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Hydrolytic and Nonenzymatic Functions of Acetylcholinesterase Comodulate Hemopoietic Stress Responses

Dan Grisaru,2* Marjorie Pick,2‡ Chava Perry,† Ella H. Sklan,‡ Ronit Almog,* Ilan Goldberg,* Elizabeth Naparstek,† Joseph B. Lessing,* Hermona Soreq,3‡ and Varda Deutsch‡

Glucocorticoid-initiated granulocytosis, excessive proliferation of granulocytes, persists after cortisol levels are lowered, suggesting the involvement of additional stress mediator(s). In this study, we report that the stress-induced acetylcholinesterase variant, AChE-R, and its cleavable, cell-penetrating C-terminal peptide, ARP, facilitate granulocytosis. In postdelivery patients, AChE-R-expressing granulocyte counts increased concomitantly with serum cortisol and AChE activity levels, yet persisted after cortisol had declined. Ex vivo, mononuclear cells of adult peripheral blood responded to synthetic ARP26 by overproduction of hemopoietically active proinflammatory cytokines (e.g., IL-6, IL-10, and TNF-α). Physiologically relevant ARP26 levels promoted AChE gene expression and induced the expansion of cultured CD34+ progenitors and granulocyte maturation more effectively than cortisol, suggesting autoregulatory prolongation of ARP effects. In vivo, transgenic mice overexpressing human AChE-R, unlike matched controls, showed enhanced expression of the myelopoietic transcription factor PU.1 and maintained a stable granulocytic state following bacterial LPS exposure. AChE-R accumulation and the consequent inflammatory consequences can thus modulate immune responses to stress stimuli. The Journal of Immunology, 2006, 176: 27–35.

Poststress leukocytosis, i.e., an increase in peripheral blood white blood cell (WBC) counts, was first described over 50 years ago (1). Increased WBC counts occur after diverse stress insults, such as shock, blood loss, parturition, and bacterial infections (2–6). Leukocytosis, a common feature of pregnancy, is more frequent in postpartum mothers (4), as a result of both the migration of mature leukocytes from vessel walls into the circulation as well as an increase in bone marrow WBC proliferation. The initiation of WBC overproduction has been attributed to elevated serum levels of cortisol (7), causing both enhanced proliferation and improved WBC maturation, predominantly toward the granulocytic lineage (8). However, the elevated levels of cortisol, such as those found following the stressful event of delivery, recede within several hours (9). Furthermore, the lifespan of granulocytes is extremely short, with 50% of the granulocytes being replaced daily by the bone marrow (10). Therefore, although cortisol may initiate the signaling pathways, ultimately leading to leukocytosis, the underlying mechanism associated with the progression of this process has remained largely unknown.

Granulocytes are known to play an important role in inflammatory responses by virtue of their ability to perform a series of effector functions that collectively represent a major mechanism of innate immunity against injury and infection (for review, see Ref. 11). Granulocytosis, following injury or infection, is associated with an increased production of proinflammatory and hemopoietic cytokines that are regulated in peripheral tissues by various factors, including acetylcholine (ACh) (12, 13). Briefly, ACh activates nicotinic α7 ACh receptors on residing tissue macrophages to attenuate the secretion of proinflammatory cytokines (14). However, how cholinergic signaling in the circulation is controlled under exposure to inflammatory reactions remains unclear. We predicted that circulating acetylcholinesterase (AChE) modulates poststress ACh levels, consequently promoting the protracted poststress granulocytosis (15, 16). The AChE gene includes a functional glucocorticoid response element in its distal promoter (17), as well as multiple putative binding sites for hemopoiesis-related transcription factors (18). Alternative splicing gives rise to the “synaptic” (AChE-S) multimers, which control ACh levels in the brain and muscles, the “erythrocytic” (AChE-E) dimers, and the stress-induced “readthrough” (AChE-R) monomers (19). AChE-R is expressed in multiple embryonic and tumor cells (20–22), where it displays morphogenic functions, and is also found in healthy, unstressed human sera, where its elevated levels reflect anxiety parameters (23).

Under stress responses, blood AChE-R undergoes C-terminal cleavage in both mice (18) and humans (24). We previously demonstrated that ARP26, a synthetic peptide with the sequence of the C terminus of AChE-R, functions in cell cultures as a hemopoietic growth factor and directs cultured CD34+ progenitors toward proliferation (18, 25). Therefore, as a working hypothesis, we postulated that circulating AChE-R and/or ARP may be involved in directing hemopoietic progenitors toward prolonged granulocytosis. The aim of the current study was to delineate the in vivo and ex vivo effects of AChE-R production to determine whether AChE-R and/or ARP26 are associated with peripartum granulocytosis.

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2 D.G. and M.P. contributed equally to the paper.

ABBREVIATIONS used in this paper: WBC, white blood cell; ACh, acetylcholine; AChE, acetylcholinesterase; AChE-S, synaptic AChE; AChE-E, erythrocytic AChE; AChE-R, readthrough AChE; TPR, transgenic; MFI, mean fluorescence intensity; RT, reverse transcriptase.
Materials and Methods

Cell sources

Fresh blood samples were collected from maternal and umbilical cords and from women patients undergoing routine blood examinations. Only healthy, medication-free patients and neonates, and only pregnancies that were uneventful up to term were included in this study. Peripheral mononuclear and CD34⁺ cells were enriched up to 85% by separation on gelatin and Ficoll-Hypaque gradients followed by purification on anti-CD34 immunomagnetic beads (Dynal Biotech) as described elsewhere (18, 26). The Tel-Aviv Sourasky Medical Center’s Ethics Committee approved the use of all human-originated material in this study.

Transgenic mice

Animal experiments were approved by the Animal Ethics Committee of The Hebrew University. Transgenic (TgR) mice expressing human AChE-R were generated by injecting a DNA construct including the proximal CMV promoter-enhancer followed by exons 2, 3, 4, pseudointron 4’, exon 5, and an SV40 polyadenylation signal, into fertilized eggs of FVB/N mice (27). This transgene presented unimpaired Mendelian inheritance over five generations (28).

To generate acute inflammation, 5 μg of LPS of Escherichia coli origin (Sigma-Aldrich) was injected i.p. in 400 μl of PBS (Biological Industries). Peripheral blood drawn from the retroorbital vein of TgR and FVB/N mice was collected in EDTA (7.5%) tubes. Marrow cells were harvested from the mouse femur bones with a 26 G needle preswashed with heparin, and kept in PBS.

Cell counts and serum tests

Plasma was separated from blood samples used for cell counts with the Coulter Gen-S analyzer (Beckman Coulter). Plasma cortisol levels were measured by ECL immunoassay (ECLA) and analyzed by ECLysys 1010/1020 (Biocode) and Ficoll-Hypaque gradients followed by purification on anti-CD34 immunomagnetic beads (Dynal Biotech) as described elsewhere (18, 26). The Tel-Aviv Sourasky Medical Center’s Ethics Committee approved the use of all human-originated material in this study.

Flow cytometric immunophenotyping and AChE-R detection

To detect AChE-R, cells were incubated with CD45-PerCP (BD Biosciences), followed by permeabilization using the Intrastain kit (DAKO) and staining with rabbit anti-human ARP26 Abs (30). Protein was detected with FITC-conjugated goat anti-rabbit Fab Ab (The Jackson Laboratory). Mean fluorescence intensity (MFI) served as a measure of AChE-R content in analyzed cells. When multiplied by the percent fractions of AChE-R-positive cells, the MFI values reflected the total content of AChE-R in the analyzed blood cell samples. Myeloid markers of maternal blood cells were analyzed by the following combination of mAbs: anti-CD15-FITC (DAKO), anti-CD33-PE (BD Biosciences), anti-CD45-PerCP (BD Biosciences), and anti-CD14-allophycocyanin (Caltag Laboratories). The corresponding MFI values reflected the amount of receptor on the surface of granulocytes and monocytes. Expanded CD34⁺ cells were analyzed by four-color flow cytometry with FITC-conjugated anti-CD15 and PE-conjugated anti-CD33, PerCP-conjugated anti-CD34, and allophycocyanin-conjugated anti-CD38 (all Abs purchased from BD Biosciences) using a FACSCalibur with CellQuest software (BD Biosciences). Relevant isotype control Abs were used to detect nonspecific background fluorescence. The total number of expanded cells for each lineage was calculated by multiplying their relative proportions by the number of viable cells in each culture.

Immunophenotyping of the hematopoietic population in bone mouse marrow and peripheral blood used the following Ab panels: Gr.1-FITC (clone RB6-8C5; Caltag Laboratories), CD11b-PE or -allophycocyanin (clone M1/70.15; Caltag Laboratories), CD45-TC (clone YW62.3; Caltag Laboratories) to detect the myeloid lineage, or CD19-FITC (clone 6D5; Caltag Laboratories), CD4-PE (clone CT-CD4; Caltag Laboratories), CD3-allophycocyanin (clone CT-CD3; Caltag Laboratories) to detect the lymphoid lineage.

Real-time RT-PCR

Total RNA was purified from bone marrow with the RNeasy kit (Qiagen), followed by treatment with DNase I (Qiagen) according to the manufacturer’s protocol. RNA quality was confirmed by electrophoresis on an agarose gel, and by inspection of the OD ratios at 260 nm/280 nm (all values were between 1.8 and 2.1).

cDNA was prepared from this RNA using the Improm II kit (Promega): for each reaction, 2.4 μl of 25 mM MgCl₂, 4 μl of 5X buffer, 1 μl of reverse transcriptase (RT), 1 μl of dNTP mix (10 mM of each), 1 μl of random hexamers (of 50 μM stock; Sigma-Aldrich), 0.5 μl of RNase inhibitor (20 U; Promega), and 2 μl of sample RNA (200 ng/μl) were mixed with diethyl pyrocarbonate water to a final volume of 20 μl. The RT program was 45°C at 42°C, 5°C at 90°C, and then maintained at 4°C.

For real-time quantitative PCR, we used the Lightcycler system (Roche) and SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences for mFOG, mGATA1, Runx1, AML1, PU1, β globin, STAT5, the housekeeping gene β-actin, and amplification conditions are listed in Table I. Primer purity of PCR products was verified by a melting curve analysis and by agarose gel electrophoresis.

Amplification reactions were performed in a final volume of 10 μl containing 1 μl of 5-fold diluted RT reaction product, 1 μl SYBR Green PCR Master Mix, 10 μM primer, and nuclelease-free water. For quantification of transcript levels, we first tested the target concentrations at which each transcript was amplifying at the log linear range, using serial dilutions of

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Table I. Primer sequences used for real-time PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Annealing Temperature in °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>GATA1+</td>
<td>5’-3’ CTCTCTCCCTCCACCTGGACGCTT</td>
<td>65</td>
</tr>
<tr>
<td>GATA1−</td>
<td>5’-3’ CTCTCTCCCTCCACCTGGACGCTT</td>
<td>65</td>
</tr>
<tr>
<td>LM02+</td>
<td>5’-3’ TGGTGGAGGGCGTCGCAATA</td>
<td>65</td>
</tr>
<tr>
<td>LM02−</td>
<td>5’-3’ CCACTGATCCTGGTCAC</td>
<td>65</td>
</tr>
<tr>
<td>RUNX1/AML1+</td>
<td>5’-3’ ACTCCTCTCTGCCTCGTCAGT</td>
<td>65</td>
</tr>
<tr>
<td>RUNX1/AML1−</td>
<td>5’-3’ GTCACATCTTTTATGACGCC</td>
<td>55</td>
</tr>
<tr>
<td>PU1+</td>
<td>5’-3’ GATGAGAACGCTGTAGAAGCAGA</td>
<td>65</td>
</tr>
<tr>
<td>PU1−</td>
<td>5’-3’ TTGGTCCTTGACGAGAACGTA</td>
<td>65</td>
</tr>
<tr>
<td>STAT5b+</td>
<td>5’-3’ GGGACTCATATGATCAGTTTGAATCC</td>
<td>65</td>
</tr>
<tr>
<td>STAT5b−</td>
<td>5’-3’ AACTGAGTCTGGATCCGAGGCTGT</td>
<td>65</td>
</tr>
<tr>
<td>Actin+</td>
<td>5’-3’ CAAATCCATCATGAAGTGTGC</td>
<td>65</td>
</tr>
<tr>
<td>Actin−</td>
<td>5’-3’ ATCTTGACCTTCTGAGGCTT</td>
<td>65</td>
</tr>
</tbody>
</table>
cDNA preparations (1/1, 1/3, 1/9, 1/81, where 1/1 corresponds to a concentration of 400 ng/μl at the reaction mix). The efficiencies for all targets were very similar (amplification of $-n^{1.8}$ per PCR cycle) when RT products were diluted 1/5.

**Immunoblots**

To detect AChE-R in circulation, plasma samples (a total of 20 μg of protein) were run on 4–20% polyacrylamide gels and electroblotted. Membranes were blocked, incubated with anti-human ARP<sub>α</sub> Abs (30), washed and incubated with HRP/anti-rabbit-conjugated Ab (Amersham Pharmacia Biotech). Peroxidase activity was detected using an ECL kit from Amersham. Blots were analyzed using the luminescence tool of Adobe Photoshop 7.0 ME (Adobe Systems).

**In situ hybridization**

In situ hybridization involved freshly isolated cells and 5'-biotinylated, 2'-O-methylated AChE cRNA probes complementary to 3'-alternative human AChE exons, as previously described (18). Labeling intensity was assessed as the percent cytoplasmic red pixels and normalized by subtraction of background signals. Confocal microscopic scans of the cells were obtained using an MRC-1024 Bio-Rad confocal microscope (Hemel Hempsted). The statistics involved ANOVA and t tests.

**Results**

**Intrapartum cortisol escalation is associated with increased granulocytic AChE-R expression**

Sixteen patients with premature rupture of membranes (without uterine contractions) at term were monitored from admission through delivery and postpartum periods (27.08 ± 14.22 and 61.82 ± 15.99 h postadmission, respectively). Cortisol levels were high prepartum (30.6 ± 8.2 vs 21.3 ± 11.2 μg/dl in an age-matched control population, p < 0.001), remained high intrapartum (32.1 ± 12.2 μg/dl; p < 0.001 compared with matched controls, repeated measures ANOVA), and were markedly lower postpartum (27.2 ± 10.6 μg/dl, Fig. 1A), receding to levels that are not statistically different from those of the matched control population (p = 0.05, compared with the intrapartum values). Serum AChE activity increased considerably as compared with controls (21.6 ± 7.2 vs 5.5 ± 1.9 nmol/min/mg protein; p < 0.001) and remained significantly higher than controls during the entire period (Fig. 1B).

The stress-induced AChE-R variant is produced by alternative splicing from the AChE gene (Fig. 1C). To explore its putative involvement with postpartum granulocytosis, we used Abs raised against the AChE-R unique C-terminal peptide, ARP, translated from pseudo-intron 4. Another cohort of 20 intrapartum women were used in this analysis. Cortisol levels were predictably elevated intrapartum as compared with an age-matched group of 48 Caucasian women (36.6 ± 4.2 vs 21.3 ± 11.2 μg/dl, p < 0.001; Fig. 1D). Intrapartum serum cortisol levels showed a direct correlation with granulocyte counts (Pearson correlation; R = 0.54, p = 0.04; Fig. 1E). This was accompanied by increased expression of AChE-R in the cytoplasm of mature WBC as detected by flow cytometry (p = 0.009; Fig. 1F). A direct, significant correlation of cortisol levels with the fraction of AChE-R-positive granulocytes (R = 0.72, p = 0.003; Fig. 1G), but not with monocytes or lymphocytes (data not shown), was consistent with the predicted role of AChE-R in postpartum granulocytosis.

**Sustained peripartum granulocytosis and correlates to granulocytic AChE-R expression**

To explore the relevance of cholinergic changes for intrapartum granulocytosis, we first studied the peripartum hemopoietic changes in blood samples. WBC counts in these patients were higher than the predelivery average and increased significantly intrapartum (p < 0.0001; Fig. 2A). Hemoglobin levels maintained the normal to low range before delivery and decreased significa-
may represent a cumulative effect of rapid production and the release of early myeloid cells from the bone marrow, on the one hand, accompanied by their rapid maturation on the other hand.

Additionally, CD14 expression on monocytes did not vary, while CD33 expression on granulocytes decreased during the intrapartum period and increased postpartum.

FIGURE 2. Peripartum blood profile. Shown are blood profile changes in patients before (PRE), during (INTRA), and following (POST) delivery. Dotted background areas represent normal blood count ranges. A, WBC counts increase during labor (above normal range) and decrease postpartum, although remaining above normal. Hemoglobin levels (Hgb) are lower below normal range during and after delivery. Platelet (Plat) counts remain stable (at normal range) during the entire period. B, Sustained leukocytosis correlates with an elevation in granulocyte (Gran), but not monocyte (Mono) or lymphocyte (Lymph) counts that remained within the normal range over the period studied. * Statistically significant differences (n = 16 patients). C, Myeloid markers. Shown are CD15 and CD33 labeling on AChE-R-positive granulocytes (upper panel) and CD14 and CD33 in monocytes (lower panel) determined by flow cytometry. Note decreases in CD15 expression in intrapartum granulocytes and decreases in CD33 expression in postpartum monocytes. D, The significant intrapartum and postpartum increase in AChE-R-positive granulocytes, but not monocytes or lymphocytes, may explain the stable serum AChE activity. Immunoblots (lower inset) of serum proteins from three patients demonstrate the presence of AChE-R. Luminescence analysis of the AChE-R blot (upper inset) shows stable presence of AChE-R in the serum of women during the peripartum period.

FIGURE 3. ARP26 operates as an inducer of AChE gene expression and potentiates myeloid expansion ex vivo. A, Human cord blood CD34+ cells treated for 24 h with the noted doses of ARP26 as the sole growth factor were subjected to in situ hybridization with probes selective for each of the noted AChE mRNA splice variants. Shown are representative micrographs of the cells. Lower panels, Cytochemical staining for AChE catalytic activity in the presence of 10^{-5} M ISO-OMPA, a selective inhibitor of BChE (center) and nuclear staining with DAPI (bottom). Note intensified brown precipitates of the AChE reaction product, mainly under 2 nM ARP26. B, Average mRNA labeling densities for 10–20 individual cells. Note the concomitant increases in all transcripts, peaking at 2 nM ARP, and the limited variance between cells. C, Flow cytometric analysis of CD34+–derived hemopoietic cells after 2 wk in liquid culture. Incubation with ARP26, but not with cortisol, ASP40, or PBAN, increased the total number of cells. The expansion index (the number of viable cells per milliliter culture divided by the number of seeded cells) was considerably higher following incubation with ARP26. The percentage of immature stem cells (left column), committed myeloid (middle column), and mature myeloid cells (right column) that developed in the presence of each supplement is indicated by numbers on the relevant dot plots. Unlabeled cells appear as black dots and double-labeled ones as green dots. Note similar patterns under the influence of cortisol and ARP26, but not those of the ASP40 and PBAN negative control peptides.
postpartum CD33 labeling markedly decreased (145 ± 89 vs 91 ± 40 MFI, p = 0.05; Fig. 2C).

A significant increase was observed in the number of granulocytes expressing cytoplasmic AChE-R, both intrapartum and postpartum as compared with prepartum (from 1.7 ± 0.6 × 10^3 cells/μl to 5.2 ± 0.5 and 4.9 ± 0.4 × 10^3 cells/μl, respectively, p = 0.05; Fig. 2D). This pattern of expression was not reflected in monocytes or lymphocytes (Fig. 2D), consistent with a selective role for AChE-R in peripartum granulocytosis. Importantly, FACS analysis highlighted a conspicuous stress-induced change in the subcellular distribution of AChE-R. Thus, granulocytes from adult controls included 33 ± 4% cells with cytoplasmic AChE-R labeling, and 63 ± 4% with surface labeling. Postpartum granulocytes showed massive increases in these numbers, to 62 ± 5 and 84 ± 6, respectively. That cytoplasmic AChE-R doubled in stressed granulocytes likely reflects changes in the choice of alternate promoters, shown to respond to both stress and glucocorticoid stimuli (31). High serum levels of AChE-R were found throughout the peripartum period (Fig. 2D, insets), supporting the notion that serum AChE activity reflected sustainable AChE-R levels, compatible with parturition anxiety (23).

ARP26 enhances AChE expression and facilitates differentiation in liquid myeloid cell cultures

Assuming a turnover number of 1 × 10^6 molecules ACh hydrolyzed/second/AChE subunit, and based on our previous findings (24), up to one-half of the AChE-R would be C-terminally cleaved in vivo to yield ARP, the AChE-R C-terminal peptide. Based on the measured ACh hydrolysis rates in the serum of postpartum mothers, we predicted a peptide concentration in the range of 5–30 nM. We further hypothesized that comparable peptide concentrations are found in the bone marrow. Therefore, we tested the ex vivo effects of ARP26, a synthetic peptide with the cleavable C-terminal sequence of AChE-R at 0.2, 2.0, and 20 nM on CD34^+ progenitors. When administered in vivo, ARP26 showed cell-penetrating capacity (29). Within 24 h from its addition to the medium of cultured CD34^+ cells, in situ hybridization followed by confocal quantification revealed elevated levels of all 3’ AChE mRNA variants (Fig. 3, A and B). This was accompanied by increased cytochemically stainable cellular ACh hydrolytic activity, reflecting accumulated AChE protein in the ARP26-treated cultured cells (Fig. 3A), and highlighting AChE-R induction in newborn CD34^+ progenitors. The enhanced activity with physiologically relevant concentrations of ARP26 reflected an increase in endogenous AChE, because the synthetic peptide has no enzymatic capacity. It also provided a possible explanation for the sustained AChE activity in peripartum sera, since 2 nM ARP26 induced AChE increases similar to those observed in cells exposed to stress-associated cortisol levels (25).

To test the long-term effect of ARP26 on myelopoietic expansion, we used flow cytometry to monitor the development of phenotypically distinct cell populations from human CD34^+ hematopoietic stem cells incubated with ARP26 over a 2-wk period. Peptide controls (e.g., ASP40, the C-terminal peptide of the primary AChE-S splice variant and PBAN, an insect negative control peptide) were used to explore the specificity of this response. Fig. 3, C and D, present the resulting cell growth and changes in the populations that emerged from a typical CD34^+ culture. Incubation with ARP26, but not with cortisol, ASP40, or PBAN, increased the total number of cells (Fig. 3C). A larger fraction of committed progenitors (CD34^+CD38^-) emerged in the presence of cortisol at stress levels (1.2 μM), as compared with a physiologically relevant concentration of ARP26 (Fig. 3D); however, the expansion index (the number of viable cells per milliliter of culture divided by the number of seeded cells) was considerably higher following incubation with ARP26 (Fig. 3C). Interestingly, increases were observed along the entire myelopoietic differentiation pathway (CD34^+CD33^+, CD33^-CD34^-CD15^+), and CD33^-CD15^-), supporting the notion that ARP26 tilts hemopoiesis toward the myeloid lineage in a cortisol-independent process, expanding the population of mature CD33^-CD15^- granulocytes and inducing increased growth of early progenitors that produce large numbers of mature granulocytes. These findings demonstrate that the ARP26-induced myelopoiesis leads preferentially toward granulocytosis.
AChE-R supports proinflammatory cytokine release from mononuclear cells

Next, we addressed the putative mechanism(s) enabling the long-term effects of ARP26. Increased AChE-R contents and AChE activity predicted lower ACh levels in the postpartum blood, which should suppress the cholinergic control over the production of proinflammatory cytokines by macrophages (13, 32). To test this prediction, we compared the levels of several inflammation/stress-associated cytokines in the plasma of intrapartum mothers to those of nonpregnant women. Elevated levels in the postpartum mothers were observed for IL-1β, IL-6, and TNF-α, all known to have proinflammatory and hemopoietic roles (33, 34) (Fig. 4A). Next, we tested whether this increase could be induced by overexpressed AChE-R in peripheral WBCs. To this end, 2.5 × 10^6 mononuclear cells/ml from adult women controls were incubated with 2 nM ARP26 (Fig. 4, B and C). Significant increases were observed 24 h later in the secretion from these cell cultures of IL-1β, IL-6, and TNF-α. In contrast, there was no change in the release of the anti-inflammatory cytokine IL-8 from cells incubated with ARP26, as compared with control cells (Fig. 4C and data not shown). Thus, the postpartum AChE-R overexpression in peripheral nucleated blood cells could be causally associated with a selective elevation of proinflammatory cytokine levels.

Transgenic AChE-R accentuates bone marrow PU.1 production

The ACHE promoter harbors abundant binding sites for stress and hemopoiesis-related transcription factors, inducing overexpression upon inflammatory insults (Fig. 5A). Both the basal levels and the response patterns to LPS of LMO2, GATA1, RUNX1, and STAT5 were similar in both FVB/N and TgR mice, whereas PU.1 levels were initially higher in TgR mice and decreased significantly in FVB/N but not in TgR mice, in response to LPS. At 72 h post-LPS injection, PU.1 levels recovered and even reached higher than baseline values in FVB/N mice, but showed some decrease in TgR mice.

FIGURE 5. Expression pattern of transcription factors pivotal for hemopoiesis following inflammatory stress of TgR mice with excess AChE-R. A. Relevant hemopoiesis-related transcription factor binding sites on the ACHE promoter. B. Expression levels of transcription factors pivotal for hemopoiesis in bone marrow extracts from FVB/N and TgR mice (n = 25 each), at different time points post-LPS injection. *, Significant differences, and the results are presented as mean ± SD (p < 0.02, n = 10 repeats), by real-time RT-PCR. Note that levels and response patterns to LPS of LMO2, GATA1, RUNX1, and STAT5 were similar in both FVB/N and TgR mice, whereas PU.1 levels were initially higher in TgR mice and decreased significantly in FVB/N but not in TgR mice, in response to LPS. At 72 h post-LPS injection, PU.1 levels recovered and even reached higher than baseline values in FVB/N mice, but showed some decrease in TgR mice.

FIGURE 6. TgR mice keep a stable granulocytic state in response to LPS. A, Immunophenotyping of the hemopoietic progenitors and the relevant transcription factors following inflammatory stress of TgR mice with excess AChE-R in peripheral WBCs. B, Shown are changes from baseline of peripheral blood immunophenotyping of Gr1^− (granulocyte) cells, CD11b^+ (monocytic) cells and CD19^+ (lymphocytic) cells (n = 10). Note the significant decrease in all cell lineages, in response to LPS injection, of FVB/N and recovery at post 48 h. In contrast, TgR mice keep a steady state in the Gr1^− (granulocyte) cells following LPS injection. The behavior of the CD11b^+ (monocytic) and CD19^+ (lymphocytic) cells show a similar pattern in TgR and FVB/N mice. Shown is the change from baseline of CFU/GM colonies. Note the prompt response in FVB/N mice in response to the drop in Gr1^− (granulocyte) cells, following LPS injection, while the TgR mice keep a low steady state (n = 10), reflecting the constant Gr1^− (granulocyte) cell counts.
were similar in FVB/N and TgR mice overexpressing AChE-R (35). In contrast, the levels of the PU.1 myelopoietically active transcription factor were significantly higher in the TgR bone marrow (Fig. 5B). To study the relevance of the AChE-R-induced increases in PU.1 for hemopoietic reactions to acute inflammation, we i.p. injected TgR mice with bacterial LPS. PU.1 expression was assessed in bone marrow extracts at different time points post-LPS injection. Following LPS exposure, PU.1 expression markedly decreased in the bone marrow of FVB/N, but not TgR, mice (Fig. 5B). At 48 h post-LPS injection, PU.1 levels recovered and even reached higher than baseline values in FVB/N mice, but showed some decrease in TgR mice (Fig. 5B). Thus, both basal PU.1 levels and its expression pattern following LPS exposure were conspicuously altered, predicting modified hemopoietic reactions to LPS insults.

**AChE-R excess changes hemopoietic LPS responses**

PU.1 is notably involved in the production of granulocyte/monocyte cells from the common myeloid progenitor (Fig. 6A) (36, 37). To test for hemopoietic changes following LPS injection, we compared peripheral blood cell counts in TgR and FVB/N mice. Peripheral blood immunophenotyping revealed an appreciable decrease in Gr1+ (granulocyte) cells, CD11b+ (monocytic) cells, and CD19+ (lymphocyte) cells 24 h following LPS injection of FVB/N mice (Table II and Fig. 6B). This decrease in all cell lines recovered partially at 48 h post-LPS injection (Table II and Fig. 6B). The response of TgR mice to LPS injection was different. Gr1+ (granulocyte) cell counts remained unchanged in TgR mice following LPS injection, while the CD11b+ (monocytic) and CD19+ (lymphocyte) cell count followed the same pattern described in the FVB/N mice (Table II and Fig. 6B). Therefore, TgR mice maintain stable granulocyte counts, despite the LPS suppressive effects. This is further supported by the bone marrow behavior following LPS injection. In FVB/N mice there is an increase in the granulocyte formation capacity. This is reflected by larger CFU/GM colony counts from cultures seeded 24 h post-LPS injection, during the nadir in the Gr1+ (granulocyte) cell counts. Decrease in CFU/GM was observed at 48 h with the recovery of these counts (Fig. 6). In contrast, TgR mice maintained a stable low CFU/GM colony count following LPS injection, reflecting their consistent Gr1+ (granulocyte) cell counts (Fig. 6). These findings support the hypothesis that the continuous AChE-R expression preserves a granulocytic steady state, even during deleterious challenges, such as reaction to LPS.

**Discussion**

The immune system maintains a complex two-way communication with the nervous system, through chemical messengers that are able to breach their independent locations (for review, see Ref. 38). The communication is mediated through neurotransmitters, cytokines, and endocrine hormones. The brain is linked to the immune system through the autonomic nervous system, including the sympathetic pathways (norepinephrine), and the parasympathetic pathways (acetylcholine). Behavioral stimuli such as stress can influence the immune response, like augmentation of delayed-type hypersensitivity in the skin (39). The question we addressed regarded the involvement of the peripheral cholinergic stress responses, and in particular, AChE gene expression in the modulation of the immune system.

**From cortisol induction to AChE-R C-terminal cleavage**

Leukocytosis can result from increased cell traffic (mobilization) from bone marrow to blood, demargination from the blood vessel walls (e.g., after intense physical exercise), and decreased exit to tissues. The signal(s) from the inflammatory site to the marrow are unknown, even though a number of humoral factors that can mobilize polymorphonuclear cells are well recognized (40). Strenuous physical stress has been used, for example, as another human model for a short stressful event that causes leukocytosis (41). Researchers found an increase in the percentage of immature band (nonsegmented) neutrophils (a shift to the left) that provided evidence for the release of neutrophils from the bone marrow. Although significant delayed granulocytosis was demonstrated, no significant increase was found in the levels of cortisol, growth hormone, complement factors, IL-6, G-CSF, IL-8, and MIP-1β. However, others were able to show increased levels of G-CSF, IL-1β, IL-6, IL-8, IL-10, TNF-α, and MIP-1β following intensive strength exercise (42, 43). Thus, different mechanisms, or different selections of mobilizers, may be involved in the delayed neutrophilia in response to strength exercise, infections or delivery, since the (patho)physiological stress mechanisms may differ considerably between these inflammation models. Our study linked the initial enhancement of AChE gene expression in hemopoietic cells with the existence of a functional glucocorticoid response element in the upstream AChE promoter (18), combined with the transient intrapartum increase in serum cortisol (44). The transient nature of cortisol elevation, however, implies that other propagating signal(s) should extend this response after the first few hours. Our current findings attribute much of this effect to the cleavable, cell-penetrating C-terminal peptide of AChE-R (29), which accumulates in the human circulation under stress (24). The ex vivo elevation by ARP26 of AChE gene expression in CD34+ progenitors further suggests that the overproduced cleavable AChE-R can autoregulate its own production, providing a tentative explanation for this prolonged phenotype. The bell-shaped dose-dependent pattern of the ARP26-induced enhancement further indicates that either too high or too low concentrations of ARP26 would fail to affect AChE gene expression in blood cells, suggesting strict dependence of this protracted process on AChE metabolism in hemopoietic cells. The specificity of ARP26 is supported by the finding that ASP40, the
C-terminal peptide of AChE-S, failed to induce such effects. Thus, extended granulocytosis reflects cellular consequences unique to the AChE-R variant, compatible with recent reports that AChE-R suppression reduces the levels of proinflammatory cytokines in both rats (45) and Cynomolagus monkeys (46) and not as previously related exclusively to the sympathetic pathways (47).

**Catalytic and nonenzymatic properties of AChE-R are most likely involved**

In addition to the function(s) of its cleavable C-terminal peptide, the effects exerted by AChE-R on the proliferation and maturation of granulocytes could be due to both the catalytic and the non-catalytic properties of AChE-R itself (16). At the catalytic level, AChE-R excess should lead to reduced ACh concentrations in the postpartum serum. This, in turn, would alleviate the control over macrophage production of proinflammatory cytokines, increasing the circulation concentration of such cytokines and inducing further proliferative and cell activation signals (13, 14, 32, 48). Myeloid cells also carry nicotinic (14) and muscarinic (35, 49) ACh receptors. However, others have reported no direct ACh effects on peripheral blood cells (13), suggesting that the cholinergic effects on them operate indirectly through cytokine production. The ARP26-induced increase in proinflammatory cytokine production is in line with this hypothesis. The fact that our current study shows direct proliferative effects for ARP26 further attributes to AChE-R the role of a precursor to its C-terminally cleaved peptide with its growth factor activities. Importantly, we found that AChE-R production following transient increases in cortisol, the reduced anti-inflammatory action due to decrease in ACh, and the C-terminal cleavage of AChE-R are additional elements in the pathway leading to protracted poststress granulocytosis.

**Acute and chronic effects of AChE-R excess**

In addition to proinflammatory cytokines, LPS induces granulocytes to produce several chemokines, possibly influencing early cell trafficking and activation steps during various pathophysiological processes (11). Typically, the extracellular release of these chemokines starts within 1 h post-LPS exposure and reaches a peak at 24 h (50). The initial release of chemokines reflects an LPS-induced transcriptional effect, but the later phase was attributed to the autocrine granulocytic release of TNF-α and IL-1β (51). By facilitating the production of proinflammatory cytokines, the AChE-R cleavable peptide ARP might therefore induce chemokines production as well, protecting the body from acute postpartum conditions such as infection. That preincubation of CD34+ progenitors with ARP facilitates their transplantation efficacy into NOD-SCID mice corroborates this conclusion (52).

The chronic and mechanistic aspects of the cholinergic effect on granulocytotic stress reactions were addressed using the TgR transgenic mice overexpressing AChE-R. TgR mice appear protected from the inflammatory challenge of LPS injection. Previous reports defined the hemopoietic response to LPS stress as initiating with a rapid fall in peripheral blood granulocytes, proceeding with a transient depletion in bone marrow myelopoiesis, and leading to a dramatic increase in myeloid stem cells (53, 54). Following LPS exposure, which induces an inflammatory stress, TgR mice maintained steady levels of granulocytes, unlike FVB/N mice, where granulocyte counts were reduced by this insult. Moreover, the bone marrow of FVB/N mice showed a decrease in CFU/GM at 48 h with the recovery of the counts. In contrast, TgR mice maintained stably low CFU/GM colony counts following LPS injection, reflecting their consistent capacity for granulocytosis. In the bone marrow of TgR, as compared with FVB/N mice, we found marked PU.1 overexpression. PU.1, the protein product of the putative oncogene Spi-1, is a hemopoietic-specific Ets factor, essential for myeloid and lymphocyte development. PU.1 is expressed at low levels in multipotent hemopoietic progenitors, where it allows differentiation into B cells. In contrast, high PU.1 concentrations induce myeloid differentiation, blocking B cell development and activating myeloid cell-specific promoters such as the M-CSF receptor (55, 56). Together with more generally expressed transcription factors, PU.1 participates in the basal regulation of TLR4, implicated in LPS-induced signaling (57). In macrophages, LPS stimulation induces PU.1 phosphorylation, altering its conformation and transcriptional activity (58). AChE-R-induced overexpression of PU.1 provides a putative mechanism that explains the prolonged parturition-associated leukocytosis.

The effects of stress on the immune system have been divided into the beneficial effect of acute stress (resistance to infections) and the deleterious effect of chronic stress (autoimmunity) (59). The involvement of AChE-R on the immune system under acute stress prolongs the beneficial effects of the hypothalamic-adrenal axis mediated through cortisol. The complexity of the LPS response likely extends far beyond AChE-R alone and involves both changes in cellular trafficking and in corticosterone regulation. Yet, we have shown here that chronically increased levels of AChE-R can maintain the immune system at high alert, causing resistance to acute stimuli such as LPS. At the same time, however, ARP activities may explain the deleterious aspects of stress (namely, chronically high cytokine levels). In conclusion, we show that acetylcholinesterase serves as a bimodal long-term modulator of the immune system when exposed to stressful events.

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**Disclosures**

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**References**