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Characterization of Resident B Cells of Vascular Walls in Human Atherosclerotic Patients

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Animal models of atherosclerosis suggest that B cells have contradictory protective or proatherogenic effects that are also subset and context dependent. To further understand the pathophysiology of human atheroma, we characterized local Ig production and functional properties of resident B cells in human arterial lesions. Ig repertoires were analyzed by RT-PCR in carotid endarterectomy samples. Cytokine, differentiation marker and transcription factor mRNA expression was studied on arterial wall lymphocytes isolated by laser capture microdissection. Ig sequence analysis revealed that individual samples each contained a limited number of B cell clones. Functional α and γ mRNAs made up the majority of H chain mRNAs in the adventitia. Clonal evolution of Ig V regions, expression of activation-induced cytidine deaminase, clonal H chain switch, and an inverted λ/κ ratio of Ig L chain usage indicated that a local differentiation process was taking place in arterial walls. Clonotypic markers revealed different plaque and adventitia Ig repertoires and a B cell recirculation between adventitia and draining lymph nodes. Microdissected mononuclear cells had an activated phenotype expressing IL-6, GM-CSF, and TNF-α, whereas IL-2, IL-4, IL-10, M-CSF, and IFN-γ were not detected. Advenitial oligoclonal resident B cells of atherosclerotic patients are mainly mature B2 (conventional) CD20− plasma blasts lacking markers of terminal differentiation to plasma cell (CD138 and Blimp-1). They present hallmarks of Ag-driven maturation and could act on inflammation and disease progression directly or by promoting polarization of other immune cells.


Atherosclerosis is characterized by a lipid-initiated chronic inflammation of vascular walls involving both innate and adaptive immune cells in the pathophysiologic process (1). Macrophages recruited in tunica intima play a crucial role during initial steps of atherosclerosis by uptaking modified lipoproteins [mainly oxidized low-density lipoproteins (OxLDL)], leading to foam cell formation, a hallmark of arterial lesions (2). Macrophages participate in inflammation by secreting cytokines, growth factors, and enzymes that increase plaque instability. The

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Abbreviations used in this article: AID, activation-induced cytidine deaminase; Be, effector B (lymphocyte); LCM, laser-capture microdissection; OxLDL, oxidized low-density lipoprotein; Treg, regulatory T cell.

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atherosclerosis in hyperlipidemic mice (18). Presence of protective IgG and IgA has been demonstrated in sera of laboratory animals immunized with OxLDL (19, 20). However, titers of anti-OxLDL IgG correlate well with atherosclerosis severity in mouse models (21–23), whereas findings in humans are controversial (24, 25). Two independent reports have demonstrated the proathero-
genomic role of B2 (conventional) lymphocytes (5, 6). This apparent discrepancy with earlier concepts is likely because spleenectomy selectively depletes B1a cells, whereas anti-CD20 Abs preferen-
tially target mature B2 lymphocytes (26, 27). Whether B2 cells exert their proatherogenic effects by reducing T lymphocyte ac-
tivation or in their own right is still an unresolved question. Re-
cently, the protective role of resident B lymphocytes in diseased arterial wall was shown to be subset and context dependent (28).

In this article, we have characterized Ig repertoires and func-
tional properties of arterial wall infiltrating B cells in advanced human atherosclerosis, with a special emphasis on the adventitial population. We demonstrate that most resident B lymphocytes expressed switched isotypes of Ig H chains (frequently and sometimes exclusively of the IgA type) that also featured hyper-
mutated V regions. B cell responses were oligoclonal with a bias for a recurrent use of some particular V region genes. Clonal diver-
sification of V regions by additional somatic mutations, clonal H chain switch, inversion of the λ/κ ratio of L chain usage, and activation-induced cytidine deaminase (AID) expression witnessed a skewed recruitment and an in situ maturation of arterial wall B cells. The resident B2-type lymphocytes were CD20<sup>-</sup> activated plasmablasts expressing IL-6, GM-CSF, and TNF-α.

Materials and Methods

Patients and surgery

Tissues from patients undergoing carotid endarterectomy were obtained from the Montpellier-Nîmes University Hospital. All patients gave written informed consent according to the university hospital ethical committee guidelines. Samples from a first group of 19 patients (7 women and 12 men, from 52 to 86 y old) were used for mRNA expression and laser-
capture microdissection (LCM) experiments. Specimens from a second group of 5 patients (1 woman and 4 men, from 66 to 77 y old) served exclusively for imaging purposes. The surgical procedure to ablate vascular lesions involved dissection though the tunica media. Therefore, the resulting tissue samples further termed “plaque” and “adventitia” contain, re-
spectively, the tunica intima and adventitia and in both cases a portion of the smooth muscle cell layer. When requested by the surgical protocol, one of the deep lateral cervical lymph nodes (n = 9) was removed and included in our analysis.

Laser-capture microdissection

Carotid endarterectomy samples (n = 6, patients 14 to 19 y old) were immediately extensively washed in PBS, immersed in RCL2 fixative (Alphelys, Plaisir, France), and embedded in paraffin, as previously de-
scribed (29). RCL2 is an alcohol based formalin-free fixative suitable for subsequent nucleic acid studies, but some difficulties may arise and cause discernible morphohistochecmic reactivity for certain Ags (30). As indicated by the manufacturer (www.alphelys.com/alph01/prod/dsc12/as/ rcl2 histórico.php) and in our hands, too, the use of anti-CD19 or anti-IgH Abs resulted in inconstant staining and precluded their validation as reliable reagents in this particular experimental setting. Tissue sections (of 7-
micron thickness) were affixed onto Superfrost glass slides, deparaffinized for 5 min in absolute ethanol at 60°C, and then stained by H&E and stained for 30 min containing 2% FCS, and stained with R&E or labeled for 30 min with fluorochrome-conjugated reagents. Whenever needed, Ag retrieval was performed, according to standard procedures, using citrate buffer. Rabbit F(ab’)<sub>2</sub> polyclonal anti-
human IgG/FITC, IgA/FITC, IgM/FITC (DakoCytomation F0315, F0316, F0317; final dilution 1/20), mouse IgG2A anti-human CD7/FITC (Beck-
man Coulter IM0585; final dilution 1/5), mouse IgG1 anti-human CD19/ APC–Alexa Fluor 750 (Beckman Coulter A94681; final dilution 1/10), or mouse IgG1 anti-human CD19/PE (Miltenyi Biotec 130-091-247; final dilution 1/10) were used for direct immunofluorescence. Specificity of the anti-lg conjugates was checked on a panel of monoclonal lymphoblastoid or neoplastic B cell lines and on normal PBMCs. These reagents were also devoid of background staining on the fibrous trabeculae of patients’ lymph nodes. After three washes in PBS, slides were counterstained with DAPI (300 nM) for 5 min, washed in PBS, and mounted in ProLong Gold antifade reagent (P36930; Invitrogen). Anti-CD3c (Conform, 2GV6; Ven-
tana), anti-CD20 (Conform, L26; Ventana), and anti-CD138 (B-A38, Ventana) mAbs were used as a first layer and immunostaining was then performed automatically with a DAKO Autostainer (Universal staining system; DakoCytomation, Trappes, France) using the streptavidin–per-
oxidase method.

RNA extraction

Portions of the plaque samples corresponding to intraplaque hemorrhage, thrombosis, or necrosis were eliminated to minimize contamination by nonresident cells. Before preparation of whole-tissue extracts, adventitia and plaque specimens (n = 13, patients 1 to 13) were thoroughly washed with PBS to eliminate blood cells and chopped into small pieces. Tissue fragments were resuspended in Tripure isolation reagent (Roche) and homogenized with a Polytron apparatus. Samples were then processed to extract RNAs according to the manufacturer’s protocol. RNAs were pre-
pared from microdissection samples using the QIAGEN QIAshredder system and RNeasy mini kits.

Reverse transcription

A total of 300 ng RNA (adventitias and plaques) or the totality of extracted RNAs (microdissections) were denaturated 5 min at 70°C and reverse transcribed in a total volume of 40 μl containing 2 μl 2–deoxynucleoside
5′-triphosphate mix (10 mM each), 8 μl 5× buffer, 4 μl 0.1M DTT, 1 μl oligo-dT primer (1 μg/μl), and 1 μl SuperScript III reverse transcriptase (Invitrogen) for 40 min at 37°C, 40 min at 40°C, and 40 min at 42°C. Finally, RT was heat inactivated for 15 min at 70°C.

PCR amplification

PCR was carried out with specific primers (see detailed list in Supplemental Fig. 1) and thermostable DNA polymerases (recombinant Taq polymerase [Invitrogen] or Ex Taq polymerase [Takara]). Reactions (50 μl) were ampliﬁed using the following protocol: one cycle of 94°C for 10 min; 26 (32 or 36) cycles of 94°C for 45 s, 55°C for 30 s, and 70°C for 1 min 50 s; and one cycle of 10 min at 72°C. In some instances, second-round PCRs were performed with specific Ig nested primers, using the same protocol with 26 cycles. All the primer pair combinations used in this study yield readily detectable amplification products in single-round PCRs (32 cycles) using total spleen cell cDNAs as templates. The RT-PCR system designed to characterize expressed Ig sequences is based on the use of 14 VH, 13 Vκ, and 15 Vλ upstream primers able to amplify almost full-length open reading frames belonging to all known V region families. Reverse primers were chosen sufﬁciently downstream to allow, when necessary, the unambiguous identiﬁcation of the classes and subclasses of expressed constant regions.

Cloning

PCR products were characterized on 2% agarose gels, puriﬁed as recom-
median by QIAquick and cloned into PCR II Topo vector (Invitrogen).
Ligation reactions were transfected to competent *Escherichia coli*, by heat shock at 42°C for 1 min, as recommended by the manufacturer. Transformed bacteria (100 μl) were plated onto Luria-Bertani Petri dishes containing 100 μg/ml ampicillin and 0.17 mg/ml X-Gal.

**Plasmid preparation and sequencing**

Plasmids were extracted from bacteria following an alkaline lysis protocol. Nucleic acid sequences were commercially purchased from MWG. Mutation analysis of the V region sequences were performed using the V Quest software of the International ImMunoGeneTics information system (IMGT, www.imgt.org; Ref. 31). Fully annotated nucleotide sequences of the adventitial Ig H and L chain cDNAs are available from the European Molecular Biology Laboratory database under accession numbers HF541880–HF541920 (www.ebi.ac.uk/embl/Access/index.html).

**Results**

**Lymphoid cells in arterial wall samples**

Plaque specimens were 3 cm long on average and consisted of most of the carotid artery circumference, which greatly differed in size for each patient. They often contained hemorrhagic, necrotic, and calcified areas. Adventitial tissue samples were more homogeneous upon macroscopic examination, had a crescent shape (5 mm length, 4 mm maximal width, and 2 mm thickness), and weighed 15–40 mg. Owing to the discontinuity and heterogeneity in inflammatory elements and fibrous content, total cell counts in specimens used for whole-tissue RNA extraction were almost impossible to achieve in intimal plaques and could be determined only indirectly in adventitial samples. For the latter, estimations made from representative H&E-stained slides used for LCM (3 patients) and DAPI-stained slides (5 patients) resulted in a range of 5–15 × 10³ nucleated cells per adventitial specimen. Cells with a lymphocyte or plasma cell morphology accounted for 10–30% (plaque specimens; nine patients) or for 30–70% (adventitial specimens; eight patients) of the total cell content. Intimal plaque immune cells were mainly found as diffuse infiltrates in the subendothelial region or in the fibrous cap. Adventitial lymphocytes were closely associated with the vasa vasorum and frequently present within the microvessel walls. In rare cases, intimal or adventitial lymphocytes were organized as small cell clusters containing up to 400 lymphoid cells (Fig. 1A). Immunofluorescence staining of plaque tissue sections by Fab’2 anti IgH (μ, γ, or α) reagents revealed bright cytoplasmic staining of occasional large cells pertaining to all three isotypic classes under study. In adventitial samples, the anti-α and anti-γ conjugates identified Ig-producing lymphoblasts mainly associated with the vasa vasorum walls (Fig. 1B). A similar staining pattern was obtained with the anti-CD19 reagent (Fig. 1B). In the five adventitial specimens studied by immunofluorescence, we found no IgM-producing cells.

**FIGURE 1.** Histology and immunostaining of arterial wall lymphoid infiltrates, Ig depositions, and LCM (representative experiments). (A) H&E staining of arterial wall tissue sections. 1, Plaque patient 22 10×; 2, Adventitias: 2a, patient 22 10×; 2b, patient 21 20×; 2c, patient 16 63×, A, adventitia; FC, fibrous cap; M, media; NC, necrotic core; NI, nodular infiltrate; VV, vasa vasorum. (B) CD19⁺ and IgA-producing lymphoblasts in the vicinity of adventitial vasa vasorum. Patient 21, upper row: DNA, CD19, and overlay magnification ×20; lower row: DNA, IgA, and overlay magnification ×40. Asterisks indicate vasa vasorum lumen. (C) Immunoglobulin depositions in arterial walls. Magnification ×10. 1, Plaques: 1a, patient 20; 1b, patient 22. 2, Adventitias: 2a, patient 21; 2b, patient 22. Anti-CD7 and anti-CD19 fluorochrome-coupled reagents do not stain tunica media under similar experimental conditions. (D) Examples of LCM of patient 15 (1: upper row) and patient 14 (2: lower row) adventitial diffuse lymphoid infiltrates at different cell densities. Before (a) and after (b) laser shot; captured cells in cap (c).
Depositions of IgM, IgG, and IgA were found mainly in tunica media in all samples under study (Fig. 1C). Most specimens were brightly stained by anti-IgA and anti-IgG reagents. Ig deposits had a lamellar organization, and counterstaining with DAPI nuclear dye indicated that there was no Ig-producing cell in this layer. Western blots confirmed that IgH chains were major components in the protein extracts of atherosclerotic arterial walls (not shown).

**B cells in arterial walls preferentially express IgA and IgG**

In the arterial walls of a first group of 13 patients, an overall assessment of H chain isotype expression was achieved by RT-PCR amplification of Ig transcripts from whole-tissue extracts using J and C region primers. All adventitias but one contained Ig mRNAs, whereas three plaques completely lacked Ig expression and two contained only trace amounts of Ig transcripts (Fig. 2). IgG and IgA mRNAs were concomitantly expressed in nine plaque specimens, together with IgM in eight cases, and a single sample contained only a minute amount of CH1 transcripts (Fig. 2). Five adventitias contained μ H chain transcripts, whereas α H chain mRNAs were present in all Ig-expressing specimens and were the sole IgH transcripts in three cases (Fig. 2). In many instances, the JH-Co amplification products appeared as doublets, owing to the difference in length between the α1 and α2 hinge regions, indicating that both isotypes were concomitantly produced. This finding was further confirmed by RT-PCR using a subclass specific primers and digestion of the amplification products with the PstI restriction enzyme that cleaves the CH1 α1 but not the CH1 α2 sequence (not shown). Nine adventitial specimens contained γ-chain transcripts (Fig. 2). Subclass-specific PCR primers identified the existence of γ1 transcripts in all of them, whereas γ2 mRNAs were found only in seven samples. Trace amounts of γ3 mRNAs were sometimes present, and there were no γ4 transcripts (not shown).

Only three adventitia and three plaque extracts, in each case, contained low amounts of δ H chain mRNAs and there were no functional ε H chain transcripts (Fig. 2). Taken together, in a majority of samples, the absence of μ and/or δ mRNAs ruled out a possible blood cell contamination, and the isotype distribution was evocative of some degree of selection among resident B lymphocytes. Further insights on IgH expression were obtained by analyzing 10 LCM samples from arterial tissues of a second group of six patients. Plaque and adventitia lymphocytes isolated by LCM all expressed at least one H chain isotype. Ig α- and γ-chain transcripts were indeed predominant, and only two specimens contained μ mRNAs (Fig. 2). Of interest, different H chain isotypes were expressed by two independent nodular cell aggregates from patient 16: μ-type transcripts were exclusively found in the adventitial one, whereas a mixture of α and γ mRNAs was present in the intimal one (Fig. 2).

**Atherosclerotic arterial walls are populated by low numbers of B cell clones**

We then examined the Ig VH and VL region repertoires of resident arterial B cells. Because IgH isotype production was rather homogeneous in intimal plaques, the samples under study were
chosen to be representative of the different H chain isotype distributions in adventitia (IgM + G + A, IgG + A, and IgA-only producers). With use of our RT-PCR amplification system of full-length VH regions, the total number of amplified bands detectable in each patient arterial sample ranged from 2 to 16 in adventitia and from 3 to 28 in plaque for the cumulated H chain isotypes (Fig. 3A). In a similar approach to assessing L chain repertoire of adventitial tissues, we found 0 to 7 bands per sample for k L chains and 2 to 10 bands per sample for l L chains (these numbers include consideration of some very weak bands) (Fig. 3B). For a given adventitia, numbers of H and L amplification products were generally in good correlation, with sometimes a slight excess of L chains that may be due either to a more efficient amplification of the shorter L chain mRNAs, to a certain degree of promiscuity in VL primers, or to the presence of non-functional transcripts (see below).

To assess their homogeneity, we cloned and sequenced a number of representative adventitial PCR products. The Ig H and L chain transcripts had an open reading frame throughout the entire V(D)J and constant regions (except for two independent l L chain mRNAs from patient 5 that contained a frameshift mutation in CDR3, resulting in the appearance of a premature stop codon in the C region) and therefore could encode functional proteins. Iterative sequencing of 18 adventitial VH PCR products yielded a single sequence in 15 cases and 2 different sequences in 3 samples. In all cases, the amplified sequences strictly corresponded to the VH

FIGURE 3. Arterial wall samples each contain a limited number of B cell clones. (A) Nested RT-PCR (32 and 26 cycles) using forward VH family–specific primers and reverse C\(\mu\), Cy, and C\(\alpha\) reverse primers. Templates are constituted by RNAs extracted from adventitias (left panel) and intimal plaques (right panel) representative of the different IgH isotype distributions in the outer arterial layer (2 in each group: IgM + G + A producers: patients 4 and 6, IgG + A producers: patients 5 and 9, IgA-only producers: patients 10 and 12). The same analysis performed on RNAs extracted from normal individual PBL is given for comparison. The presence of IgM and IgA transcripts in patient 5 samples is likely due to the better sensitivity of nested of VH-CH PCRs compared with the single-round JH-CH PCRs. (B) Analysis of L chain repertoire in adventitias by single-round RT-PCR (32 cycles) using forward V\(k\) or V\(\lambda\) family–specific primers and respective C\(k\) or C\(\lambda\) reverse primers. Patients are analyzed in the same order as in Fig. 3A.
(sub) family specified by the upstream oligonucleotide. A more limited evaluation of H chain PCR product complexity was achieved on randomly selected intimal VH3-CH bands from patient 4 and resulted in the retrieval of only a single sequence in each assay. Therefore, >80% of the VH amplification products were homogeneous, making the enumeration of DNA bands on agarose gels a fair estimation for the number of B cell clones actually present in the arterial wall samples. DNAs of 13 adventitial VL bands (4 κ and 9 λ) were also cloned and sequenced: 50% corresponded to a single sequence and 50% to two sequences. However, several duplicated sequences were explained by some promiscuity within VL oligonucleotides leading to the redundant amplification of certain L chain transcripts. In all adventitias but one (patient 5), there was a majority of Ig λ over Ig κ clones (overall ratio, 70.6% λ versus 29.4% κ). Taken together, these results indicate that each arterial wall sample contained a limited number of B cell clones.

Adventitial B cells preferentially express a limited set of hypermutated VH regions

When results from all patients under study were aggregated, the majority of expressed V regions belonged to the VH3, VH4, and VH5 gene families (Supplemental Fig. 2). Nevertheless, the observed frequencies were not significantly different from those expected from random gene usage. However, a preferential usage of a small number of specific individual family members accounted for the overrepresentation of VH3 sequences: The VH3-23 gene that is normally expressed by 6–7% of blood B cells (32) contributed to 30% of cumulated adventitial VH3 sequences and was present in the arterial wall samples of four of the six analyzed patients. VH3-66 was found in three instances and VH3-73 twice. Thus, more than one-half (58%) of the VH3-IgH sequences came from only three individual VH genes. Conversely, no particular DH or JH region appeared to be preferentially expressed.

Whereas only two μ H chain mRNAs contained unmodified VH, DH, and JH germline regions, all the other Ig transcripts featured extensive somatic modifications. In the γ, α, and one μ mRNAs, the number of mutations ranged from 7 to 39 per sequence (average, 21) for the sole VH portion. Somatic diversification was further extended by N and P insertions and numerous DH mutations (Supplemental Fig. 2). The VL regions were also targets for somatic mutations (7–21 per V region in κ-chains and 9–21 in functional λ-chains) (Supplemental Fig. 2).

Arterial wall B cells present hallmarks of local adaptive maturation and selection

Expression of AID was detected in three adventitias (whole-tissue extracts from patients 3 and 12 and some microdissected cells from patient 16) and two plaques (patients 4 and 6) (Fig. 4, Supplemental Fig. 3). Of note, in patient 16, AID was expressed in the adventitial diffuse cell infiltrate (sample 16b) but was not detected within B cell clusters producing IgM only (adventitial sample 16a) or IgG and IgA (plaque sample 16c). Transcripts resulting from a clonal H chain class switch event were evidenced in the tunica adventitia of patient 9: A fully identical VH-DH-JH sequence was present in the arterial wall samples of four of the six analyzed patients. VH3-66 was found in three instances and VH3-73 twice. Thus, more than one-half (58%) of the VH3-IgH sequences came from only three individual VH genes. Conversely, no particular DH or JH region appeared to be preferentially expressed.

FIGURE 4. Markers of cellular differentiation in microdissected lymphoid cells. Nested RT-PCR (32 and 26 cycles) performed on total RNAs obtained from (A) whole adventitial (ADV) or plaque (PLQ) tissue extracts and (B) microdissected lymphoid cells, using specific primer pairs. Reactions resulting in undetectable amplification product were further amplified up to 50 cycles in the secondary rounds and remained negative (not shown). CT, Control reaction in the absence of RNA template.
associated either with a Cy1 or with a Cy2 region (Supplemental Fig. 4). In the same sample, two clonally related IgA1 mRNAs featured a common CDR 3 sequence resulting from a VH3-23-DH2-15-JH4 rearrangement whose formation has necessitated the incorporation of 13 nontemplate nucleotides and 2 mutations in the D region. However, they were representative of two divergent subclones having accumulated independent mutations in FRs and CDR 1 and 2 regions (23 differences leading to 13 aa replacements; Supplemental Fig. 4). Two different IgA1 transcripts from patients 4 and 9 shared the same nucleotide sequence in their VH3-23 portion, including seven identical mutations in CDR2 and a germline CDR1. Yet their CDR3 regions were dissimilar, using two different DH regions (Supplemental Fig. 4). Within L chain transcripts of different patients, it was also possible, in two cases, to identify common CDR3s either at the DNA or at the protein level (Supplemental Fig. 4). In both instances, the V genes that contributed the final CDR3 sequences were different.

In a limited number of patients, using oligonucleotides specific to individual CDR3 sequences as clonotypic identifiers, we could design RT-PCRs that allowed detection of the traffic of certain lymphocyte clones between adventitia and available draining lymph nodes (Fig. 5). We were able to reclone, from patient 6 lymph node RNAs, a number of VDJ cDNAs corresponding to the 06_59A and 06_60A prototypes already identified in adventitia. Whereas all the 06_59A related sequences remained fully identical, members of the 06_60A family presented signs of ongoing clonal maturation (Supplemental Fig. 4). Of note, the inserted Arg60 in somatically diversified 06_60A family sequences is specified by different codons that may indicate a strong selective pressure for the maintenance of this mutation. Using this approach, we also found that plaque and adventitia repertoires were essentially different (Fig. 5 and data not shown), thus confirming the observed difference in H chain isotype expression.

Most adventitial B lymphocytes are representative of late stages of differentiation and express proinflammatory cytokines

Immunohistochemical staining of RCL2-fixed arterial wall tissue sections by anti-CD3 and anti-CD20 Abs revealed 0.5–2% and < 1% positive cells, respectively, in plaque samples and even lower percentages in adventitia, whereas no cell reacted with anti-CD138 in both arterial layers (data not shown). When compared with the pattern obtained on formalin-fixed tissues, anti-CD19 or anti-IgH staining of RCL2-treated specimens appeared rather inefficient, identifying only a small proportion of the potentially positive resident lymphocytes. In addition, for there was some uncertainty about arterial wall B lymphocyte surface CD marker detection by immunohistochemical techniques (11, 33, 34) and because anti-Ig reagent use could also identify non-B cells bearing Ig-loaded FcRs and was complicated by the widespread presence of Ig deposits, we decided to microdissect cells in lymphoid infiltrates by simply selecting them on the basis of morphological criteria. The LCM specimens (five adventitias and five plaques) corresponding to 100–2500 cell equivalents were surprisingly homogeneous in term of lineage marker expression: Samples all contained transcripts coding for Igs (Fig. 2) and the short isoform of CD79b, whereas CD3e, CD11b, or CD11c mRNAs were under the threshold of detection in our reactions (Fig. 4). This finding indicated a predominance of B lymphocytes within the LCM samples because T and monocytic cell lineage markers (CD3e and CD11b transcripts) could be detected in the arterial wallwhole-tissue extracts (Supplemental Fig. 3). CD20 transcripts were not detected in the captured cells (Fig. 4) in accordance with the occasional CD20 mRNA expression in adventitial total extracts (3 of 13; Supplemental Fig. 3). Moreover, this finding was confirmed by immunohistochemistry experiments showing a very low percentage of lymphocytes reacting with the anti-CD20 Ab in the corresponding samples (not shown). However, CD20 mRNAs were expressed in the total extracts of 7 of 13 plaque specimens (Supplemental Fig. 3), indicating that some sampling could be linked to the LCM protocol (see Discussion). CD138 mRNAs were not found in LCM lymphocytes, a confirmatory result of data obtained on total arterial wall samples showing that expression of this molecule was infrequent in atherosclerotic lesions (Fig. 4, Supplemental Fig. 3). CD5 and CD27 transcripts were also absent from microdissected mononuclear cells (Fig. 4), whereas they were likely contributed by other cell types in total arterial extracts (Supplemental Fig. 3). Finally, B cell differentiation reciprocal and antagonistic transcription factors BCL6 (B-cell lymphoma 6) and Blimp-1 (B lymphocyte–induced maturation protein 1) were not expressed by LCM cells (with one exception for the latter). In summary, adventitial B cells mostly express switched IgH isotypes with a hypermutated V region, and their phenotype is compatible with their classification as plasmablasts.

Within the complex array of cytokine transcripts present in whole-tissue extracts (Supplemental Fig. 3), LCM of arterial wall lymphocytes allowed identification of a more restricted pattern of expression. Several adventitial and plaque LCM samples that featured B cell–related mRNAs also contained transcripts coding for the proinflammatory cytokines IL-6, GM-CSF, and TNF-α (Fig. 6). Conversely, IL-1β, IL-2, IL-4, IL-10, IL-12, IFN-γ, MC SF, TGF-β, lymphotoxin A, and lymphotoxin B mRNAs were never detected within microdissected cells (Fig. 6 and data not shown).

**Discussion**

In atherosclerosis, lymphocyte infiltration in the adventitia has been known for a long time (35, 36), but description of its B cell component is more recent (7–13). Adoptive transfer experiments showed that vascular wall B lymphocytes constitutively home within arterial regions prone to lesion development (6, 7, 15, 28), indicating a high degree of specialization. However, many properties of these resident B cells remain to be described. Herein, we addressed the question of local Ig production for all H chain classes. Of interest, IgA-producing lymphocytes are an integral feature of the local immune response and are predominant in adventitia, where they are frequently associated with IgG-expressing cells, whereas IgM-producing cells are often missing. Of note, elevated levels of serum IgA and C4 were independently associated with severe atherosclerosis (corresponding to the clinical status of
patients studied herein), in multivariate analysis during a retrospective study, whereas serum IgG and IgM were not (37). IgA is definitely the dominant isotype produced by mammalian organisms but is mainly found in mucosal tissues; its presence in the arterial wall is more surprising. Whether IgA achieves, in this peculiar location, immune protection while avoiding deleterious inflammation and local tissue damage, as it does in the gut, remains to be determined. Both IgA1 and IgA2 are produced in inflammation and local tissue damage, as it does in the gut, remains to be determined. Both IgA1 and IgA2 are produced in arteriosclerosis lesions (40) and arterial wall IgM may be derived in part from local biosynthesis, as evidenced by metabolic labeling (41). By immunofluorescence, we have found deposits of the three main Ig classes mainly within arterial tunica media, whereas other studies in animal models have found Ig deposition with roughly the same localization (7) or in the acellular base of the lesion with roughly the same localization (7) or in the acellular base of the lesion with a reciprocal relationship to CD68⁺ macrophages (42). Other reports have demonstrated a higher Ig content within intimal plaque than in tunica media (40, 43). Besides technical issues related to the modes of Ig detection, variations in the anatomic localization of arterial specimens (abdominal aorta versus carotid), differences may also arise in relation to the evolutive stage of the disease because we observed, in a group of organ donors, that milder arterial lesions had less prominent Ig deposits in the media (not shown; M. Hamze, C. Desmetz, and P. Guglielmi, unpublished observations).

Diversity of Ig expression within the arterial wall has been evaluated only in humans but was differently appreciated. Walton et al. (10) found a polyclonal BCR repertoire in 24 of 25 cases of abdominal aneurysms. In contrast, Burioni et al. (44) demonstrated an oligoclonal expansion and evolution of local IgG1/k-producing intimal lymphocytes, interpreted as signs of Ag-driven maturation. Interestingly, VH3-23 and VH3-73 were the most represented VH genes in 4 of the 6 intimal plaques examined in this latter study, but their CDR3 regions generated by the recombination process differed from those we found in adventitialis. We show herein that low numbers of B cell clones populate the adventitial specimens. However, this conclusion needs to be balanced by taking in account the patchy nature of lymphocyte infiltration in arterial lesions. Moreover, because structure and inflammatory cell composition of the diseased arterial wall may differ according to anatomic location (15), extrapolation of results to a whole organism has to be made with caution. The question of clonality of resident T cells in atherosclerotic lesions has also been addressed, with contrasting results. Initial data pointed to a polyclonal origin for T cells in plaques (45, 46), but more recent studies demonstrated that resident T cells were locally activated by OxLDL and heat shock protein, using a restricted TCR repertoire (47, 48).

BCR sequence analysis indicated that only a minority of adventitial B cells used germline VDJ regions and could therefore be classified as innate-like B lymphocytes. Accordingly, LCM isolated B cells did not feature the CD5 and CD27 markers present, respectively, on B1a and human marginal zone subpopulations. By far, mutated VDJ sequences identified a great proportion of resident B cells as fully differentiated B2 lymphocytes expressing switched isotypes. However, many of these B cells were not yet committed toward a memory or plasma cell phenotype because they respectively lacked CD27 and CD138 and Blimp-1 expression. The absence of CD20 expression by adventitial B cells producing inflammatory cytokines may allow their classification as plasmablasts or early plasma cells (49).

Evidence of local differentiation of B lymphocytes gathered by the current study includes recurrent preferential use of certain VH genes, clonal diversification by additional somatic mutations, similarities in CDR sequences between independent B cells, clonal H chain switch, and inversion of the λ/κ ratio of L chain use. AID that mediates somatic hypermutation and H chain class switch is, at first glance, inconstantly present in arterial wall samples. However, this enzyme is normally expressed during limited steps of B cell differentiation, and its detection in samples that are snapshots of long-lasting lesions indicates that it likely plays a role in the natural history of the disease. Taken together, our results suggest the existence of an adaptive selection, and it is tempting to speculate that resident adventitial B lymphocyte differentiation is an Ag-driven process. Indeed, preliminary experiments in our laboratory with genetically engineered reconstructed adventitial Abs indicate that they react with discrete components of arterial wall cell extracts. It is interesting that a reconstructed Ab fragment, originally produced in atherosclerotic plaque, was shown to react with human transgelin type 1 expressed by arterial fibrobyte-like cells, and to cross-react with outer membrane proteins of Proteus mirabilis and Klebsiella pneumoniae (50).

B cells traffic regionally between adventitia and cervical lymph nodes. Despite limitations due to the human model (only a restricted portion of the arterial wall and a single lymph node can be examined per patient) and the low number of lymphocytes under study, clonally related B cells were easily found in different anatomic locations. It remains to be determined if B cells expressing isotypes other than IgA also circulate and if vascular wall lymphocytes can home in more distant lymphoid organs. In an individual arterial lesion, both CH and VH region expression differed between plaque and adventitia, confirming the limited inflammatory cell traffic observed between these compartments in mouse atherosclerosis (15).

Animal models have shown that B lymphocytes affect atherosclerosis physiopathology by different mechanisms. Natural
anti-OxLDL IgM produced by B1a cells attenuate, whereas B2 cells (directly or indirectly) promote atherosclerosis (5, 6, 26). However, adventitial B lymphocytes of young mice may be also early responders to atherogenic signals, limiting macrophage accumulation in intimal plaque through local production of anti-inflammatory cytokines (28). Results herein were obtained in aged patients having advanced end-stage lesions and suggest a different adaptation of B lymphocytes to this different context. Human arterial wall B cells produce GM-CSF, which is known to be a regulator of lesional dendritic cell accumulation and differentiation (51). GM-CSF is one of the general determinants of macrophage polarization toward the proinflammatory M1 (classical inflammatory macrophage) phenotype (52, 53) and is also clearly associated with IL-17–producing Th17 cell pathogenicity (54, 55). However, there is yet no unambiguous demonstration of an instrumental role for Th17 lymphocytes in atherosclerosis pathophysiology, and their involvement is still unclear. Resident B cells produce IL-6 and TNF-α. Elevated serum levels of these cytokines are generally found in patients having a poor prognosis or more severe arterial lesions (55, 56). Animal models designed to decipher the role of IL-6, TNF-α, and GM-CSF in atherosclerosis have not yet been fully conclusive because experimental settings (feeding diet, location of the lesions, and sex of the animals) largely determine the outcome of lesion evolution (57–64). In this study, we show that IL-6, TNF-α, and GM-CSF production is inconstant but characteristic of resident B cells, whereas the lack of expression of the other tested cytokines is a permanent feature. Whether the observed differences also reflect discrete stages of arterial lesion evolution remains to be determined. Indeed, different cell types can synthesize these cytokines, but IL-6–producing B cells were recently demonstrated to be major contributors to the pathogenesis of well-known Th1 cell–mediated autoimmune conditions (65). Finally, the pattern of cytokine production by resident B cells described in this article does not fit well with the classification of effector B (Be) lymphocytes (66) in Be1 B cells (producing the proinflammatory Th1 type cytokines IFN-γ, IL-2, and IL-12) and Be2 B cells (producing the Th2-type cytokines IL-4, IL-6, and IL-10). Rather, resident B lymphocytes have an activated phenotype close to the one observed after in vitro B cell combined stimulation of the CD40 and BCR pathways (67).

Data herein are indeed compatible with B cells being a major component of the adventitial lymphocyte population within the severe carotid lesions of many patients, in agreement with the observations indicating that B cell parietal infiltration increases with progression of atherosclerosis (11, 15). The tertiary lymphoid structures within those grafted hearts. B lymphocytes also contributed to the pathogenicity of atherosclerosis in apolipoprotein E-knockout mice. Arterioscler. Thromb. Vasc. Biol. 18: 375–380. Data herein are indeed compatible with B cells being a major component of the adventitial lymphocyte population within the severe carotid lesions of many patients, in agreement with the observations indicating that B cell parietal infiltration increases with progression of atherosclerosis (11, 15). The tertiary lymphoid structures within those grafted hearts. B lymphocytes also contributed to the pathogenicity of atherosclerosis in apolipoprotein E-knockout mice. Arterioscler. Thromb. Vasc. Biol. 18: 375–380.

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