

# Paracrine Regulation of Germinal Center B Cell Adhesion through the c-Met–Hepatocyte Growth Factor/Scatter Factor Pathway

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## Summary

T cell-dependent humoral immune responses are initiated by the activation of naive B cells in the T cell areas of the secondary lymphoid tissues. This primary B cell activation leads to migration of germinal center (GC) cell precursors into B cell follicles where they engage follicular dendritic cells (FDC) and T cells, and differentiate into memory B cells or plasma cells. Both B cell migration and interaction with FDC critically depend on integrin-mediated adhesion. To date, the physiological regulators of this adhesion were unknown. In the present report, we have identified the *c-met*-encoded receptor tyrosine kinase and its ligand, the growth and motility factor hepatocyte growth factor/scatter factor (HGF/SF), as a novel paracrine signaling pathway regulating B cell adhesion. We observed that c-Met is predominantly expressed on CD38<sup>+</sup>CD77<sup>+</sup> tonsillar B cells localized in the dark zone of the GC (centroblasts). On tonsil B cells, ligation of CD40 by CD40-ligand, induces a transient strong upregulation of expression of the c-Met tyrosine kinase. Stimulation of c-Met with HGF/SF leads to receptor phosphorylation and, in addition, to enhanced integrin-mediated adhesion of B cells to both VCAM-1 and fibronectin. Importantly, the c-Met ligand HGF/SF is produced at high levels by tonsillar stromal cells thus providing signals for the regulation of adhesion and migration within the lymphoid microenvironment.

Antigen-specific B cell differentiation, the process by which naive B cells develop into memory cells or plasma cells, requires multiple interactions of B cells with other cells, such as T cells and follicular dendritic cells (FDC)<sup>1</sup>, and with the extracellular matrix (ECM), that take place within distinct microenvironmental compartments of the lymphoid tissues (1–6). After their initial activation in the extrafollicular T cell (paracortical) area, germinal center (GC) founder cells migrate into B cell follicles where they initiate the formation of GCs (7, 8). Once in the germinal center (GC), the B cells first pass the dark zone where they undergo rapid clonal expansion and somatic hypermutation in their IgV genes (9–13). Mutated B cells then progress to centrocytes and move to the basal light zone of the GC. Here they reencounter antigen, presented as low levels of immune complexes on FDC, and undergo affinity selection

(14–16). Whereas low-affinity mutants and autoreactive mutants die by apoptosis, high-affinity mutants internalize antigen and process it on their migration pathway to the apical light and outer zones of the GC. In these areas, the affinity-selected B cells present antigen to antigen-specific GC T cells (17–19). Cognate T–B interaction results in expansion and Ig isotype switching of high-affinity B cells (20, 21), that mature into memory B cells or plasma cells and receive signals mediating their export from the lymphoid organ (1). Adhesion regulation, particularly regulation of lymphocyte integrin function, is believed to be fundamental to the control of cell migration and microenvironmental homing during this B cell differentiation process (22, 23).

Integrins are a widespread family of heterodimeric ( $\alpha\beta$ ) transmembrane glycoproteins that can function as cell–ECM and cell–cell adhesion receptors (for review see reference 24). In the immune system they are involved at multiple levels, including interaction of lymphoid precursors with stromal cells during lymphopoiesis, lymphocyte homing, and antigen presentation. Importantly, adhesion receptors of the integrin family have recently been implicated in B cell differentiation. Integrins, specifically  $\alpha 4\beta 1$ , were shown to be involved in adhesion and terminal differentiation of

<sup>1</sup>Abbreviations used in this paper: BCR, B cell antigen receptor; CD40L, CD40 ligand; ECM, extracellular matrix; FDC, follicular dendritic cells; GC, germinal center; HGF/SF, hepatocyte growth factor/scatter factor; PKC, protein kinase C; SAC, *Staphylococcus aureus* Cowans strain; VCAM-1, vascular cell adhesion molecule 1.

precursors during B-lymphopoiesis in the bone marrow in vitro (25, 26), and in B cell adhesion to FDC during GC reactions (27–29). In vivo experiments with  $\alpha 4$  null chimeric mice, confirmed a key role for this integrin in early B cell development (30). Together, these studies indicate that integrin mediated adhesion plays an important role in the control of several steps of B cell development, including migration and adhesion during antigen-specific differentiation. However, the physiological regulators of lymphocyte integrin activity during B cell differentiation remain unknown.

In a survey of the molecular pathways that might regulate B cell adhesion, we explored the possible role of the *c-met*-encoded receptor tyrosine kinase and its ligand hepatocyte growth factor/scatter factor (HGF/SF). The HGF/SF-*c-Met* pathway has been shown to regulate growth, motility and morphogenesis of epithelial and endothelial cells (31–36), which requires tight regulation of adhesion and de-adhesion. Furthermore, this pathway mediates invasion and migration of tumor cells (37–39), a process reminiscent of lymphocyte migration. Here, we identify the HGF/SF-*c-Met* pathway as a novel molecular pathway in antigen-specific B cell differentiation, which is involved in the regulation of integrin-mediated B cell adhesion.

## Materials and Methods

**Antibodies.** Mouse monoclonal antibodies used were anti-*c-Met*, DO24 (IgG2a) (Upstate Biotechnology, Lake Placid, NY); anti-CD38, OKT-10 (IgG1) (American Type Culture Collection [ATCC], Rockville, MD); FITC-conjugated anti-CD38, HIT2 (IgG1) (Caltag Laboratories, Burlingame, CA); biotin-conjugated anti-CD38, HIT2 (IgG1) (Caltag); anti- $\beta 1$  integrin (CD29), 4B4 (IgG1) (Coulter Immunology, Hialeah, FL); anti- $\alpha 4$  integrin (CD49d), HP2/1 (IgG1) (Immunotech, Marseille, France); anti- $\alpha 5$  integrin (CD49e), SAM-1 (IgG2b) (40) (a gift from A. Sonnenberg, NKI, Amsterdam, The Netherlands); anti- $\alpha 4\beta 7$ , Act-1 (IgG1) (41) (a gift from A. Lazarovits, University of Western Ontario, London, Canada); anti-ICAM-1 (CD54), RR1/1 (IgG1) (42) (a gift from T. Springer, Harvard University, Boston, MA); anti-HGF/SF, 24612.111 (IgG1) (R&D Systems, Abingdon, UK); anti-CD3, OKT-3 (IgG2a) (ATCC); anti-DRC-1, R4/23 (IgM) (DAKO, Glostrup, Denmark); anti-CD19, HD37 (IgG1) (DAKO); and anti-phosphotyrosine, PY-20 (IgG2b) (Affiniti, Nottingham, UK). Polyclonal antibodies used were rabbit anti-*c-Met*, C-12 (IgG) (Santa Cruz Biotechnology, Santa Cruz, CA); goat anti-HGF/SF (R&D Systems); FITC-conjugated rabbit anti-IgD (DAKO); RPE-conjugated goat anti-mouse (Southern Biotechnology, Birmingham, AL); AP-conjugated goat anti-mouse (total, IgG1, or IgG2a) (Southern Biotechnology); biotin-conjugated rabbit anti-mouse (DAKO); biotin-conjugated goat anti-mouse (DAKO); biotin-conjugated rabbit anti-goat (Vector Laboratories, Burlingame, CA); HRP-conjugated goat anti-rabbit (DAKO); and HRP-conjugated goat anti-rabbit (DAKO). In addition we used a rat monoclonal anti-CD77, 38.13 (IgM) (43) (provided by J. Wiels, Institute Gustave-Roussy, Villejuif, France); and RPE-Cy5-conjugated streptavidin (DAKO).

**Cell Lines.** The epidermoid carcinoma cell line A431, the lung fibroblast cell line MRC-5, and the B cell lines Raji, Namalwa, Daudi, Ramos, JY, and Nalm-6 were obtained from ATCC and

cultured in RPMI 1640 (Gibco BRL/Life Technologies, Paisley, UK) supplemented with 10% FCS (Integro, Zaandam, The Netherlands). The Burkitt's lymphoma line EB4B (44) was provided by R. Jefferis (University of Birmingham, Edgbaston, UK) and cultured in 10% FCS/RPMI 1640.

**B Cell Isolation and Culturing.** B cells were isolated as described previously (29). Total B cell fractions were >97% pure as determined by FACS<sup>®</sup> analysis.

B cells were cultured in Iscove's medium (Gibco BRL/Life Technologies) containing 10% FCS, 0.5% BSA, 50  $\mu$ g/ml human transferrin (Sigma, Bornem, Belgium) and 5  $\mu$ g/ml bovine pancreas insulin (Sigma). Some media were supplemented with 50 ng/ml phorbol-12-myristate-13-acetate (PMA; Sigma).

For CD40 ligation, B cells were cultured on irradiated (7,000 rad) CD40L-transfected or, as a control, wild-type L cells (45) (provided by J. Banchereau, Schering Plough, Dardilly, France). In specific experiments, culture media were supplemented with either pansomycin cells of *Staphylococcus aureus* strain Cowan I (0.002%; Calbiochem Novabiochem, La Jolla, CA), rabbit anti-human Ig-coated beads (2  $\mu$ g/ml) (BioRad Laboratories, Hercules, CA), recombinant human IL-2 (100 U/ml) (Eurocetus, Amsterdam, The Netherlands), recombinant human IL-4 (100 U/ml) (Genzyme Diagnostics, Cambridge, MA), or recombinant human IL-6 (1,000 U/ml) (CLB, Amsterdam, The Netherlands).

**T Cell Isolation and Culturing.** Tonsillar T cells were isolated as described for the B cell isolation, except that after the second Ficoll-Isopaque density gradient centrifugation the pellet was collected, washed, and resuspended in shock medium. The remaining B cells were removed by using a MACS magnetic cell separator (Miltenyi Biotec, Bergisch Gladbach, Germany) using anti-CD19. The T cell fraction was >98% pure as determined by FACS<sup>®</sup> analysis.

**Stromal Cell Isolation and Culturing.** Tonsillar stromal cells were isolated as described (46). The cells were cultured in 100-mm petri dishes (Costar, Cambridge, MA) containing 10% FCS/RPMI 1640. After 4 d nonadherent cells were removed.

**FDC Isolation and Culturing.** FDC were isolated as described (29). The cells were cultured in Iscove's medium containing 10% Fetal Clone I serum (HyClone Laboratories, Logan, UT). These FDC-enriched cell cultures contained 10–15% DRC-1-positive cells.

**Transfections.** *c-Met* transfected Namalwa cells (Nam<sup>met</sup>) were obtained by electroporating Namalwa cells with the eukaryotic expression plasmid pA71d containing full-length *c-met* cDNA (a gift from G. Hartmann and E. Gherardi, University of Cambridge, Cambridge, UK). After 2 d in culture, transfectants were selected in culture medium containing 250  $\mu$ g/ml hygromycin (Sigma). *c-Met* positive cells were sub-cloned by using a FACStar<sup>plus</sup><sup>®</sup> flow cytometer (Becton Dickinson, Mountain View, CA).

**Immunoprecipitation and Western Blot Analysis.** For analysis of tyrosine phosphorylation of the *c-Met* protein, cells were incubated overnight in serum-free RPMI 1640. Nam<sup>met</sup> or EB4B cells were incubated in serum-free RPMI 1640 in the presence or absence of 200 ng/ml HGF/SF (R&D Systems). After 5 min at 37°C the cells were solubilized in ice-cold 2 $\times$  lysis buffer containing 20 mM Tris-HCl (pH 8), 250 mM NaCl, 20% glycerol, 2% NP-40, 20  $\mu$ g/ml aprotinin (Sigma), 20  $\mu$ g/ml leupeptin (Sigma), 4 mM sodium orthovanadate (Sigma), 10 mM EDTA, and 10 mM NaF. After 1 h at 4°C the insoluble nuclear material was removed by centrifugation at 1  $\times$  10<sup>4</sup> g at 4°C for 20 min after which the supernatant was precleared with protein A-Sepharose CL-4B (Pharmacia Biotech) for 45 min at 4°C. *c-Met* was precipitated with rabbit anti-*c-Met* coupled to protein A-Sepharose at 4°C

for at least 2 h. The immune complexes were washed with lysis buffer and diluted in Laemmli sample buffer containing final concentrations of 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 100 mM 2-mercaptoethanol (BioRad Laboratories), and 0.001% bromophenol blue. After boiling for 5 min, the samples were subjected to 8% SDS-PAGE. Western blotting was performed as described previously (48).

For analysis of c-Met in total cell lysates, cells were lysed in 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% NP-40, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 1 mM sodium orthovanadate, 2 mM EDTA, and 5 mM NaF for 1 h at 4°C. After centrifugation at  $1 \times 10^4 g$  and 4°C for 20 min, the supernatant was diluted in Laemmli sample buffer, boiled for 5 min and subjected to 8% SDS-PAGE. Western blotting was performed as described previously (48).

**FACS<sup>®</sup> Analysis.** Expression of c-Met on tonsillar B cell subpopulations was studied using a triple staining technique (47). Staining was measured by using a FACSCalibur<sup>®</sup> flow cytometer (Becton Dickinson).

**Immunohistochemistry.** Expression of c-Met in tonsillar tissue was analysed by single and double staining. For single staining cryostat tonsil sections were fixed in acetone for 10 min, washed in PBS and preincubated with 10% normal goat serum (Sera Lab, Sussex, UK) in PBS for 15 min. After incubating with the primary antibody for 1 h, endogenous peroxidases were blocked with 0.1% NaN<sub>3</sub>, 0.3% H<sub>2</sub>O<sub>2</sub>, PBS for 10 min. Subsequently, the sections were stained with biotin-conjugated rabbit anti-mouse for 30 min, followed by an incubation with HRP-conjugated avidin-biotin complex for 30 min. Substrate was developed with 3,3'-amino-9-ethylcarbazole (Sigma) for ~10 min. Tissue sections were counterstained with Haematoxylin (Merck, Darmstadt, Germany).

Double staining was performed as described for the single staining, except that a cocktail of primary antibodies was used, which was detected by either a cocktail of AP-conjugated goat anti-mouse and HRP-conjugated goat anti-rabbit, or a cocktail of AP-conjugated goat anti-mouse IgG2a and HRP-conjugated goat anti-mouse IgG1. The second color was developed with Fast Blue BB (Sigma) for ~10 min.

**Adhesion Assays.** 96-well flat-bottom plates (Costar) were coated overnight with 5  $\mu$ g/ml human fibronectin (CLB) or 0.2  $\mu$ g/ml recombinant human sVCAM-1 (R&D Systems) at 4°C. After blocking the plates with 4% BSA, RPMI 1640 (2 h at 37°C), B cells that had been pre-incubated with HGF/SF (R&D Systems), in the presence or absence of monoclonal antibodies, for 30 min at 37°C, were added. Then, the plates were centrifuged (3 min 800 rpm, no brake) and incubated at 37°C for 25 min. After washing the wells, the bound cells were fixed with

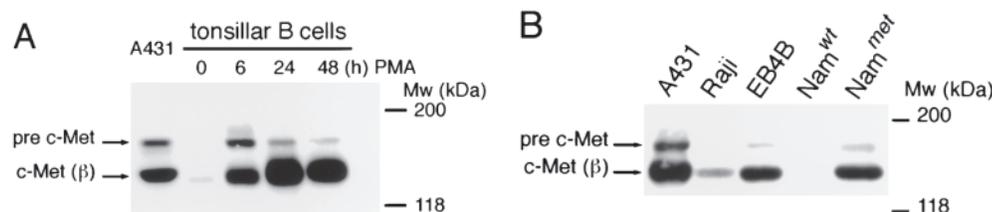
10% neutral buffered formalin solution (Sigma) and stained with Giemsa (Merck). Bound cells were quantified by using a color CCD camera (Sony) and NIH Image 1.60 software on an Apple Quadra 840AV.

**c-Met ELISA.** 96-well EIA/RIA plates (Costar) were coated overnight with mouse anti-HGF/SF immunoglobulins at 4°C. Then, the plates were washed and blocked with 4% BSA, PBS for 1 h at 37°C. Next, the wells were incubated with culture supernatants or with a HGF/SF concentration series for 2 h at 37°C, followed by an incubation with goat anti-c-Met immunoglobulins for 1 h at 37°C. Subsequently, the wells were incubated with biotin-conjugated rabbit anti-goat immunoglobulins for 60 min at 37°C followed by HRP-conjugated avidin-biotin complex (DAKO) for 1 h at 37°C. Substrate was developed with 1,2-phenylenediamine (Fluka Chemical, Buchs, Switzerland) in 50 mM KH<sub>2</sub>PO<sub>4</sub>, 50 mM Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O (pH 5.4) containing H<sub>2</sub>O<sub>2</sub>. The reaction was stopped with 1 N H<sub>2</sub>SO<sub>4</sub> and the results were analysed at 492 nm using a microplate reader (BioRad Laboratories).

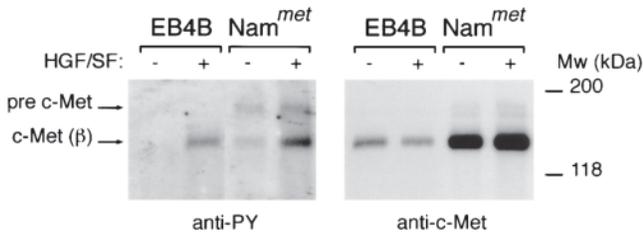
**RNA Isolation and RT-PCR.** Total RNA was isolated with RNAsol (Cinna/Biotex Laboratories, Houston, TX) according to manufacturers description. First-strand cDNA synthesis was performed on total RNA by a standard reverse transcription reaction, using Moloney leukemia virus reverse transcriptase (Gibco BRL/Life Technologies) and p(dN)<sub>6</sub> random hexamers (Pharmacia Biotech). PCR was performed with Taq DNA Polymerase (Gibco BRL/Life Technologies), 200  $\mu$ M dNTPs (Pharmacia Biotech) and 1.5 mM MgCl<sub>2</sub> in 1 $\times$  PCR Buffer (both Gibco BRL/Life Technologies). Primers used were HGF-1 (5'-CGACAGTGT-TTCCCTTCTCG-3') in combination with HGF-3 (5'-GGT-GGGTGCAGACACAC-3'), or 5' $\beta$ 2M (5'-ATCCAG CGTACT-CCAAAGATT-3') in combination with 3' $\beta$ 2M (5'-CATGTC-TCGATCCCACTTAAC-3'). PCR was started with a 5 min denaturation step at 95°C, after which amplification was performed in 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 1 min and elongation at 72°C for 2 min. After a final elongation step for 10 min at 72°C, samples were cooled on ice and analysed by electrophoresis in a 1.5% agarose TBE gel containing ethidium bromide.

## Results

*The c-Met Receptor Tyrosine Kinase Is Expressed by Activated Human Tonsillar B Cells as well as by Several B Cell Lines.* Expression of c-Met by human tonsillar B cells and by a panel of B cell lines was assessed by Western blotting and by FACS<sup>®</sup> analysis. On Western blot, c-Met expression



**Figure 1.** c-Met expression on human B cells. (A) PMA induces c-Met expression on tonsillar B cells. (B) Several B cell lines constitutively express c-Met. A431 (positive control) is an epidermoid carcinoma cell line. Raji, EB4B and Namalwa (Nam<sup>wt</sup>) are Burkitt's lymphoma cell lines. Nam<sup>met</sup> are c-Met-transfected Namalwa cells. In both A and B the Western blot of the cell lysates was stained with anti-c-Met. The c-Met precursor (*pre-c-Met*) and c-Met  $\beta$  chain (*c-Met* ( $\beta$ )) are indicated.



**Figure 2.** Tyrosine phosphorylation of c-Met on B cells in response to HGF/SF. c-Met on EB4B and Nam<sup>met</sup> B cells becomes phosphorylated on tyrosine residues upon triggering with HGF/SF. c-Met was precipitated with anti-c-Met antibodies and the Western blot was consecutively stained with anti-phosphotyrosine- or with anti-c-Met antibodies. The c-Met precursor (*pre-c-Met*) and c-Met  $\beta$  chain are indicated.

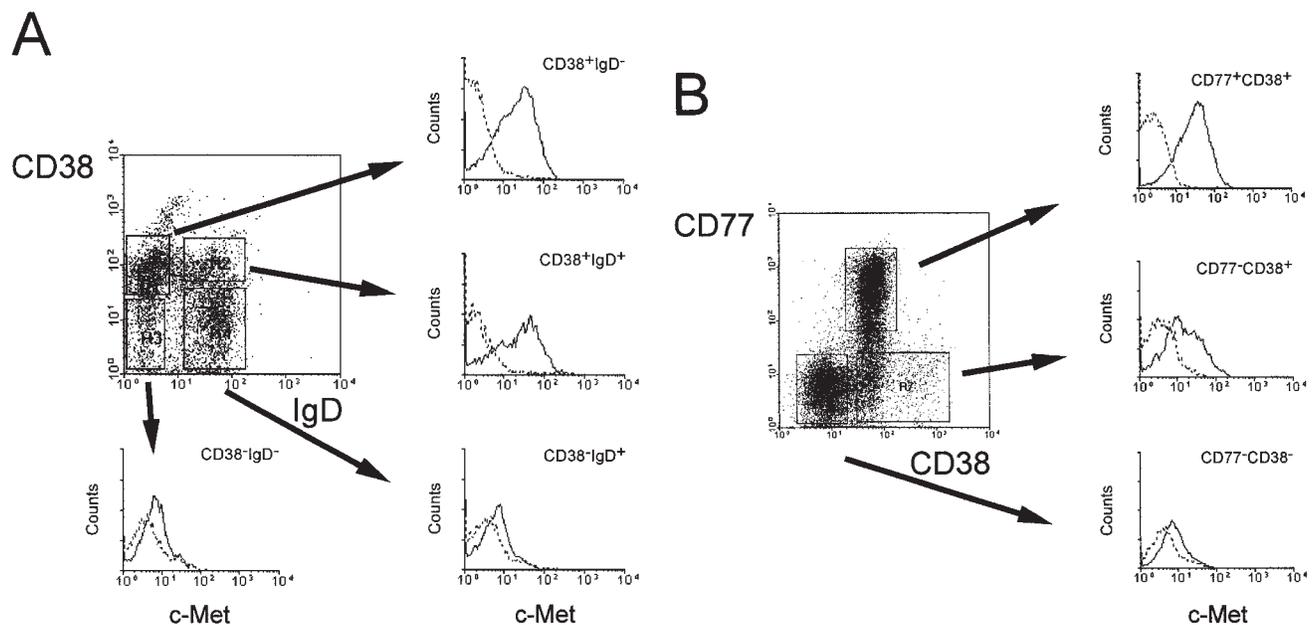
was hardly detectable in freshly isolated tonsillar B cells, but we observed a strong induction of c-Met (and the c-Met precursor [*pre-c-Met*]) upon stimulation with the phorbol-ester PMA (Fig. 1 A). Furthermore, constitutive expression of c-Met was found in the Burkitt's lymphoma cell lines Raji and EB4B, but not in the Burkitt's lymphoma cell line Namalwa (Fig. 1 B) nor in the B cell lines Daudi, Ramos, JY, or Nalm-6 (data not shown). As positive controls, c-Met expression of the epidermoid carcinoma cell line A431 and of Namalwa cells stably transfected with c-Met (Nam<sup>met</sup>) are shown (Fig. 1 B).

*The c-Met Receptor on B Cells Is Functional.* The above findings clearly show that B cells can express c-Met and, hence, might potentially be triggered via the HGF/SF-c-Met pathway. To demonstrate that the c-Met receptor on B

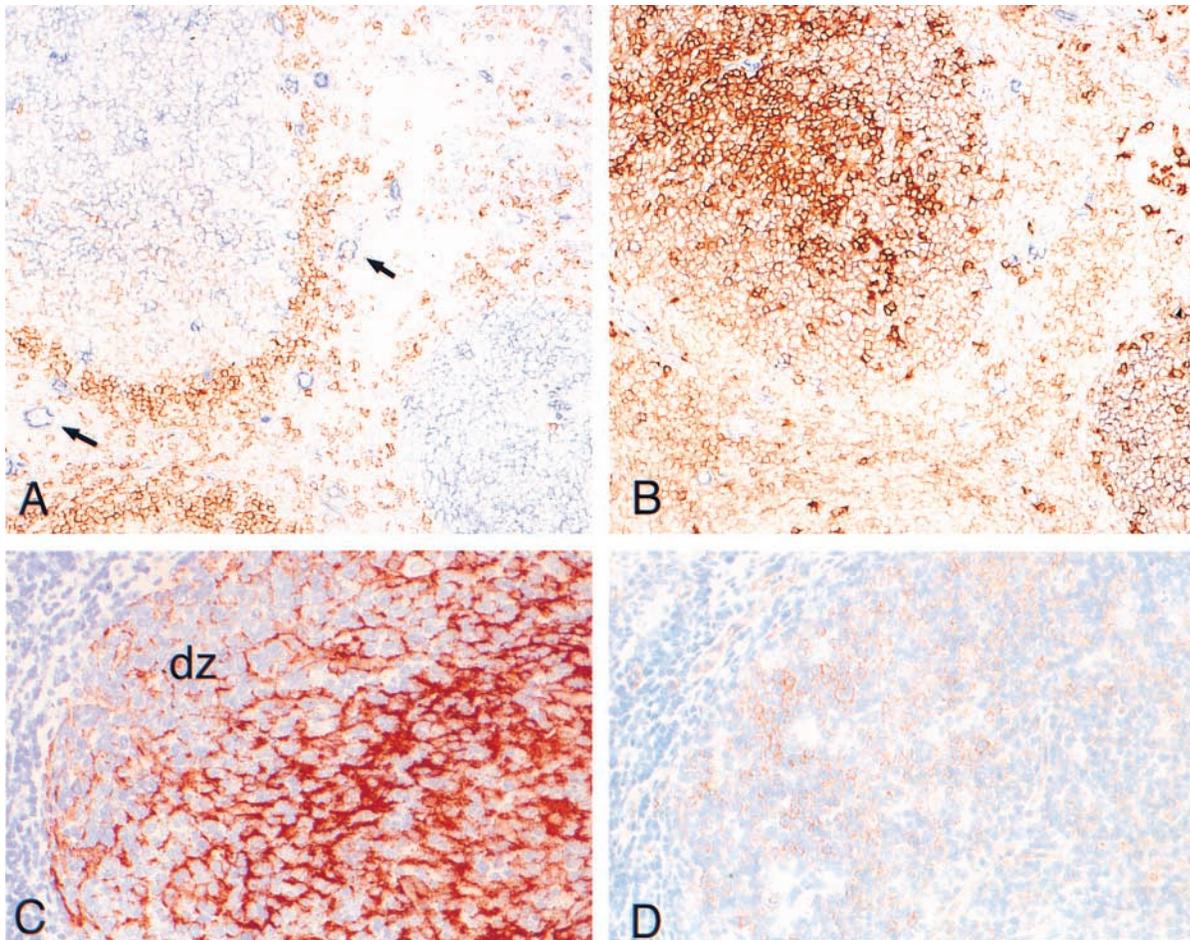
cells can indeed be functionally activated by HGF/SF, we studied c-Met receptor phosphorylation on tyrosine residues in response to HGF/SF. As is shown in Fig. 2, HGF/SF stimulation of EB4B cells as well as of Nam<sup>met</sup> B cells resulted in an enhanced tyrosine phosphorylation of c-Met. This indicates that the HGF/SF-c-Met pathway on B cells is capable of signaling.

*c-Met Receptor Expression on Human Tonsillar B Cell Subsets.* To investigate whether c-Met induction is a physiological phenomenon, that occurs also during antigen-specific B cell differentiation in vivo, we assessed the expression of c-Met on human tonsillar B cell subsets using FACS<sup>®</sup> triple staining. The subsets studied, recently defined by Pascual et al. (13), were: the naive B cell subset, IgD<sup>+</sup>CD38<sup>-</sup> (Bm1-2); two GC B cell subsets, IgD<sup>-</sup>CD38<sup>+</sup>CD77<sup>+</sup> centroblasts (Bm3), and IgD<sup>-</sup>CD38<sup>+</sup>CD77<sup>-</sup> centrocytes (Bm4); and an IgD<sup>-</sup>CD38<sup>-</sup> memory B cell subset (Bm5). Fig. 3 shows that c-Met is expressed by CD38<sup>+</sup>CD77<sup>+</sup> centroblasts (Bm3) and by a part of the CD38<sup>+</sup>CD77<sup>-</sup> subset. This finding is supported by immunohistochemical studies on frozen sections of human tonsillar tissue: as is shown in Fig. 4, c-Met is predominantly expressed by lymphocytes within the dark zone of the GC, which contains rapidly dividing centroblasts and low numbers of FDC. These results mean that c-Met induction in vivo, occurs in GC-cells at a pre-selection stage, i.e., cells that have recently been recruited by antigen plus antigen-specific T lymphocytes in the T cell-rich extrafollicular microenvironment.

*CD40 Ligation Induces a Transient Expression of c-Met on Tonsillar B Cells.* Ligation of the B cell antigen receptor (BCR) and CD40 plays a key role in the initiation of a T



**Figure 3.** Expression of c-Met on tonsillar B cell subsets. (A) Germinal center (CD38<sup>+</sup>) B cells express the c-Met tyrosine kinase, while naive (IgD<sup>+</sup>CD38<sup>-</sup>) and memory (IgD<sup>-</sup>CD38<sup>-</sup>) tonsillar B cells are c-Met negative. Tonsillar B cells were triple-stained with anti-CD38, anti-IgD, and either anti-c-Met (*solid line*) or control antibodies (*dotted line*). (B) c-Met is expressed on centroblasts (CD38<sup>+</sup>CD77<sup>+</sup>) and on a subset of CD38<sup>+</sup>CD77<sup>-</sup> GC cells. Tonsillar B cells were triple stained with anti-CD38, anti-CD77, and either anti-c-Met (*solid line*) or control antibodies (*dotted line*).



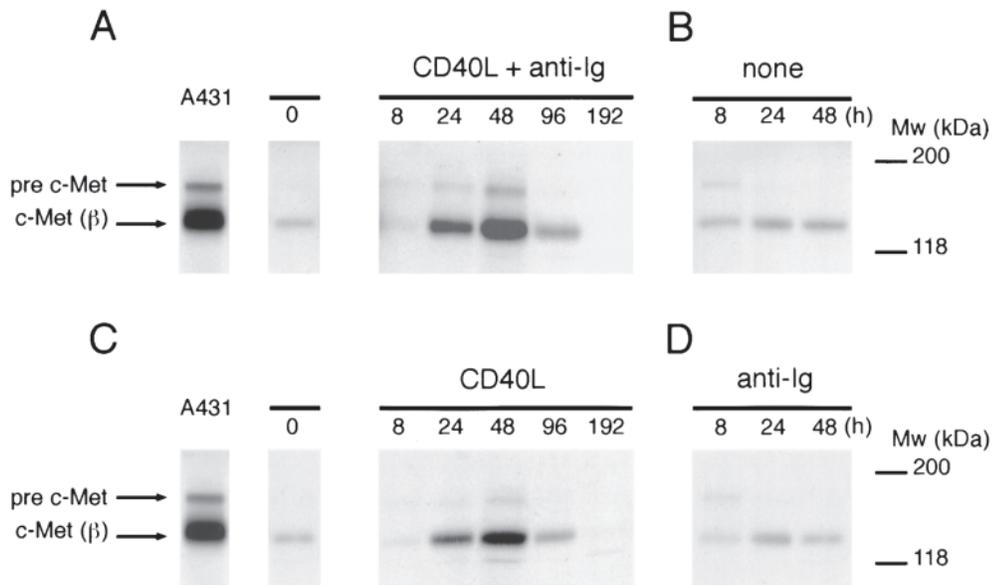
**Figure 4.** *c-Met* expression in the human tonsil. (A) Immunohistochemical double-staining for *c-Met* (blue) and IgD (red). *c-Met* is expressed by GC cells and by vascular endothelium (arrows). Prominent IgD expression is present on B cells of the mantle zones. (B) Serial section of A, stained for *c-Met* (blue) and CD38 (red). There are virtually no single *c-Met*-positive (blue) lymphocytes. Part of the GC cell show double staining (pink) (most clearly visible in the GC at the lower-right of the picture). A and B are not counterstained. (C) Immunohistochemical single-staining for DRC-1 (red) showing the FDC-network of the GC. The FDC poor area at the left handside represents the GC-darkzone (dz). (D) serial section of C, stained for *c-Met* (red). *c-Met*-positive cells are predominantly present in the GC-darkzone. C and D are counterstained with hematoxylin (blue).

cell dependent B cell response and initiates the GC reaction (1, 49–51). In view of the expression of *c-Met* on centroblasts, i.e., on recent GC immigrants, we hypothesized that these receptors might also regulate *c-Met* expression. To address this hypothesis, the biological conditions for B cell activation were mimicked in vitro. Tonsillar B cells were cultured on CD40 ligand (CD40L) transfected L cells or, as a control, on wild-type L cells, in the presence or absence of BCR stimuli (anti-Ig antibodies or *Staphylococcus aureus* Cowans strain I [SAC]). As is shown in Fig. 5 A, concurrent ligation of CD40 and the BCR induced a strong transient induction of *c-Met* in human tonsillar B cells, peaking at 48 h. Single triggering of CD40 also strongly induced *c-Met* (Fig. 5 C) but single ligation of the BCR did not induce *c-Met* expression above control levels (untransfected L cells and medium alone) (Fig. 5, B and D). In approximately half of the experiments, concurrent CD40 and BCR stimulation resulted in a *c-Met* induction that was stronger than after CD40 ligation alone, suggesting synergy

between the CD40 and BCR pathways. Stimulation by various cytokines including IL-2, IL-4, and IL-6 did not induce *c-Met* (data not shown).

These data clearly identify CD40–CD40L as a major pathway for induction of *c-Met* in B cells.

*HGF/SF Induces Integrin-mediated Adhesion of c-Met-positive B Cells to VCAM-1 and Fibronectin.* Cell motility and morphogenesis, major functions of the *c-Met*–HGF/SF pathway, are dependent on tightly controlled cell adhesion. This prompted us to study whether the *c-Met*–HGF/SF pathway might regulate B cell adhesion. Since *c-Met*-positive B cells represent a subset of tonsillar B cells that cannot be readily purified by negative selection procedures, we addressed this question by using Namalwa cells transfected with *c-met* cDNA (Nam<sup>met</sup>). The expression of *c-Met* in this B cell lymphoma line and the wild-type control (Nam<sup>wt</sup>) are shown in Fig. 1 B. We observed that HGF/SF induces a strongly augmented adhesion to both vascular cell adhesion molecule 1 (VCAM-1) and fibronectin of *c-met* trans-



**Figure 5.** Induction of *c-Met* expression on tonsillar B cells by CD40 and BCR ligation. *c-Met* expression by tonsillar B cells cultured on (A) CD40L-transfected L cells plus anti-Ig antibodies; (B) wild-type L cells; (C) CD40L-transfected L cells; or (D) wild-type L cells plus anti-Ig antibodies. Western blots of the cell lysates were stained with anti-*c-Met* antibodies. The *c-Met* precursor (*pre-c-Met*) and the *c-Met*  $\beta$  chain (*c-Met* ( $\beta$ )) are indicated. In the absence of CD40 stimulation (B and D), no viable B cells were recovered at 96 and 192 h.

fectured Namalwa B cells (*Nam<sup>met</sup>*) (Fig. 6, A and B). This effect of HGF/SF on B cell adhesion was dose dependent and was not observed upon stimulation of wild-type Namalwa cells (*Nam<sup>w</sup>*). An increased adhesion in response to HGF/SF to both VCAM-1 and fibronectin was also observed with the Burkitt's lymphoma cell lines Raji and EB4B (data not shown).

To identify the adhesion receptors on *Nam<sup>met</sup>* responsible for enhanced VCAM-1 and fibronectin binding, antibody blocking experiments were performed. *Nam<sup>met</sup>* expresses  $\alpha 4\beta 1$  and  $\alpha 4\beta 7$ , which both are receptors for VCAM-1 and for an alternatively spliced segment (CS-1) of fibronectin, but expresses no detectable level of the fibronectin receptor  $\alpha 5\beta 1$  (data not shown). We observed that adhesion to VCAM-1 and fibronectin was completely blocked by mAbs against both the  $\alpha 4$  and  $\beta 1$  integrin chain (Fig. 6, C and D). Since *c-Met* stimulation by HGF/SF did not lead to increased  $\alpha 4\beta 1$  expression (and also did not upregulate or induce  $\alpha 4\beta 7$  or  $\alpha 5\beta 1$ ) (data not shown), these results indicate that the *c-Met*-HGF/SF pathway enhances B cell adhesiveness through activation of the  $\alpha 4\beta 1$  integrin.

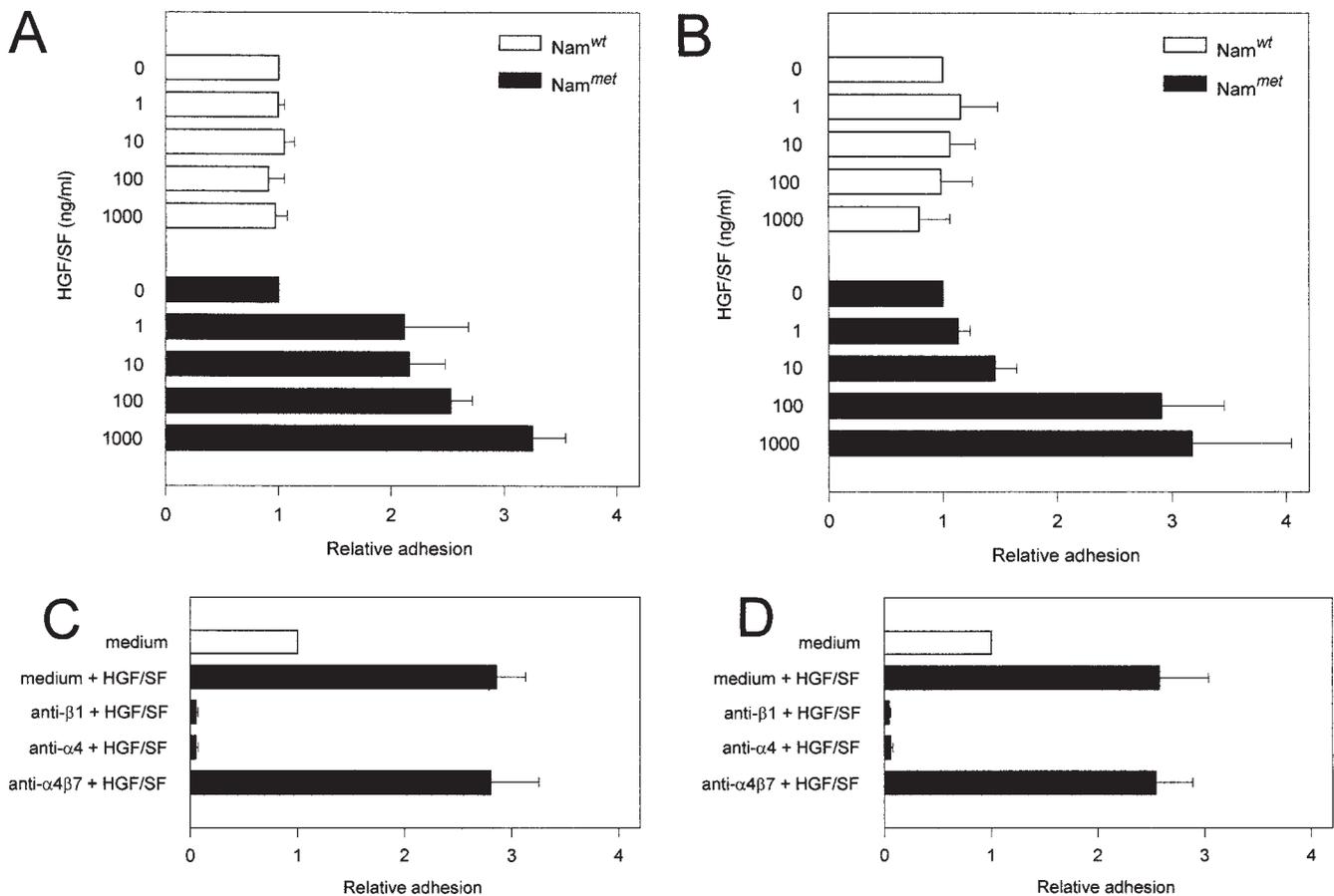
*Tonsillar Stromal Cells Produce High Levels of HGF/SF.* All together, the above data strongly favor a functional role of the *c-Met*-HGF/SF pathway in B cell differentiation, namely in the regulation of B cell adhesiveness. However, obviously, an *in vivo* biological role in this process would require the availability of HGF/SF within the lymphoid tissue microenvironment. This prompted us to (a) assay the production of HGF/SF by primary cultures of various tonsillar cell populations, and (b) study the expression of HGF/SF mRNA within these cell populations by RT-PCR. Determinations of HGF/SF production by ELISA demonstrate that high levels of HGF/SF are produced by primary cultures of tonsillar stromal cells, including cultures of FDC-enriched tonsillar cell subfractions (Fig. 7 A). By contrast, tonsillar T- or B-lymphocytes, cultured in the presence or absence of various mitogenic stimuli, did not

produce detectable levels of HGF/SF. Consistent with these results, in RT-PCR studies HGF/SF mRNA was exclusively detectable in tonsillar stromal cells (Fig. 7 B).

## Discussion

The products of proto-oncogenes are important regulatory molecules that exert a wide range of effects on basic cellular functions such as the control of cell growth and differentiation. The *c-met* proto-oncogene product is a receptor tyrosine kinase (52, 53) that binds HGF/SF, a mesenchymally derived cytokine with pleiotropic biological effects on proliferation, cell motility and morphogenesis of epithelial, endothelial, and myogenic cells (31–36, 54). More recently, the *c-Met*-HGF/SF pathway has also been implicated in the proliferation and differentiation of early hematopoietic progenitor cells (55–58) and in monocyte-macrophage differentiation (59). Here, we demonstrate that the *c-Met*-HGF/SF pathway is also operative during T cell-dependent B cell differentiation, where it is involved in the control of lymphocyte integrin function on B cells, a process regulating adhesion and homing of B cells within the lymphoid microenvironment.

We observed that stimulation of tonsillar B cells with phorbol ester PMA leads to a rapid *c-Met* induction (Fig. 1 A). Induction of *c-Met* expression upon protein kinase C (PKC) stimulation by phorbol ester has previously also been reported in epithelial cell lines (60). In addition, we observed constitutive expression of *c-Met* on several B cell lymphoma lines (Fig. 1 B). The *c-Met* receptor on these B cell lines is signaling competent, as triggering by HGF/SF resulted in enhanced tyrosine phosphorylation of *c-Met* (Fig. 2). These findings present the first direct evidence for expression of a functional *c-Met* receptor on B lymphocytes. Indirect evidence for a role of *c-Met* in B cells has previously been provided by Delaney et al. (61), who demonstrated that HGF/SF enhances immunoglobulin produc-



**Figure 6.** The HGF/SF-c-Met pathway regulates  $\alpha 4\beta 1$  integrin-mediated adhesion to sVCAM-1 and fibronectin. (A) Effect of HGF/SF on the binding of c-Met transfected ( $Nam^{met}$ ) and control ( $Nam^{wt}$ ) B cells to sVCAM-1. (B) Effect of HGF/SF on the binding of  $Nam^{met}$  and  $Nam^{wt}$  B cells to fibronectin. (C) Effect of anti- $\beta 1$  (4B4), anti- $\alpha 4$  (HP2/1), and anti- $\alpha 4\beta 7$  (Act-1) integrin antibodies on the binding of  $Nam^{met}$  B cells to sVCAM-1. (D) Effect of anti- $\beta 1$  (4B4), anti- $\alpha 4$  (HP2/1), and anti- $\alpha 4\beta 7$  (Act-1) integrin antibodies on the binding of  $Nam^{met}$  B cells to fibronectin. Cells were pre-incubated with HGF/SF in the presence or absence of anti-integrin monoclonal antibodies. The results are expressed as relative (compared with the control cells not incubated with HGF/SF) adhesion. Error bars represent the standard deviation of triplicate wells.

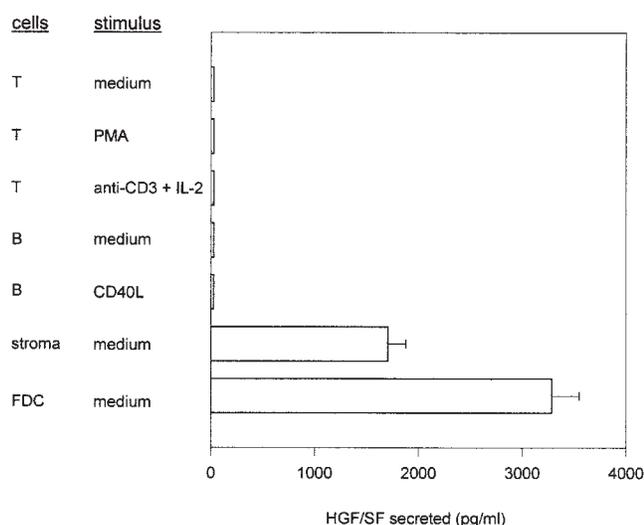
tion by murine B cells. However, as c-Met expression was not studied and whole splenocyte cultures were used, indirect effects of HGF/SF were not ruled out. For T cells, Shaw and colleagues reported that HGF/SF stimulated the adhesion and migration of the memory subset. However, these target cells appeared not to express c-Met (62).

One of the key findings of our study is that concurrent CD40 and BCR ligation induces a strong transient expression of c-Met on B cells in vitro (Fig. 5). Presumably, BCR and CD40 mediated signals are also instrumental in the physiological induction of c-Met. This is suggested by the fact that c-Met is expressed in vivo on a subset of tonsillar centroblasts ( $CD38^+CD77^+$ ) (Figs. 3 and 4). Centroblasts are the offspring of B cells that have recently been activated at extrafollicular sites by antigen plus accessory signals provided by antigen-specific T cells (1). These signals critically involve CD40/CD40L interactions: patients with x-linked hyper-IgM syndrome (due to mutated and consequently defective CD40L) do not develop GC and blocking of the CD40/CD40L pathway in mice leads to complete inhibition of GC reactions (49–51, 63, 64). Our results strongly

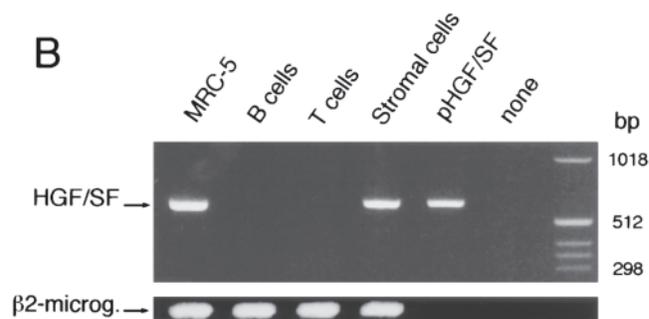
suggest that c-Met induction is directly linked to the initiation of the B cell immune response. Indeed, we observed that dual ligation of CD40 and the BCR also induces c-Met on naive ( $IgD^+CD38^-$ ) B cells (our own unpublished observation).

As both the migratory and morphogenic responses to HGF/SF are critically dependent on cell adhesion, could regulation of the c-Met-HGF/SF pathway also have a regulatory role in B cell adhesion? This idea is supported by the finding that HGF/SF augments adhesion of c-Met transfected Namalwa B cells as well as the c-Met expressing B cell lines Raji and EB4B to VCAM-1 and fibronectin (Fig. 6 and data not shown). This HGF/SF induced adhesion was mediated through activation of the integrin  $\alpha 4\beta 1$ . Previous studies from our own and from other laboratories have shown an important role for  $\alpha 4\beta 1$  in GC formation (27–29). In the GC,  $\alpha 4\beta 1$  mediates B cell adhesion to VCAM-1 on FDC, an interaction that regulates the formation of the microenvironment required for the affinity selection of GC B cells. Apart from establishing physical contact between B cells and FDC,  $\alpha 4\beta 1$  presumably contributes directly to

**A**



**B**



**Figure 7.** Expression of HGF/SF protein and mRNA by tonsillar stromal cells and lymphocytes. (A) HGF/SF secretion by cultured T cell, B cells, stromal cells, and FDC enriched tonsillar cells. ELISAs were performed to determine HGF/SF concentrations in the culture media (lower limit of detection 400 pg/ml). T and B cells were stimulated as indicated. (B) Expression of HGF/SF mRNA in T cells, B cells, tonsillar stromal cells, and, as a positive control, in the lung fibroblast cell line MRC-5. The RT-PCR was performed on total RNA, a plasmid containing full-length human HGF/SF cDNA (pHGF/SF), or on water. Primers used were HGF/SF-specific or, as a control,  $\beta$ 2-microglobulin specific.

the B cell selection process itself, as signaling through the  $\alpha$ 4 $\beta$ 1-VCAM-1 pathway costimulates rescue of GC B cells from apoptosis (6, 29, 47). Furthermore,  $\alpha$ 4 $\beta$ 1 also regulates cell adhesion to fibronectin (65), an important substrate for cell migration.

The above data strongly favor a functional role of the c-Met-HGF/SF pathway in B cell differentiation, namely in the regulation of B cell adhesiveness. Obviously, however, an *in vivo* biological role in this process requires the availability of HGF/SF within the lymphoid microenvironment. Interestingly, we indeed observed production of high levels of HGF/SF as well as expression of HGF/SF mRNA by tonsillar stromal cells. In contrast, tonsillar T- or B-lymphocytes were HGF/SF negative (Fig. 7).

Adhesion regulation is believed to be fundamental to the control of cell migration and microenvironmental homing during lymphocyte differentiation. This migration and homing within tissues, like recruitment from the blood, presumably is determined by an organized display of adhesive ligands and regulatory factors, specific for a given microenvironment (23). Thusfar, most studies on the regulation of integrin-mediated adhesion have focussed on cytokines of the chemokine family (66). Chemokines, which bind to G protein linked 7-transmembrane serpentine receptors (67, 68), have been shown to mediate chemotaxis and rapid functional activation of leukocyte integrins on myeloid cells, mac-

rophages, and lymphocytes (69–73). HGF/SF belongs to the family of plasminogen-related growth factors (74), that also includes macrophage stimulating protein. These molecules, that are structurally unrelated to the chemokines, have the basic domain organization and mechanism of activation of the blood proteinase plasminogen, i.e., they are characterized by the presence of a kringle domain(s), an activation domain, and a serine proteinase domain. Our present data strongly support a physiological role of HGF/SF in the regulation of B cell adhesiveness, microenvironmental homing and in the morphogenesis of the GC. Local production of HGF/SF has been demonstrated surrounding blood vessels in inflammation (75, 76), and in this paper we demonstrate that HGF/SF is produced by FDC-enriched cell populations. In view of the pleiotropic effects of HGF/SF on other cell types, HGF/SF may have additional, as yet unknown, roles in antigen-specific B cell differentiation. In particular, cross-talk between integrins and c-Met-signaling pathways triggered by FDC might contribute to B cell survival (6, 29, 47). Interestingly, like the chemokines, HGF/SF has a high affinity for heparin, which is present on cell surfaces and in the ECM in the form of heparan sulphate proteoglycans. This heparan sulphate binding limits diffusion, thus allowing the development of chemotactic gradients and the localization of proadhesive activity to the appropriate lymphoid microenvironment (77).

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