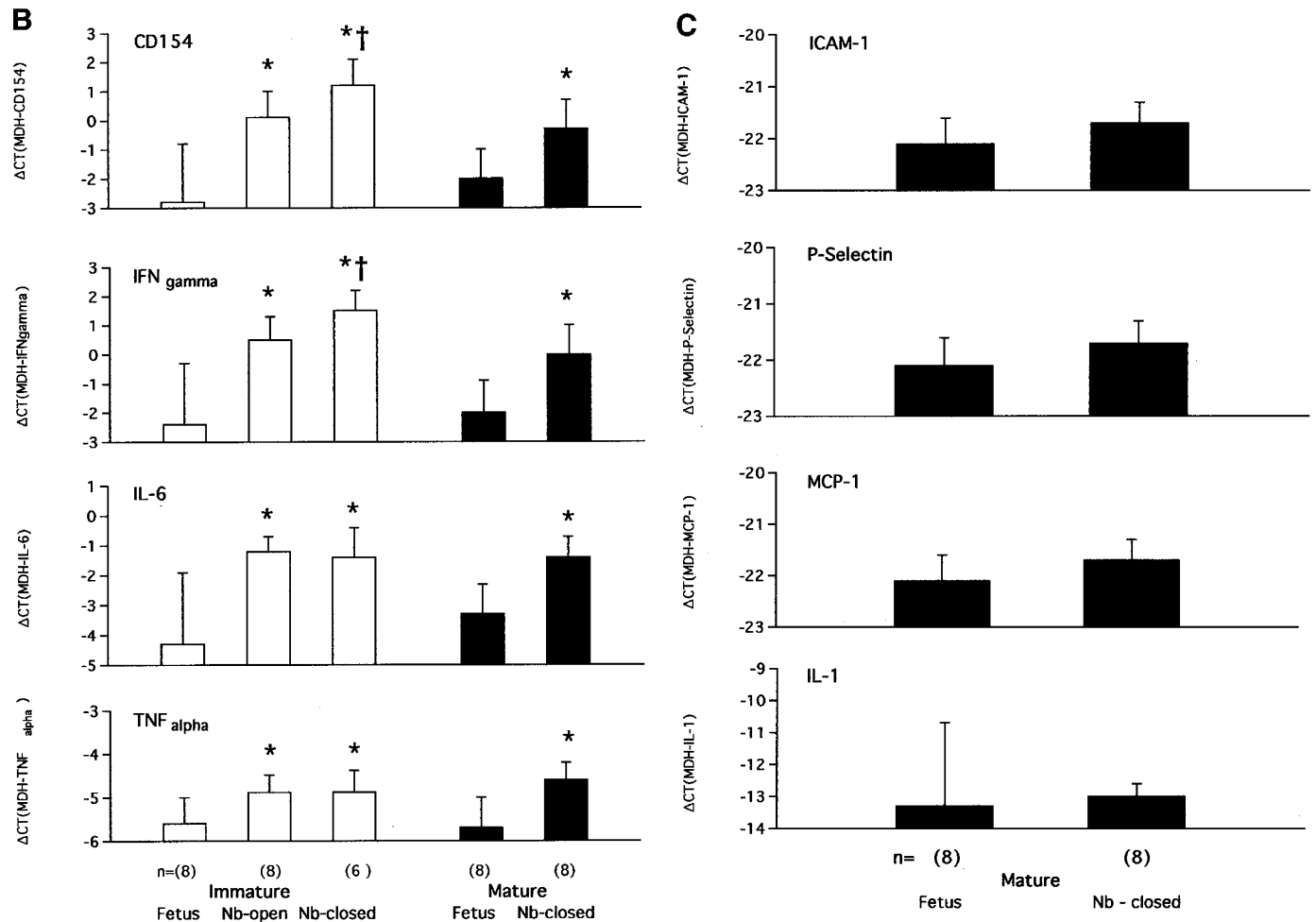

Fig. 2b. (*B*) Immunomodulatory genes.

Fig. 2c. (*C*) Genes not expressed during ductus closure. *$p < 0.05$ *vs* fetal ductus; †$p < 0.05$ *vs* newborn-open. *N*, number of ductus examined.

cantly associated with the presence of multiple endothelial cell layers or mounds (Fig. 6). Similarly, the presence of CD68$^+$ cells in the subendothelial space was significantly associated with a thickened subendothelial zone (Fig. 7).

Resident CD68$^+$ mononuclear cells were present in the adventitia and outer media of the fetal and newborn ductus. CD68$^+$ cells increased in number and invaded deeper into the muscle media after birth (Fig. 8A). The increased postnatal invasion of CD68$^+$ cells only occurred after ductus closure and loss of luminal flow (Fig. 8A). The extent of vasa vasorum penetration into the muscle media correlated with the extent of CD68$^+$ cell invasion [Fig. 8, *B* (immature) and *C* (mature)].

DISCUSSION

During atherosclerotic remodeling, monocytes/macrophages (10) appear to be required for the remodeling process, whereas lymphocytes appear to modulate its progression (28). We found that after postnatal ductus constriction, monocytes/ macrophages and, to a lesser extent, T-lymphocytes, adhere to the ductus lumen and the extent of their recruitment correlates with the degree of neointimal and vasa vasorum remodeling. We hypothesize that mononuclear cell recruitment is as nec-

essary for ductus remodeling as it is for atherosclerotic remodeling.

Leukocyte recruitment occurs through a process that involves both leukocyte rolling and adhesion. Rolling is mediated by the interaction of selectins (P-, E-, and L-) with their glycoconjugate ligands, whereas firm adhesion is mediated by the interactions of integrins (β2 subfamily and VLA4) with their respective endothelial ligands (ICAM-1 and VCAM-1) (9). During atherosclerosis, mononuclear cell recruitment and vascular remodeling are dependent on the induced expression of P-selectin, E-selectin, ICAM-1, and VCAM-1 (21–23,29, 30). In contrast, during ductus closure, there is a more limited pattern of adhesion molecule expression: neither P-selectin nor ICAM-1are expressed; only E-selectin and VCAM-1 are increased (Figs. 2, 3, and 4).

In the face of physiologic shear forces, both P- and E-selectin appear to be necessary for leukocyte recruitment (21,31). However, under low-flow conditions, the interaction of VCAM-1 with the VLA4 integrin can mediate rolling and adhesion of monocytes without the need for selectins or β2 integrins (32–34). The VLA4/VCAM-1 interaction is less effective in supporting rolling and adhesion of lymphocytes
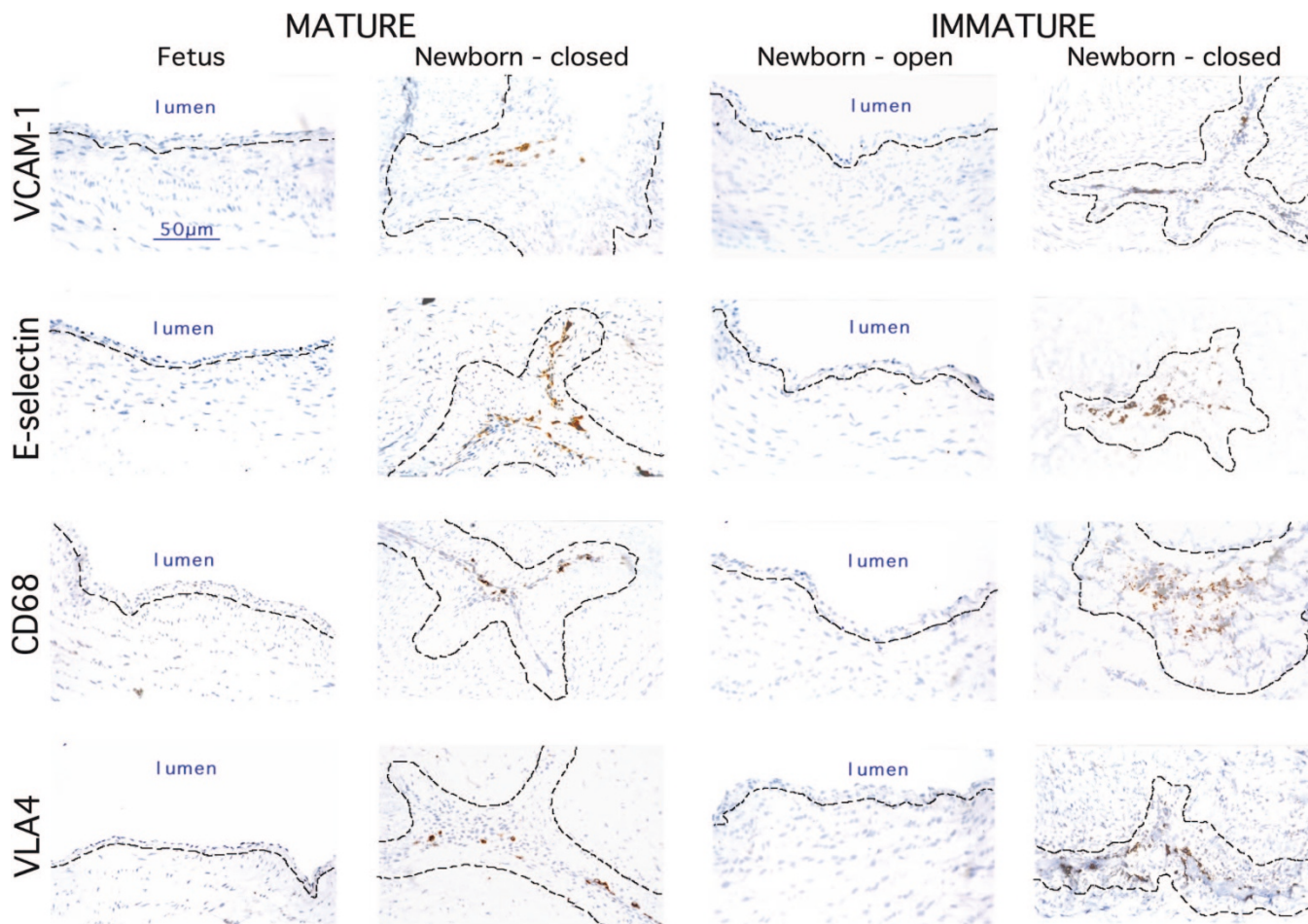

Figure 3. VCAM-1 and E-selectin expression and recruitment of CD68$^+$ and VLA4$^+$ cells are increased during ductus closure. Ductus from mature fetuses (175 d gestation), mature newborns (1- to 2-d-old full-term), immature newborns (6 d old, with or without a patent ductus). Positive immunoreactivity = *brown stain*. Cell nuclei counterstained with hematoxylin (*blue*). *Dashed black line* = internal elastic lamina (identified by phase-contrast microscopy). Horizontal bar represents 50 μm. Lumen indicates the open lumen of the ductus. See Figures 4 and 5 for quantitation.
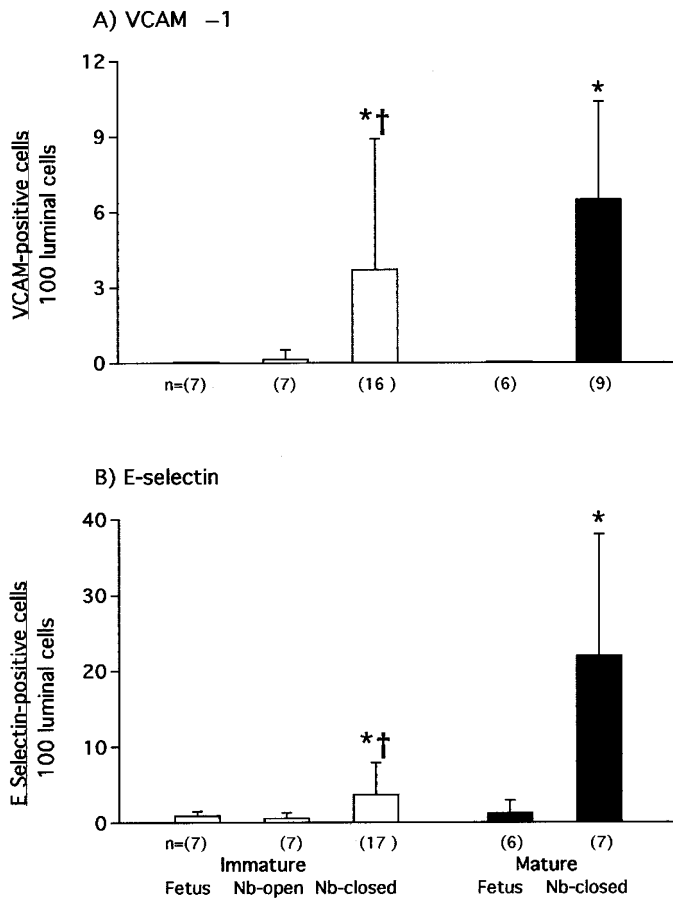
Figure 4. Relationship between the degree of ductus constriction and VCAM-1 and E-selectin expression. The number of cells lining the ductus lumen that are positive (by immunohistochemistry) for VCAM-1 (*A*) and E-selectin (*B*) are expressed per 100 lumen-lining cells. *$p < 0.05$ *vs* fetal ductus; †$p < 0.05$ *vs* newborn-open. *N*, number of ductus examined.

Figure 5. Relationship between the degree of ductus constriction and mononuclear cell adhesion to the ductus lumen. The number of mononuclear cells [expressing VLA4 (*A*), CD68 (*B*), CD14 (*C*), or CD3 (*D*) by immunohistochemistry] that adhere to the ductus lumen are expressed per 100 lumen-lining cells. *$p < 0.05$ *vs* fetal ductus; †$p < 0.05$ *vs* newborn-open. *N*, number of ductus examined.

(35,36) and is completely incapable of supporting neutrophil adhesion because VLA4 is not present on neutrophils (27). These findings help to explain why monocytes/macrophages and, to a lesser extent, T cells adhere to the ductus endothelium after ductus closure (Figs. 3 and 5), and why neutrophils, which normally contribute to most inflammatory conditions, are notably absent. We examined the ductus at two time points after birth (immature newborn = 6 d, mature newborn = 1–2 d); it is possible that other inflammatory cells, in addition to monocyte/macrophages, may play a role during later phases of ductus remodeling.

If mononuclear cell recruitment is necessary for ductus remodeling, then the current studies also help to explain why tight ductus constriction and loss of luminal flow are essential for ductus remodeling and permanent closure (1,2). Decreased luminal flow has a direct effect on VCAM-1 expression (37), promotes cytokine-induced VCAM-1 expression (see below), and enables the interaction of VCAM-1 with VLA4 to support the adhesion of mononuclear cells to the ductus wall in the absence of P-selectin and $\beta2$ integrin/ICAM-1 interactions.

After postnatal ductus closure, profound hypoxia develops in the ductus wall; this does not occur if the neonatal ductus remains patent after birth (1,2). Our measurements of VEGF
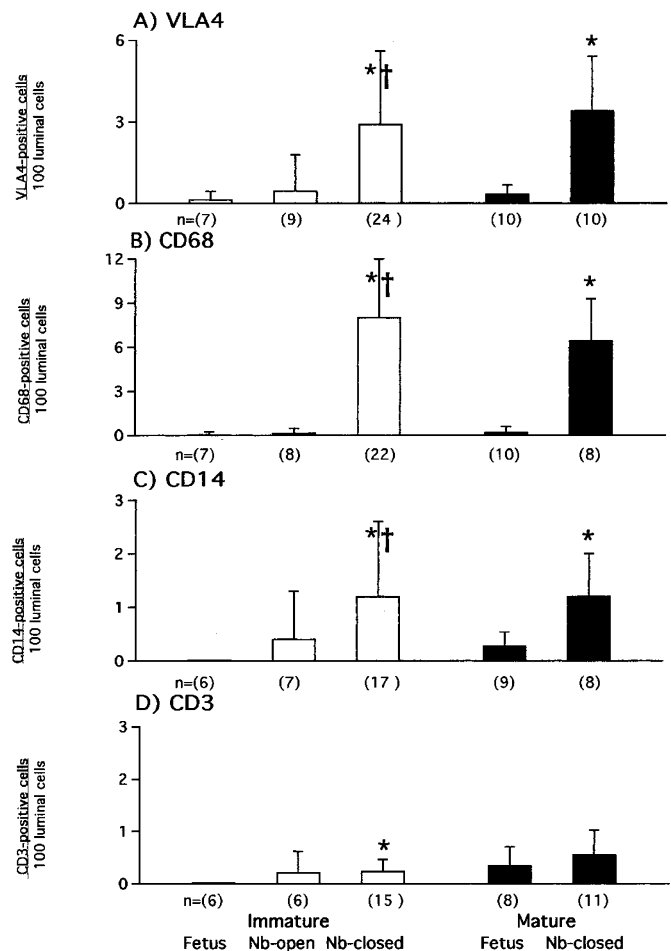
gene expression (mRNA) in the fetal and neonatal baboon ductus are consistent with these observations. Several of the cytokines induced during ductus closure [TNF-α, IFN-γ, CD154 (Fig. 2)], are capable of promoting VCAM-1 and E-selectin expression. We hypothesize that ductus wall hypoxic ischemia induces the production of these and the other cytokines (IL-8, macrophage colony stimulating factor-1, and IL-6) known to affect vessel remodeling (10–14,16–20). The pattern of gene expression during ductus closure differs in some respects from that seen during atherosclerosis. Several cytokines (IL-1 and MCP-1) that determine the extent of atherosclerotic remodeling are not expressed during ductus closure (Fig. 2*C*).

To study the changes in RNA expression that occur during ductus closure, we used preterm newborns whose ductus had closed spontaneously. Because spontaneous ductus closure is a relatively infrequent event (25–30%) in the preterm newborn baboon (1), we used several pharmacological methods (prostaglandin inhibition with or without nitric oxide inhibition) to ensure an adequate number of closed ductus samples for our immunohistochemical studies. Al-
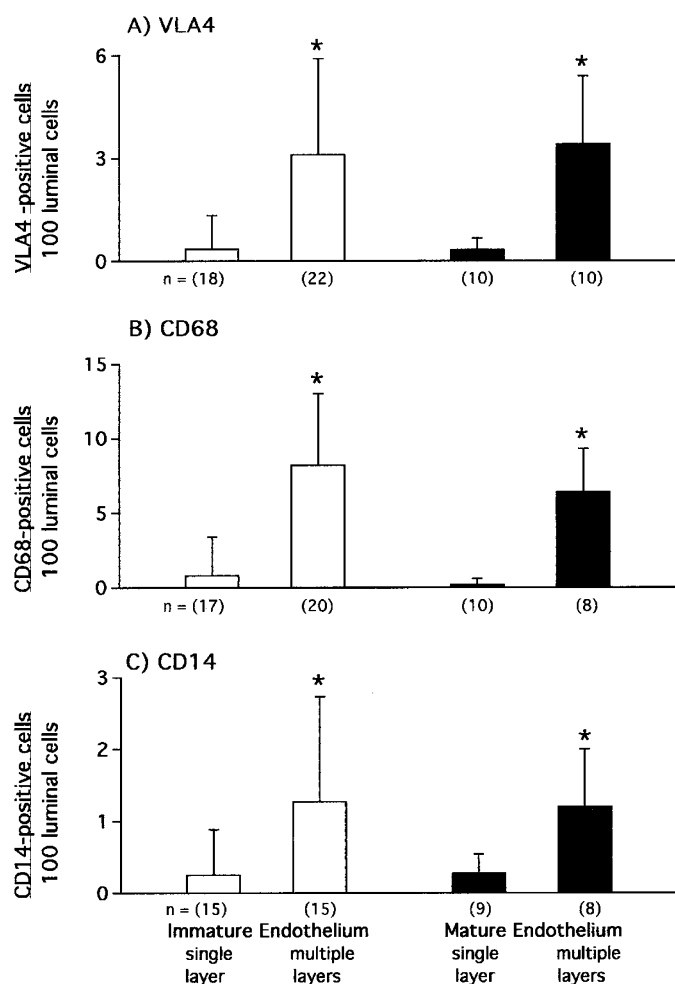
A) VLA4

B) CD68

C) CD14

Figure 6. Luminal endothelial accumulation is associated with mononuclear cell adhesion to the ductus lumen. Fetal and neonatal ductus from immature and mature baboons were categorized as having either single or multiple (three or more) layers of CD31$^+$ luminal endothelial cells. The vertical axis represents the number of mononuclear cells [expressing VLA4 (*A*), CD68 (*B*), or CD14 (*C*)] that adhere to the ductus lumen in each group and is expressed per 100 cells lining the ductus lumen. *$p < 0.05$ *vs* ductus with single layer of endothelial cells. *N*, number of ductus examined.

Figure 7. Fetal and newborn ductus from immature ($n = 37$) and mature ($n = 18$) baboons were examined for the presence of CD68$^+$ cells in the subendothelial zone. In 24/37 immature ductus and 8/18 mature ductus, a subendothelial zone of normal thickness ($\leq$20 μm) occupied $\geq$50% of the lumen's circumference (immature = 73 + 16% of luminal circumference, mature = 66 $\pm$ 8% of luminal circumference). In 34/37 immature ductus and 18/18 mature ductus, the subendothelial zone had regions where the thickness exceeded 20 μm (thickened subendothelial zones). When they were present, the thickened subendothelial zones encircled 48 $\pm$ 27% (immature) and 60 $\pm$ 28% (mature) of the luminal circumference. The vertical axis represents the number of subendothelial zones (expressed as percentage incidence) that contain CD68$^+$ cells. CD68$^+$ cells were present in 79% (immature) and 88% (mature) of the thickened subendothelial zones [number of CD68$^+$ cells/ thickened zone = 3.0 $\pm$ 2.5 (immature), 5.8 $\pm$ 4.9 (mature)]; the number of CD68$^+$ cells in the thickened zone correlated with the extent that the thickened subendothelial zone encircled the lumen (number of CD68$^+$ cells *vs* percentage of luminal circumference surrounded by thickened subendothelium: immature: $r = 0.68$, $p < 0.05$; mature: $r = 0.84$, $p < 0.05$). In contrast, when normal thickness ($\leq$20 μm) subendothelial zones (that encircled more than 50% of the luminal circumference) were examined for the presence of CD68$^+$ cells, CD68$^+$ cells were present in only 8% (immature) and 0% (mature) of the normal thickness zones. *$p < 0.05$ *vs* number of normal thickness subendothelial zones with CD68$^+$ cells. *N*, number of subendothelial zones examined.

though inhibition of prostaglandin and nitric oxide production could have altered the inflammatory response in the ductus (38,39), we believe the observed changes are related to ductus closure rather than prostaglandin or nitric oxide inhibition. The same inflammatory and remodeling changes are seen whether or not the ductus close spontaneously, close following prostaglandin inhibition by itself, or close after combined prostaglandin and nitric oxide inhibition. They occur only in the areas of the ductus that are tightly constricted. They are not apparent in the open ends of the ductus near the aortic and pulmonary junctions, nor are they observed in ductus that remain patent despite receiving the same pharmacological treatment protocol.

We hypothesize that the inflammatory response that follows postnatal constriction and loss of luminal flow may be responsible for ductus remodeling and permanent closure. Future studies that interrupt the inflammatory pathway will test this hypothesis more directly.
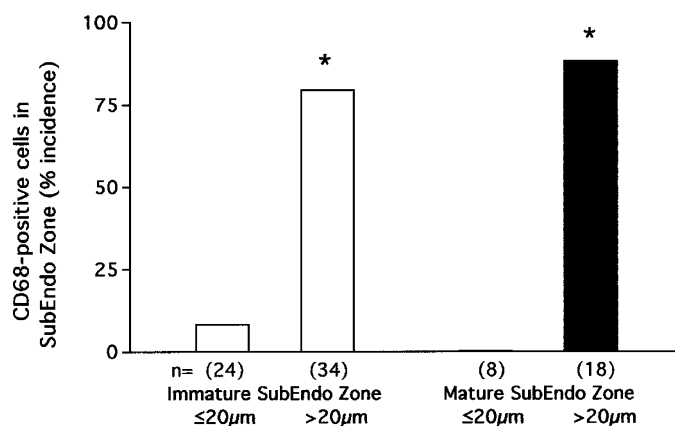
On the other hand, it is possible that the inflammatory response may have the opposite effects on permanent ductus closure if the ductus fails to constrict after birth. Although the expression of inflammatory cytokines appeared to be related primarily to the degree of ductus constriction, the postnatal expression of several of the cytokines was increased even in the presence of persistent ductus patency (Fig. 2). Several of these cytokines have potent vasodilatory activities (*e.g.* TNF-α, IL-6). After postnatal ductus constriction, ischemia of the ductus wall usually produces cell death and loss of ductus vasoreactivity (1,40). However, when the ductus fails to constrict, and remains patent after birth, it retains its responsiveness to vasoactive stimuli (41). In the presence of persistent ductus vasoreactivity, the postnatal induction of vasodilatory cytokines may play a role in preventing ductus closure after birth. Because these cytokines work through nonprostaglandin-mediated pathways (*e.g.* TNF-α, IL-6) (42–45), their presence may explain why indomethacin (which produces ductus constriction primarily by inhibiting prostaglandin production) becomes less effective in constricting the ductus with increasing postnatal age (46).
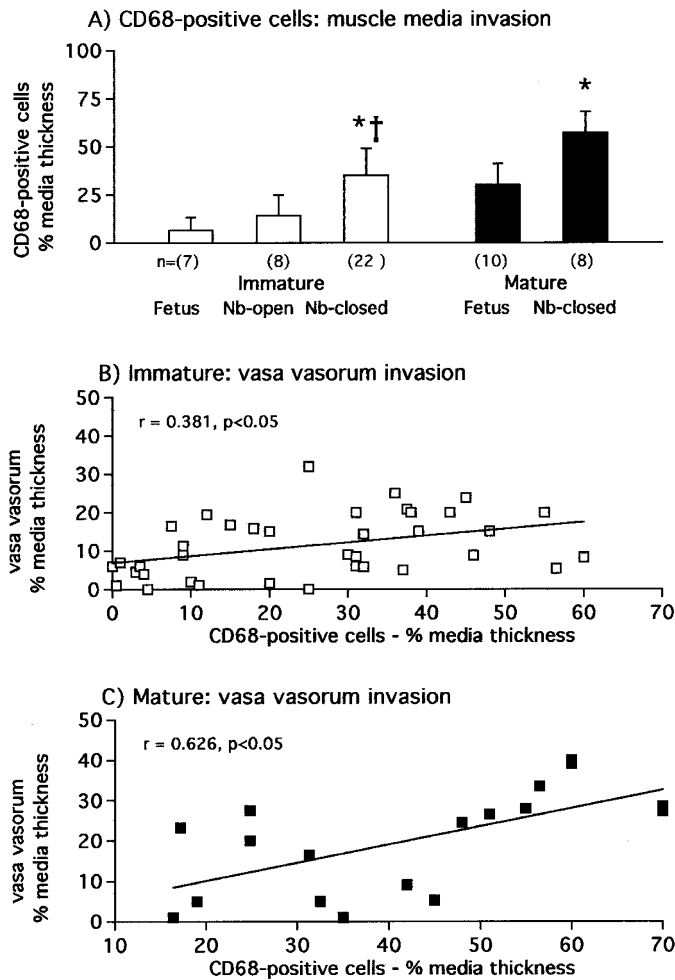
Figure 8. (*A*) Relationship between the degree of ductus constriction and invasion of the muscle media by CD68+ mononuclear cells [expressed as percentage of muscle media thickness containing CD68+ cells (see "Methods")]. *$p < 0.05$ *vs* fetal ductus; †$p < 0.05$ *vs* newborn-open. *N*, number of ductus examined. (*B* and *C*) Fetal and neonatal ductus were stained for CD68 and CD31. There was a significant relationship between the percent of muscle media containing CD68+ cells and the percentage of muscle media containing CD31+ vasa vasorum. (*B*) Immature: $r = 0.38$, $n = 37$, $p < 0.05$. (*C*) Mature: $r = 0.63$, $n = 18$, $p < 0.05$.

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