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This study investigates the mechanism(s) underlying the immunoregulatory activities of placenta-derived human amnion epithelial cells (hAEC). The working hypothesis is that NAD\(^+\) and ATP, along with ectoenzymes involved in their metabolism, play a significant role in hAEC-mediated immune regulation. Proof of principle of the hypothesis was obtained by analyzing the interactions between hAEC and the main human leukocyte populations. The results obtained indicate that hAEC constitutively express a unique combination of functional ectoenzymes, driving the production of adenosine (ADO) via canonical (CD39, CD73) and alternative (CD38, CD203a/PC-1, CD73) pathways. Further, the picture is completed by the observation that hAEC express A1, A2a, and A2b ADO receptors as well as ADO deaminase, the enzyme involved in ADO catabolism. The contribution of the purinergic mediator to immunomodulation was confirmed by exposing in vitro different immune effector cells to the action of primary hAECs. B cells showed an enhanced proliferation and diminished spontaneous apoptosis when in contact with hAEC. T cell proliferation was partially inhibited by hAEC through ADO production, as confirmed by using specific ectoenzyme inhibitors. Further, hAEC induced an expansion of both T and B regulatory cells. Last, hAEC inhibited NK cell proliferation. However, the involvement of ADO-producing ectoenzymes is less apparent in this context. In conclusion, hAEC exert different in vitro immunoregulatory effects, per se, as a result of interactions with different populations of immune effector cells. These results support the view that hAEC are instrumental for regenerative medicine as well as in therapeutic applications for immune-related diseases.

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Stem cells have gained acceptance in cell-based therapies, and their “off-the-shelf” availability has been recognized as being essential for the emerging field of regenerative medicine. The placental membrane in direct contact with the fetus is a tissue of fetal origin (amnion) and has been used in a variety of disorders (1, 2). Amnion tissue consists of cuboidal epithelial cells firmly adherent to a thick basal membrane and an abundant extracellular matrix with interspersed fibroblast-like cells. To our knowledge, our group was the first to identify and describe the nature of stem cells of human amnion epithelial cells (hAEC) (3). We developed efficient techniques to isolate hAEC with epithelial characteristics and a heterogeneous expression of membrane surface Ags (4). The hAEC phenotype includes classic epithelial adhesion molecules, such as EpCAM (CD325) and integrin subunits (CD29 and CD49f). On the contrary, markers routinely used to define and identify stromal cells are not detectable (4, 5). The surface markers commonly observed on embryonic stem cells (e.g., globoseries glycolipids SSEA-3 and -4 and keratan sulfate-associated Ags TRA 1-60 and 1-81) are consistently expressed, perhaps contributing to their multilineage differentiation potential (1, 3, 6). Unlike pluripotent cells, hAEC are not immortal; they do not express telomerase and maintain a normal karyotype, without resulting in tumor formation upon transplantation (3, 7). hAEC have shown preclinical and clinical efficacy (1, 8). Over the past years, hAEC have been suggested as a useful cell type for the treatment of different life-threatening models of inborn error of diseases (9–11). The results on transplants confirmed that hAEC are capable of a functional maturation into hepatic cells in addition to their characteristic immune-modulatory and anti-inflammatory properties (12). In fact, the same cells maintain immunosuppressive properties in animal models of autoimmune/inflammatory disease (13–15). Their safety and the absence of immune rejection were confirmed after transplantation in immune-competent mouse models as well as in human volunteers and patients (16–18).
Materials and Methods

hAEC generation and culture

Discarded placentae from uncomplicated cesarean resections at 37–42 wk of gestational age were obtained from Karolinska University Hospital (Stockholm, Sweden) after the approval by the Institutional Review Board. Placental hAEC were isolated as previously described (4). Briefly, amnion membrane was surgically removed from the surface of the placenta and washed to remove blood cells. TrypLE (Life Technologies) digestion (30 min) was specifically performed to release epithelial cells without altering cell surface Ags, as previously described (3, 4). Viability after isolation ranged from 90 to 97%, whereas after cryopreservation it ranged from 85 to 94%. hAEC were seeded on Gelatin 0.2% and maintained for few days (7). Standard culture media consisted of DMEM supplemented with 10% FBS, 1 mM nonessential amino acids, 4 mM L-glutamine, 55 μM 2-ME (all from Sigma-Aldrich), and antimycotic/antibacterial. Mismatches in HLA expression are recognized by immune cells and are generally followed by rejection. The characteristic expression of noncanonical HLA-Ib (i.e., HLA-G) was proposed as a possible explanation for long-term engraftment and survival of hAEC in allogenic or xenogenic settings (12). Among immunosuppressive molecules, adenosine (ADO) has been recently identified to play an important role in different physiological and pathological settings. ADO is produced from the catabolism of mono- and di-nucleotides (ATP and NAD\(^+\)) and from their byproducts (ADP, AMP ribose [ADPR], and AMP). The canonical pathway of ADO production starts from CD39, an ecto-nucleoside triphosphate diphosphohydrolase that converts ATP to ADP and then into AMP. The latter molecule is then converted to ADO by the 5'-nucleotidase CD73 (19). CD203a/PC-1 was recently identified as a hinge ectoenzyme for its ability to convert both ADP and ADPR to ADO (20). Expression of ADORA on NK cells inhibits cytotoxicity and IFN-γ release (23, 26, 27), whereas engagement of ADORA and ADORA2b with the hAEC.

Flow cytometry

Evaluation of surface expression of ectoenzymes was performed by hAEC by means of anti-CD39 PE-Cy7 mAb (eBiosciences) and a panel of mAbs generated in our laboratory and FITC or allophycocyanin conjugates by Aczon (Bologna, Italy): anti-CD38 (clone IB4), anti-CD45 (clone CB73), and anti-CD203a/PC-1 (clone 3E8, kindly provided by J. Goding), and anti-CD157 (clone SY/11.BS). FITC- or allophycocyanin-conjugated irrelevant isotype-matched mAbs were purchased from Beckman Coulter. Expression of ADO deaminase (ADA)/CD26 complex was evaluated using PE-conjugated anti-CD26 mAb (clone CB26), locally generated.

Expression of ADOR was evaluated on hAEC as well as on CD4\(^+\) T and B lymphocytes and NK cells isolated from five normal donors (see above), using rabbit polyclonal Abs anti-ADORA1 (LifeSpan Biosciences), anti-ADORA2a, and anti-ADORA2b (Thermo Scientific). PE-conjugated goat anti-rabbit Ig (Beckman Coulter) was used as secondary reagents.

Cells were run on a Gallios cytometer and analyzed using Kaluza software (Beckman Coulter). Data were shown as the percentage of positive cells or mean relative of fluorescence intensity (MRFI), calculated as mean fluorescence obtained with specific mAb normalized to mean fluorescence obtained with irrelevant isotype-matched mAb.

ADO production by hAEC

Adherent hAEC (3 × 10\(^5\) cells) maintained at 37°C and 5% CO\(_2\) in 24-well plates (Costar Corning) were exposed to PBS supplemented with 0.1% glucose and in the presence (or absence) of AMP, ATP, or NAD\(^+\) (50 μM). Supernatants were collected after 5, 30, or 45 min, and acetinol (ACN; Sigma-Aldrich) was immediately added to supernatants at a 1:2 ratio to stabilize ADO. Samples were then centrifuged at 12,000 × g, and supernatants were collected and stored at −80°C until use. The presence of AMP, ATP, and NAD\(^+\) was investigated by HPLC analysis.

HPLC analysis

Chromatographic analysis was performed with an HPLC System (Beckman Gold 126/166 NM; Beckman Coulter) equipped with a reverse-phase column (Synergi Fusion C18, 5 μm; 150 × 4.5 mm; Phenomenex). The nucleotides and nucleosides were separated using a mobile-phase buffer (pH 5.1) (0.125 M citric acid and 0.025 M KH\(_2\)PO\(_4\)) along with 8% ACN over 10 min at a flow rate of 0.8 ml/min. UV absorption spectra were measured at 254 nm. HPLC-grade standards used to calibrate the signals were dissolved in 0.2 μM of sterile-filtered AIF V serum-free medium (Invitrogen, Paisley, U.K.) (pH 7.4) and injected in a buffer volume of 20 μl. The retention times (in min) of standards were as follows: AMP, 2.15; NAD\(^+\), 2.8; and ADO, 5.56. Peak integration was performed using Gold software (Beckman Coulter). ACN-treated hAEC supernatants were evaporated by Speed-Vac (Eppendorf), reconstituted in mobile-phase buffer, and analyzed by HPLC. The assessment of the percentage of EICC-positive cells was determined by two independent pathologists on both sets of slides. Analyses were performed using ImageJ software (https://imagej.net).
Cell proliferation assay

Cell proliferation was assessed using CFSE dilution assay. Briefly, CD4+ T cells, B cells, or NK cells were stained with 1 μg/mL CFSE (Invitrogen), incubated for 15 min at 37°C, washed, and then cultured in RPMI medium supplemented with 10% FBS. Cells were kept at 37°C and 5% CO2, alone or in the presence of specific stimuli. T cells were treated with beads coated with anti-CD3/anti-CD28 mAb (T cell activation/expansion kit; Miltenyi Biotec), and B cells were stimulated with 2.5 μg/mL CpG 2006 (TIB Biomol), 100 ng/mL CD40-L, and 20 ng/mL rhIL-4 (both from Immunotools). NK cells were cultured by adding 20 ng/mL rhIL-15 (Immunotools). Stimulated cells were cultured in 96-well flat-bottom plates (Costar Corning) in the presence of absence of hAEC (at hAEC/effector cell ratios ranging from 1:1 to 1:8). The same experiments were performed in the presence or absence of human fibroblasts (Lonza), tested as control. In some experiments, T cells were treated before culture for 30 min with the following specific inhibitors of ectoenzymes: 10 μM MMRS 1706 (32) (a specific antagonist of A2a receptor; Sigma-Aldrich), 100 nM α-β methyl ADP (29) (APCP, a CD73 inhibitor; Sigma Aldrich), and 300 μM β-γ-methyl ATP (30) (a CD203a/PC-1 inhibitor; Sigma-Aldrich). The optimal concentration of each inhibitor has been already determined in previous studies (22). hAEC were used with effector cells at 1:1 hAEC/effector cell ratio. In another set of experiments, effector cells (T and NK cells) were treated before culture for 30 min with the following specific inhibitors of ectoenzymes: 10 μM MMRS 1706 (32) (a specific antagonist of A2a receptor; Sigma Aldrich), 300 μM α-β methyl ADP (29) (APCP, a CD73 inhibitor; Sigma Aldrich), and 1 μM MRS 1706 (32) (a specific antagonist of A2b receptor; Tocris).

Effector cells were harvested after 6 d, washed, and then stained with PE-conjugated anti-CD4, anti-CD19, or anti-CD56 mAbs (Beckman Coulter). After additional washes, cells were run on a Gallios cytometer, and CFSE dilution was analyzed gating on PE+ cells, using Kaluza software (Beckman Coulter). Data were expressed as the percentage of proliferating cells.

Apoptosis

PB B lymphocytes were cultured for 5 d in RPMI 1640 medium supplemented with 10% FBS in the presence (or absence) of hAEC (1:1 B cells/hAEC ratio). Apoptosis level was measured on B lymphocytes by flow cytometry using PE-Annexin V Apoptosis Detection Kit I (BD Pharmingen), following manufacturer’s protocol. Alive cells were detected as Annexin V−/7AAD− cells. Dead cells were identified as Annexin V+/7AAD+ cells and shown as the percentage of total cells.

Expansion of regulatory cell subsets

B lymphocytes or CD4+ T cells were isolated from PB of three different normal donors and cultured in 24-well plates (Costar Corning) at 500,000 cells per well in RPMI supplemented with 10% FBS, in the presence or absence of hAEC (1:1 lymphocytes/hAEC ratio). PB lymphocytes were challenged with the substrates specific for each ectoenzyme by flow cytometry using PE–Annexin V Apoptosis Detection Kit I (BD Pharmingen), following manufacturer’s protocol. Alive cells were detected as Annexin V−/7AAD− cells. Dead cells were identified as Annexin V+/7AAD+ cells and shown as the percentage of total cells.

Statistical analysis

Statistical analysis was performed using Prism 5.03 software (GraphPad Software). Gaussian distribution of data was analyzed using the Kolmogorov–Smirnov test. The Student’s t test or Mann–Whitney U test was used to compare data, depending on data distribution. The significance ranges as follows: *p < 0.05 (significant), **p < 0.005, and ***p < < 0.0005.

Results

Expression of ectoenzymes and ADOR on hAEC

The expression of different ectoenzymes on the surface of hAEC was measured by flow cytometry on cells obtained from 17 different donors. All the samples showed high levels of CD38, CD203a/PC-1, CD39, CD73, and CD157 (Fig. 1A). Inset shows a representative FACS analysis. Data are summarized in Supplemental Table I. All molecules were expressed by the whole cell population, with the exception of CD38, expressed only by a fraction of hAEC (percentage of positive cells ± SD: 47.89 ± 7.9). No difference was observed between freshly isolated and cryopreserved hAEC (data not shown). All the analyzed primary amnion-derived cells are equipped with a complete ectoenzymatic machinery, able to produce ADO through the canonical as well as the alternative pathway.

Because hAEC are cultured in the presence of rhEGF, we asked whether these molecules could alter the expression of the adenosinergic ectoenzymes. To address this issue, we performed an independent experiment on four different batches of hAEC, cultured for 3 d in the presence (or absence) of rhEGF. As shown in Fig. 1B, rhEGF significantly upregulates the expression of CD73, whereas the expression of CD38, CD39, and CD203a/PC-1 was not affected. Inset shows a representative staining for CD73 expression on hAEC treated or not treated with rhEGF.

One question was whether these cells are simply a producer or if they may also intervene in ADO metabolism. Thus, we analyzed the expression of ADOR and ADA on hAEC. A constitutive expression of ADOR, ADORA1, ADORA2a, and ADORA2b was detected on hAEC. Moreover, hAEC express surface CD26, the carrier of ADA that metabolizes ADO to inosine (Fig. 1C). Inset shows a representative experiment. Data are summarized in Supplemental Table I.

The expression of ADOR and CD26 was also analyzed on resting and activated immune effector cells. Data obtained were consistent with previous reports (33, 34) and are summarized in Supplemental Table I and in Supplemental Fig. 1. Indeed, resting T lymphocytes express low levels of all ADOR and CD26. The expression of all ADOR was significantly upregulated by activated lymphocytes, whereas the expression of CD26 was not influenced by T cell activation. Resting B lymphocytes commonly express low levels of ADORA1, ADORA2a, and CD26, whereas ADORA2b is highly expressed. This pattern of expression was not significantly affected by B cell stimulation.

Resting NK cells expressed high levels of ADOR and CD26. At variance with T lymphocytes, such expression was significantly downregulated in NK cells after activation. Again, CD26 expression was not significantly affected by activation.

Functional analysis of the distinct ectoenzymes

For functional analysis of the distinct ectoenzymes, hAEC were challenged with the substrates specific for each ectoenzyme by testing the production of the final substrate. Data are summarized in Table I. Exposure of hAEC to AMP (substrate of CD73) was followed by the production of ADO. AMP conversion is a time-dependent process: ADO production is detectable within 5 min and reaches two peaks after 30 and 45 min. The conversion of ADO into ADO resulted in a significant reduction of the initial AMP concentration, both at 30- and 45-min incubation (Fig. 2A).

To a lesser extent, hAEC can also produce ADO from ATP and NAD+ in a time-dependent manner. As shown in Fig. 2B, ADO produced by ATP conversion was detectable after 5 min and significantly increased after 30 and 45 min. Similarly, AMP conversion was detectable after 5 min and significantly increased after 30 and 45 min. Again, the initial concentration of NAD+ decreased after 30 and 45 min (Fig. 2C).

The roles of CD39 and CD203a/PC-1 in the production of ADO were assessed by performing experiments similar to those described above, in the presence of CD39 inhibitor POM-1. ADO production was detected after 15 min. As expected, POM-1 did not
affect ADO production from AMP (mediated by CD73) (Fig. 2A). In contrast, the production of ADO from ATP (mediated by CD39 or CD203a/PC-1 in cooperation with CD73) decreases after exposure to POM-1 (Fig. 2B). These findings suggested that both CD39 and CD203a/PC-1 are involved in the process. Lastly, POM-1 treatment does not influence ADO production starting from NAD+ (Fig. 2C), being the processes mediated by CD38 and CD73 in sequence. All these data are summarized in Table I.

The ability of hAEC to produce ADO from different substrates was further confirmed in situ by EICC. Cells were counterstained with H&E (Fig. 3A1, 3A2) to confirm epithelial morphology. ADO production was detected on all hAEC samples after exposure to substrates specific for each ectonucleotidase activity: ATP for CD39 (Fig. 3A3), AMP for CD73 (Fig. 3A5), and TPP for CD203a/PC-1 (Fig. 3A7). Positive controls (endogenous phosphatases) were assessed using β-GP substrate (Fig. 3A9), whereas negative controls were obtained using the same substrate in the presence of levamisole (Fig. 3A10). ADO production was reduced in the presence of specific inhibitors: POM-1 (CD39 inhibitor, Fig. 3A4); APCP (CD73 inhibitor, Fig. 3A6), and β-γ-methyl ATP (CD203a/PC-1 inhibitor, Fig. 3A8). The analysis of the reactivities obtained in the EICC tests showed a significant decrease of the overall positive cells following incubation with specific inhibitors (Fig. 3B). Namely, ATP positivity was reduced by ∼80% after POM-1 incubation. AMP positivity was reduced by ∼85% following APCP incubation. TPP positivity was fully abolished following pretreatment with β-γ-meATP. Tetramisole incubation reduced the reactivity by ∼65% (Fig. 3C).

**hAEC and T cell proliferation**

The functional relevance of different ectoenzymes on the surface of hAEC to their immunomodulatory activity was tested on activated human T lymphocytes. T cell proliferation assay was performed in the presence or absence of hAEC at different E:T cell ratios.

CD4+ T cells were stimulated with beads coated with anti-CD3 and anti-CD28 mAbs. As shown in Fig. 4A, the proliferation of CD4+ T lymphocytes was significantly reduced in the presence of hAEC at 1:1, 1:2, 1:4, and 1:8 hAEC/CD4 ratios. Inset shows a...
representative experiment. Additional experiments were performed using hAEC and human fibroblasts, tested as negative control. As shown in Supplemental Fig. 2A, T cell proliferation was inhibited in the presence of hAEC but not in the presence of human fibroblasts, at 1:1, 1:2, 1:4, and 1:8 ratios. These data proved unambiguously that hAEC-mediated inhibition of T cell proliferation was not due to a competition for nutrients and represented a specific feature of hAEC.

Next, we performed additional experiments in the presence of specific inhibitors of adenosinergic ectoenzymes. T cell proliferation was significantly reduced in the presence of hAEC, at a 1:1 ratio, and the inhibition was partially reverted in the presence of

Table I. Functional characterization of adenosinergic ectoenzymes expressed on hAEC

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>ATP</th>
<th>p</th>
<th>NAD⁺</th>
<th>p</th>
<th>AMP</th>
<th>p</th>
<th>ADO</th>
<th>p</th>
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<tbody>
<tr>
<td>CD73</td>
<td></td>
<td></td>
<td>70.7 ± 1.9⁷</td>
<td></td>
<td>29.2 ± 1.9</td>
<td></td>
<td>17.6 ± 5.5⁵</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>37.6 ± 3.9</td>
<td></td>
<td>39.6 ± 3.5</td>
<td>NS</td>
<td>1.9 ± 1.68</td>
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<tr>
<td>POM-1</td>
<td></td>
<td></td>
<td>2.5 ± 0.3</td>
<td></td>
<td>1.6 ± 0.4</td>
<td></td>
<td>2.2 ± 0.54</td>
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<tr>
<td>CD203a/PC-1 CD39</td>
<td></td>
<td></td>
<td>98 ± 1.68⁶</td>
<td></td>
<td>1.9 ± 0.05</td>
<td>NS</td>
<td>91.9 ± 5.8⁶</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>2.5 ± 0.3</td>
<td>NS</td>
<td>1.6 ± 0.4</td>
<td></td>
<td>2.2 ± 0.54</td>
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<tr>
<td>POM-1</td>
<td></td>
<td></td>
<td>2.5 ± 0.3</td>
<td>NS</td>
<td>1.6 ± 0.4</td>
<td></td>
<td>2.2 ± 0.54</td>
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<tr>
<td>CD38</td>
<td></td>
<td></td>
<td>97.7⁶</td>
<td></td>
<td>91.7 ± 4.2²</td>
<td></td>
<td>93.8 ± 2.9⁶</td>
<td></td>
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<tr>
<td>Control</td>
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<td></td>
<td>2.2 ± 0.02</td>
<td></td>
<td>1.9 ± 0.04</td>
<td>NS</td>
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<tr>
<td>POM-1</td>
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<td></td>
<td>1.9 ± 0.04</td>
<td>NS</td>
<td>2.2 ± 0.02</td>
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*Substrate; results are expressed as pmol/300,000 cells.

FIGURE 2. Kinetics of ADO production by hAEC. ADO production was investigated in supernatants collected at 5, 30, and 45 min of incubation from six different batches of hAEC treated with 50 μM AMP (A), 50 μM ATP (B), and 50 μM NAD⁺ substrates (C). White and gray columns indicated the concentration of substrates and ADO, respectively. Light gray and black columns indicated the concentration of substrates and ADO, respectively, in the presence of POM-1 (inhibitor of CD39). Data are expressed as picomoles of ADO or substrate/3 × 10⁵ cells.
APCP, β-γ methyl ATP, and POM-1. Addition of kuromanin (CD38 inhibitor) did not significantly rescue the functional effects (Fig. 4B). Inset shows a representative experiment.

Because rhEGF upregulated CD73 expression, we investigated whether the same treatment could influence the hAEC-mediated inhibition of T cell proliferation. To this aim, independent experiments were performed using four different hAEC samples exposed (or not) to rhEGF. As shown in Fig. 4C, T cell proliferation (percentage of proliferating cells ± SD: 97 ± 1.2) was significantly inhibited by untreated hAEC at 1:1, 1:2, 1:4, and 1:8 hAEC/CD4 ratios. This inhibition was significantly increased using hAEC cultured in the presence of rhEGF (as compared with untreated hAEC), at 1:1 and 1:2 but not at 1:4 and 1:8 hAEC/CD4 ratios. Inset shows a representative experiment. All these results are summarized in Supplemental Table II.

To dissect the contribution of each ADOR to hAEC-mediated inhibition of T cell proliferation, we performed additional experiments using specific antagonists of A1 (ZM241385), A2a (MRS1706), or A2b (DPCPX) ADOR. As shown in Fig. 4D, T cell proliferation was significantly inhibited by hAEC at a 1:1 ratio. This inhibition was significantly reverted by pretreating T cells with specific antagonists of A1 and A2b (but not of A2a) ADOR. Thus, ADO produced by hAEC inhibits T cell proliferation mainly through A1 and A2b ADOR.

**hAEC and B cell proliferation**

B cells were stimulated using CpG 2006, CD40L, and IL-4 in the presence (or absence) of hAEC. As shown in Fig. 5A, B cell proliferation was increased in the presence of hAEC, added at 1:1, 1:2, 1:4, and 1:8 hAEC/B cell ratios. The effects were further increased in the presence of APCP and β-γ methyl ATP. Addition of POM-1 resulted in a partial reversion of the hAEC effect. Inset shows a representative experiment. Results are summarized in Supplemental Table II.

**hAEC and NK cell proliferation**

NK cells were stimulated with IL-15 in the presence (or absence) of hAEC. As shown in Fig. 5B, NK cell proliferation was significantly reduced in the presence of hAEC at a 1:1 ratio. The effects were even more apparent at 1:2, 1:4, and 1:8 hAEC/NK cell ratios. Because a linear decrease of NK cell proliferation was not observed, we have performed additional experiments at lower ratios (1:8 to 1:64 hAEC/NK cell ratios, Supplemental Fig. 2B).
Lastly, NK cell proliferation was further decreased in the presence of APCP and POM-1, whereas β-g methyl ATP did not induce statistically significant effects. Inset shows a representative experiment. Results are summarized in Supplemental Table II.

Additional experiments were performed using specific antagonists of ADOR. As shown in Fig. 5C, NK cell proliferation was significantly inhibited by hAEC at a 1:1 ratio. NK cell proliferation was partially but significantly restored by pretreating these cells with specific inhibitors of A1 and A2A ADOR but not using a specific inhibitor of A2B ADOR. These data suggested that NK cell proliferation was dampened by ADO produced by hAEC, mainly through the interaction with A1 and A2A ADOR.

hAEC secreted IL-21 and rescued PB B cells from apoptosis

Because B cell proliferation was increased in the presence of hAEC, we tested whether hAEC may rescue unstimulated B cells from apoptosis through the expression of activating receptors (e.g., CD40L) or secretion of cytokines (e.g., IL-21), both crucial for signaling in B lymphocytes. Data are summarized in Table II.

As shown in Fig. 6A, the percentage of viable resting B cells was significantly increased in the presence of hAEC as compared to the control. This effect was mediated by hAEC secreted IL-21, as shown by the increase in the percentage of surviving B cells in the presence of IL-21. Data are summarized in Supplemental Table II.
with the same cultures in the absence of hAEC. Consequently, the percentage of dead cells was significantly reduced in the presence of hAEC. Inset shows a representative experiment.

IL-21 production and CD40L expression were tested on six different batches of cryopreserved hAEC. The results obtained indicate that most hAEC produce IL-21 (percentage of positive cells 6\pm SD: 75\pm 10), whereas only a small portion of these cells express CD40L (6.0\pm 3.1\%) (Fig. 6B). Inset shows a representative staining with anti-IL-21 mAb performed on six different batches of hAEC.

hAEC promoted the expansion of regulatory cell subsets

The picture of the influence of hAEC on immune cells was completed by assessing whether hAEC are able to induce the expansion of B and T regulatory cells in vitro. Data are summarized in Table II.

The percentage of CD4^+CD25^{high}CD127^{low} regulatory T cells (Treg) was significantly increased in the presence of hAEC. The percentage of Treg was significantly upregulated in the presence of hAEC, also when T lymphocytes were activated with polyclonal stimuli. Similar results were obtained with CD19^+CD24^{high}CD38^{high} Breg. The percentage of Breg was significantly increased in the presence of hAEC, starting from samples of PB unstimulated or activated B lymphocytes (Fig. 6C). A representative experiment performed on resting T and B lymphocytes is shown in Fig. 6D.

Discussion

Human amnion epithelial cells are a relatively new source of multipotent cells, and a phenotypic map of the molecules expressed by these cells is not completed. However, different groups highlighted the presence of immunomodulatory molecules, among which are noncanonical HLA class I molecules (12) or 5^-ectonucleotidase enzyme (CD73), commonly referred to as characteristic of mesenchymal stromal cells (35).

The experience acquired from studying tolerogenic environments, such as placenta, prompts investigations of the components of the adenosinergic pathways (21, 22). ADO, a potent agent involved in inflammation and immunosuppression, is generated in...
the extracellular milieu using ATP, metabolized by CD39 to AMP and then to ADO by CD73 (36). CD39 and CD73 have been previously identified on some tumor cells but are also expressed by regulatory cell subsets and immune effectors, such as T, B, and NK cells (37). The pyridine nucleotide NAD⁺ may also lead to ADO production through the concerted action of CD38, whose product ADPR is metabolized by CD203a/PC-1 to AMP (21), later degraded by the bottleneck ectoenzyme CD73. CD38/CD203a/CD73 is an alternative pathway, confirmed to be present in human placenta and cornea (38, 39) and in pathological environments, such as the one of myeloma (40).

Full-term placenta provides cells characterized by a homogenic coexpression of adenosinergic ectoenzymes. We have been able to confirm that hAEC are endowed with ectoenzymes of the canonical (CD39, CD73) as well as of the alternative pathway (CD38, CD203a/PC-1, CD73). Both pathways are functional and have a role in the immune modulation mediated by hAEC. The specific effect on all identified ectoenzymes was assessed using specific inhibitory compounds, as previously tested and titrated (28, 30, 41). Another original observation stands on the presence of ADOR on hAEC, suggesting a circuit of production and use of ADO in these cells. ATP and NAD⁺ were released into the extracellular space, and ADO thereby generated play roles in the control of proliferation and apoptosis of different sets of immune cells. Indeed, ADO has been shown to inhibit dendritic, T, and NK cells, and to promote the generation of Treg and M2 macrophages in vitro (23, 42). Different studies addressed an important role of ADO in the release of immunomodulatory molecules by macrophages (43–45).

Results presented in this article support the hypothesis that ADO generated extracellularly by hAEC is involved in the inhibition of T cell proliferation. Indeed, a selective inhibition of molecules of both adenosinergic pathways and blockade of A1 and A2B ADOR using selective antagonists led to a partial recovery in T cell proliferation. Because ADOR antagonists failed to completely reverse the effects of extracellular ADO, we may hypothesize the existence of an intracellular uptake of ADO. Indeed, extracellular ADO homeostasis is maintained by a bi-directional transport through equilibrative nucleoside transporters located in the plasma membrane in the direction of concentration gradients. ADO entering cells through equilibrative nucleoside transporters is phosphorylated by a low-Km ADO kinase into AMP, which may accumulate inside the cell and activate specific AMP-dependent protein kinase (46). In addition, extracellular ADO also mediates signals through membrane-anchored G protein–coupled ADOR (47). Finally, the partial block of ADOR may increase the concentration of extracellular AMP, favoring the mononucleotide internalization either through an unknown receptor or by gradient concentration, inducing a positive effect on the AMPK/mTOR/p70S6K/ rpS6 protein axis. Consequently, it is possible to observe an increased proliferation and thus a partial reversal of the inhibition of T cell proliferation. Thus, the effects observed on T lymphocytes are at least in part mediated by both pathways of ADO production, but other immunosuppressive molecules, such as HLA-G, likely play additional roles (12) (F. Morandi, S. Strom, and R. Gramignoli, manuscript in preparation).

In this study, we demonstrated that hAEC promote an increase of Treg number in vitro, and such an effect might be secondary, at least in part, to the production of ADO from hAEC. Indeed, it has been demonstrated that ADO may promote an expansion of Treg after engagement of ADORA2a (48). Our data suggest that hAEC-derived ADO may be involved in the reduction of T cell responses in vivo, as observed in murine models of experimental
autoimmune encephalomyelitis after treatment with hAEC (15). Indeed, hAEC induced a decrease of IL-17A and an increase of the number of Treg in blood and Th2 cells in peripheral lymphoid organs (15).

Notably, we measured a different effect on human NK cells. NK cell proliferation was slightly influenced by hAEC presence and mostly at low NK/hAEC ratios (1:4 to 1:64). Because NK cells express ADOR at a high level, it is likely that a large number of hAEC is necessary to quickly saturate all the ADOR, particularly A1 and A2A receptors that are mainly involved in such inhibition (as witnessed by experiments performed using selective antagonists). Moreover, NK cells express high levels of the CD26/ADA complex. ADA allows the conversion of ADO into inosine, neutralizing the ADO inhibitory effects.

The results obtained after coculturing hAEC with B lymphocytes delineate another scenario. In the presence of polyclonal stimuli, hAEC significantly increased B cell proliferation, which is further increased after a selective inhibition of the CD73 and CD203a/PC-1 ectoenzymes. B cell proliferation, per se, remains unaltered in the presence of hAEC (data not shown). These observations suggest that ADO produced by hAEC may counterbalance B cell activation by acting on ADORA1, which guides cell differentiation, survival, and proliferation by decreasing cAMP (49). B cell activation and survival can be supported by other molecules expressed or released by hAEC. Out of these, NAD+ and ATP permeate activated hemichannels and mediate paracrine effects in cells by increasing the intracellular Ca2+ levels (50). Furthermore, extracellular nucleotide concentrations can potentially favor (or suppress) the local immune responses, depending on their concentration as well as the relative abundance of ectonucleotidases and P2 receptor subtypes on immune cells (51, 52). Such biphasic effects of extracellular nucleotides could be partially explained in light of the peculiar impact of inhibitors of ectonucleotidases on the increased B cell proliferation observed after cocultures with hAEC. B cells express different levels of ADOR in comparison with T lymphocytes (prevalently, ADORA2b). Differently from T lymphocytes, the expression of ADOR was not increased upon activation of B cells, suggesting a different effect of hAEC-derived ADO. Indeed, the

FIGURE 6. hAEC rescued B lymphocytes from apoptosis through IL-21 production and CD40L expression and induced expansion of regulatory cell subsets. The percentage of (spontaneous) apoptotic B lymphocytes in the presence (white columns) or absence (gray columns) of hAEC. Dead cells are quantified by flow cytometry as Annexin V+/7AAD+ cells. Data are expressed as the percentage of positive cells. Mean of 10 different experiments using different batches of primary hAEC is shown in (A). The p values are indicated where differences are statistically significant. Inset shows a representative experiment. (B). IL-21 production and surface CD40L expression were investigated by flow cytometry on six different batches of hAEC. Data are expressed as the percentage of positive cells. Mean ± SD is shown. Inset shows a representative experiment of IL-21 production. Gray profiles indicated staining with irrelevant isotype-matched mAb, whereas black profiles indicated staining with specific mAb. (C) CD19+ and CD4+ lymphocytes isolated from normal donors were cultured for 5 d in the presence or absence of 1) hAEC (at 1:1 lymphocytes/hAEC ratio) and 2) polyclonal stimuli. The percentage of CD19+CD24hiCD38hi Breg and CD4+CD25hiCD127lo Treg was evaluated by flow cytometry and gating on CD19+ and CD4+ lymphocytes, respectively. Results are expressed as the percentage of positive cells. A representative experiment performed on T and B cells is shown in (D).
lack of CD26 expression (a molecule witnessing the function of ADA) may lead to an increase of pericellular ADO and, consequently, increased effects on B cells. Another original observation is that cocultivation of hAEC with resting peripheral B lymphocytes is followed by a rescue from apoptosis of the latter cells. CD40L is normally expressed by activated T lymphocytes, and it is effective in overcoming apoptosis induced by cross-linking of Ag receptor (53). CD40L is detectable on follicular helper T (54) and dendritic cells (55) and has been reported supporting B cell maturation in the germinal center. However, this ligand is expressed in $4\%$ of hAEC. Another molecule involved in the control of B cell differentiation is IL-21, which can modulate proliferation, differentiation, and apoptosis in B lymphocytes, depending on costimulatory signals (56). Most of the hAEC analyzed express intracellular IL-21, suggesting that they are able to support the in vitro survival of resting B lymphocytes as well as B cell proliferation in the presence of polyclonal stimuli. These data should be taken into account in hAEC-based treatments of autoimmune diseases related to B cell activation because preclinical experiments in animal models were successful (13–15).

Finally, in this study we also demonstrated an expansion of Bregs driven by hAEC. The effective role of ADO in such context is still to be elucidated, taking into account that ADO induces the expansion of CD39$^{hi}$CD73$^{+}$ Bregs through ADOra1/A2a (57). A reasonable inference is that ADO may induce the expansion of CD19$^{+}$CD24$^{hi}$CD38$^{hi}$ Bregs as well.

In conclusion, hAEC constitutively express a complete set of cell surface nucleotidases, previously scattered on different cells, and were constitutively identified for the first time, to our knowledge, on a single cell type. The products of such ectoenzymes modulate important functions of immune effector cells. Such effects of hAEC in vitro on immune effector cells are summarized in Fig. 7. These conclusions are relevant in regenerative medicine, in which the adoption of therapeutic protocols based on hAEC infusion in immunocompetent recipients, as well as in patients with immune-related disorders, may constitute an effective treatment. Large numbers of hAEC can be isolated by full-term placenta, readily banked, and potentially available “off the shelf” in medical centers worldwide, similarly to what happens for bone marrow cells. For such reasons, the Karolinska Institute is in the process of creating the first GMP bank of human amnion-derived cells. Regulatory approvals, a preliminary step to start the transfer to patients with liver and other diseases, are ongoing.

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Disclosures
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References

