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Casein α s1 Is Expressed by Human Monocytes and Upregulates the Production of GM-CSF via p38 MAPK

Stefan Vordenbäumen,* Achim Braukmann, † Klaudia Petermann, ‡ Andrea Scharf, ‡
Ellen Bleck,* Anna von Mikecz, ‡ Joachim Jose, ‡ and Matthias Schneider*

Caseins are major constituents of mammalian milks that are thought to be exclusively expressed in mammary glands and to function primarily as a protein source, as well as to ameliorate intestinal calcium uptake. In addition, proinflammatory and immunomodulatory properties have been reported for bovine caseins. Our aim was to investigate whether human casein α s1 (CSN1S1) is expressed outside the mammary gland and possesses immunomodulatory functions in humans as well. For this purpose, CSN1S1 mRNA was detected in primary human monocytes and CD4+ and CD8+ T cells, but not in CD19+ B cells. CSN1S1 protein was traceable in supernatants of cultured primary human CD14+ monocytes by ELISA. Similarly, CSN1S1 mRNA and protein were detected in the human monocytic cell lines HL60, U937, and THP1 but not in Mono Mac 6 cells. Moreover, permeabilized human monocytes and HL60 cells could be stained by immunofluorescence, indicating intracellular expression. Recombinant human CSN1S1 was bound to the surface of Mono Mac 6 cells and upregulated the expression of GM-CSF mRNA in primary human monocytes and Mono Mac 6 cells in a time- and concentration-dependent manner. A similar increase in GM-CSF protein was found in the culture supernatants. CSN1S1-dependent upregulation of GM-CSF was specifically blocked by the addition of the p38 MAPK inhibitor ML3403. Our results indicated that human CSN1S1 may possess an immunomodulatory role beyond its nutritional function in milk. It is expressed in human monocytes and stimulates the expression of the proinflammatory cytokine GM-CSF. The Journal of Immunology, 2011, 186: 000–000.

Since the late 1980s, milk proteins of various species have been reported to have physiological functions beyond providing a digestive substrate; in particular, immunomodulatory properties were discovered (1). Caseins are the main protein constituents of milk in most mammalian species (2). In humans, they represent ~30% of the protein fraction (3). Caseins were believed to mainly function as an amino acid source (4) and to ameliorate the uptake of trace elements and calcium from the intestine of infants (5, 6). Polypeptides in milk may be degraded by proteases within milk and the digestive tract or survive the digestive process in an intact form (1). Research has focused on fermented streams and identified a number of immunomodulatory effects (7–11), such as the modulation of leukocyte adhesion (12), chemotactic properties (13–16), inhibition of cell growth (17, 18), modulation of the innate immune response of intestinal cells (19), amelioration of experimental inflammation (20), and chaperone-like activities (21). Most of these investigations were focused on bovine casein-derived peptides. Furthermore, bovine casein preparations are often used to induce experimental inflammation in animal models by injection (22–25). The mammary gland was believed to be the sole site of expression for caseins (26), but a transgenic-mouse model showed elevated serum levels of human GM-CSF expressed under the control of the rodent αs1-casein gene (27). Additionally, increased casein gene expression was found in the blood of patients with multiple sclerosis, in lymph nodes of mice with experimental encephalomyelitis in the reconvalescence phase (28), and in benign prostate hyperplasia of humans (29). These observations suggested a more widespread expression of the protein in the context of an unknown physiological function.

Depending on the species, there are three or four evolutionarily related casein genes. Their products are differentiated based on their electrophoretic properties and the capacity to precipitate in the presence of calcium into the calcium-sensitive α and β caseins on the one hand or the insensitive κ caseins on the other hand (30, 31). Across species, the highly conserved organization of the casein genes is remarkable (31). Situated within this cluster are the genes of the histatin/statherin family, which possess antimicrobial properties, and the gene of the follicular dendritic cell-secreted protein, which activates B cells (31). Because of this highly conserved organizational structure, it was suggested that caseins have evolved to provide combined nutritional and immune-modulating functions (31).

In the current study, we analyzed whether human casein α S1 (CSN1S1) may be expressed in blood cells and influences inflammatory processes by modulating GM-CSF expression in monocytes. GM-CSF is a well-known immunomodulatory cytokine that stimulates various functions in innate and adaptive immune responses (32, 33) and plays a key role in inflammatory and autoimmune disorders (34).

Materials and Methods

Isolation of cells

Cells were isolated from peripheral blood, collected into EDTA tubes (BD Vacutainer, BD Biosciences, Plymouth, U.K.), of five healthy donors or...
from buffy coat leukocytes, as indicated. Cell subsets were separated by magnetic cell sorting with the following bead: Abs: anti-CD14 for monocytes, anti-CD8 and anti-CD4 for T cells, and anti-CD19 for B cells (Miltenyi Biotec, Bergisch Gladbach, Germany). Human tumor cell lines were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cell lines were seeded at 1 × 10^5/mL, except for Mono Mac 6 cells, which were seeded at 0.5 × 10^5/mL. The study was approved by the local ethics committee.

**Stimulation experiments**

Mono Mac 6 cells and human monocytes were cultured in RPMI 1640 + GlutaMAX supplemented with 10% heat-inactivated FBS, 50 IU/mL penicillin, and 50 μg/mL streptomycin (Invitrogen, Karlsruhe, Germany). Mono Mac 6 medium was also supplemented with 2 mM nonessential amino acids, 1 mM sodium pyruvate, and 10 μg/mL insulin (Invitrogen). LPS (Sigma-Aldrich, Munich, Germany), TNF-α, and IFN-γ (Cell Sciences, Canton, MA) were used at the indicated concentrations. Thirty micrograms per milliliter of polymyxin was added to culture media to exclude LPS effects, as indicated (Sigma-Aldrich, Munich, Germany). For experiments, 1 × 10^5/mL monocytes or 0.5 × 10^5/mL Mono Mac 6 cells were seeded onto culture plates. LPS contamination was determined by Endoimmune Limulus Ameobocyte Lysate Complement Genotyping Endpoint assay (Hycult Biotechnology, Uden, The Netherlands), according to the manufacturer’s instructions. Recombinant human CSN1S1 (rCSN1S1; Calbiochem, Darmstadt, Germany) was added to cultured cells at different concentrations and for various periods of time. For inhibition of casein effects, the indicated concentrations of cell-permeable inhibitors (Table I; all from Merck, Darmstadt, Germany) were added to cultured cells after 6 h of incubation at room temperature and were harvested after an additional 24 h. Viability of cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium-assay (Promega, Mannheim, Germany), according to the manufacturer’s instructions, and staining of cells with trypan blue (Invitrogen). In all experiments, vitality of cells was unaffected and was >90%, unless indicated otherwise. Overall RNA production was measured using Nanodrop 1000 (Thermo Scientific, Wilmington, DE). Proliferation of cells was assessed by methyl-[3H]thymidine 185 MBq (5 mCi) assay (Hartmann Analytic, Braunschweig, Germany), according to the manufacturer’s instructions.

**Western blot**

Western blot was carried out as described previously (35). Briefly, after stimulating Mono Mac 6 cells for 24 h with 10 μg/mL rCSN1S1, total-cell proteins were prepared for SDS-PAGE on a 12.5% gel. Electroblotting was carried out using a polyvinylidene difluoride membrane (Porablot; Macherey-Nagel, Düren, Germany). Membranes including the same samples were blocked with an Ab against p38-MAPK (GeneTex, Eching, Germany), according to the manufacturer’s instructions, and staining of cells with trypan blue (Invitrogen). In all experiments, vitality of cells was unaffected and was >90%, unless indicated otherwise. Overall RNA production was measured using Nanodrop 1000 (Thermo Scientific, Wilmington, DE). Proliferation of cells was assessed by methyl-[3H]thymidine 185 MBq (5 mCi) assay (Hartmann Analytic, Braunschweig, Germany), according to the manufacturer’s instructions.

**Real-time PCR**

RNA was isolated with an RNeasy Mini Kit (Qiagen, Hilden, Germany), and reverse transcription was performed using QuantiTect Reverse Transcription (Qiagen), according to the manufacturer’s instructions. PCR with real-time measurement of fluorescence was carried out on the 7300 Real-time PCR system (Applied Biosystems, Foster City, CA) with 0.3 μM gene-specific, exon-spanning primers (Table II, Tib Molbiol, Berlin, Germany) in at least triplicates using a QuantiTect SYBR green PCR Kit (Qiagen). Results were quantified relative to GAPDH (Table II, Tib Molbiol) as an internal reference and relative to reference RNA (Stratagene, La Jolla, CA) as an external standard, using the ∆∆CT method.

**ELISA**

Quantikine human GM-CSF ELISA (R&D Systems, Wiesbaden, Germany) and IL-6 and IL-1 ELISA (R&D Systems, Minneapolis, MN) were used to measure proteins in the supernatants of cell cultures, according to the manufacturers’ instructions. Determinations were carried out at least in triplicates. Absorbance was measured at 450 nm using the ANASO 2001 ELISA reader (Anthus Mikrosysteme, Krefeld, Germany). Human recombinant casein α (kindly provided by Y.K. Kim, Korea Research Institute of Bioscience) (36) was used for production of a polyclonal antisemirum in rabbit (ModiQuest, Nijmegen, The Netherlands). For detection of CSN1S1 by ELISA, microtiter plates were coated with 0.3 μg/mL anti-CSN1S1 in coating buffer containing 10.6 mg/ml Na2CO3 and 8.4 mg/ml NaHCO3, adjusted to pH 9.6, overnight at 4°C. Plates were washed three times in PBS, blocked with 2% BSA in PBS & Tween for 1 h, and then washed three times with PBS and Tween. Supernatants were added undiluted for 24 h at 4°C, 0.3 μg/mL anti-CSN1S1 in PBS plus Tween supplemented with 2% BSA was added for 1 h at room temperature. After additional washing with PBS and Tween, appropriate amounts of streptavidin (DakoCytomation, Glostrup, Denmark) were added for 1 h, and the plates were washed with PBS, 3.5,5’-5’-tetramethylbenzidine (Sigma T 8665) was added for 30 min before stopping the reaction with H2SO4, and the absorption was read at 450 nm.

**Immunocytochemistry**

Mono Mac 6 cells were incubated at a density of 0.5 × 10^5/mL for 1 h with or without 10 μg/mL rCSN1S1 in microtiter plates. Cells were washed twice with PBS (Invitrogen) in 5-ml tubes (BD Biosciences), transferred onto glass slides, and fixed in 3% paraformaldehyde for 15 min at room temperature. After washing for 10 min in PBS and 2 min in Tris buffer (pH 7.4), slides were transferred to a humid chamber. Peroxidase-blocking reagent (DakoCytomation, Ely, U.K.) was added for 10 min, followed by washing with Tris. Finally, Ab diluent and an HRP-labeled anti-His-tag Ab (Dianova, Hamburg, Germany) diluted 1:1000 were added for 30 min before visualization of the HRP reaction with Liquid diaminobenzidine + substrate chromogen system (DakoCytomation). Photographs were taken using and Axioskop 2 plus and AxioCam (Carl Zeiss, Jena, Germany, Intas Science Imaging Instruments, Götttingen, Germany).

**Immunofluorescence**

Freshly isolated and washed primary monocytes from buffy coat leukocytes and HL60 cells suspended in PBS were transferred to glass slides and fixed in 3.5% paraformaldehyde for 15 min at room temperature. After washing for 10 min in PBS and 2 min in Tris buffer, pH 7.4, slides were transferred to a humid chamber. Peroxidase-blocking reagent (DakoCytomation, Ely, U.K.) was added for 10 min, followed by washing with Tris. Finally, Ab diluent and an HRP-labeled anti- His-tag Ab (Dianova, Hamburg, Germany) diluted 1:1000 were added for 30 min before visualization of the HRP reaction with Liquid diaminobenzidine + substrate chromogen system (DakoCytomation). Photographs were taken using and Axioskop 2 plus and AxioCam (Carl Zeiss, Jena, Germany, Intas Science Imaging Instruments, Götttingen, Germany).

### Table 1. Inhibitors of signal transduction

<table>
<thead>
<tr>
<th>Second Messenger</th>
<th>Function</th>
<th>Inhibitor</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein kinase B</td>
<td>Regulation of cell survival and proliferation</td>
<td>Akti-1/2</td>
<td>(54)</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transduction of multiple cytokines (e.g., IL-6, IL-10)</td>
<td>PPYLY5T-KTS</td>
<td>(55)</td>
</tr>
<tr>
<td>P38 MAP</td>
<td>Regulation of cellular differentiation and response to stress</td>
<td>ML3403</td>
<td>(56)</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Regulation of development, adaptive, and immune responses</td>
<td>6-amino-4-(4'-phosphonyl)aminobenzamide</td>
<td>quinazoline</td>
</tr>
<tr>
<td>JAK2</td>
<td>Associates with cytokine receptors like GM-CSF family</td>
<td>Hexabromocyclodexhane</td>
<td>(57, 58)</td>
</tr>
<tr>
<td>Protein kinase A</td>
<td>Regulation of immune response by inhibition of Ag-induced T and B cell activation</td>
<td>Myr-GRTGRRNA-IIH2</td>
<td>(59)</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Regulation of cell proliferation and apoptosis</td>
<td>PD98059</td>
<td>(60)</td>
</tr>
</tbody>
</table>
Data presentation

Data are presented as error bars representing mean and SD or dot-plots representing single measurements. Data comparison was carried out using the two-sided t test; \( p < 0.05 \) was considered significant.

Results

Detection of CSN1S1 in leukocytes

To determine whether CSN1S1 is expressed in human peripheral blood leukocytes, blood of five healthy donors was used to isolate CD14+ monocytes, CD4+ and CD8+ lymphocytes, and CD19+ B cells. Total RNA was isolated, and the amount of specific mRNA was quantified by RT-PCR using sequence-specific primers (Table II). Expression of CSN1S1 mRNA was detectable in CD14+, CD4+, and CD8+ cells, with some variations, but not in CD19+ B cells (Fig. 1a). In the next step, we intended to determine whether expression of the protein is also found in cells and tested the human promyelocytic leukemia cell line HL60, the histiocytic lymphoma cell line U937, and the acute monocytic leukemia cell lines THP1 and Mono Mac 6. Equal amounts of CSN1S1 mRNA were found in HL60, U937, and THP1 cells, but no specific mRNA was detectable in Mono Mac 6 cells (Fig. 1a). To analyze CSN1S1 protein secretion by cells producing CSN1S1-specific mRNA, a polyclonal anti-CSN1S1 Ab was used to perform an ELISA with supernatants of cultured primary human CD14+ monocytes, CD4+ and CD8+ lymphocytes, and CD19+ B cells. Detectable amounts of protein were found in the supernatants of CD14+ monocytes only (Fig. 1b). Similarly, supernatants of the cultured leukocytic cell lines HL60, U937, THP1, and Mono Mac 6 were tested. CSN1S1 protein was detected in HL60 and U937 cells but not in THP1 or Mono Mac 6 cells (Fig. 1b). Because of interindividual variations in the amount of CSN1S1 mRNA expressed by monocytes and lymphocytes (Fig. 1a), we were interested in determining whether stimulation of cells may lead to modulation of CSN1S1 gene expression and protein secretion. Therefore, CD14+ monocytes ofuffy coat leukocytes were stimulated with 100 ng/ml LPS, 50 ng/ml TNF-\( \alpha \), or 1000 U/l IFN-\( \gamma \) for 24 h, and specific mRNA production and protein secretion into supernatants were assessed by PCR and ELISA, respectively (Fig. 1c). There was a significant increase in CSN1S1 mRNA following stimulation with LPS, whereas no apparent changes were observed for TNF-\( \alpha \), and a nonsignificant decrease occurred after IFN-\( \gamma \) stimulation. Similarly, CSN1S1 protein was detectable in significantly increased amounts in the supernatants of LPS-stimulated cells, whereas decreased concentrations were observed after TNF-\( \alpha \) and IFN-\( \gamma \) stimulation (Fig. 2c). Furthermore, there was no protein detectable by ELISA in the supernatants of naive or stimulated CD4+ and CD8+ cells, and no change in mRNA levels was observed following stimulation (data not shown).

Table II. Primer sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5'–3'</th>
</tr>
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<tbody>
<tr>
<td>GM-CSF</td>
<td>Fw: CACTGCTGTGAGATGAAATG</td>
</tr>
<tr>
<td></td>
<td>Rv: AATCTGGGTTGACACAGGAG</td>
</tr>
<tr>
<td>CSF2RA</td>
<td>Fw: CCCCCCTCTCGAGACAGCAC</td>
</tr>
<tr>
<td></td>
<td>Rv: TACCTGAGCGCTGGTTCCAC</td>
</tr>
<tr>
<td>CSN1S1</td>
<td>Fw: TCCAGCACTACAGTTCAGGAG</td>
</tr>
<tr>
<td></td>
<td>Rv: CTGGAAGAGCACGTGACAT</td>
</tr>
<tr>
<td>IL-6</td>
<td>Fw: CAGGGGTTGATTCATCGCCT</td>
</tr>
<tr>
<td></td>
<td>Rv: GGGCTCAAGAAGAAAGATC</td>
</tr>
<tr>
<td>IL-1b</td>
<td>Fw: TTCTGCTGTAGAGGTGCTG</td>
</tr>
<tr>
<td></td>
<td>Rv: CCAGCGACGAGACATCGG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Fw: ATGAGCCACCCAGG</td>
</tr>
<tr>
<td></td>
<td>Rv: CCTCACAGCTCCCTCAC</td>
</tr>
</tbody>
</table>

To confirm intracellular expression of CSN1S1 protein, permeabilized HL60 cells and primary human monocytes were incubated with a primary polyclonal anti-CSN1S1 Ab, followed by a secondary Alexa Fluor 488-labeled Ab (Fig. 3), and confocal laser scanning microscopy was carried out. By this means, both cell types showed a predominant presence of intracellular CSN1S1 in...
the cytoplasmic compartment, with some nuclear staining observed in HL60 cells. There was no staining detectable if only the secondary Ab or an appropriate isotype control was used.

**CSN1S1 mediates upregulation of GM-CSF in a time- and dose-dependent fashion in monocytic cells**

In the next step, we intended to analyze a putative function of CSN1S1 and, therefore, tried to assess its ability to upregulate the expression of the proinflammatory cytokine GM-CSF, as well as the corresponding receptor subunit α (human CSF2 receptor α alias GM-CSF receptor α [CSF2RA]). Mono Mac 6 cells, which showed no production of CSN1S1 mRNA or protein, were stimulated with different concentrations of rCSN1S1 in the presence of polymyxin to exclude any LPS-mediated effect on gene expression (37–39). GM-CSF mRNA was monitored by RT-PCR, and GM-CSF protein in the cell supernatants was analyzed by ELISA. Although only minute amounts of mRNA and protein were detectable with nonstimulated cells, a significant concentration-dependent upregulation of GM-CSF mRNA and protein was detectable after the addition of rCSN1S1 (Fig. 2a). Similarly, CSF2RA mRNA was increased in a concentration-dependent manner in stimulated cells (Fig. 2b). To determine whether the upregulation of GM-CSF was time dependent, Mono Mac 6 cells were stimulated with rCSN1S1 for different periods of time before RT-PCR and ELISA experiments were performed. Although the production of the specific mRNA peaked after 6 h of stimulation and declined thereafter, the amount of protein increased constantly. Again, nonstimulated cells showed only little GM-CSF expression throughout the experiment (Fig. 2c). A significant increase in CSF2RA mRNA was detectable at 48 h (Fig. 2d).

**CSN1S1 upregulates the expression of GM-CSF in human monocytes**

Consequently, we were interested in whether CSN1S1, in addition to its effect on a monocytic cell line, has a stimulating effect on GM-CSF expression in primary human CD14+ monocytes. To this end,uffy coat CD14+ monocytes were incubated with different concentrations of rCSN1S1 in the presence of polymyxin. Cells...
were harvested after 24 or 48 h, and mRNA was analyzed by RT-PCR or protein in supernatants by ELISA (Fig. 4). Nonstimulated cells showed scarce expression of GM-CSF mRNA. rCSN1S1 markedly upregulated GM-CSF mRNA amounts. This was also the case for LPS, with the maximum effect noted with 200 ng/ml. Significantly higher amounts of specific mRNA were measured following stimulation with 0.01 mg/ml rCSN1S1 in comparison with the maximum stimulatory effect of LPS at a concentration of 200 ng/ml LPS (Fig. 5). Additionally, the vitality of cells was assessed by tetrazolium assay and staining of cells with trypan blue. Vitality was >90% up to a concentration of 200 ng/ml LPS, when maximum stimulation of GM-CSF expression was noted; it declined thereafter (mean: 86% at 400 ng/ml; 80% at 600 ng/ml; 76% at 1000 ng/ml). Then we assessed whether blockage of LPS would result in neutralization of rCSN1S1’s effects on GM-CSF mRNA expression by adding 30 μg/ml polymyxin B, which binds and neutralizes LPS, to the culture medium (Fig. 5). The addition of polymyxin led to a complete absence of GM-CSF mRNA in LPS-stimulated Mono Mac 6 cells. In contrast, rCSN1S1 stimulation caused a significant upregulation of GM-CSF mRNA in the presence of polymyxin (Fig. 5).

**FIGURE 4.** Human buffy coat monocytes, isolated by positive selection using CD14-marked magnetic beads, were stimulated with the indicated concentrations of rCSN1S1 for 24 or 48 h. a, Expression of GM-CSF mRNA were assessed by real-time PCR (left y-axis), and amounts of GM-CSF peptide in the supernatants were measured by ELISA (right y-axis). Significant differences between unstimulated and stimulated cells, as determined by the t test, are indicated: *p < 0.05; †p < 0.0005. b, Expression of CSF2RA.

CSN1S1 upregulates IL-1 and IL-6 in monocytic cells

Because GM-CSF was upregulated following CSN1S1 stimulation of monocytic cells, we hypothesized that other proinflammatory cytokines may be regulated by CSN1S1 stimulation as well. To this end, human CD14+ monocytes and Mono Mac 6 cells were incubated for 24 h with 10 μg/ml rCSN1S1. Specific mRNA and protein secretion into the supernatants were measured by RT-PCR and ELISA, respectively. Marked increases in IL-1 and IL-6 mRNA were observed in stimulated monocytes and Mono Mac 6 cells (Fig. 6). Furthermore, IL-1 and IL-6 were barely detectable in supernatants of unstimulated cells, but they showed a marked increase following CSN1S1 stimulation, which was statistically significant in all cells except for IL-6 ELISA in Mono Mac 6 cells; however, a tendency toward higher concentration was observed (Fig. 6).

CSN1S1 inhibits proliferation and overall RNA expression in monocytic cells

In the next step, it was assessed whether the cell proliferation of Mono Mac 6 cells was altered in response to rCSN1S1 and by different concentrations of LPS and whether these effects could be differentially influenced by the addition of polymyxin, as suggested by the above results. For this purpose, cells were incubated for 72 h with rCSN1S1 in the presence or the absence of LPS-blocking polymyxin to control for any LPS-mediated effect (Fig. 7a). Proliferation was measured by [3H]thymidine incorporation. In the absence of polymyxin, the addition of rCSN1S1 to Mono Mac 6 cells led to a significant reduction in [3H]thymidine incorporation, indicating a reduced cell proliferation. This was also the case for cells incubated with high (200 ng/ml) or low (2.5 ng/ml) concentrations of LPS. When 30 μg/ml polymyxin was added to culture medium, the suppressive effect of LPS on cell proliferation was completely abolished. However, the addition of polymyxin had no effect on the reduction of cell proliferation induced by rCSN1S1. To determine the status of cellular-expression activity following CSN1S1 stimulation, Mono Mac 6 cells were incubated with rCSN1S1 or LPS in the presence or absence of polymyxin (Fig. 7b), and total RNA content was measured by Nanodrop. Without the addition of polymyxin, total RNA content markedly decreased following stimulation with rCSN1S1 or different concentrations of LPS, whereas polymyxin addition completely abolished this effect (Fig. 7b).
centrations of LPS. This was less pronounced with the lowest concentration of LPS used (2.5 ng/ml). Similarly, as observed above for the reduction in cell proliferation, the addition of polymyxin to the culture medium abrogated the LPS-induced effects, whereas the effect of rCSN1S1 on the decrease in total RNA content was sustained. Altogether, the elimination of LPS-mediated effects by polymyxin and the sustained cellular response to rCSN1S1, despite polymyxin, excluded that contaminating LPS is responsible for the results observed after exposure of the cells to CSN1S1.

**CSN1S1 is bound by monocytic cells**

In a first experiment to elucidate whether the effect of CSN1S1 could be mediated by targeting a cell-surface receptor, we analyzed its binding to monocytic cells. Mono Mac 6 cells were incubated with His-tagged rCSN1S1 and subsequently stained with an HRP-coupled anti–His-tag Ab (Fig. 8a). Cells that were not incubated with rCSN1S1 before addition of the anti–His-tag Ab served as a negative control (Fig. 8b). As can be seen in Fig. 8, binding of rCSN1S1 was detectable in the majority of cells (Fig. 8a), whereas no signal was found in cells without CSN1S1 incubation (Fig. 8b).

**CSN1S1-mediated upregulation of GM-CSF is blocked by p38 MAPK inhibitor**

In an attempt to identify a putative signal-transduction pathway responsible for CSN1S1-mediated upregulation of GM-CSF, Mono Mac 6 cells were incubated with rCSN1S1 in the presence of diverse inhibitors of second messengers known to be involved in pathways of immunomodulation or cellular differentiation (Table I). Polymyxin was added to the culture medium to exclude any effects of LPS. GM-CSF mRNA was measured by RT-PCR, and the corresponding protein was determined by ELISA in the cell supernatants. Addition of ML3403, a specific inhibitor of p38 MAPK, caused a significant and dose-dependent downregulation of GM-CSF mRNA expression, which reached statistical significance with the higher concentration of ML3403 used (Fig. 9a). Similarly GM-CSF protein measurements in the supernatants were lower in the presence of ML3403 in Mono Mac 6 cells (Fig. 9a). There was no change in GM-CSF mRNA expression with any of the other inhibitors tested compared with stimulation with rCSN1S1 alone (Fig. 9b). To determine whether rCSN1S1-mediated upregulation of GM-CSF in primary human monocytes is also reduced by ML3403, buffy coat CD14+ monocytes were incubated with rCSN1S1 in the presence of ML3403. Again, GM-CSF mRNA and protein content in the supernatants were measured. A dose-dependent inhibition of GM-CSF mRNA upregulation was detectable when cells were incubated with ML3403. This was paralleled by a reduced GM-CSF protein content in the supernatants, which were found to be significantly lower in the presence of ML3403 (Fig. 9c). To confirm activation of the p38 MAPK pathway by CSN1S1, Mono Mac 6 cells were stimulated for 24 h with 10 μg/ml rCSN1S1 in the presence of polymyxin, and Western blotting of cell lysates was carried out using an anti-phospho p38-MAPK Ab (P-p38) and an Ab staining p38-MAPK, irrespective of the phosphorylation status (p38) (Fig. 9d). Although the p38 Ab stained lysates of stimulated and unstimulated cells equally, the P-p38 Ab exclusively stained lysates of cells stimulated with CSN1S1, providing evidence for the activation of the p38 MAPK pathway.

**Discussion**

Proteins of the casein family can be found in most mammalian species (2). Consistent with the notion that their function is mainly nutritional (5, 6), the sole site of expression was believed to be the mammary gland. The organization and orientation of the diverse casein genes within their genomic cluster is highly conserved (31). Fractions of casein peptides, gained by in vivo or in vitro fermentation to simulate digestive processes, were shown to possess immunomodulatory and other functions, including...
opioid-like activity and inhibition of the angiotensin-converting enzyme (1, 7–9). This is consistent with the assumption that caseins have evolved to exhibit immunomodulatory and nutritional properties, which were derived from the fact that the casein genes lie within a region of genes relevant for both tasks (31). Observations on the expression of CSN1S1 outside of the mammary gland (28) led us to hypothesize that the intact, unfermented peptide might be expressed by human cells relevant to the immune system.

We initially demonstrated the expression of specific mRNA, the presence of CSN1S1 in the supernatants of CD14+ monocytes and leukocytic cell lines, and the intracellular presence of the protein in monocytes. The use of exon-spanning, gene-specific primer pairs made amplification of a sequence other than CSN1S1 highly unlikely. Although specific mRNA was detected in T cells and monocytes, the protein was detectable in the supernatants of monocytes only. This might be explained by a low sensitivity of our assay or, among the cells tested, monocytes may solely secrete the peptide. Furthermore, based on the mRNA quantification, the protein level might have been expected to be greater. Variations in extracellular processing rendering the protein undetectable for our Ab or inhibitors of protein translation, such as the presence of microRNA, are possible explanations. Interestingly, in Mono Mac 6 cells, which display a predominantly monocytic phenotype, neither CSN1S1 mRNA nor peptide was detected, whereas the promyelocytic and histiocytic cell lines, HL60 and U937, respectively, displayed both. On one hand, unphysiological gene transcription and translation in tumor cell lines could be responsible for this finding. On the other hand, it is tempting to speculate that CSN1S1 gene expression may vary according to the stage of cellular differentiation within monocytic cells. However, further evidence is needed to confirm either hypothesis. Additionally, HL60 cells and CD14+ monocytes, which were deemed to produce the protein based on the results of the RT-PCR and ELISA experiments, could be stained with an anti-CSN1S1 Ab. Overall, these results suggest that CSN1S1 may be produced by human monocytes.

To study CSN1S1-mediated effects, we used high concentrations of rCSN1S1 compared with the concentration detected in the supernatants of untreated monocyte cultures at 24 h. It is unknown...
whether the concentrations used for stimulation are reached in vivo. The use of high concentrations of rCSN1S1 may be justified by the fact that, by this means, effects on cells can be studied more easily. Furthermore, CSN1S1 concentrations may be higher in certain tissues or microenvironments, which is suggested by the finding of increased CSN1S1 expression in prostate hyperplasia or in lymph nodes of mice suffering from encephalomyelitis (28, 29). Various aspects of the mechanisms by which CSN1S1 acts on the cells. First, we demonstrated binding of CSN1S1 on monocytic cells; however, this finding does not prove the presence of specific binding sites, because unspecific attachment may occur. Early experiments using bovine casein and human and animal leukocytes and other cell types suggested that casein binding receptors exist (13–15, 47), but this finding cannot easily be transferred to human caseins because sequence homology is weak (31). For instance, the identity between the human CSN1S1 mRNA (accession code NM_001890; http://www.ncbi.nlm.nih.gov/Genbank) and the homologs of bovine (X00564), rat (NM_138874), and mouse (NM_007784) is 57.6, 49.8, and 47.8%, respectively (determined by the European Molecular Biology Open Software Suite Align tool at the European Bioinformatics Institute, http://www.ebi.ac.uk). Evidence about activation of specific intracellular mechanisms stems from our observation that inhibition of CSN1S1-mediated effects (e.g., upregulation of GM-CSF mRNA and protein) is accomplished by the addition of a p38 MAPK inhibitor but not any of the other inhibitors tested. Additionally, an anti–p-p38 MAPK Ab stained Mono Mac 6 cell lysates following CSN1S1 stimulation, whereas no staining was detectable by Western blotting in unstimulated cell lysates following stimulation with LPS. Finally, although all cells were treated essentially the same, we cannot exclude that activation of cells, rather than interindividual variation, may be a cause of varying CSN1S1 mRNA levels observed in untreated monocytic cultures of healthy donors (Fig. 1a).

Because of known immunomodulatory effects of bovine caseins (7), we were further interested in the potential effects of human CSN1S1 on immune cells and investigated human monocytes and Mono Mac 6 cells, which did not express the CSN1S1 gene in our experiments. GM-CSF plays a key role in inflammatory and autoimmune disorders (32–34, 40–43). It is produced by a variety of cells, including monocytes, and mainly acts on granulocytes, macrophages, and monocytes (34). There is ongoing research about the stimuli controlling GM-CSF expression (34). Interestingly, bovine caseins were shown to induce the secretion of M-CSF in murine myeloid cells (18). The present data demonstrated that human CSN1S1 leads to increased expression and secretion of GM-CSF in human monocytic cells and monocytes. Additionally, monocytic cells showed decreased proliferational activity and displayed reduced total RNA content, which suggests reduced overall gene expression. It is tempting to speculate that these observations hint at a differentiation of monocytic cells, induced by CSN1S1, to take up specialized tasks. In fact, these changes may be due to CSN1S1 or be mediated by the induction of GM-CSF expression and subsequent autocrine stimulation of monocytic cells. The latter is suggested by the observation that human blood monocytes differentiate into dendritic or macrophage-like cell types when stimulated with GM-CSF (33, 44, 45). Furthermore, direct stimulation of monocytes with GM-CSF induced upregulation of the otherwise constitutively expressed CSF2RA (46). After stimulation with CSN1S1, expression of the GM-CSF receptor CSF2RA was increased in Mono Mac 6 cells, but not in primary human monocytes, in which a rather constitutive expression was observed. Possible explanations for these findings are that stimulation with CSN1S1 acts by a different mechanism on CSF2RA gene expression than direct stimulation by GM-CSF. Additionally, activation of inflammatory mechanisms in monocytic cells by CSN1S1 is further supported by the finding that IL-1β and IL-6, both well-known proinflammatory mediators, are upregulated as well.

Following these observations, we wanted to gain insight into the mechanisms by which CSN1S1 acts on the cells. First, we demonstrated binding of CSN1S1 on monocytes; however, this finding does not prove the presence of specific binding sites, because unspecific attachment may occur. Early experiments using bovine casein and human and animal leukocytes and other cell types suggested that casein binding receptors exist (13–15, 47), but this finding cannot easily be transferred to human caseins because sequence homology is weak (31). For instance, the identity between the human CSN1S1 mRNA (accession code NM_001890; http://www.ncbi.nlm.nih.gov/Genbank) and the homologs of bovine (X00564), rat (NM_138874), and mouse (NM_007784) is 57.6, 49.8, and 47.8%, respectively (determined by the European Molecular Biology Open Software Suite Align tool at the European Bioinformatics Institute, http://www.ebi.ac.uk). Evidence about activation of specific intracellular mechanisms stems from our observation that inhibition of CSN1S1-mediated effects (e.g., upregulation of GM-CSF mRNA and protein) is accomplished by the addition of a p38 MAPK inhibitor but not any of the other inhibitors tested. Additionally, an anti–p-p38 MAPK Ab stained Mono Mac 6 cell lysates following CSN1S1 stimulation, whereas no staining was detectable by Western blotting in unstimulated cells, confirming our results. Based on our findings that CSN1S1 binds to the surface of monocytes and mediates phosphorylation of p38 MAPK and that CSN1S1’s effects are inhibited by the addition of the p38 MAPK inhibitor ML3403, which binds to activated and inactivated p38 MAPK (48), a receptor-mediated indirect activation of the p38 MAPK pathway is a conceivable mechanism of CSN1S1 signal transduction. Interestingly, the p38 MAPK is a critical mediator of the inflammatory response (49) and has a role in LPS-, TNF-α-, and IL-1–mediated upregulation of GM-CSF (50–53).

Because recombinant proteins may be contaminated with LPS, we had to exclude that the observed effects were mediated by LPS rather than rCSN1S1. We demonstrated that the addition of polymyxin, a naturally occurring antibiotic with potent LPS-blocking properties (37–39), to the culture medium completely inhibited the effects of LPS on GM-CSF gene expression. This was also true for the LPS concentration with the greatest stimulatory effect (200 ng/ml). Furthermore, rCSN1S1 led to a significantly greater upregulation of GM-CSF gene expression than did 200 ng/ml LPS, whereas 0.01 mg/ml rCSN1S1 was determined to contain only 0.7 ± 0.31 ng/ml LPS. Finally, a significant increase in GM-CSF mRNA was demonstrated after stimulation with rCSN1S1, despite the presence of polymyxin. Thus, the effects observed were specifically related to rCSN1S1 rather than contaminating LPS.

FIGURE 8. Stimulation of Mono Mac 6 cells for 1 h with His-tagged rCSN1S1 and then with HRP-marked anti–His-tag Ab. Brown dots indicate binding of CSN1S1 to Mono Mac 6 cells (a) but not to cells without incubation of CSN1S1 before the addition of the secondary Ab (negative control) (b). Original magnification ×360.
In conclusion, we collected evidence that CSN1S1, in addition to its nutritional properties in milk, may be a leukocyte-derived peptide and possess immunomodulatory properties.

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Disclosures
The authors have no financial conflicts of interest.

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