EXPERIMENTAL STUDY

Differential expression of somatostatin receptor subtypes in human peripheral blood mononuclear cell subsets

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Abstract

Background: Somatostatin (SS)-binding sites have been demonstrated in human lymphoid tissues and peripheral blood cells. However, not much is known with respect to the SS receptor subtype (sst) expression pattern and the expression of SS itself in the immune system.

Objective: The aim of this study was to evaluate the mRNA expression of the five known sst (sst_{1-5}) in peripheral blood mononuclear cell (sub)populations. Moreover, the expression of the mRNAs encoding SS and the SS-like peptide cortistatin (CST) in immune cell subsets was studied.

Methods: RT-PCR and quantitative PCR were performed to evaluate sst, SS and CST mRNA expression in cells in the basal or activated state. Fluorescence-activated cell sorter (FACS) analysis using fluorescent SS was performed to visualize sst protein on cell membranes.

Results: B- and T-lymphocytes selectively expressed sst_3 mRNA. sst_3 expression in B-lymphocytes was significantly lower compared with T-lymphocytes. Unstimulated, freshly isolated monocytes did not express any sst mRNA. Upon activation, monocytes selectively expressed sst_2 mRNA, whereas T-lymphocyte activation upregulated sst_3 expression. sst_2 mRNA expression on monocytes was confirmed by FACS analysis. B- and T-lymphocytes did not express SS mRNA, while both cell types expressed CST mRNA. CST mRNA expression was downregulated following T-lymphocyte activation.

Conclusion: We demonstrate for the first time unequivocally that human peripheral blood B- and T-lymphocytes selectively express sst_3, whereas monocytes do not express sst. However, upon activation, monocytes are induced to express sst_{2A}. No expression of SS mRNA was detected in any cell type, whereas all cell types expressed CST mRNA. The differential expression of sst and CST mRNA in lymphocytes and monocytes suggests a functional significance for the CST–sst interaction in immune cells, but further studies should be performed to evaluate the significance of sst and CST in these cells.

European Journal of Endocrinology **150** 565–577

Introduction

Somatostatin (SS) is a small neuropeptide that is widely distributed throughout the body and has numerous functions. In the endocrine system it was originally characterized as an inhibitor of growth hormone secretion by the pituitary (1). SS also inhibits hormone secretion from the gastrointestinal tract (2, 3), whereas in the nervous system it functions as a neurotransmitter (4). A growing body of evidence suggests a role for SS in the immune system as well (5, 6). SS acts on its target cells through specific G-protein coupled transmembrane receptors. Initially, these receptors were identified as binding sites for radiolabelled ligands (7), usually SS or the more stable synthetic analogue octreotide. Later on, the genes of five different subtypes were isolated. These SS receptor subtypes (sst) are being characterized with respect to binding affinities for SS analogues, coupling to effector systems for signal transduction and tissue distribution of mRNA expression (8).

Although there are numerous studies demonstrating the presence of SS-binding sites or effects of SS in lymphoid tissues, immune cells or lymphoid cell lines, the data obtained so far do not present a clear view of the function of SS in the immune system. A first step towards understanding should be the elucidation of the expression patterns of SS and its sst in the immune system. *In situ* autoradiography and *in vivo* scintigraphy have repeatedly demonstrated the presence of SS-binding sites in human lymphoid tissues (9–16); however, it is not yet evident which sst are expressed. It is also not clear how receptor expression is regulated in the immune tissues and in immune cells in the peripheral blood.

Reports in the literature on this issue have been quite equivocal. SS-binding sites in peripheral blood mononuclear cells (PBMCs) were described by Bathena *et al.* (17) in 1981. However, Hiruma *et al.* (18) were unable to detect SS-binding sites on resting PBMCs; only after mitogen activation were such binding sites detected. Similarly, mRNA for sst subtype 2 (sst_2) could barely be detected in unstimulated human PBMCs, but was readily detected after mitogen stimulation (19). In contrast, sst_5 was reported to be responsible for growth inhibition of PMBCs by SS analogues (20). Additionally, several human lymphoid cell lines were shown to bear SS-binding sites (21). Such cell lines can also express mRNA for several sst (19, 22) and functionally respond to SS or SS analogues (22–24). These data, however, yield by no means a conclusive notion of the expression pattern of sst in human immune cells *in vivo*. Comparison with data obtained on PBMCs and other lymphoid tissues from rat (25) or mouse (26) demonstrate species-related differences and add to the confusion as to which sst are expressed in human immune cells.

Therefore, in the present study we set out to clarify the issue whether or not human PBMC subsets in the circulation express sst and their natural ligand SS. Moreover, we investigated the expression of the mRNA encoding a recently discovered SS-like peptide, cortistatin (CST), which we previously found to be widely expressed in cells and tissues from the human immune system (27). CST shows high structural resemblance to SS (28) and binds with high affinity to all five sst (29). Accordingly, we isolated human PBMCs and determined by RT-PCR whether SS, CST or sst were expressed, and whether the expression pattern is altered by mitogen activation. Moreover, we separated the human PBMC cell fraction into subpopulations and determined the expression patterns in T-lymphocytes, B-lymphocytes and monocytes. By quantitative PCR (Q-PCR) we subsequently studied the regulation of the sst and CST mRNA expression in PBMCs and monocytes. To gain more insight into the cell-type-specific expression of SS and its receptors in the human immune system, a comparison was made with the expression levels of sst in several other cell lines and primary human immune cell types.

Materials and methods

Cells, media and reagents

PBMCs were isolated from 20–40 ml blood from eight different healthy donors using Ficoll density-gradient separation immediately after blood withdrawal. After Ficoll separation, PBMCs were kept on ice to prevent changes in mRNA expression during further treatment of the cells. PBMCs or isolated subpopulations were either used directly for mRNA analysis or cultured for 24 h at 37 °C in a 5% CO_2 incubator in RPMI medium supplemented with 10% heat-inactivated fetal calf serum (Life Technologies), 10^5 U/l penicillin and 1.25 mg/l Fungizone. PBMCs of three different donors were used for further culture. As mitogenic stimuli, we added either 2 μg/ml phytohaemagglutinin (PHA), 3 μg/ml concanavalin A (ConA), 1 μg/ml lipopolysaccharide (LPS) or 10 μg/ml pokeweed mitogen (PWM). After 24 h the cultured cells were harvested for mRNA isolation as described below.

For fluorescent ligand-binding experiments (see below) cell populations enriched for lymphocytes or monocytes were used. Enrichment for lymphocytes was achieved by allowing the monocytes among the PBMCs to adhere to culture dishes and subsequently collecting the non-adherent lymphocyte fraction for further culturing. Monocytes were isolated by performing a Percoll density gradient following a standard Ficoll density gradient as described previously (30).

Separation of PBMCs into subpopulations

Subpopulations of PBMCs were isolated by means of magnetic beads coated with specific antibodies (Dynal AS, Oslo, Norway). For T-lymphocyte isolation, beads coated with CD2 or CD3 antibody were used, while CD19-antibody-coated and CD14-antibody-coated beads were used for B-lymphocyte and monocyte isolation respectively. To distinguish between T-helper cells and cytotoxic T-cells, beads coated with respectively CD4 or CD8 antibodies were used. Isolation of cells was performed following the manufacturer's instructions. After separation procedures, cells were either used for RT-PCR or cultured for 24 h as described above.

RT-PCR

RT-PCR was performed as described previously (31). Briefly, poly A^+ mRNA was isolated using Dynabeads Oligo $(dT)_{25}$ (Dynal) from cell samples. cDNA was synthesized using the poly A^+ mRNA, which was eluted from the beads in 40 μl H_2O for 10 min at 65 °C, using Oligo $(dT)_{12-18}$ Primer (Life Technologies). One-twentieth of the cDNA library was used for each amplification by PCR using primer sets specific for human SS, sst_{1-5}, CST and hypoxanthine phosphoribosyl transferase (hprt) (see Table 1A) as a control. As positive controls for SS, CST and hprt, cDNA of human brain RNA (Invitrogen, Groningen, The Netherlands) was used. As positive control for sst_{1-5}, DNA of a BLCL-BSM cell line (an EBV-transformed B-cell line) was used. The PCR reaction was carried out in a DNA thermal cycler with a heated lid (Applied Biosystems, Nieuwerkerk aan de Ijssel, The Netherlands). After an initial denaturation at 94 °C for 5 min, the samples were subjected to 40 cycles of denaturation at 94 °C for 1 min, annealing for 2 min at 60 °C and extension for 1 min at 72 °C. After a final extension for 10 min at 72 °C, 10 μl aliquots of the resulting PCR products were analysed by electrophoresis on 1.5% agarose gels stained with

Table 1 Primers used for the RT-PCR and Q-PCR. The sequences of the primers for sst_1 and sst_4 are derived and adapted from (60), for sst_5 from (61), for CST from (62) and all other primers and probes were designed by use of the Primer3! software (http://www-genome.wi.mit.edu/genome_software/other /primer3. html) and the appropriate GenBank entries.

	Sequence (5′–3′)[a]	PCR product (bp)
A. RT-PCR		
sst_1 (forward)	ATGGTGGCCCTCAAGGCCGG	318
sst_1 (reverse)	CGCGGTGGCGTAATAGTCAA	
sst_{2A} (forward)[b]	GCCAAGATGAAGACCATCAC	414
sst_{2A} (reverse)	GATGAACCCTGTGTACCAAGC	
sst_3 (forward)	CCAACGTCTACATCCTCAACC	314
sst_3 (reverse)	TCCCGAGAAGACCACCAC	
sst_4 (forward)	ATCTTCGCAGACACCAGACC	321
sst_4 (reverse)	ATCAAGGCTGGTCACGACGA	
sst_5 (forward)	CGTCTTCATCATCTACACGG	226
sst_5 (reverse)	CCGTCTTCATCATCTACACGG	
Somatostatin (forward)	GATGCTGTCCTGCCGCCTCCAG	349
Somatostatin (reverse)	ACAGGATGTGAAAGTCTTCCA	
CST (forward)	GCAAATTCGCTCTAAACACAGGA	173
CST (reverse)	TTGGGAAGGAGGAGAGGAAAGAT	
hprt (forward)	CAGGACTGAACGTCTTGCTC	413
hprt (reverse)	CAAATCCAACAAAGTCTGGC	
B. Q-PCR		
sst_{2A} (forward)	ATGCCAAGATGAAGACCATCAC	171
sst_{2A} (reverse)	TGAACTGATTGATGCCATCCA	
sst_{2A} (probe)	TGGCTCTGGTCCACTGGCCCTTTG	
sst_3 (forward)	CTGGGTAACTCGCTGGTCATCTA	86
sst_3 (reverse)	AGCGCCAGGTTGAGGATGTA	
sst_3 (probe)	CGGCCAGCCCTTCAGTCACCAAC	
CST (forward)	GGAGAGAAGCTCCAGTCAGC	198
CST (reverse)	GGTCCACTCAAACCACCAA	
CST (probe)	TATGCTCGCTGTCTCGGCCG	
hprt (forward)	TGCTTTCCTTGGTCAGGCAGTAT	109
hprt (reverse)	TCAAATCCAACAAAGTCTGGCTTATATC	
hprt (probe)	CAAGCTTGCGACCTTGACCATCTTTGGA	

[a] The position is given of the 5′ nucleotide of the primer relative to the first nucleotide of the coding region in the cDNA sequence.
[b] The sst_2 primers are specific for sst_{2A}. In initial experiments we also included primers that amplify both sst_{2A} and sst_{2B}, but the sst_{2B} specific product was never found (unpublished results).

ethidium bromide. The identity of the products was confirmed by direct sequencing using an ABI Prism 3100 Genetic Analyser (Applied Biosystems) according to the manufacturer's protocol (data not shown). Several controls were included in the RT-PCR experiments. To ascertain that no detectable genomic DNA was present in the polyA$^+$ mRNA preparation (since the sst genes are intron-less), the cDNA reactions were also performed without reverse transcriptase (RT) and amplified with each primer pair. Amplification of the cDNA samples with the hprt-specific primers served as positive control for the quality of the cDNA. To exclude contamination of the PCR reaction mixtures, the reactions were also performed in the absence of the DNA template in parallel with cDNA samples. No PCR products were formed in the samples without RT or in the absence of cDNA template (not shown). As a positive control for the PCR reactions of the sst receptor subtypes, 0.1–0.001 μg human genomic DNA, representing approximately 30 000 to 300 copies of sst template, was amplified in parallel with the cDNA samples. As a positive control for the PCR of the hprt, SS and CST cDNA, aliquots of a cDNA sample known to contain SS, CST and hprt mRNA were amplified,

because these primer pairs did enclose introns in the genomic DNA.

Q-PCR

Q-PCR was performed employing a nuclease assay (32) for real-time detection of amplification (33, 34) with an ABI Prism 7700 Sequence Detection System (i.e. thermal cycler and real-time fluorescence detector; Perkin Elmer, Nieuwerkerk aan de Ijssel, The Netherlands). New primer sets were designed for use in quantitative PCR of sst_{2A}, sst_3, hprt and CST with accompanying probes for the detection of the PCR products (Table 1B). These were obtained from BioSource (Nivelles, Belgium).

In each experiment, standard curves for each primer set were included. Known amounts of genomic DNA containing sst_{2A} and sst_3 or dilutions of a pool of hprt or CST containing cDNAs were amplified (in duplicate or triplicate) together with the unknown cDNA samples. A standard curve was constructed by plotting the Ct vs the logarithm of the starting quantity. With these standard curves and the measured Ct of the unknown cDNA samples the starting amounts in the cDNA

samples were determined in duplicate or triplicate. To correct for differences in the efficiency of RNA isolation and cDNA synthesis the amounts of sst_{2A}, sst_3 and CST were divided by the amount of hprt in a given cDNA sample. Since the absolute copy number of hprt templates in the control cDNAs used for the hprt standard curve is not known, the starting amounts are given in arbitrary units.

Fluorescent SS binding

To detect SS-binding sites on PBMCs the fluorescein-labelled SS analogue fluosomatostatin (Fluo-SS) (Advanced Bioconcept, Montreal, Canada) was used. Cells were washed with PBS containing 0.5% BSA and incubated at room temperature with 50 nmol/l Fluo-SS or with 50 nmol/l Fluo-SS and 5000 nmol/l $D\text{-trp}^8\text{-SS14}$. After 30 min incubation an appropriate dilution of phycoerythrin (PE)-labelled CD3, CD19 (Becton Dickinson, Erembodegem, Belgium) or CD14 antibodies (Beckman Coulter, Mijdrecht, The Netherlands) was added. Following a further 15 min incubation the cells were washed, resuspended in PBS containing 0.5% BSA and 20 mmol/l sodium azide and analysed on a fluorescence-activated cell sorter (FACS) (FACScan Cytometer; Becton Dickinson). As a positive control for ligand binding, a cell line stably transfected with sst_{2A} was used.

Results

sst, SS and CST mRNA in PBMCs

To determine whether or not sst mRNA is expressed in the PBMC cell fraction of circulating blood we started isolation of the cells immediately after blood withdrawal and were able to lyse the cells within 2 h. This guarantees that the RNA expression pattern of the studied cells resembles that of the live cells in the circulation as closely as possible. In doing so, we consistently found expression of sst_3 mRNA in all eight PBMC samples by RT-PCR (Fig. 1). In some experiments a very weak sst_{2A} PCR product could be detected as well, but only after 40 cycles when the detection limit for sst_{2A} was better than 1% of the population. None of the other sst subtypes could be detected, nor mRNA for SS itself (Table 2). However, we detected the mRNA for CST in all cell samples (Table 2). In some experiments also a PCR specific for sst_{2B} was performed, but this subtype was never detected (results not shown).

After culturing the eight isolated PBMC preparations for 24 h in medium alone or in medium containing the mitogenic agents PHA, ConA, PWM or LPS the expression of sst_{2A} mRNA was clearly induced (Fig. 1; Table 2). The expression pattern of the other sst and of SS remained unchanged (sst_3 detectable, others not, no SS).
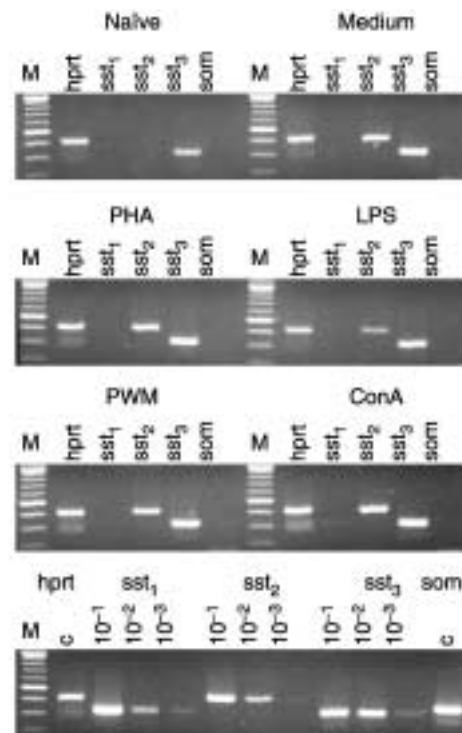

Figure 1 RT-PCR analysis of SS (som) and SS receptor expression in naïve and cultured PBMCs. PBMCs were assayed for expression of mRNA immediately after isolation (naïve) or after 24 h culturing in culture medium alone (medium) or with the addition of PHA, LPS, PWM or ConA. Control DNA samples contained either cDNA (lanes c) from a control cell line known to express hprt and SS mRNA, or different amounts of genomic DNA (lanes 10^{-1}, 10^{-2} and 10^{-3} μg) comprising 30 000, 3000 and 300 copies of sst genes respectively. All mock-reverse-transcribed samples and all controls with no added template showed no PCR products and none of the samples expressed sst_4 or sst_5 (not shown). Marker lanes (M) contain a 100 bp DNA ladder. This Figure shows mRNA expression of one healthy donor as representative of the eight donors used.

Expression in T-lymphocytes, B-lymphocytes and monocytes

To determine in which cell-type sst_{2A} and sst_3 receptors are expressed, we separated the PBMC cell population into its main subpopulations of T-lymphocytes, B-lymphocytes and monocytes by use of paramagnetic beads coated with appropriate antibody. The isolated cell populations were subsequently assayed by RT-PCR (Fig. 2; Table 2). The results demonstrated that sst_3 was expressed by $CD3^+$ T-lymphocytes and by $CD19^+$ B-lymphocytes, but not by $CD14^+$ monocytes. None of the other sst or SS was expressed in these three subpopulations, whereas the expression of CST mRNA was found in all subpopulations evaluated (Table 2). Isolation of T-lymphocytes by CD2-antibody-coated instead of CD3-antibody-coated beads yielded similar results. Culturing the isolated cell populations during

Table 2 Expression of somatostatin receptor subtype, CST and SS mRNA in PBMCs ($n = 8$), purified peripheral blood T-lymphocytes ($n = 4$), B-lymphocytes ($n = 4$) and monocytes ($n = 4$).

	sst_1	sst_{2A}	sst_3	sst_4	sst_5	CST	SS
PBMCs							
0 h	–	–	+	–	–	+	–
24 h medium	–	+	+	–	–	+	–
24 h PHA	–	+	+	–	–	+	–
24 h ConA	–	+	+	–	–	ni	–
24 h PWM	–	+	+	–	–	ni	–
24 h LPS	–	+	+	–	–	ni	–
T-lymphocytes (CD3$^+$)							
0 h	–	–	+	–	–	+	–
24 h medium	–	–	+	–	–	+	–
24 h ConA	–	–	+	–	–	+	–
B-lymphocytes (CD19$^+$)							
0 h	–	–	+	–	–	+	–
24 h medium	–	–	+	–	–	+	–
24 h PWM	–	–	+	–	–	+	–
Monocytes (CD14$^+$)							
0 h	–	–	–	–	–	+	–
24 h medium	–	+	–	–	–	+	–
24 h LPS	–	+	–	–	–	+	–

ni = not investigated.

24 h in medium alone or in medium containing ConA, PWM or LPS induced sst_{2A} in the monocyte fraction only (Fig. 2). sst_3 expression remained confined to T- and B-lymphocytes, whereas the other sst and SS were still undetected in all cell populations studied (Fig. 2; Table 2). In the same way we determined the expression pattern of unstimulated and ConA-stimulated CD4$^+$ and CD8$^+$ T-lymphocytes and found in all populations a selective expression of sst_3 mRNA (data not shown).

Quantification of sst_{2A}, sst_3 and CST mRNA expression

To quantify the induction of sst_{2A} mRNA after activation we performed a Q-PCR on cDNA of PBMCs from four donors and on purified monocytes from two donors before and after activation (24 h culturing in medium). The sst_{2A} and sst_3 mRNA expression (in arbitrary units) corrected for the amount of hprt in the different samples is shown in Fig. 3. In all PBMC samples there was a significant induction of sst_{2A} mRNA levels after activation. In the monocyte populations purified from two of the donors the induction was even larger. In three of the four donors also the sst_3 expression in PBMCs was slightly increased after activation. Among the donors the expression levels of both sst_{2A} (after activation) and sst_3 showed a broad range.

Since the very low amount of sst_{2A} mRNA in naïve cells cannot be determined with the same accuracy as the higher copy numbers, it is difficult to express the induction as a fold of the level in naïve cells. However, the sst_{2A} copy number obtained after RNA isolation and cDNA synthesis per million PBMCs increased from approximately 1000 (or less) to 25 000–150 000. In contrast, the number of sst3 copies obtained per million naïve PBMCs was approximately 7000–15 000. From three donors of PBMCs, T- and B-lymphocytes were isolated and Q-PCR was performed to quantify the expression of sst_{2A}, sst_3 and CST mRNA in these cell subsets as well.

As already demonstrated by RT-PCR, T- and B-lymphocytes were found to express sst_3 selectively. After 24 h of culture, expression of sst_3 mRNA was upregulated approximately 1.5-fold in T-lymphocytes. After a 24 h incubation with PHA, sst_3 mRNA levels were upregulated approximately 2.5-fold when compared with control cells (Fig. 4). In B-lymphocytes, on the other hand, we were not able to detect expression of sst_3 mRNA by Q-PCR, even after culture and stimulation with PHA (data not shown). This is probably due to very low expression levels of sst_3 in B-lymphocytes and probably the conditions or primers for RT-PCR are more sensitive to detect these low levels.

For comparison we determined the expression levels of sst_{2A} and sst_3 in several other normal human
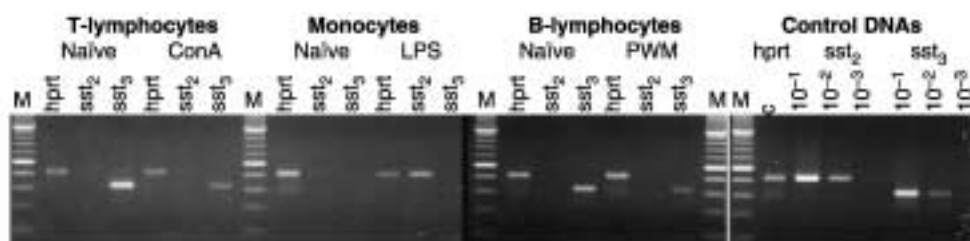

Figure 2 RT-PCR analysis of sst$_2$ and sst$_3$ expression in naïve and cultured purified T-lymphocytes, monocytes and B-lymphocytes. Cells were assayed for expression of mRNA immediately after isolation (naïve) or after 24 h culturing in culture medium with the addition of ConA, LPS or PWM. Control DNA samples contained either cDNA from a control cell line (lane c) or different amounts of genomic DNA (lanes 10^{-1}, 10^{-2} and 10^{-3} μg) comprising 30 000, 3000 and 300 copies of sst genes respectively. All mock-reverse-transcribed samples and all controls with no added template showed no PCR products and none of the samples expressed sst$_1$, sst$_4$ or sst$_5$ (not shown). Marker lanes (M) contain a 100 bp DNA ladder. Results represent an example of the findings in cell separations from four individual healthy donors.
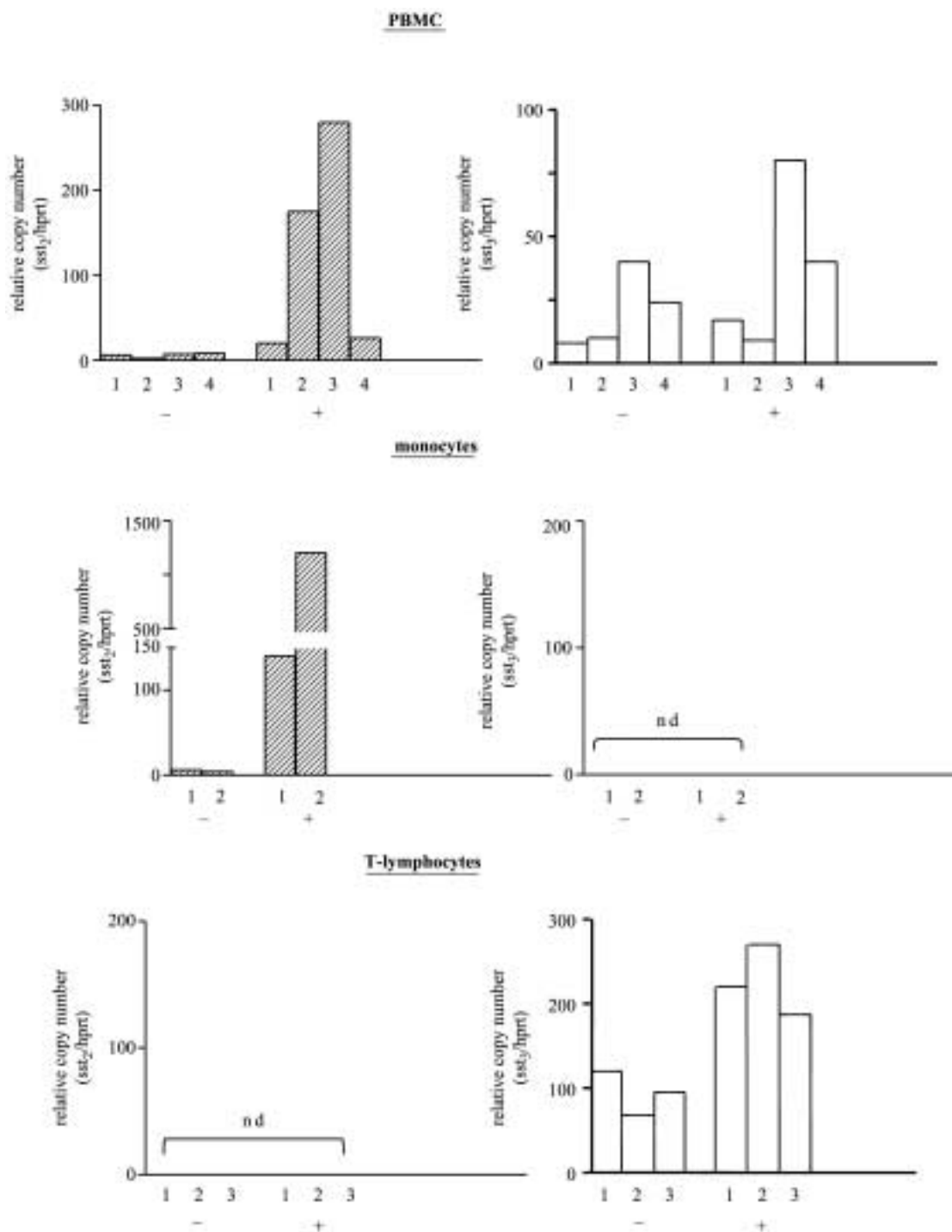
Figure 3 Quantitative PCR analysis of sst_{2A} and sst_3 mRNA expression in human immune cells. sst_{2A} and sst_3 levels were corrected for the amount of hprt detected in each sample and are given in arbitrary units. PBMCs from four donors, monocytes from two donors and T- lymphocytes from three donors were analysed in naïve ($-$) and activated ($+$) states. Hatched bars represent sst_{2A} mRNA, white bars represent sst_3 mRNA. nd = not detected.

immune cells, in leukaemic cells and in lymphoid cell lines (Fig. 5). In freshly dispersed normal human thymocytes the sst_3 expression levels were similar to the levels found in PBMCs, whereas there was more sst_{2A} mRNA than in naïve PBMCs. In normal human bone marrow cells, the expression of sst_{2A} was much higher. Although the expression level of sst_{2A} in human haematological tumour cells is highly variable, sst_{2A} was present in both tumour cell populations studied. sst_3 was just detectable in normal bone marrow
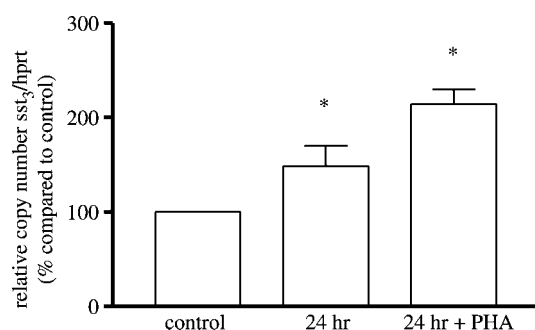
Figure 4 Expression of sst_3 mRNA in T-lymphocytes. This figure represents the expression of sst_3 mRNA in T-lymphocytes. sst_3 mRNA levels were corrected for hprt levels and bars represent the percentage of upregulation compared with control cells. Expression levels of sst_3 mRNA were evaluated in freshly isolated T-lymphocytes (control), in T-lymphocytes after 24 h of culture (24 hr) and after 24 h of culture with addition of PHA (24 hr + PHA). Results are the mean of three independent experiments using cell samples from three different donors. *$P < 0.01$ when compared with control.

cells and tumour cells studied. In immortalized T- and B-cell lines, sst_{2A} and sst_3 were also both present. However, these cell lines contained approximately 10–20 times more hprt mRNA per cell than naïve
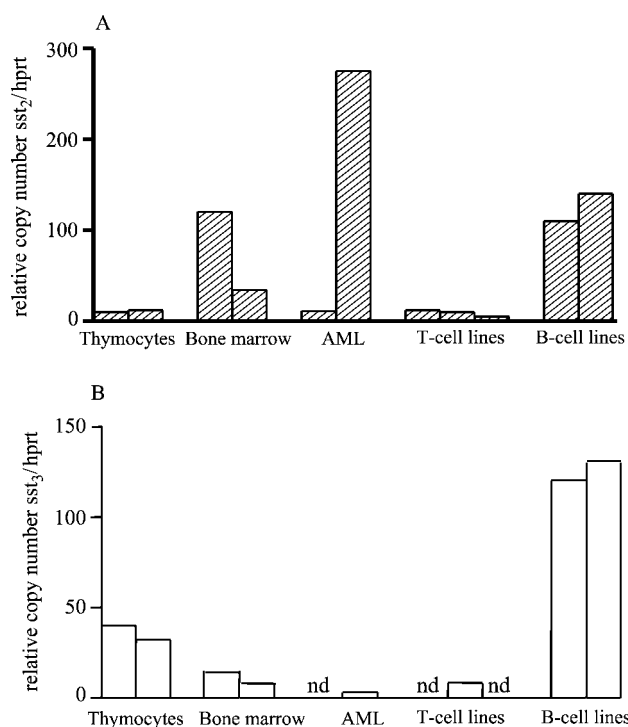

Figure 5 Expression of sst_{2A} and sst_3 mRNA in human immune cells and cell lines. sst_{2A} and sst_3 levels were corrected for the amount of hprt detected in each sample and are given in arbitrary units. (A) Expression of sst_{2A} in cell suspensions from normal human thymocytes ($n = 2$), normal human bone marrow ($n = 2$), acute myeloid leukaemia (AML) cells ($n = 2$), established T-lymphoid cell lines Jurkat, HPB-ALL and Peer, and two EBV-transformed B-cell lines. (B) Expression of sst_3 mRNA was evaluated in the same preparations. nd = not detected.

PBMCs. Therefore the absolute numbers of sst_{2A} and sst_3 mRNA copies obtained per million cells were much higher than in PBMCs. In established T-cell lines the amount of sst_{2A} was at least 10 times higher than in naïve PBMCs (10 000–60 000 copies obtained per million cells), while sst_3 expression was comparable with PBMCs. In transformed B-cell lines both sst_{2A} and sst_3 levels were much higher than in naïve PBMCs (500 000–800 000 copies obtained per million cells, i.e. more than 500 and 35 times higher for sst_{2A} and sst_3 respectively).

Finally, we evaluated the expression of CST mRNA in T- and B-lymphocytes by Q-PCR. T-lymphocytes expressed CST, and CST mRNA levels were downregulated when the cells were incubated with PHA (Fig. 6A). CST expression in B-lymphocytes could only be detected in two out of three donors and in these two donors we found an upregulation after 24 h of culture. However, when the cells were cultured for 24 h with addition of PHA, CST levels returned to basal levels (Fig. 6B).

Fluo-SS ligand binding to monocytes

To confirm that, besides sst mRNA, the protein could also be detected as ligand-binding sites, we performed FACS analysis on cell populations of naïve PBMCs, PHA-activated non-adherent PBMCs (i.e. enriched for T- and B-lymphocytes) and on LPS-activated Percoll gradient-isolated monocytes (purity 70–90%). The purity of monocytes was determined by FACS analysis, using CD14, CD68, CD71, CD26, CD1a, CD80, CD86 and HLADR antibodies (data not shown). The cells of the purified monocyte fraction were positive for CD14, CD26, CD71, CD86 and HLA-DR (70–90%), in agreement with the CD-marker expression pattern of monocytes (35). Using the fluorescent SS14 analogue we were able to detect sst only in the activated monocyte-enriched cell population, but not in the naïve PBMC fraction or in the activated non-adherent PBMC fraction. The signal was specific for SS because it was displaced by addition of excess unlabelled SS14 analogue (Fig. 7).

Discussion

In the present study we have determined for the first time unequivocally that only sst_3 is expressed at significant levels in human PBMCs, resembling the *in vivo* situation as closely as possible. This expression is found in B-lymphocytes, as well as in T-lymphocytes, but not in monocytes. Expression of sst_3 mRNA in B-lymphocytes was significantly lower compared with the expression of this sst in T-lymphocytes. Freshly isolated monocytes derived from peripheral blood do not express any of the five sst, but upon activation these cells were shown to express sst_{2A} mRNA. Previously,

A   T- lymphocytes
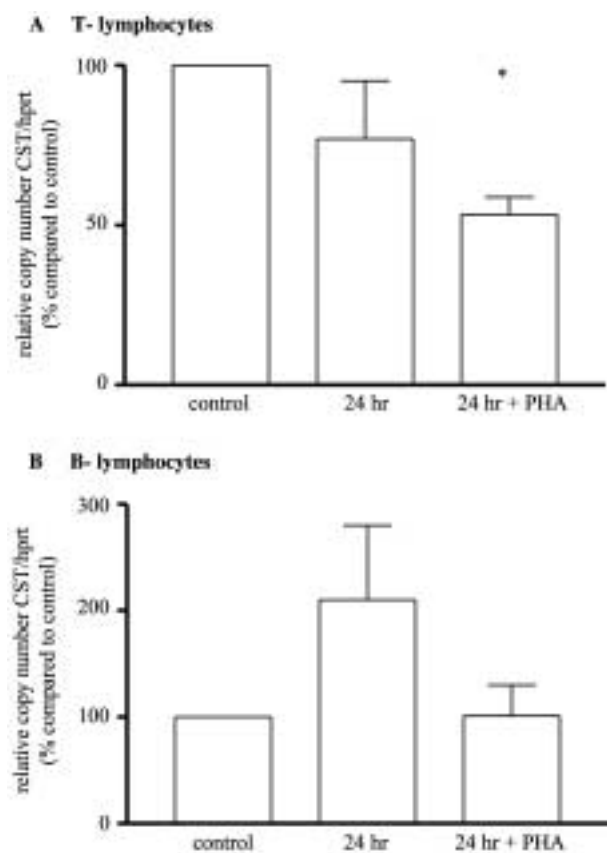

B   B- lymphocytes

Figure 6 Expression of CST mRNA in human B- and T-lympho-cytes. Expression of CST mRNA was determined in unstimulated cells (control), after 24 h of culture (24 hr) and after 24 h of culture with addition of PHA (24 hr + PHA). (A) Expression of CST mRNA in T-lymphocytes (mean of three donors). $*P < 0.001$ when compared with control cells. (B) Expression of CST mRNA in B-lymphocytes (mean of two donors). Bars represent the percentage of upregulation when compared with control.

we demonstrated that monocytes, after a Ficoll and Percoll density-gradient centrifugation, express a very low amount of sst_{2A} mRNA (36). This suggests that already minimal activation, caused by the Percoll den-sity-gradient centrifugation step, of the cells results in upregulation of sst_{2A} mRNA. Stimulation of T- and B-lymphocytes with non-specific mitogens did not alter the expression pattern of receptor subtypes. SS mRNA was undetected in all cell populations studied, whereas we detected CST mRNA in lymphocytes. In a previous study we demonstrated expression of the SS-like peptide CST in monocytes and its functionally derived cells, i.e. macrophages and dendritic cells (36). Since the sensi-tivity of our assay was set at detection of expression in approximately 10% of the cell population, it is not known whether all T- and B-lymphocytes express sst_3, or whether sst_3 expression is restricted to a subset of the T- and B-lymphocytes. Separation of T-lymphocytes into the major subpopulations of $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T-cells showed sst_3 mRNA to be

present in both. Using Fluo-SS derivative in FACS ana-lysis we were not able to detect sst on the T- or B-lym-phocyte populations. This could suggest that the expression of sst_3 in these cells is very low or restricted to a very minor subset of the lymphocytes or, alterna-tively, that the Fluo-SS derivative has altered specificity for sst_3. Moreover, previous studies have demonstrated that in the cerebellum high levels of sst_3 receptors could be detected, while radioligand-binding studies failed to detect sst_3-binding sites in this region (37, 38). Recent studies have shown that sst can form either homo- or heterodimers with other sst or other G-protein coupled receptors (39, 40). Heterodimeriza-tion of sst_3 with sst_2 resulted in a 100-fold lower affinity for sst_3-selective agonists, compared with cells selec-tively expressing sst_3 (39). However, heterodimerization of sst_3 with other sst can be ruled out in lymphocytes, as no other sst are expressed. Therefore, heterodimeri-zation with other members of the G-protein coupled receptor family might provide a potential explanation for the difficulty in detecting sst_3-binding sites in our studies. Further studies will have to elucidate this issue.

A number of studies have shown functional effects of SS on human lymphocytes (6, 20, 41, 42), providing evidence for the presence of functional sst on these cells. Corroborating our data, sst_3 was recently shown to be present in human enriched lymphocyte fractions, which also contained functional SS-binding sites (43). Our data obtained with Q-PCR on PBMC samples reveal that the number of sst_3 cDNA copies obtained per million cells is approximately 15 000 or less. Assuming that the combined efficiency of RNA isolation and cDNA synthesis is in the order of 10%, this suggests that only a subpopulation of the T-lymphocytes (which comprise more than 50% of the PBMC population) express sst_3.

Freshly isolated monocytes do not express sst_{2A} or sst_3 at detected levels, but can be induced to express sst_{2A} receptor mRNA. The presence of sst on these acti-vated monocytes was confirmed using Fluo-SS and FACS analysis. Any of the non-specific stimuli we applied was sufficient to trigger this induction. Expression of sst_{2A} was in some samples detectable as early as 1 h after stimulation. These observations suggest that sst_{2A} upregulation is a very early event in activation of monocytes. By Q-PCR we showed that the level of sst_{2A} induction varies among different donors, but that it is very substantial in all cases.

Translating our results with naïve and *in vitro*-acti-vated immune cells from the peripheral circulation to the situation for immune cells present in lymphatic tis-sues, it seems very likely that the receptor-binding sites found with radiolabelled SS (analogues) are present on immune cells from the monocytic lineage. Whether the lymphocytes after migration into the tissues also express sufficient amounts of sst to be visualized in autoradiog-raphy is uncertain, since T-lymphocyte-rich regions in lymphoid tissues were reported to be sst negative and
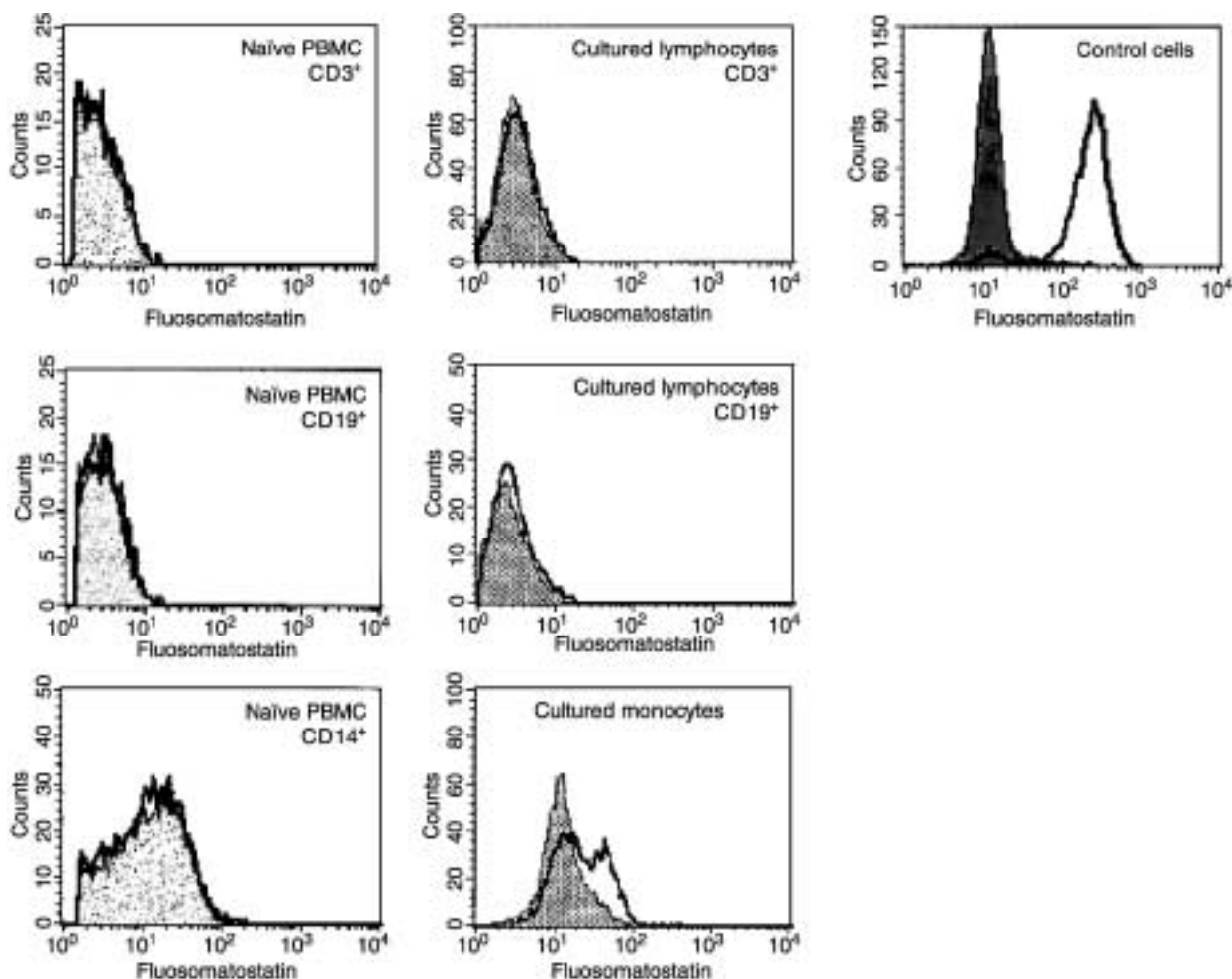
Figure 7 FACS analysis of Fluo-SS binding to T- or B-lymphocytes or to monocytes. Naïve PBMCs or an enriched lymphocyte population cultured for 24 h in the presence of PHA were labelled with Fluo-SS and PE-conjugated CD3 or CD19 antibodies to analyse binding of SS to T- and B-lymphocytes respectively. Monocytes were analysed by labelling the naïve PBMCs with Fluo-SS and PE-conjugated CD14 antibody and by labelling an enriched monocyte population cultured for 24 h in the presence of LPS with Fluo-SS. The grey areas represent the fluorescent signal remaining after displacement of Fluo-SS by unlabelled SS, the solid line shows the amount of fluorescence after labelling with Fluo-SS. These data are examples of one of the experiments carried out and represent the data found in three independent healthy donors.

germinal centres of secondary lymphatic follicles rich in B-lymphocytes were shown to be receptor positive by autoradiography (10, 44). Our recent studies using a sst_{2A}-specific antibody for immunohistochemistry confirmed the presence of the sst_{2A} receptor on cells of the monocytic lineage and the absence of this receptor from T-lymphocytes in human rheumatoid synovium (13) and sarcoid granuloma tissue biopsies (12). However, we cannot rule out the possibility that one or more of the receptor subtypes is upregulated by other, more specific stimuli than the ones we applied. Furthermore, cells other than lymphocytes or monocytes/macrophages in lymphoid tissues are likely to express SS and sst as well, such as has been shown in endothelial cells in inflammatory lesions (13) and in thymic epithelial cells from normal human thymus (31).

Knowing these results, we may now understand much better the data described so far in the literature. Whether or not sst were detected in PBMCs presumably depended on the extent to which monocytes were activated during isolation of the PBMCs (17–19). Moreover, our results show that sst mRNA expression in established cell lines may not necessarily be representative for human immune cells *in vivo*. Most of the lymphoid cell lines studied by Tsutsumi *et al.* (19) expressed mRNA for sst_{2-5}, with sst_2 being the major subtype. Our own studies of a panel of lymphoid cell lines revealed only sst_{2A} and sst_3 or no SS receptor expression at all (26, 45). The incongruity possibly can be explained by differences in assay sensitivity. Nevertheless, it is evident that many lymphoid cell lines express sst_{2A} as the major subtype, which is

certainly at variance with lymphoid cells in the circulation. Our comparison of human PBMCs and human lymphoid cell lines also revealed that in immortalized cell lines the expression level of sst_{2A} is similar to or much higher than in activated PBMCs. Since also the amount of hprt RNA per cell was much higher in the cell lines than in PBMCs, presumably due to the enhanced proliferative capacity of the immortalized cells, the absolute numbers of sst_{2A} and sst_3 mRNA copies per cell are 10- to 800-fold higher in the immortalized cell lines.

Results reported in studies concerning the function of SS and its receptors in immune cells are not conclusive as to which sst mediate the different effects. On the other hand, expression of sst_2 receptors has been found to be of major interest in the treatment of patients with growth hormone-secreting pituitary adenomas, carcinoids and other gastroenteropancreatic tumours (46–48). Treatment of these patients with SS analogues has demonstrated an improvement of clinical symptoms due to the inhibition of the secretion of hormones or bioactive peptides by the tumour cells (48). In addition, reduction of tumour mass has been observed in selected cases. Apart from their important role in therapy, sst can also play a role in diagnosis of disease. Radiolabelled SS analogues are used for visualization of neuroendocrine tumours (49), but also, diseases involving cells of the human immune system, like Hodgkin's and non-Hodgkin's lymphomas and sarcoidosis, can be visualized by a gamma camera after binding of radiolabelled SS analogues to sst_2 receptors present in affected tissues in these diseases (16, 50, 51). These studies have thus shown that sst can play a major role in diagnosis and therapy of various diseases.

In vitro culturing of human PBMCs has demonstrated effects of SS or its analogues on numerous (immune) functions of these cells. However, several conflicting results regarding effects of SS (or its analogue octreotide) on proliferation, differentiation or cytokine production have been reported (discussed below), which possibly can be attributed to different experimental designs but do not clarify the function of SS in the immune system.

sst_{2A} receptors are usually associated with inhibition of hormone secretion (52, 53). Also, for LPS-activated human monocytes, it has been shown that SS inhibits the secretion of pro-inflammatory cytokines (54), although another study reported stimulation of secretion (55). Notwithstanding these conflicting accounts, the biological significance of the early induction of sst_{2A} during activation of monocytes might be that through this receptor the inflammatory response could be modulated (decreased) immediately it is invoked. The presence of SS-binding sites in rheumatoid arthritis or in granulomatous diseases (15, 16, 25) and the effects of SS in arthritis (56) also suggest that SS may act in inflammation. Consistent with the suggestion that sst_{2A} could mediate these effects are our recent studies

demonstrating the presence of sst_{2A} by immunohistochemistry in cells of the monocytic lineage in human rheumatoid synovium and sarcoid granulomas (12, 13).

The identification of SS-binding sites in haematological malignancies and the ability of SS (or octreotide) to inhibit proliferation of these malignant cells *in vitro* (18, 57) hints at a role for SS in the regulation of proliferation and/or differentiation during haematopoiesis. The presence of sst_{2A} in normal human bone marrow cells, where differentiation and proliferation take place, as well as in tumour cells from haematological origin might suggests that sst_{2A} is involved in regulating the balance between proliferation and differentiation. Moreover, recent studies have shown that SS, acting via sst_2, induces migration of human haematopoietic progenitor cells (58). These findings suggest that sst_2 on human immune cells may play a role in homing of the cells during differentiation or in immune reactions by chemotaxis.

In human PBMCs, inhibition of proliferation of mitogen-activated lymphocytes has been reported (20, 41, 42), although contrasting results have also been obtained with enriched lymphocyte fractions (43) and with a lymphoid cell line (22). In the latter studies proliferation could be stimulated by SS. Because lymphocytes selectively express sst_3 mRNA, we suggest that this receptor subtype mediates the responses observed in this cell type. Interestingly, sst_3 has been shown to be involved in SS-induced apoptosis, resulting in growth inhibition of the cell population (59). Our finding that sst_3 is the main subtype expressed in mature lymphocytes and, moreover, the upregulation of sst_3 mRNA in T-lymphocytes during culture and by activation with PHA, suggests that this receptor subtype may have an important function in these cells. Mature B- and T-lymphocytes in the circulation are resting cells that start proliferation after activation. It is therefore conceivable that in mature B- and T-lymphocytes sst_3 may have an important function in regulating the balance between proliferation and apoptosis after activation. However, with respect to the possible role of sst_3 in mature B-lymphocytes we could only detect sst_3 mRNA by RT-PCR, and not by Q-PCR. This suggests that sst_3 mRNA expression in B-lymphocytes is very low and therefore it may be hypothesized that sst_3 in B-lymphocytes plays a less important regulatory role, when compared with T-lymphocytes, in which we found clear regulation of sst_3 mRNA expression.

Equally important for the understanding of the function of SS and its receptors in lymphoid tissues is knowledge of which cells produce the agonistic peptide SS. We demonstrated that PBMCs do not synthesize SS mRNA, not even after activation. We therefore assume that SS is synthesized in other cells in the lymphoid tissues, e.g. in thymic epithelial cells (31) or in nerve endings, or perhaps in endothelial cells. Another possibility is that lymphocytes or monocyte-derived cells do express SS mRNA after more specific stimulation *in vivo*, conceivably after

migration from the periphery into lymphoid tissues or to sites of inflammation. On the other hand, the detection of CST mRNA in various tissues and cells of the human immune system (27) and the detection of CST mRNA in the present study in T- and B- lymphocytes may suggest that CST, rather than SS, may play a regulatory role acting via the sst in these immune cells. However, in monocytes, macrophages and dendritic cells, CST mRNA was upregulated upon activation with LPS (27), whereas in B- and T-lymphocytes we found in general a downregulation of the CST mRNA expression upon activation. The significance of this downregulation in T- and B-lymphocytes as well as the functional significance of CST in these cells remains to be determined. We hypothesized that the downregulation of endogenous CST expression may be a direct feedback mechanism following the upregulation of sst_3, in order to regulate responsiveness to the peptide. This suggests that CST and sst_3 may interact in regulation of the number of T-cells. Although we have now extensively demonstrated the expression of different sst and their ligands in cells of the human immune system *in vitro*, the functional significance of SS and its receptors in the human immune system *in vivo* remains largely unknown. As discussed above, previous studies have shown different (conflicting) results. Further studies will have to demonstrate the functional significance of SS or SS-like peptides and their receptors *in vivo* and the potential implications for treatment of immunological diseases.

Summarizing, we have shown for the first time that human B- and T-lymphocytes in the peripheral blood selectively express sst_3, whereas circulating monocytes do not express sst. Expression of sst_3 mRNA can be upregulated by activation in T-lymphocytes, while sst_3 expression levels in B-lymphocytes are probably very low, suggesting a less important role of these receptors in B-lymphocytes, when compared with T-lymphocytes. Upon stimulation with non-specific mitogens the monocytes, but not the lymphocytes, are induced to express sst_{2A} receptors. No expression of SS mRNA was detected, not even after stimulation. In line with the expression of CST mRNA in monocytes, macrophages and dendritic cells, CST mRNA is expressed in T- and B-lymphocytes as well. The differential and cell-type-specific regulation of the expression of sst_{2A} and sst_3 receptors in human PBMC, in combination with data on functional studies of SS on immune cells, suggests that the function of SS, or more presumably CST, and its receptors in the human immune system may be found in regulating cytokine secretion in inflammatory responses and in regulating the balance of proliferation and apoptosis of (activated) lymphocytes in immune responses.

Acknowledgement

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