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

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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: 592–601.**

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 fragments 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

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Abbreviations used in this paper: CSF2RA, human CSF2 receptor α alias GM-CSF receptor α; CSN1S1, human casein α s1; DIC, differential interference contrast; MM6, Mono Mac 6; Mo, monocytes; P-p38, anti-phospho p38-MAPK Ab; rCSN1S1, recombinant human casein α S1.

from buffy coat leukocytes, as indicated. Cell subsets were separated by magnetic cell sorting with the following beaded 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 1×10^6/ml, except for Mono Mac 6 cells, which were seeded at 0.5×10^6/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 (CellSciences, 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, 1×10^6/ml monocytes or 0.5×10^6/ml Mono Mac 6 cells were seeded onto culture plates. LPS contamination was determined by Endoclear *Limulus* Amebocyte Lysate Chromogenic 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-[^{3}H]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 incubated with an Ab against p38-MAPK (GeneTex, Eching, Germany) or an Ab against phosphorylated p38-MAPK (AIBN361512; Abs-online, Aachen, Germany), each diluted 1:1,000 overnight at 4°C. After appropriate washing procedures, the membranes were incubated with a 1:10,000 dilution of HRP-conjugated anti-rabbit IgG (Sigma-Aldrich). Proteins were visualized via ECL substrate (Santa Cruz Biotechnology, Santa Cruz, CA) and detected by a CCD camera (Intas Chemilux ECL Imager, Intas Science Imaging Instruments, Göttingen, Germany).

Real-time PCR

RNA was isolated with an RNeasy Mini Kit (Qiagen, Hilden, Germany), and reverse transcription was performed using QantiTect 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 QantiTect 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 –ΔΔCT 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 Anthos 2001 ELISA reader (Anthos 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 antiserum 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 Na_2CO_3 and 8.4 mg/ml $NaHCO_3$, 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. 3,5,3′,5′-tetramethylbenzidine (Sigma T 8665) was added for 30 min before stopping the reaction with H_2SO_4, and the absorption was read at 450 nm.

Immunocytochemistry

Mono Mac 6 cells were incubated at a density of 0.5×10^6/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, Gö ttingen, 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 (pH 7). After washing for 10 min in PBS and 0.1% Triton X-100 and additional washing procedures in PBS, cells were incubated for 1 h with a primary anti-CSN1S1 Ab from rabbit (MaxPab Rabbit anti-CSN1S1, Abnova, Taipei, Taiwan) and Rabbit IgG (DakoCytomation) or PBS as negative controls. After appropriate washing procedures with PBS, 1.3 μg/ml a secondary, Alexa Fluor 488-marked goat-anti-rabbit Ab and TO-PRO-3 (Invitrogen) were applied for 30 and 15 min, respectively. Images of intracellular indirect staining of CSN1S1 were obtained with a confocal laser scanning microscope (Fluoview 2.0, IX70, inverted microscope; Olympus, Melville, NY) using a ×60 oil objective (UPlanFl; Olympus). Alexa Fluor 488 and the DNA stain TO-PRO-3 were excited at 488 and 647 nm, and emission was detected between 510 and 550 nm and at 660 nm, respectively.

Table I. *Inhibitors of signal transduction*

Second Messenger	Function	Inhibitor	Ref.
Protein kinase B	Regulation of cell survival and proliferation	Akti-1/2	(54)
STAT3	Signal transduction of multiple cytokines (e.g., IL-6, IL-10)	PpYLKTK-mts	(55)
P38 MAPK	Regulation of cellular differentiation and response to stress	ML3403	(56)
NF-κB	Regulation of development, adaptive, and immune responses	6-amino-4-(4-phenoxyphenylethylamino) quinazoline	(56)
JAK2	Associates with cytokine receptors like GM-CSFR family	Hexabromocyclohexane	(57, 58)
Protein kinase A	Regulation of immune response by inhibition of Ag-induced T and B cell activation	Myr-GRTGRRNAI-NH2	(59)
ERK1/2	Regulation of cell proliferation and apoptosis	PD98059	(60)

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. 1*a*). In the next step, we intended to determine whether expression of the protein is also found in cell lines 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. 1*a*). 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. 1*b*). 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. 1*b*). Because of interindividual variations in the amount of CSN1S1 mRNA expressed by monocytes and lymphocytes (Fig. 1*a*), we were interested in determining whether stimulation of cells may lead to modulation of CSN1S1 gene expression and protein secretion. Therefore, $CD14^+$ monocytes of buffy coat leukocytes were stimulated with 100 ng/ml LPS, 50 ng/ml TNF-α, or 1000 U/l IFN-γ for 24 h, and specific mRNA production and protein secretion into supernatants were assessed by PCR and ELISA, respectively (Fig. 1*c*). There was a significant increase in CSN1S1 mRNA following stimulation with LPS, whereas no apparent changes were observed for TNF-α, and a nonsignificant decrease occurred after IFN-γ stimulation. Similarly, CSN1S1 protein was detectable in significantly increased amounts in the supernatants of LPS-stimulated cells, whereas decreased concentrations were observed after TNF-α and IFN-γ stimulation (Fig. 2*c*). Furthermore, there was no protein detectable by ELISA in the supernatants of native or stimulated $CD4^+$ and $CD8^+$ cells, and no change in mRNA levels was observed following stimulation (data not shown).

Table II. *Primer sequences*

Primer	Sequence 5′–3′
GM-CSF	Fw: CACTGCTGCTGAGATGAATGA
	Rv: AATCTGGGTTGCACAGGAAG
CSF2RA	Fw: CCCTTCTCTCTGACCAGCAC
	Rv: TTACTGAGCCTGGGTTCCAC
CSN1S1	Fw: TCCAGCATCAGTTCATCGAG
	Rv: CTGGAAAGGCACTTGGACAT
IL-6	Fw: CCTTCCAAAGATGGCTGAAA
	Rv: CAGGGGTGGTTATTGCATCT
IL-1b	Fw: GGGCCTCAAGGAAAAGAATC
	Rv: TTCTGCTTGAGAGGTGCTGA
GAPDH	Fw: CCAGCCGAGCCACATCGCTC
	Rv: ATGAGCCCCAGCCTTCTCCAT

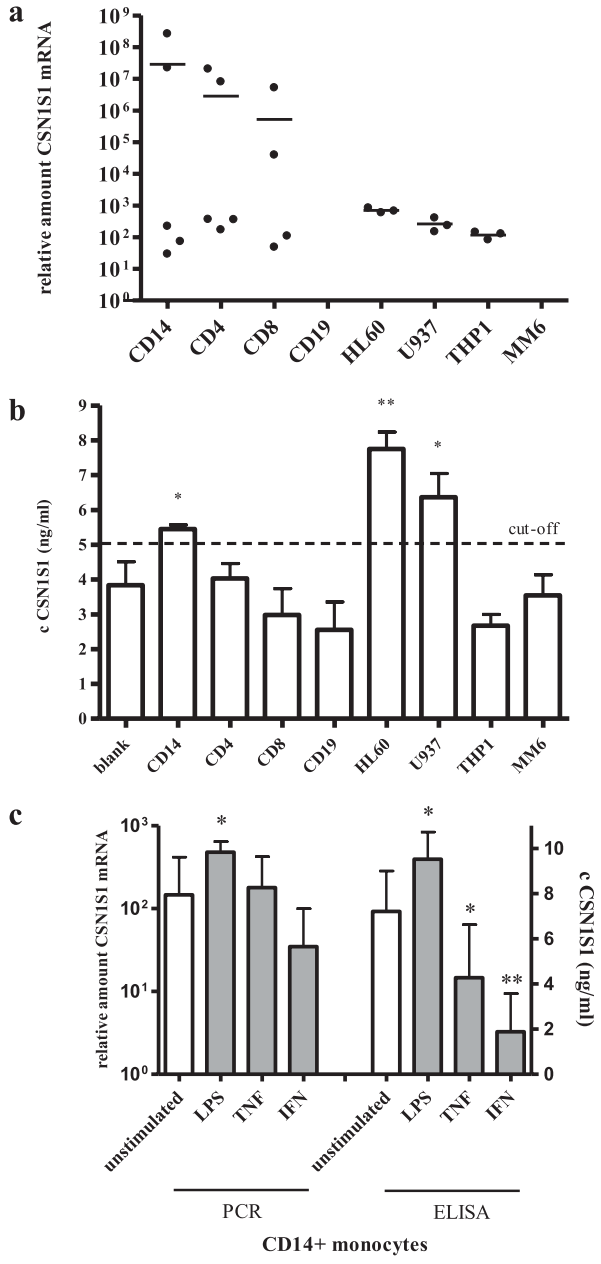

FIGURE 1. Measurement of CSN1S1 mRNA by RT-PCR (*a*) and protein by ELISA (*b*) in primary human cells of five healthy donors isolated via magnetic beaded Abs for CD14, CD4, CD8, and CD19 and in the leukocytic cell lines HL60, U937, THP1, and Mono Mac 6 (MM6). Supernatants and mRNA were collected after 24 h. In *a*, symbols represent mean individual measurements, and horizontal lines represent median value of the group. In *b*, the threshold of detection (mean + 2 times SD of the negative control [blank]) is depicted by the horizontal dashed line (cut-off). Means and SD are given. Significant elevations above the threshold, as determined by the *t* test, are indicated: $*p < 0.05$; $**p < 0.005$. *c*, PCR and ELISA of buffy coat naive (open bars) or stimulated (shaded bars) human CD14 cells (stimulated with 100 ng/ml LPS, 50 ng/ml TNF-α, or 1000 U/l IFN-γ for 24 h). Mean and SD of six experiments. Significant differences compared with unstimulated cells are indicated: $*p < 0.05$; $**p < 0.005$.

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

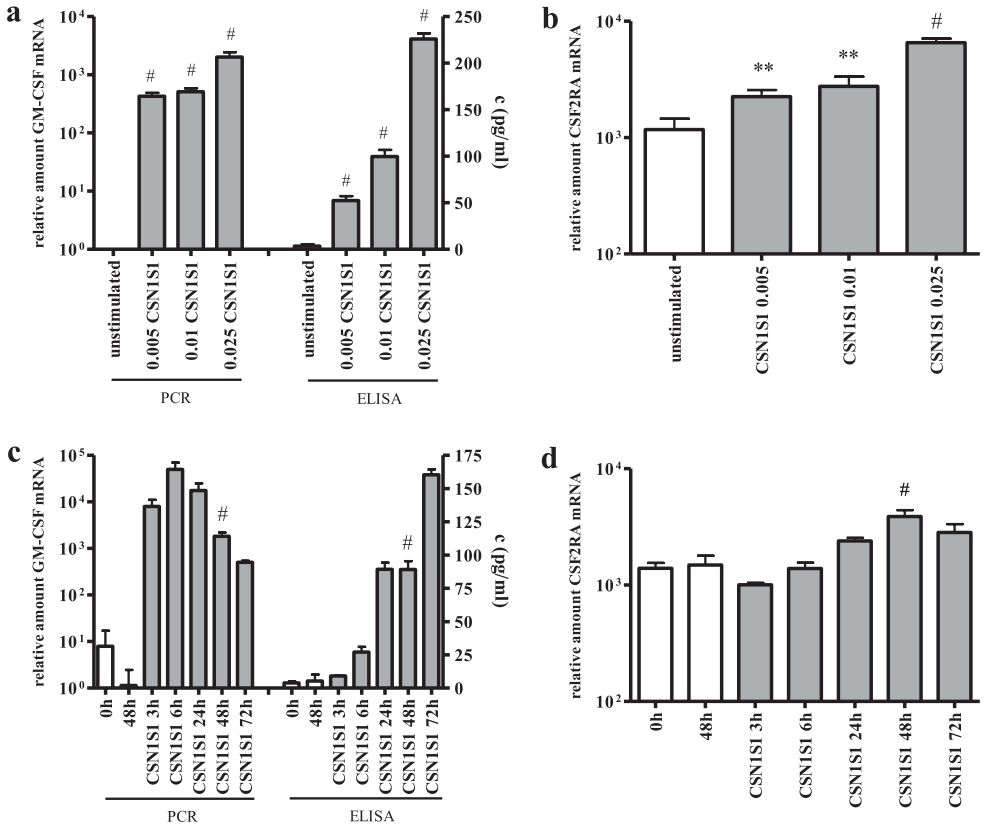

FIGURE 2. Mono Mac 6 cells were stimulated with 0.005, 0.01, or 0.025 mg/ml rCSN1S1. Cells were harvested after 72 h. *a*, Expression of GM-CSF mRNA was assessed by real-time PCR (PCR, *left y-axis*), and amounts of GM-CSF protein in the supernatants were measured by ELISA (*right y-axis*). *b*, Expression of GM-CSF receptor (CSF2RA) mRNA was measured by PCR. Mono Mac 6 cells were stimulated with 0.01 mg/ml CSN1S1 for the indicated periods of time. *c*, Expression of GM-CSF mRNA was assessed by PCR (*left y-axis*), and amounts of GM-CSF protein were assessed by ELISA (*right y-axis*). *d*, Expression of CSF2RA mRNA was measured by PCR. Values are given as mean plus SD. Stimulated cells were compared with unstimulated cells using the *t* test. $^{**}p < 0.005$; $^{\#}p < 0.0001$.

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. 2*a*). Similarly, CSF2RA mRNA was increased in a concentration-dependent manner in stimulated cells (Fig. 2*b*). 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. 2*c*). A significant increase in CSF2RA mRNA was detectable at 48 h (Fig. 2*d*).

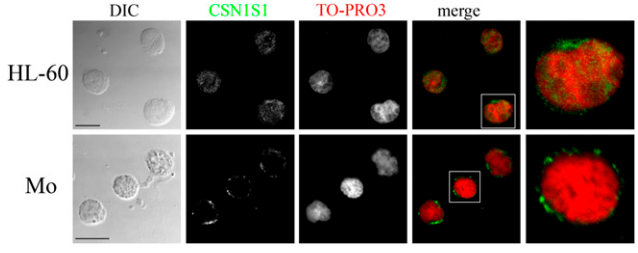

FIGURE 3. Confocal microscopy images of indirect immunofluorescence using a primary anti-human casein α s1 Ab from rabbit (CSN1S1) and a secondary Alexa Fluor 488-labled goat anti-rabbit Ab (green) in Triton X-100–permeabilized HL60 cells and primary $CD14^+$ monocytes (Mo). The DNA stain TO-PRO-3 (red) was used to demonstrate the predominant cytoplasmic staining. Merged images and magnifications of single cells are depicted. Microscopy was carried out on Olympus Fluoview 2.0, IX70, inverted microscope using a ×60 oil-immersion objective. Original magnification ×600. Scale bars, 12 μm. DIC, differential interference contrast.

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, buffy coat $CD14^+$ monocytes were incubated with different concentrations of rCSN1S1 in the presence of polymyxin. Cells

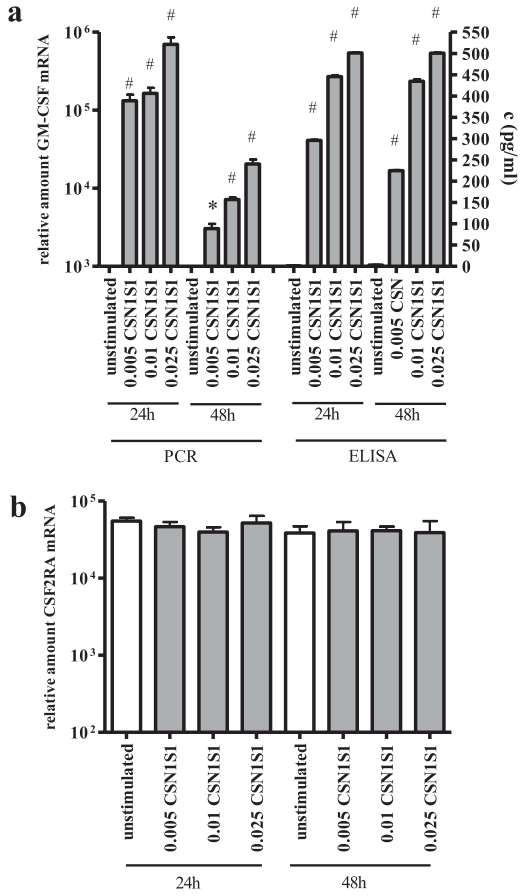

FIGURE 4. Human buffy coat monocytes, isolated by positive selection using CD14-marked magnetic beads, were stimulated with the indicated concentrations of rCSN1S1 for 24 or 48 h. *a*, Expression of GM-CSF mRNA were assessed by real-time PCR (*left y-axis*), and amounts of GM-CSF peptide in the supernatants 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.

were harvested after 24 or 48 h, and mRNA was analyzed by RT-PCR or protein in supernatants by ELISA (Fig. 4*a*). Although nonstimulated cells showed minimal presence of specific mRNA, a concentration-dependent upregulation of GM-CSF mRNA was measured after 24 h. Cells harvested after 48 h similarly showed a significant upregulation of GM-CSF mRNA, but to a lesser extent than after 24 h. In accordance with these results, measurements of GM-CSF protein in the supernatants indicated scarce expression in nonstimulated cells. rCSN1S1 led to increased GM-CSF protein measurements in supernatants after 24 and 48 h in a concentration-dependent manner. Additionally, the expression of CSF2RA in monocytes was determined by RT-PCR after rCSN1S1 stimulation. Irrespective of the concentration of rCSN1S1 used or the length of stimulation, CSF2RA mRNA was expressed at equal amounts (Fig. 4*b*).

CSN1S1-mediated upregulation of GM-CSF is not caused by LPS

Recombinant proteins produced in *Escherichia coli* may be contaminated with LPS to various degrees. Furthermore, LPS is known to stimulate upregulation of GM-CSF in leukocytes. Therefore, we excluded that the observed effects were caused by contamination with LPS rather than by a specific property of CSN1S1. First, the LPS content of rCSN1S1 was determined by the *Limulus* amebocyte lysate assay; 0.7 ± 0.31 ng/ml LPS in 0.01 mg/ml rCSN1S1 was found. Mono Mac 6 cells were incubated with 0.01 mg/ml rCSN1S1 or increasing concentrations of LPS, and the expression of GM-CSF mRNA was measured (Fig. 5). 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).

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. 7*a*). Proliferation was measured by [^{3}H]thymidine assay. In the absence of polymyxin, the addition of rCSN1S1 to Mono Mac 6 cells led to a significant reduction in [^{3}H]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. 7*b*), and total RNA content was measured by Nanodrop. Without the addition of polymyxin, total RNA content markedly decreased following stimulation with rCSN1S1 or different con-

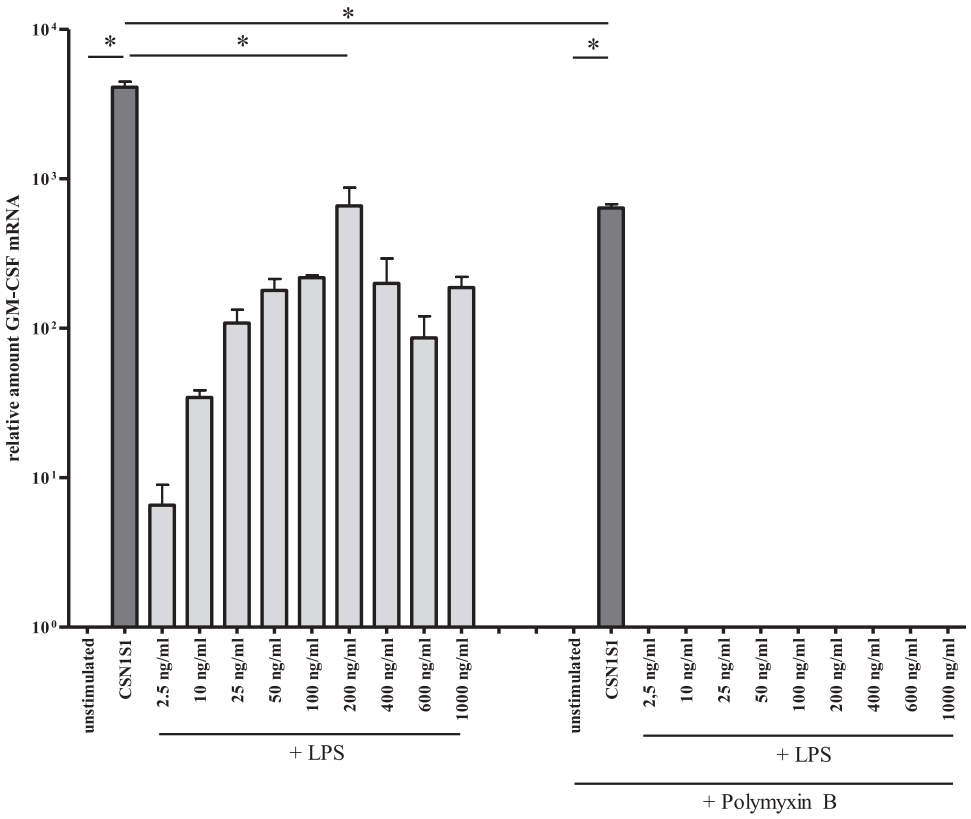

FIGURE 5. Relative amount of GM-CSF mRNA, as determined by real-time PCR in unstimulated Mono Mac 6 cells and cells stimulated with rCSN1S1 and indicated concentrations of LPS in the absence and presence of 30 μg/ml polymyxin B. Significant differences, as determined by the *t* test, are indicated: $*p < 0.005$.

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. 8*a*). Cells that were not incubated with rCSN1S1 before addition of the anti–His-tag Ab served as a negative control (Fig. 8*b*). As can be seen in Fig. 8, binding of rCSN1S1 was detectable in the majority of cells (Fig. 8*a*), whereas no signal was found in cells without CSN1S1 incubation (Fig. 8*b*).

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. 9*a*). Similarly GM-CSF protein measurements in the supernatants were lower in the presence of ML3403 in Mono Mac 6 cells (Fig. 9*a*). There was no change in GM-CSF mRNA expression with any of the other inhibitors tested compared with stimulation with rCSN1S1 alone (Fig. 9*b*). 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. 9*c*). 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. 9*d*). 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

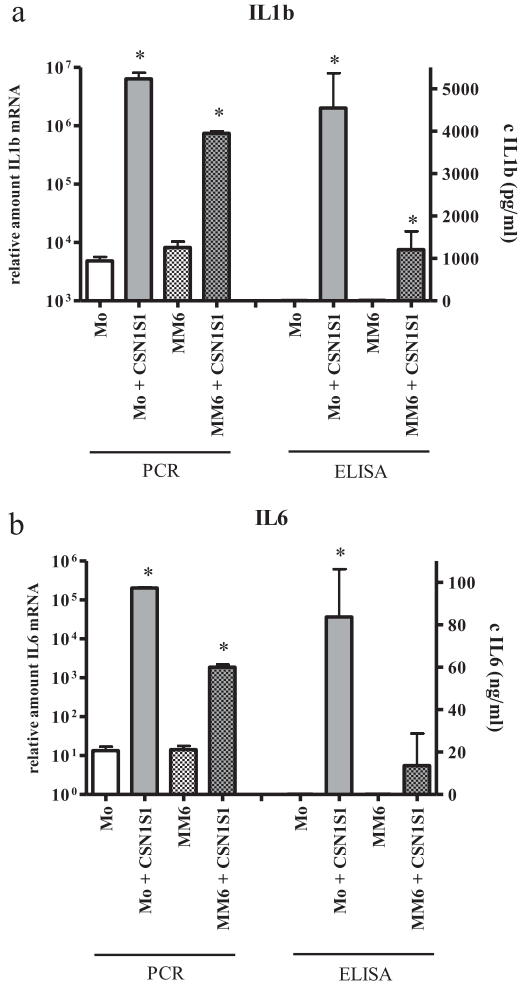

FIGURE 6. Primary human CD14$^+$ monocytes (Mo) or Mono Mac 6 cells (MM6) were stimulated for 24 h with 10 μg/ml CSN1S1 (gray bars) or were left untreated (light bars). PCR or ELISA was carried out to measure IL-1b (*a*) and IL-6 (*b*). Mean and SD of at least three experiments. Significant differences compared with untreated controls, as determined by the *t* test, are indicated: $^*p < 0.05$.

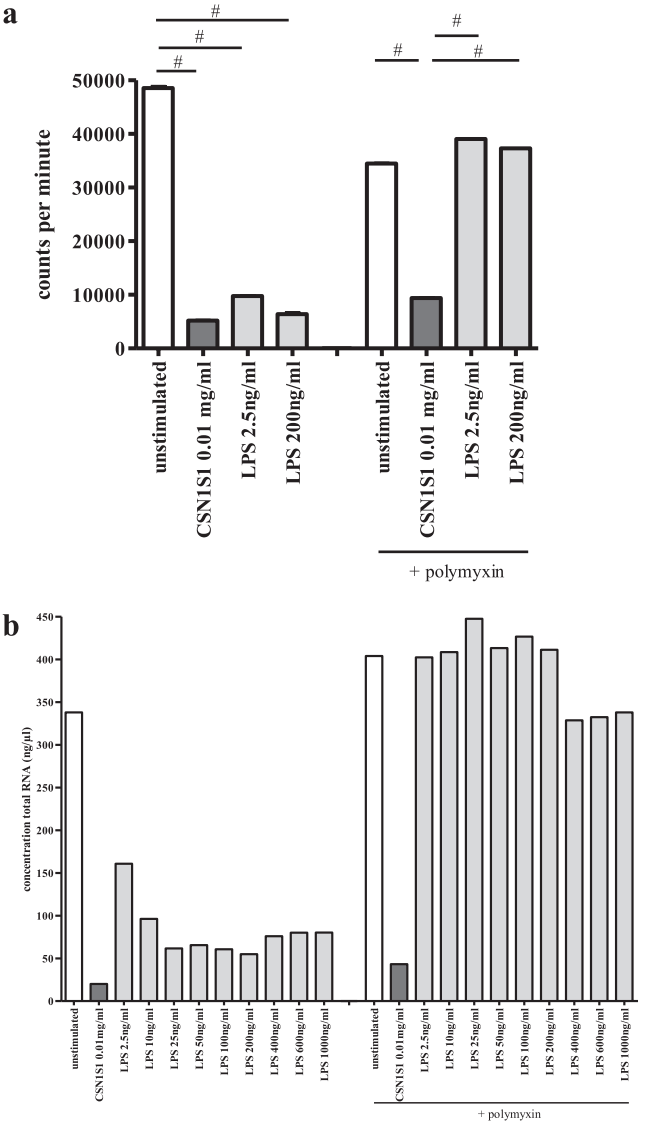

FIGURE 7. Mono Mac 6 cells were stimulated for 72 h with 0.01 mg/ml CSN1S1 or LPS in the presence and absence of 30 μg/ml polymyxin. *a*, Proliferational activity determined by [^{3}H]thymidine assay. cpm are given as mean and SD. Significant differences, as determined by the *t* test, are indicated: $^\#p < 0.0005$. *b*, Mean concentrations of total RNA, as determined by Nanodrop.

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

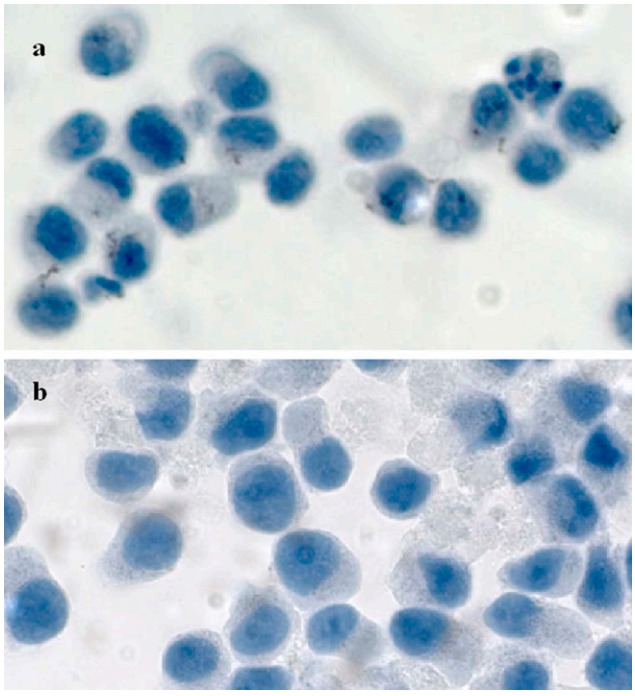

FIGURE 8. Stimulation of Mono Mac 6 cells for 1 h with His-tagged rCSN1S1 and then with HRP-marked anti–His-tag Ab. Brown dots indicate binding of CSN1S1 to Mono Mac 6 cells (*a*) but not to cells without incubation of CSN1S1 before the addition of the secondary Ab (negative control) (*b*). Original magnification ×360.

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 tissues or cells, in addition to monocytes, may contribute to the overall CSN1S1 level. Finally, monocytes may be regulated to increase CSN1S1 production. To further elucidate the latter, we stimulated $CD14^+$ monocytes with LPS, TNF-α, and IFN-γ and demonstrated increased CSN1S1 mRNA expression and protein secretion 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 monocyte cultures of healthy donors (Fig. 1*a*).

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-1b 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 acted 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 cattle (X00564), rat (NM_138874), and mouse (NM_007784) is 57.6, 49.8, and 47.8%, respectively (determined by the Euopean 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 monocytic cells 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, 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.

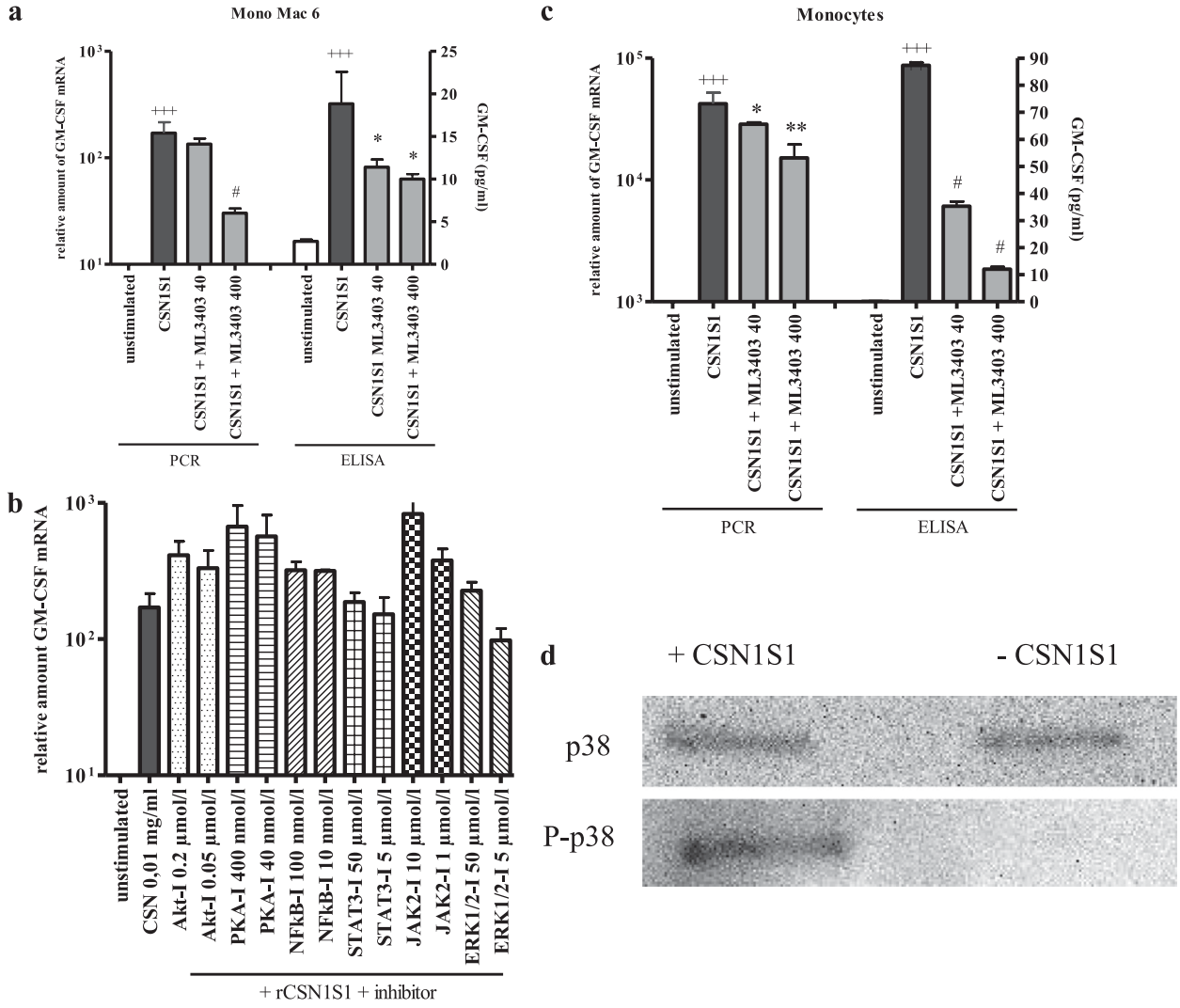

FIGURE 9. Mono Mac 6 cells were incubated with 0.01 mg/ml rCSN1S1 and the indicated concentrations of inhibitors of second messengers (ML3403 for p38 MAPK, Akti-1/2 for protein kinase B [Akt-I], Myr-GRTGRRNAI-NH2 for protein kinase A [PKA-I], 6-amino-4-[4-phenoxyphenylethylamino] quinazoline for NF-κB [NF-κB-I], PpYLKTK-mts for STAT3 [STAT3-I], hexabromocyclohexane for JAK II [JAK2-I], or PD98059 for ERK1/2 [ERK1/2-I]) for 24 h. *a*, GM-CSF mRNA was assessed by PCR (*left y-axis*), and GM-CSF protein was measured in supernatants by ELISA (*right y-axis*) in Mono Mac 6 cells stimulated with CSN1S1 and different concentrations of ML3403. *b,* GM-CSF mRNA assessed by PCR in cells stimulated with CSN1S1 and inhibitors in the indicated concentrations. *c*, GM-CSF mRNA was assessed by PCR (*left y-axis*), and GM-CSF protein was measured in supernatants by ELISA (*right y-axis*) in primary human $CD14^+$ monocytes stimulated with CSN1S1 and different concentrations of ML3403. *a* and *c*, Data comparison was by the *t* test between unstimulated cells and cells stimulated with CSN1S1 ($^{+++}p < 0.0005$), as well as between cells stimulated with CSN1S1 alone or in combination with the indicated inhibitors ($^{*}p < 0.05$; $^{**}p < 0.005$; $^{\#}p < 0.0005$). *d*, Western blot of cell lysates demonstrating staining of unphosphorylated p38, irrespective of CSN1S1 stimulation (p38), but exclusive staining of activated, phosphorylated p38 MAPK (P-p38) in lysates from CSN1S1-stimulated cells.

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.

References

1. Britton, J. R., and A. J. Kastin. 1991. Biologically active polypeptides in milk. *Am. J. Med. Sci.* 301: 124–132.
2. Jenness, R., and C. Holt. 1987. Casein and lactose concentrations in milk of 31 species are negatively correlated. *Experientia* 43: 1015–1018.
3. Goldfarb, M. 1999. Two-dimensional electrophoresis and computer imaging: quantitation of human milk casein. *Electrophoresis* 20: 870–874.
4. Anderson, G. H., and A. Aziz. 2006. Multifunctional roles of dietary proteins in the regulation of metabolism and food intake: application to feeding infants. *J. Pediatr.* 149: 74–79.
5. Donida, B. M., E. Mrak, C. Gravaghi, I. Villa, S. Cosentino, E. Zacchi, S. Perego, A. Rubinacci, A. Fiorilli, G. Tettamanti, and A. Ferraretto. 2009. Casein phosphopeptides promote calcium uptake and modulate the differentiation pathway in human primary osteoblast-like cells. *Peptides* 30: 2233–2241.
6. Lönnerdal, B. 1997. Effects of milk and milk components on calcium, magnesium, and trace element absorption during infancy. *Physiol. Rev.* 77: 643–669.
7. Clare, D. A., G. L. Catignani, and H. E. Swaisgood. 2003. Biodefense properties of milk: the role of antimicrobial proteins and peptides. *Curr. Pharm. Des.* 9: 1239–1255.
8. Ferranti, P., M. V. Traisci, G. Picariello, A. Nasi, V. Boschi, M. Siervo, C. Falconi, L. Chianese, and F. Addeo. 2004. Casein proteolysis in human milk: tracing the pattern of casein breakdown and the formation of potential bioactive peptides. *J. Dairy Res.* 71: 74–87.

9. Fiat, A.-M., D. Migliore-Samour, P. Jollès, L. Drouet, C. Bal dit Sollier, and J. Caen. 1993. Biologically active peptides from milk proteins with emphasis on two examples concerning antithrombotic and immunomodulating activities. *J. Dairy Sci.* 76: 301–310.
10. Meisel, H., and R. J. FitzGerald. 2003. Biofunctional peptides from milk proteins: mineral binding and cytomodulatory effects. *Curr. Pharm. Des.* 9: 1289–1295.
11. Shah, N. P. 2000. Effects of milk-derived bioactives: an overview. *Br. J. Nutr.* 84 (Suppl. 1): S3–S10.
12. Aihara, K., H. Ishii, and M. Yoshida. 2009. Casein-derived tripeptide, Val-Pro-Pro (VPP), modulates monocyte adhesion to vascular endothelium. *J. Atheroscler. Thromb.* 16: 594–603.
13. Katagiri, T., I. Adachi, T. Terao, and T. Osawa. 1980. Alpha-casein-binding proteins of guinea pig macrophage membranes and their possible roles in chemotaxis. *J. Biochem.* 87: 1421–1430.
14. Lewis, S. L., and D. E. Van Epps. 1983. Demonstration of specific receptors for fluoresceinated casein on human neutrophils and monocytes using flow cytometry. *Inflammation* 7: 363–375.
15. Solymossy, M., Z. Nagy, G. Tóth, and F. Antoni. 1986. Interaction of casein with human polymorphonuclear cells. *Biochem. Med. Metab. Biol.* 35: 293–301.
16. Van Epps, D. E., A. D. Bankhurst, and R. C. J. Williams, Jr. 1977. Casein-mediated neutrophil chemotaxis: a parallel between surface binding and chemotaxis. *Inflammation* 2: 115–123.
17. Pocoví, C., C. Conesa, C. Barbana, M. D. Pérez, M. Calvo, and L. Sánchez. 2009. Comparison of the activity of human and bovine milk on two cell lines. *J. Dairy Res.* 76: 308–316.
18. Ramos-Mandujano, G., B. Weiss-Steider, B. Melo, Y. Córdova, E. Ledesma-Martínez, S. Bustos, O. Silvestre, I. Aguiñiga, N. Sosa, I. Martínez, et al. 2008. Alpha-, beta- and kappa-caseins inhibit the proliferation of the myeloid cell lines 32D cl3 and WEHI-3 and exhibit different differentiation properties. *Immunobiology* 213: 133–141.
19. Jørgensen, A. L., H. R. Juul-Madsen, and J. Stagsted. 2010. Colostrum and bioactive, colostral peptides differentially modulate the innate immune response of intestinal epithelial cells. *J. Pept. Sci.* 16: 21–30.
20. Hatori, M., K. Ohki, S. Hirano, X. P. Yang, H. Kuboki, and C. Abe. 2008. Effects of a casein hydrolysate prepared from *Aspergillus oryzae* protease on adjuvant arthritis in rats. *Biosci. Biotechnol. Biochem.* 72: 1983–1991.
21. Bhattacharyya, J., and K. P. Das. 1999. Molecular chaperone-like properties of an unfolded protein, alpha(s)-casein. *J. Biol. Chem.* 274: 15505–15509.
22. Bicer, S., P. J. Reiser, S. Ching, and N. Quan. 2009. Induction of muscle weakness by local inflammation: an experimental animal model. *Inflamm. Res.* 58: 175–183.
23. Lotem, J., and L. Sachs. 1985. Independent regulation of myeloid cell growth and differentiation inducing proteins: in vivo regulation by compounds that induce inflammation. *Int. J. Cancer* 35: 93–100.
24. Noursadeghi, M., M. C. M. Bickerstaff, J. Herbert, D. Moyes, J. Cohen, and M. P. Pepys. 2002. Production of granulocyte colony-stimulating factor in the nonspecific acute phase response enhances host resistance to bacterial infection. *J. Immunol.* 169: 913–919.
25. Skeel, A., and E. J. Leonard. 1994. Action and target cell specificity of human macrophage-stimulating protein (MSP). *J. Immunol.* 152: 4618–4623.
26. Johnsen, L. B., L. K. Rasmussen, T. E. Petersen, and L. Berglund. 1995. Characterization of three types of human alpha s1-casein mRNA transcripts. *Biochem. J.* 309: 237–242.
27. Uusi-Oukari, M., J.-M. Hyttinen, V.-P. Korhonen, A. Västi, L. Alhonen, O. A. Jänne, and J. Jänne. 1997. Bovine alpha s1-casein gene sequences direct high level expression of human granulocyte-macrophage colony-stimulating factor in the milk of transgenic mice. *Transgenic Res.* 6: 75–84.
28. Otaegui, D., S. Mostafavi, C. C. A. Bernard, A. Lopez de Munain, P. Mousavi, J. R. Oksenberg, and S. E. Baranzini. 2007. Increased transcriptional activity of milk-related genes following the active phase of experimental autoimmune encephalomyelitis and multiple sclerosis. *J. Immunol.* 179: 4074–4082.
29. Xu, K., M.-T. Ling, X. Wang, and Y.-C. Wong. 2006. Evidence of a novel biomarker, alphas1-Casein, a milk protein, in benign prostate hyperplasia. *Prostate Cancer Prostatic Dis.* 9: 293–297.
30. Ginger, M. R., and M. R. Grigor. 1999. Comparative aspects of milk caseins. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 124: 133–145.
31. Rijnkels, M. 2002. Multispecies comparison of the casein gene loci and evolution of casein gene family. *J. Mammary Gland Biol. Neoplasia* 7: 327–345.
32. al-Janadi, N., A. al-Dalaan, S. al-Balla, and S. Raziuddin. 1996. CD4+ T cell inducible immunoregulatory cytokine response in rheumatoid arthritis. *J. Rheumatol.* 23: 809–814.
33. Belardelli, F., and M. Ferrantini. 2002. Cytokines as a link between innate and adaptive antitumor immunity. *Trends Immunol.* 23: 201–208.
34. Hamilton, J. A. 2002. GM-CSF in inflammation and autoimmunity. *Trends Immunol.* 23: 403–408.
35. Werz, O., J. Klemm, O. Rådmark, and B. Samuelsson. 2001. p38 MAP kinase mediates stress-induced leukotriene synthesis in a human B-lymphocyte cell line. *J. Leukoc. Biol.* 70: 830–838.
36. Kim, Y. K., B. H. Chung, S. Yoon, K.-K. Lee, B. Lönnerdal, and D.-Y. Yu. 1997. High-level expression of human alpha(s)1-casein in *Escherichia coli*. *Biotechnol. Tech.* 11: 675–678.
37. Cardoso, L. S., M. I. Araujo, A. M. Góes, L. G. Pacífico, R. R. Oliveira, and S. C. Oliveira. 2007. Polymyxin B as inhibitor of LPS contamination of *Schistosoma mansoni* recombinant proteins in human cytokine analysis. *Microb. Cell Fact.* 6: 1–6.
38. Kokkola, R., A. Andersson, G. Mullins, T. Östberg, C.-J. Treutiger, B. Arnold, P. Nawroth, U. Andersson, R. A. Harris, and H. E. Harris. 2005. RAGE is the major receptor for the proinflammatory activity of HMGB1 in rodent macrophages. *Scand. J. Immunol.* 61: 1–9.
39. Landmann, R., S. Link, S. Sansano, Z. Rajacic, and W. Zimmerli. 1998. Soluble CD14 activates monocytic cells independently of lipopolysaccharide. *Infect. Immun.* 66: 2264–2271.
40. Alvaro-Gracia, J. M., N. J. Zvaifler, C. B. Brown, K. Kaushansky, and G. S. Firestein. 1991. Cytokines in chronic inflammatory arthritis. VI. Analysis of the synovial cells involved in granulocyte-macrophage colony-stimulating factor production and gene expression in rheumatoid arthritis and its regulation by IL-1 and tumor necrosis factor-alpha. *J. Immunol.* 146: 3365–3371.
41. Campbell, I. K., A. Bendele, D. A. Smith, and J. A. Hamilton. 1997. Granulocyte-macrophage colony stimulating factor exacerbates collagen induced arthritis in mice. *Ann. Rheum. Dis.* 56: 364–368.
42. Jang, J., D.-S. Lim, Y.-E. Choi, Y. Jeong, S.-A. Yoo, W.-U. Kim, and Y.-S. Bae. 2006. MLN51 and GM-CSF involvement in the proliferation of fibroblast-like synoviocytes in the pathogenesis of rheumatoid arthritis. *Arthritis Res. Ther.* 8: R170.
43. Moss, S. T., and J. A. Hamilton. 2000. Proliferation of a subpopulation of human peripheral blood monocytes in the presence of colony stimulating factors may contribute to the inflammatory process in diseases such as rheumatoid arthritis. *Immunobiology* 202: 18–25.
44. Baron, C. L., S. M. Scholl, H. Bausinger, D. Hanau, P. Pouillart, B. Goud, and J. Salamero. 1999. Two distinct cell populations are obtained from human blood monocytes cultured with M-CSF, GM-CSF and IL-4. *Eur. J. Cancer* 35: 39–40.
45. Conti, L., and S. Gessani. 2008. GM-CSF in the generation of dendritic cells from human blood monocyte precursors: recent advances. *Immunobiology* 213: 859–870.
46. Prevost, J. M., J. L. Pelley, W. Zhu, G. E. D'Egidio, P. P. Beaudry, C. Pihl, G. G. Neely, E. Claret, J. Wijdenes, and C. B. Brown. 2002. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and inflammatory stimuli up-regulate secretion of the soluble GM-CSF receptor in human monocytes: Evidence for ectodomain shedding of the cell surface GM-CSF receptor alpha subunit. *J. Immunol.* 169: 5679–5688.
47. Hira, T., H. Hara, F. Tomita, and Y. Aoyama. 2003. Casein binds to the cell membrane and induces intracellular calcium signals in the enteroendocrine cell: a brief communication. *Exp. Biol. Med. (Maywood)* 228: 850–854.
48. Munoz, L., E. E. Ramsay, M. Manetsch, Q. Ge, C. Peifer, S. Laufer, and A. J. Ammit. 2010. Novel p38 MAPK inhibitor ML3403 has potent anti-inflammatory activity in airway smooth muscle. *Eur. J. Pharmacol.* 635: 212–218.
49. O'Sullivan, A. W., J. H. Wang, and H. P. Redmond. 2009. The role of P38 MAPK and PKC in BLP induced TNF-alpha release, apoptosis, and NFkappaB activation in THP-1 monocyte cells. *J. Surg. Res.* 151: 138–144.
50. Grund, E. M., D. Kagan, C. A. Tran, A. Zeitvogel, A. Starzinski-Powitz, S. Nataraja, and S. S. Palmer. 2008. Tumor necrosis factor-(alpha) regulates inflammatory and mesenchymal response via mitogen-activated protein kinase kinase, p38, and nuclear factor kB in human endometriotic epithelial cells. *Mol. Pharmacol.* 73: 1394–1404.
51. Hashimoto, S., K. Matsumoto, Y. Gon, S. Maruoka, K. Kujime, S. Hayashi, I. Takeshita, and T. Horie. 2000. p38 MAP kinase regulates TNF alpha-, IL-1 alpha- and PAF-induced RANTES and GM-CSF production by human bronchial epithelial cells. *Clin. Exp. Allergy* 30: 48–55.
52. Koch, A., M. Giembycz, K. Ito, S. Lim, E. Jazrawi, P. J. Barnes, I. Adcock, E. Erdmann, and K. F. Chung. 2004. Mitogen-activated protein kinase modulation of nuclear factor-kappaB-induced granulocyte macrophage-colony-stimulating factor release from human alveolar macrophages. *Am. J. Respir. Cell Mol. Biol.* 30: 342–349.
53. Meja, K. K., P. M. Seldon, Y. Nasuhara, K. Ito, P. J. Barnes, M. A. Lindsay, and M. A. Giembycz. 2000. p38 MAP kinase and MKK-1 co-operate in the generation of GM-CSF from LPS-stimulated human monocytes by an NF-kappa B-independent mechanism. *Br. J. Pharmacol.* 131: 1143–1153.
54. Song, G., G. Ouyang, and S. Bao. 2005. The activation of Akt/PKB signaling pathway and cell survival. *J. Cell. Mol. Med.* 9: 59–71.
55. Tangye, S. G., M. C. Cook, and D. A. Fulcher. 2009. Insights into the role of STAT3 in human lymphocyte differentiation as revealed by the hyper-IgE syndrome. *J. Immunol.* 182: 21–28.
56. Sumbayev, V. V., and I. M. Yasinska. 2006. Role of MAP kinase-dependent apoptotic pathway in innate immune responses and viral infection. *Scand. J. Immunol.* 63: 391–400.
57. Geijsen, N., L. Koenderman, and P. J. Coffer. 2001. Specificity in cytokine signal transduction: lessons learned from the IL-3/IL-5/GM-CSF receptor family. *Cytokine Growth Factor Rev.* 12: 19–25.
58. Ihle, J. N., and D. G. Gilliland. 2007. Jak2: normal function and role in hematopoietic disorders. *Curr. Opin. Genet. Dev.* 17: 8–14.
59. Skålhegg, B. S., A. Funderud, H. H. Henanger, T. T. Hafte, A. C. Larsen, A. K. Kvissel, S. Eikvar, and S. Ørstavik. 2005. Protein kinase A (PKA)—a potential target for therapeutic intervention of dysfunctional immune cells. *Curr. Drug Targets* 6: 655–664.
60. Mebratu, Y., and Y. Tesfaigzi. 2009. How ERK1/2 activation controls cell proliferation and cell death: Is subcellular localization the answer? *Cell Cycle* 8: 1168–1175.