Effects of Siglec on the expression of IL-10 in the macrophage cell line RAW264

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Abstract Interleukin-10 (IL-10) expression was significantly elevated upon stimulation with lipopolysaccharide (LPS) when the sialic acid-recognizing Ig-superfamily lectin Siglec-5 or -9 was overexpressed in RAW264 cells. During the course to clarify the mechanism for this activation, we found that IL-10 promoter proximal region up to −500 bp led to transactivation similar to that up to −1,500 bp. Among the transcription factors that activate the mouse IL-10 promoter so far reported, the level of C/EBP β was increased in Siglec-9-expressing cells. Transient expression of the C/EBP β major isoform LAP led to an increase in the expression of IL-10 in Siglec-9-expressing cells, but not in mock-transfected control RAW264 cells upon stimulation with LPS, as assessed by either a luciferase assay or the production of IL-10. Without LPS, the IL-10 promoter was activated by transiently expressed LAP in Siglec-9-expressing cells, however, the magnitude of transactivation was less than that with the LPS stimulation. The knockdown of C/EBP β down-regulated the production of IL-10. Taken together, these results suggest that one of the reasons for the stimulation of IL-10 expression in Siglec-9-expressing cells may be an increase in intracellular C/EBP β level.

Keywords Interleukin-10 · Siglec · C/EBP β · Lipopolysaccharide

Introduction

Innate immunity functions as a pathogen sensor and contributes to protection against pathogens and the establishment of adaptive immunity. A group of transmembrane proteins, Toll-like receptors (TLRs), recognizes components of bacteria, viruses, and fungi as a pattern-recognition receptor to activate the first line of defense against pathogens (Kondo et al. 2012; West et al. 2006). For example, lipopolysaccharide (LPS) was recognized by TLR4 and induced strong immune responses including inflammation.

Siglecs are sialic acid-recognizing Ig-superfamily lectins mainly expressed in immune cells (Crocker et al. 2012; Pillai et al. 2012). Members of CD33-related Siglecs have been shown to down-regulate both innate and acquired immune responses, and this may occur via cytosolic immunoreceptor tyrosine-based inhibitory motifs (ITIMs). We previously reported that Siglec-9, a member of CD33-related Siglecs, inhibited the production of proinflammatory cytokines and enhanced that of anti-inflammatory cytokine interleukin-10 (IL-10), induced by various ligands for TLRs. The ITIMs of Siglec-9 were previously shown to play an indispensable role in modulating the expression of cytokines (Ando et al. 2008).

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CCAAT/enhancer-binding protein (C/EBP) family proteins are a member of the basic region-leucine zipper (bZIP) proteins including C/EBP α , β , γ , δ , and ζ (Nerlov 2008). C/EBPs play central roles in many physiological processes such as liver metabolism, adipogenesis, and hematopoiesis, and these transcription factors form complexes with a wide variety of other transcription factors. Among the C/EBPs, C/EBP β plays an important role in adipose differentiation and innate immunity. Three isoforms of C/EBP β have been identified: LAP*, LAP, and LIP, and these are translated from the same mRNA, but from different initiation codons (Descombes and Schibler 1991). LAP and LAP* were shown to be transactivators, whereas LIP is a repressor. The balance between LAP and LIP was reported to have a marked effect on C/EBP β -mediated transcriptional regulation (Li et al. 2008; Schroeder-Gloeckler et al. 2007). IL-10 is produced by macrophages upon LPS stimulation, which seems to constitute a negative feedback regulation of inflammation by its potent anti-inflammatory activity (Saraiva and O'Garra 2010). IL-10 promoter contained several binding sites for C/EBP β , which were required for LPS-induced transactivation of IL-10 (Liu et al. 2003). It is also reported that C/EBP β was induced by LPS with or without IFN- γ stimulation (Bradley et al. 2003; Ruffell et al. 2009), which was shown to be necessary for the production of IL-10 in macrophages (Ruffell et al. 2009). In the present study, the role of C/EBP β in the increased expression of IL-10 in Siglec-9-expressing cells was studied.

Materials and methods

Reagents

LPS from *Escherichia coli* 0111:B4 was purchased from Sigma Aldrich (St Louis, MO, USA). Anti-C/EBP β (sc-150) and anti-actin (AC-15) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and R&D Systems (Minneapolis, MN, USA), respectively. The OptEIA ELISA set for IL-10 was from BD PharMingen (San Jose, CA, USA).

Plasmid construction

To construct the mouse IL-10 promoter-luciferase reporter plasmid, the promoter region was amplified

from mouse genomic DNA by PCR using the following forward primers: 5'-CATACGCGTATCAGCCCTCTCGGGGTTTC-3' (for -500) 5'-CATACGCGTACGTGTAGCATTGCCCCC-3' (for -1,000), 5'-CATACGCGTGATCTCTGATCTACAGCAGTGTGTC-3' (for -1,500) and reverse primer: 5'-CATCTCGAGGATGGAGCTCTCTTTTCTGCAAGG-3' (*Mlu*I and *Xho*I sites used for cloning are underlined). The amplified DNA fragments were introduced into the pGL3-basic vector (Promega, Madison, WI, USA).

The expression plasmid for the isoforms of mouse C/EBP β : LAP*, LAP, and LIP were constructed by inserting PCR fragments into pcDNA4 (Invitrogen, Carlsbad, CA, USA). The primers used were as follows. Forward primers: 5'-CATAAGCTTACCATGCACCGCCTGCTGGC-3' for LAP*, 5'-CATGAATTCGCCATGGAAGTGGCCAAC-3' for LAP and 5'-CATGAATTCACCATGGCGGCCGGTTTC-3' for LIP; common reverse primer: 5'-CATCTCGAGGCA GTGGCCCCGCCGAGG-3' (*Hind*III or *Eco*RI and *Xho*I sites used for cloning are underlined), in which the endogenous stop codon was omitted to add the His tag at the C-terminus.

Cells

The macrophage cell line RAW264 and its derivative expressing Siglec-9 was described previously (Ando et al. 2008). These cells were maintained in RPMI1640 (Nissui, Tokyo, Japan) containing 10 % heat-inactivated fetal calf serum (PAA Laboratories, Pasching, Austria), 0.03 % L-glutamine (Wako, Osaka, Japan), 5×10^{-5} M 2-mercaptoethanol (Wako), 100 U/ml penicillin G (Wako), and 100 μ g/ml streptomycin (Wako).

Knockdown of C/EBP β by small interfering RNA (siRNA)

The following double-stranded RNA oligonucleotides were used as siRNA: 5'-CAGCCGGGCCUGAGUA AUCACUUA-3' (siRNA1) and 5'-CGCGAGAGCUC AGCACCCUGCGGAA-3' (siRNA2). These siRNAs (50 nM) were introduced into RAW264 cells (1.5×10^4) seeded in 96-well plates using Lipofectamine 2000 (Invitrogen) by reverse transfection as recommended by the supplier, and medium was changed after 24 h. Cells were then stimulated by LPS (500 ng/ml) at 48 h post-transfection. The

supernatant was harvested 8 h after stimulation, and the amount of IL-10 was determined by ELISA. Cells were lysed with SDS-sample buffer and used for western blotting analysis to confirm the reduction of C/EBP β .

Luciferase assay

Cells (3×10^5) were seeded into 24-well plates and were transfected with reporter plasmids using FuGENE HD (Promega) after 24 h culture. The plasmids containing luciferase reporter (1.8 μ g) were transfected with that containing Renilla luciferase expressed by the thymidine kinase (TK) promoter (0.2 μ g). The medium was changed 24 h after transfection, and LPS (500 ng/ml) was added 48 h post-transfection followed by 8 h stimulation. Reporter assays were performed with the dual luciferase assay kit (Promega) according to the manufacturer's instructions. Luciferase activity was measured with a luminometer (Atto, Tokyo, Japan), and normalized to Renilla luciferase activity. In some experiments, cells were transfected with 1.0 μ g LAP expression vector (Dohda et al. 2004) along with the luciferase reporter (0.9 μ g) and TK-Renilla control (0.1 μ g) vectors.

Chromatin immunoprecipitation (ChIP) assay

Cells (1.0×10^7) were stimulated with LPS (500 ng/ml) for the indicated period and cross-linked with 1 % formaldehyde in phosphate-buffered saline at 37 °C for 10 min. Harvested cells were suspended in a lysis buffer [1 % SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), and 1 mM phenylmethylsulfonyl fluoride (PMSF)] and sonicated. The samples were precleared with salmon sperm DNA-conjugated protein A agarose (Upstate Biotechnology, Lake Placid, NY, USA) in a dilution buffer [0.01 % SDS, 1.1 % Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 16.7 mM NaCl, 1 mM PMSF] and incubated with 2 μ g of anti-C/EBP β antibody. The precipitates with salmon sperm DNA-conjugated protein A agarose were washed, and DNA was eluted with an elution buffer (1 % SDS in 0.1 M NaHCO₃) and incubated at 65 °C for 24 h in the presence of 0.4 M NaCl. DNA samples were digested with proteinase K at 45 °C for 2 h followed by purification with a kit (QIAquick Nucleotide Removal Kit, Qiagen, Tokyo, Japan). Immunoprecipitated DNA and input DNA were

Fig. 1 The expression of IL-10 was mainly regulated by the region up to 500 bp upstream from the transcriptional start site. RAW264 cells that expressed Siglec-9 were transfected with luciferase reporter constructs containing truncated IL-10 promoter/enhancer regions (−1,500 to +65, −1,000 to +65, and −500 to +65, in which +1 indicates the transcriptional start site). Forty-eight hours after the transfection, cells were stimulated with 500 ng/ml of LPS for 8 h, and luciferase activity was then measured. Data are expressed as mean value with standard deviation of four independent experiments. *Open bar* mock-transfected control cells; *solid bar* Siglec-9-expressing cells. * $p < 0.05$ versus mock-transfected control cells

analyzed by real-time PCR with LightCycler (Roche Diagnostics, Tokyo, Japan) using 5'-TCCGAGTCAG-CAAGAAATATCGGACG-3' (as a forward primer) and 5'-TTCCTGAGGCAGACAGCTGTTCTATG-3' (as a reverse primer) to amplify the fragment located in the IL-10 promoter region from −452 to −276. Data are shown as the mean $\pm$ standard deviation. Statistical significances were determined by Student's t test.

Results

IL-10 production was mainly regulated within 500 bp upstream region in Siglec-9-expressing cells

The promoter proximal regions of IL-10 were cloned and fused to the luciferase reporter gene. The recombinant plasmids containing three different IL-10-promoter flanking regions were introduced in RAW264 cells, and reporter activity was subsequently measured (Fig. 1). Although stimulation with LPS was supposed to be essential for the production of IL-10, basal levels of luciferase activity were observed in mock-transfected control cells without stimulation. A

markedly higher activity level was observed either with or without the LPS stimulation in Siglec-9-expressing cells. In this case, luciferase activity appeared to be independent of the size of the promoter/enhancer region: similar activity levels were observed in the fragment up to -500 bp and that up to $-1,500$ bp. These results suggest that a major control region, except for a far upstream region (Saraiva and O'Garra 2010), for the production of IL-10 in Siglec-9-expressing cells resides up to -500 bp in the IL-10 promoter/enhancer region.

C/EBP β regulated IL-10 production in Siglec-9-expressing cells

The binding sites for C/EBP β and Sp1 were reported in this region (up to -500 bp) of the mouse IL-10 gene (Saraiva and O'Garra 2010). The deletion of a DNA sequence between -423 and -362 containing C/EBP binding sites caused a marked decrease in luciferase activity (data not shown). Thus, we analyzed the effects of C/EBP β on the production of IL-10. The expression levels of C/EBP β were measured in mock-transfected and Siglec-9-expressing cells. LAP and LIP levels increased in both cell types after the stimulation with LPS, and LAP and LIP levels in Siglec-9 cells were almost twice that of control cells after the stimulation (Fig. 2). On the other hand, LAP* was not abundant. These indicated that C/EBP β was involved in the increased level of IL-10 produced in Siglec-9-expressing cells. Thus, LAP, a major form of C/EBP β , was transiently expressed and luciferase

Fig. 2 The expression level of C/EBP β was higher in Siglec-9-expressing cells with LPS stimulation. Cells were stimulated with 500 ng/ml of LPS for the indicated period and the whole cell lysate was subjected to western blotting using the anti-C/EBP β antibody. Representative of four independent experiments is shown

Fig. 3 Transient expression of LAP enhanced IL-10 promoter activity in Siglec-9-expressing cells. Cells were transfected with the expression vector for LAP or the empty vector (pcDNA4) along with the luciferase vector ($-1,500$ to $+65$) and TK-Renilla. Forty-eight hours post-transfection, cells were stimulated with 500 ng/ml of LPS for 8 h, and luciferase activity was then measured. Data are expressed as mean value with standard deviation of three independent experiments. Open bar mock-transfected control cells solid bar Siglec-9-expressing cells. * $p < 0.05$ versus empty

activity was measured with the reporter construct containing the IL-10 promoter/enhancer region (Fig. 3). In the mock-transfected control cells, the expression of LAP down-regulated reporter activity either in the presence or absence of the LPS stimulation. On the other hand, LAP modestly stimulated the activity in Siglec-9-expressing cells either in the presence or absence of LPS, suggesting that LAP only enhanced promoter activity in Siglec-9-expressing cells. To clarify the mechanism underlying this activation, the derivatives of C/EBP β , His-tagged LAP*, LAP and LIP were separately expressed under similar conditions to those shown in Fig. 3. Figure 4a shows the expression levels of the C/EBP β derivatives; the amount of His-LAP was threefold that of endogenous LAP in Siglec-9-expressing cells. IL-10 was near the minimum detection levels in mock-transfected control cells in the absence of the LPS stimulation, and only a low level of IL-10 was produced in Siglec-9-expressing cells (Fig. 4b). In the presence of LPS stimulation, IL-10 production was weakly induced in mock-transfected control cells, while strong induction was observed for Siglec-9-expressing cells. On the other hand, stimulation seemed relatively weak in luciferase assay (Fig. 1), as reported (Liu et al. 2003), which may be due to the

Fig. 4 Transient expression of LAP increased IL-10 production in Siglec-9-expressing cells. **a** Cells were transfected with the expression vectors for His-tagged C/EBP β isoforms (LAP*, LAP, or LIP) and were analyzed by western blotting after 48 h. *Endogenous LAP*, **Endogenous LAP, ***endogenous LIP. The addition of the C-terminal tag increased the molecular weight of transfected C/EBP β s. empty, pcDNA4 vector used as a control. **b** Forty-eight hours after transfection of the expression vectors, cells were stimulated with 500 ng/ml of LPS for 8 h, and the amount of IL-10 in the supernatant was measured by ELISA. Data are expressed as mean value with standard deviation of three independent experiments. *Open bar* mock-transfected control cells *solid bar* Siglec-9-expressing cells. * $p < 0.05$ versus empty

fact that luciferase plasmids contained just the promoter proximal region without other factors such as far upstream enhancers and epigenetic information (Saraiva et al. 2005; Zhang et al. 2006). IL-10 production that was induced by LPS stimulation was reduced by LAP*, LAP, and LIP in mock-transfected control cells, however it increased by almost 1.7-fold in Siglec-9-expressing cells with LAP. LIP reduced the expression in a parallel experiment using Siglec-9-expressing cells, while LAP* did not have a strong effect (Fig. 4b). These results suggested that LAP stimulated the production of IL-10 in Siglec-9-expressing cells as with the case of luciferase reporter assay shown in Fig. 3.

Fig. 5 Knockdown of C/EBP β reduced IL-10 production in Siglec-9-expressing cells. **a** Confirmation of C/EBP β knockdown. The expression of C/EBP β isoforms was analyzed by western blotting. Scramble siRNA was used as a control. **b** Forty-eight hours after the transfection of siRNA, cells were stimulated with 500 ng/ml of LPS for 8 h, and the amount of IL-10 produced was measured. Data are expressed as mean value with standard deviation of five independent experiments. *Open bar* mock-transfected control cells *solid bar* Siglec-9-expressing cells. * $p < 0.05$ versus scramble

We then determined the effects of C/EBP β on IL-10 production in an inverse experimental set using siRNA (Fig. 5). In mock-transfected control cells, LAP levels were reduced to almost 50 % by specific siRNAs compared with those of scramble RNA-transfected cells, and a low level of IL-10 production was observed in the presence of the LPS stimulation. LAP expression levels in Siglec-9-expressing cells were reduced to 20–30 % by siRNAs compared with scramble RNA. IL-10 production was decreased by the knockdown, and the extent of the reduction appeared to be approximately proportional to the decrease in LAP either in the presence or absence of the LPS stimulation.

Fig. 6 Siglec-9 enhanced the binding of C/EBP β to the IL-10 promoter *in vivo* with the LPS stimulation. Cells were stimulated with 500 ng/ml of LPS for the indicated period and subjected to ChIP analysis with the anti-C/EBP β antibody. The DNA fragment of –452 to –276 containing C/EBP β binding sites was amplified and measured by real-time PCR. Data are expressed as mean value with standard deviation of four independent experiments. *Open bar* mock-transfected control cells *solid bar* Siglec-9-expressing cells. * $p < 0.05$ versus 0 h; ** $p < 0.05$ versus mock-transfected control cells

C/EBP β binding to the IL-10 promoter region was higher in Siglec-9-expressing cells upon the LPS stimulation

We then investigated whether the binding of C/EBP β to the IL-10 promoter/enhancer region was increased in Siglec-9-expressing cells by ChIP analysis. As shown in Fig. 6, the binding of C/EBP β was almost equal between control and Siglec-9-expressing cells without the LPS stimulation, but increased with the LPS stimulation in both cells. C/EBP β binding to the IL-10 promoter in Siglec-9-expressing cells was almost threefolds that of control cells 6 h after LPS stimulation. This result was in accordance with the increased amount of intracellular C/EBP β observed in Siglec-9-expressing cells, as shown in Fig. 2. Among the C/EBPs, binding of C/EBP β and δ was reported at this site (Liu et al. 2003). However, no obvious changes were observed in C/EBP δ binding between these two cell types (data not shown).

Discussion

C/EBP β and δ as well as Sp1 are known to activate the expression of mouse IL-10 (Saraiva and O'Garra 2010), and stimulation by LPS is a prerequisite for this activation. As shown in Figs. 3 and 4, the LPS stimulation was necessary for the production of IL-10 in both mock-transfected and Siglec-9-expressing cells, and an increase in C/EBP β levels may have primarily led to the enhanced production of IL-10 in Siglec-9-expressing cells. The expression of C/EBP β was previously shown to be regulated at the transcriptional level upon TLR stimulation, and enhanced CREB binding at the C/EBP β promoter was reported as the reason for the induction of C/EBP β in macrophages (Niehof et al. 1997). Thus, we examined the expression level of CREB by RT-PCR; however, our preliminary results did not support the enhanced expression of CREB as the reason for the higher induction of C/EBP β in Siglec-9-expressing cells. Therefore, the mechanism how C/EBP β level increased in Siglec-9-expressing cells remains to be elusive.

Siglecs negatively control innate immunity by recruiting tyrosine phosphatase SHP-1 and -2 through their ITIMs (Crocker et al. 2012; Pillai et al. 2012). However, we could not demonstrate direct link between increased IL-10 production and the recruitment of SHPs. In the present study, an increase in intracellular C/EBP β by transient transfection stimulated the production of IL-10 only in Siglec-9-expressing cells (Fig. 4); therefore, Siglec-9-expressing cells may possess an additional mechanism other than the increase of C/EBP β protein, which contributes to the enhanced production of IL-10. The expression of IL-10 could be regulated by various factors: for instance, tyrosine phosphorylation of STAT3 was important for the IL-10 production under a specific condition (Ziegler-Heitbrock et al. 2003). However, it has not been assessed whether recruitment of SHPs affects the phosphorylation status because cells contain large amount of SHP molecules, possibly more than the levels that Siglecs can sequester from cytosol. Phosphorylation may affect transcriptional activity of C/EBPs, but major phosphorylation sites were serine/threonine and not tyrosine (Nerlov 2008).

Thus, the additional roles of Siglecs in increased production of IL-10 are remained to be investigated for further study.

In addition to transcription factors, epigenetic changes such as modifications of chromatin may also affect the expression of IL-10. We found that histone H4 acetylation, a hallmark for active chromatin, around the C/EBP β site was higher in Siglec-9-expressing cells (Shoji et al.; unpublished results). The mechanistic reason currently remains unknown; however, such a difference may be linked to the strong and early production of IL-10 in Siglec-9-expressing cells in addition to the increased level of C/EBP β .

LPS and several RNA viruses induced the expression of mouse Siglec-E in bone marrow-derived macrophages (Boyd et al. 2009) and Siglec-G in peritoneal macrophages (Chen et al. 2013), respectively. Considering our results of relatively shared feature of human ITIM-containing CD33-related Siglecs (Siglec-9 and Siglec-5; Ando et al. 2008), Siglecs may be involved in the termination of inflammation by IL-10 production. Further studies are needed to clarify the complete mechanisms and physiological roles for the increase in IL-10 production by Siglecs in more detail.

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