

## Chapter 2

# IL-6 and Inflammatory Diseases

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**Abstract** Interleukin-6 (IL-6) is a multifunctional cytokine that plays key roles not only in the immune system but also in a variety of biological processes. It is a primary regulator of both acute and chronic inflammations. Moreover, it has proven an excellent target for clinical treatment, as the anti-IL-6 receptor antibody has been successfully used against autoimmune disorders such as rheumatoid arthritis, juvenile idiopathic arthritis, and Castleman's disease. In fact, it could be argued that IL-6 is the best example of basic cytokine research extending into clinical application. Here, we summarize IL-6 and its biological functions, with particular emphasis on inflammation and chronic inflammatory diseases, and a recently discovered inflammation control mechanism, the inflammation amplifier (formerly known as the IL-6 amplifier). We also describe a recent finding that indicates neural stimulations can modulate the activation of the inflammation amplifier at local blood vessels, creating a gate for the influx of immune cells into the central nervous system, which suggests the entry of immune cells into target organs can be artificially manipulated by local neural stimulation.

**Keywords** Chronic inflammation • Interleukin-6 • Neuroimmune interaction • NF- $\kappa$ B • STAT3 • The inflammation amplifier

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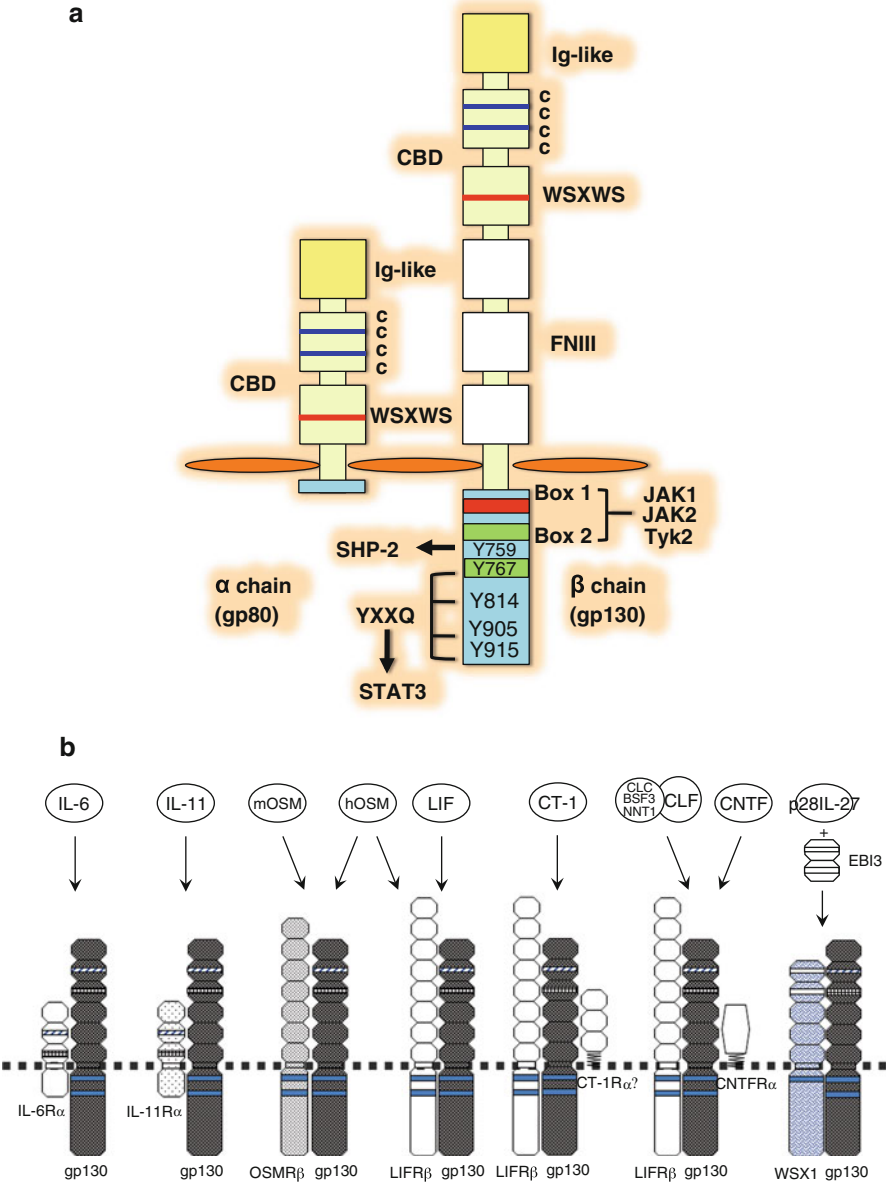
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## 2.1 General Aspects of IL-6 and Its Signal Transduction

### 2.1.1 *Discovery of IL-6, Its Receptor Subunits, and Related Signal Transduction Molecules*

We now know that IL-6 has a wide variety of biological roles in numerous systems including the immune, nervous, and endocrine systems (Kamimura et al. 2003; Taga and Kishimoto 1997). As a result, many research groups from different fields had independently sought the molecular cloning of IL-6, which led to a number of different names for the same molecule. We give special consideration to Kishimoto and Hirano, as they were the first to publish on the matter (Hirano et al. 1986). Hirano who is one of authors originally found IL-6 to be a soluble factor present in the culture supernatant of cells in pleural effusion and isolated from patients with pulmonary tuberculosis. IL-6 was seen to induce B-cell growth and antibody production, which is why it was originally named B-cell stimulatory factor 2, or BSF2. Other groups subsequently molecularly identified factors such as interferon (IFN) $\beta$ 2, which was later recognized to be BSF2 based on its 26-kDa mass and comparisons of cDNA sequences. Eventually, the research community settled on the name IL-6.

The IL-6 receptor consists of a ligand-binding IL-6-receptor chain (IL-6R, gp80, or CD126) and signal-transducing subunit gp130 (CD130). After the molecular cloning of IL-6R by Kishimoto's group, it was revealed that IL-6R has a cytoplasmic tail that was considered too short to transmit intracellular signaling (Yamasaki et al. 1988). They also later found a protein of 130 kDa co-precipitated with IL-6–IL-6R complexes, gp130 (Taga et al. 1989). Through cDNA cloning, they showed gp130 has a long cytoplasmic domain and is an essential signal-transducing component of the IL-6 receptor (Hibi et al. 1990). The extracellular regions of gp130 and IL-6R contain a four-cysteine motif and a WSXWS motif, which constitute cytokine-binding domains, whereas the intracellular domain of gp130 has the box regions responsible for Jak kinase binding (Murakami et al. 1991) and multiple tyrosine residues that are phosphorylated upon ligand binding (Fig. 2.1a). In addition to the receptor components, it was also successfully identified a key transcription factor for IL-6 signaling, STAT3 (Akira et al. 1994), a transcription factor that leads to IL-6 expression, C/EBP $\beta$  (Akira et al. 1990), and factors that negatively regulate the IL-6 signal including SOCS family molecules (Yoshimura et al. 2007). They also revealed that gp130 forms homodimers upon IL-6 binding, which activates tyrosine kinase activity (Murakami et al. 1993), and had a central role in the development of the anti-IL-6R antibody, which is currently a popular treatment against autoimmune diseases. Hirano's group also did a comprehensive study of the intracellular signaling, finding Gab1 and Gab2 are adaptor molecules for gp130-mediated ERK signaling (Nishida et al. 1999; Takahashi-Tezuka et al. 1998), and established mutant versions of gp130 knock-in mice to dissect gp130 signaling in vivo (Ohtani et al. 2000). One knock-in strain, F759 mice, spontaneously developed rheumatoid arthritis-like joint disease (Atsumi et al. 2002) to provide an excellent



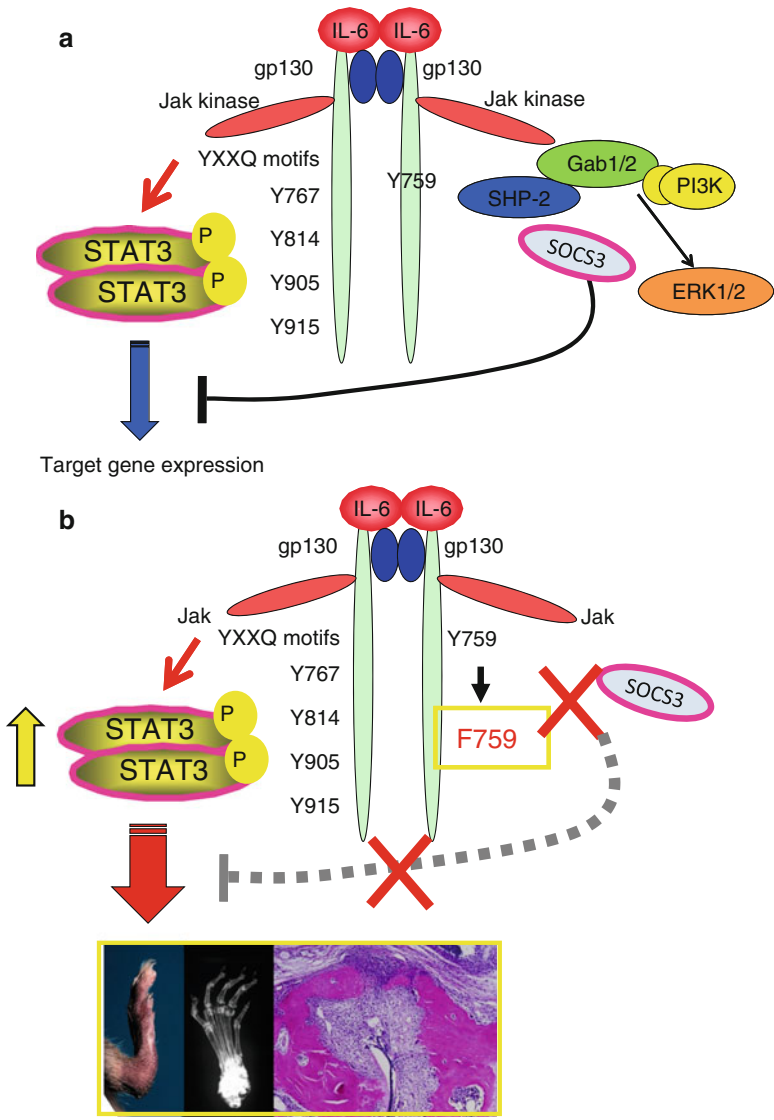
**Fig. 2.1** Components of the interleukin (IL)-6 receptor. **(a)** The IL-6 receptor  $\alpha$ -chain (gp80, *left*) and  $\beta$ -chain (gp130, *right*), and their conserved motifs. IL-6 binding to cytokine-binding domain (CBD) transduces intracellular signaling through gp130, which has a relatively long cytoplasmic tail with multiple tyrosine residues that can be phosphorylated by JAK family kinases. **(b)** gp130 is shared by many cytokines besides IL-6, including IL-11, LIF, CNTF, OSM, CT-1, CLC/NNT1/BSF3, neupoietin/CT-2, IL-27, and IL-35. These IL-6 family members all use gp130 to transmit signal transduction

model for inflammation and would eventually lead to the discovery of the inflammation amplifier (described later), a novel inflammation-inducing mechanism (Murakami et al. 2011; Ogura et al. 2008; Sawa et al. 2006).

### 2.1.2 *Signal Transduction of IL-6*

Experiments using mutated versions of the IL-6 signal transducer gp130 revealed important regions within its cytoplasmic tail, named box 1 and box 2, that are conserved among other cytokine receptors and subsequently led to the identification of the binding regions for JAK kinases (Murakami et al. 1993; Murakami et al. 1991) (Fig. 2.1a). gp130 contains six tyrosine residues in its cytoplasmic domain. All but the first are phosphorylated by JAK kinases and are involved in signal transduction emanating from the IL-6 receptor. Structural analyses have revealed that IL-6 first binds to IL-6R, and that this complex is then presented in gp130 to form a high-affinity hexamer with two IL-6 and IL-6R molecules each. The ligand binding makes the gp130 homodimer formation bend, bringing them into close proximity to enable intracellular signaling (Boulanger et al. 2003; Murakami et al. 1993; Skinotis et al. 2005). The main signaling pathway induced by the IL-6 receptor is mediated by the activation of STAT3 via YXXQ motifs located in the gp130 cytoplasmic region. Upon IL-6 binding to the receptor, the four distal tyrosine residues of gp130 are phosphorylated by JAK kinases to form binding sites for STAT3. The gp130-bound STAT3 is then tyrosine phosphorylated by JAK kinases and subsequently forms a dimer that translocates into the nucleus, where it induces the transcription of its target genes (Fig. 2.2a). One such gene is a negative regulator of cytokine signaling, SOCS3. SOCS3 binds to the JAK-kinase-phosphorylated second tyrosine residue of gp130 (Y759 in human gp130) and works as a negative feedback molecule for IL-6 signaling. SOCS3 actually binds to JAK kinases and the Y759 of gp130 simultaneously to form a high-affinity complex that mediates the inhibition of the signaling in a noncompetitive manner (Babon et al. 2012). This SOCS3-mediated negative feedback prevents immune dysregulation. As discussed next, mice devoid of this second tyrosine (F759 mice) show prolonged and enhanced STAT3 activation by IL-6 and exhibit spontaneous development of a rheumatoid arthritis-like joint disorder with age (Fig. 2.2b) (Atsumi et al. 2002). The second tyrosine of gp130 is also known to induce the SHP2/Gab/Ras/ERK pathway in some cell types (Nishida et al. 1999; Takahashi-Tezuka et al. 1998). This pathway is reported to protect mice from gastric adenoma by inducing the tissue-protecting trefoil factor, pS2/TFF1 (Tebbutt et al. 2002).

In addition to these conventional IL-6 signal transduction pathways, we recently identified a novel role of IL-6 signaling in assisting NF- $\kappa$ B signaling to synergistically induce the transcription of proinflammatory genes. We named this signaling the “inflammation amplifier” (see Sect. 2.5). Although gp130 is expressed in most cell types, IL-6R expression is relatively limited. However, IL-6R can be solubilized from the cell membrane by both alternative splicing and shedding via the action of ADAM family proteases (Chalaris et al. 2011). Theoretically, the presence



**Fig. 2.2** Intracellular signaling pathways of gp130. **(a)** Signal transduction from wild-type gp130. Tyrosine 759 is involved in the gp130-mediated ERK pathway and is essential for the SOCS3-mediated negative feedback loop. The distal four tyrosine residues form the YXXQ motif required for STAT3 binding. **(b)** Signal transduction from a gp130 F759 mutant. Because of the absence of the SOCS3-binding site (i.e., Y759), STAT3 activation is prolonged, and F759 mice suffer from autoimmune arthritis that clinically resembles rheumatoid arthritis (*bottom pictures*)

of soluble IL-6R enables IL-6 to signal into cells that express gp130 in vivo, which is the basis for multiple functions of IL-6. The soluble form of gp130 is also present naturally and acts as an antagonist for signaling mediated by IL-6/soluble IL-6R complexes, but not for IL-6/membrane IL-6R complexes (Jostock et al. 2001).

### 2.1.3 *The IL-6 Family*

gp130 is shared by many cytokines (Fig. 2.1b) in addition to IL-6, including IL-11, LIF, CNTF, OSM, CT-1, CLC/NNT1/BSF3, neuropoietin/CT-2, IL-27 and IL-35 (Collison et al. 2012; Derouet et al. 2004; Murakami et al. 2004). These IL-6 family members all use gp130 to transmit signal transduction. Some require a second signal-transducing subunit, namely, LIFR $\beta$  or OSMR $\beta$ . IL-27, a heterodimeric protein that consists of IL-27p28 and EBI3, was recently shown to act as an antagonist against gp130 signaling (Stumhofer et al. 2010). Despite sharing the signal transducer gp130, the biological functions of these IL-6 family members only partially overlap (Kamimura et al. 2003; Murakami et al. 2004). As described in detail next, IL-6, for example, has a nonredundant role in promoting inflammation, particularly chronic inflammation.

### 2.1.4 *Regulation of IL-6 Expression*

It is well known that a large amount of IL-6 is secreted in response to inflammatory stimuli such as Toll-like receptor ligands and proinflammatory cytokines including IL-1, IL-17, and tumor necrosis factor (TNF)- $\alpha$  to combat infections and, finally, to promote inflammation. The promoter region of IL-6 contains a NF- $\kappa$ B-binding site in addition to response elements for various transcription factors such as C/EBP $\beta$ . NF- $\kappa$ B activation is essential for IL-6 expression, which is greatly compromised in cells lacking vital components for NF- $\kappa$ B signaling such as IKK $\gamma$ /NEKO (Lee et al. 2012; Ogura et al. 2008). In addition, the activation of STAT3 is found to synergistically enhance IL-6 production when NF- $\kappa$ B activation is induced by other inflammatory stimuli such as IL-17 and TNF- $\alpha$ . Although a precise molecular mechanism of this synergistic effect remains elusive, the concomitant activation of NF- $\kappa$ B and STAT3 for the amplification of IL-6 expression in non-immune cells (inflammation amplifier, discussed in Sect. 2.5) is considered central for the induction and maintenance of inflammatory disease conditions.

IL-6 production can be stimulated by routine activities such as physical exercise, which results in a larger amount of IL-6 production in skeletal muscle than other cytokines (Pedersen et al. 2001). Running upregulates plasma IL-6 levels in mice to stimulate the secretion of glucagon-like peptide-1 (GLP-1), an insulin-regulating hormone, in intestinal L cells to improve insulin secretion and glycemia, suggesting IL-6 is a key regulator for glucose homeostasis, which when disrupted can cause metabolic syndromes (Ellingsgaard et al. 2011). IL-6 levels are also modulated by social interactions and stress in humans. Competitive social events, for example, increase baseline IL-6 levels (Chiang et al. 2012). Experimentally imposed stress paradigms in humans have been shown to cause potential increases in IL-6 circulation (Steptoe et al. 2007). Anxiety also promotes IL-6 production in humans (O'Donovan et al. 2010). In addition, it is known that serum IL-6 levels are controlled by a circadian cycle in humans, with a biphasic pattern that peaks at

about 0800 and 2100 and bottoms out at about 1900 and 0500 (Vgontzas et al. 2005). How these mental and daily events influence IL-6 production is not well defined at the molecular level, however. Because mental conditions can influence the immune system and potentially trigger relapse of autoimmune disorders (Srivastava and Boyer 2010), the elucidation of the molecular mechanisms underlying the neuroimmune interactions that induce pro-inflammatory cytokines including IL-6 may open a new avenue for the treatment of many chronic inflammatory diseases. One example can be seen in our discussion of the inflammation amplifier and its neural-mediated activation in endothelial cells (see Sect. 2.5), as inflammation may be induced via accumulation of immune cells in affected tissues of the central nervous system.

Posttranscriptional regulation of IL-6 is also important for controlling IL-6 levels and thus maintaining immune homeostasis in both the steady state and disease conditions. For example, the RNase *zc3h12a*, also known as regnase-1, is a Toll-like receptor (TLR)-inducible gene that controls IL-6 mRNA decay. Accordingly, *zc3h12a*-deficient mice show highly increased production of IL-6 and IL-12p40, leading to the development of autoimmunity (Matsushita et al. 2009). The stability of regnase-1 is controlled by IKK complex-mediated phosphorylation, which causes ubiquitination and degradation, and regnase-1 mRNA is targeted by regnase-1 itself. Thus, IL-6 mRNA levels can be finely tuned by RNases. Another example of the posttranscriptional control of IL-6 is its dependency on micro RNA (miR). *Let-7a* directly inhibits IL-6 expression whereas IL-6 is shown to activate NF- $\kappa$ B, which in turn represses *let-7a* levels and promotes IL-6 production. This positive feedback loop is considered important for maintaining the transformed state in certain cancer cells (Iliopoulos et al. 2009). IL-6 mRNA is also targeted by miR-26. A zinc-finger protein with an RNA interacting motif, *Zcchc-11*, induces the addition of uridines to the miR-26 3'-end, which abrogates IL-6 repression by miR-26 (Jones et al. 2009). In addition, IL-6R mRNA levels are suppressed by miR-124 in hepatocellular cancer cell lines, and it has been shown that systemic delivery of miR-124 prevents hepatocellular carcinogenesis without any side effects (Hatzia Apostolou et al. 2011). The regulation of IL-6 production can be achieved at a translational level as well. An RNA-binding protein, KSRP, which is known to have the ability to degrade mRNA with AU-rich elements, also participates in translational silencing. In KSRP-depleted cells, IL-6 mRNA is redistributed to polysomes, a phenomenon associated with increased production of IL-6. This translational silencing effect is dependent on the 3'-untranslated region of IL-6 mRNA (Dhamija et al. 2011).

## 2.2 Biological Functions of IL-6 in Inflammation and Disease

As already mentioned, gp130 plays an important role in the signal transduction by IL-6 that regulates a variety of biological functions. Because space is limited here, we discuss the function of IL-6 by focusing only on recent findings about its role in inflammation and disease. Its other biological functions are summarized elsewhere (Kamimura et al. 2003; Taga and Kishimoto 1997).



### ***2.2.1 IL-6 as a Pro-inflammatory Mediator***

It is widely accepted that CD4<sup>+</sup> T cells produce a large amount of cytokines that promote both acute and chronic inflammations. When activated, effector CD4<sup>+</sup> T cells can be divided into different subsets based on their cytokine profiles. A relatively new subset, type 17 helper CD4<sup>+</sup> T cells (Th17), mainly produce IL-17 and are responsible for the development of various autoimmune disorders, at least in mice (Nishihara et al. 2007; Veldhoen et al. 2006). Upon antigen stimulation of undifferentiated naïve CD4<sup>+</sup> T cells, IL-6 and another cytokine, transforming growth factor (TGF)- $\beta$ , direct these cells to express the transcription factor Ror $\gamma$ t, which is necessary for Th17 differentiation (Ivanov et al. 2006). TGF- $\beta$  is dispensable for Th17 generation in certain culture conditions (Ghoreschi et al. 2010). STAT3 activation emanating from the gp130 YXXQ motifs, but not the gp130 Y759-mediated SHP2/ERK pathway, is important for IL-6 activation (Fig. 2.2a). Accordingly, mice lacking gp130-STAT3 signaling in T cells have a substantially decreased number of Th17 cells (Nishihara et al. 2007). IL-6 was also reported to contribute to human Th17 differentiation (Zielinski et al. 2012). Interestingly, in the absence of TGF- $\beta$ , IL-6, together with IL-21, promotes naïve helper T cells to differentiate toward follicular helper T cells (Tfh), which requires the transcription factor Bcl-6 (Nurieva et al. 2009). Tfh plays an important role in T-cell–B-cell cooperation, resulting in enhanced formation of germinal centers and high-affinity antigen-specific immunoglobulin secretion. Because many cell types are known to produce IL-6, the cellular source of the IL-6 that promotes Th17 or Tfh differentiation and subsequent pathogenic inflammation *in vivo* remains a matter of debate. In a murine model of multiple sclerosis, IL-6 from a subset of B cells is shown to contribute to persistent inflammation in the central nervous system (Barr et al. 2012). It was also recently shown that deficiency of IL-6 and IL-21 in mice fails to induce Tfh cell-dependent immune responses against viral infection, and that IL-6 from follicular B cells is important for Tfh development (Karnowski et al. 2012). Although many studies have noted the significance of IL-6 production and its effect in immune cells, our recent findings (see Sect. 2.5) suggest nonimmune cells, including endothelial cells and fibroblasts, also make an important contribution to IL-6 production, particularly during inflammation. To formally demonstrate the functional cellular source(s) of IL-6 during inflammation, a conditional knockout of the IL-6 gene in mice such as the one established recently should be examined (Quintana et al. 2013).

### ***2.2.2 IL-6 as an Anti-inflammatory Mediator***

Under certain conditions, IL-6 exhibits an anti-inflammatory role in myeloid cells, such as dendritic cells and macrophages. Exposure to IL-6 before stimulation with microbial products [e.g., lipopolysaccharide (LPS)] inhibits major histocompatibility complex (MHC) class II expression and pro-inflammatory mediators in bone marrow-derived dendritic cells *in vitro* (Park et al. 2004). In fact, because IL-6 is



present at low levels under normal conditions, IL-6-deficient mice show a higher MHC class II expression on dendritic cells than that in wild-type mice, whereas mice with enhanced IL-6 signaling caused by the loss of the SOCS3-binding site in gp130 (F759 mice) show a lower level (Park et al. 2004). Mechanistically, IL-6 reduces the level of cystatin C, an endogenous inhibitor of cathepsins, thereby increasing cathepsin S activity and subsequent degradation of MHC class II components in IL-6-treated dendritic cells (Kitamura et al. 2005). Similarly, prolonged action of IL-6 has been shown to mimic the anti-inflammatory effects of IL-10, which also activates STAT3, in macrophages (Yasukawa et al. 2003). Moreover, the anti-inflammatory effects of IL-6 are manifested in a murine model of allergic asthma. In this model, IL-6-deficient mice show exaggerated lung inflammation whereas lung-specific overexpression of IL-6 reduced the disease symptoms (Wang et al. 2000). Importantly, IL-6 stimulation is also known to suppress T-cell-receptor-mediated signaling via SOCS3 molecules (Atsumi et al. 2009). Thus, direct IL-6 stimulation in certain immune cell populations can induce an anti-inflammatory signal.

### 2.2.3 *IL-6 and Cancer*

As already mentioned, gp130, the signal-transducing receptor subunit of IL-6, is expressed in almost all cell types in the body, whereas the expression of the IL-6-binding subunit, IL-6R, is more restricted. IL-6R expression is abundant in immune cells but not in nonimmune cells such as fibroblasts. However, shedding of IL-6R is detectable under normal conditions. The resulting soluble forms of IL-6R enable IL-6 to transmit intracellular signaling in cell types expressing gp130 alone, a phenomenon sometimes called IL-6 trans-signaling (Waetzig and Rose-John 2012). Because IL-6 is a well-known growth factor for cancer cells, it is likely that cells that do not express IL-6R exploit IL-6 trans-signaling for survival and expansion. Upon genotoxic stimuli such as those caused by drugs used for chemotherapy, IL-6 is produced from thymic endothelial cells in response to DNA damage response such that the resulting microenvironment provides a chemo-resistant niche for cancer cells. Therefore conventional chemotherapy can at the same time lead to tumor suppression and potentially create a tumor-promoting environment in an IL-6-dependent manner (Gilbert and Hemann 2010). It has been reported that excess body weight is correlated with an increase of a risk for cancer-associated death in humans, particularly hepatocellular carcinoma (Calle et al. 2003). One possible mechanism for this outcome has come from the observation that the risk of hepatocellular carcinoma was higher in high-fat diet mice. Interestingly, tumor-bearing mice fed with the high-fat diet showed elevated levels of IL-6 and TNF- $\alpha$  in their serum and around the tumor area. Similarly, it was seen in IL-6-deficient animals that obesity induces tumor progression via production of IL-6 (Park et al. 2010). Cancer progression is generally ascribed to primary tumor growth and secondary metastasis. One mechanism, termed tumor-self seeding, describes the ability of circulating tumor cells to infiltrate an established tumor and enrich it, potentially

causing tumor growth and the breeding of metastatic cells. IL-6 functions as an autocrine factor in tumor cells and also acts as an attractant for the circulating tumor cells. In fact, knockdown of IL-6R or gp130 significantly inhibits the seeding activity of cancer cells (Kim et al. 2009). IL-6 also promotes breast cancer by suppressing miR-200c, which leads to constitutive activation of NF- $\kappa$ B and JNK2. Activated JNK2 in turn phosphorylates HSF1, which induces demethylation of the IL-6 promoter that facilitates transcription. This signaling circuit is present in human cancer cells and a mouse model of ErbB2-mediated tumorigenesis (Rokavec et al. 2012).

### 2.2.4 IL-6 and Infectious Diseases

Chronic viral infection including human immunodeficiency virus (HIV)-1 infection often impairs the function of T cells. In such cases, T cells produce fewer effector cytokines, leading to the persistence of the virus. Recently, it was shown, by studying the lymphocytic choriomeningitis virus in mice, that IL-7 prevents this effect by downregulating SOCS3. Remarkably, this effect was found to depend on IL-6, as the beneficial effect of recombinant IL-7 administration on T-cell expansion and viral clearance was diminished in IL-6-deficient mice (Pellegrini et al. 2011). Although the mechanism of action by IL-6 remains elusive, it is possible that IL-7 is produced from nonimmune cells including fibroblasts by IL-6 stimulation, just as in an arthritis model (Sawa et al. 2006), or from hepatocytes in a manner dependent on type I interferon (IFN) molecules (Sawa et al. 2009). Another report indicates an important role of IL-6 in murine chronic viral infection. Upon chronic infection with the lymphocytic choriomeningitis virus, IL-6 is produced. This production enhances the Tfh responses known to promote the germinal center reaction and subsequent antibody production from B cells, and therefore is particularly important for viral control. Experiments using bone marrow chimera mice have suggested that the main source of IL-6 is irradiation-resistant stroma cells, rather than irradiation-sensitive immune cells (Harker et al. 2011). Human herpesvirus 8 (HHV8), also known as Kaposi sarcoma-associated herpesvirus, encodes a molecule similar to IL-6 called viral IL-6 (vIL-6). vIL-6 is reported to directly bind to gp130 and transmit signals in the absence of IL-6R, and it is widely accepted that it plays a significant role in the pathology of HHV8-associated diseases such as multicentric Castleman's disease (MCD). vIL-6 transgenic mice have been seen to spontaneously develop plasma cell-type MCD symptoms. Interestingly, the diseased phenotype was abrogated when the mice were crossed with IL-6-deficient mice, indicating that endogenous mouse IL-6 is required for vIL-6-mediated MCD pathology. Therefore, it may be that a combination of classic signaling mediated by endogenous IL-6 and vIL-6-mediated direct stimulation of gp130 is required for MCD (Suthaus et al. 2012). The effect of IL-6 on the control of pathogen infection is, of course, not limited to viruses. The obligate intracellular parasite *Toxoplasma gondii* can infect and persist in neurons, subsequently leading to chronic encephalitis.

Using a conditional knockout of gp130 in neurons, it was demonstrated that IL-6 signaling in neurons protects against apoptosis during the infection, which would explain why synapsin-Cre gp130<sup>fl/fl</sup> mice are unusually susceptible to *Toxoplasma* encephalitis (Handel et al. 2012). gp130-mediated signaling is also important in astrocytes during *Toxoplasma* encephalitis because astrocyte-specific deletion of gp130 in mice using GFAP-Cre failed to control the parasites and the mice died of encephalitis (Drogemuller et al. 2008).

### 2.3 IL-6 Signaling and Human Diseases: Genetic Evidence

From the studies described here, it should come as no surprise to learn that IL-6 is involved in autoimmune diseases and infections associated with inflammation. In addition, accumulating evidence has revealed that a certain level of inflammation is evident in many diseases that had not been hitherto considered associated with immune cells or inflammatory mediators: these include metabolic syndromes, such as obesity and atherosclerosis, and neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease.

Genome-wide association studies have found a genetic link between the gene polymorphisms of IL-6 and its signaling molecules and human disorders. For example, a polymorphism located in the IL-6 promoter at -174 has been reported to associate with many diseases, including the -174G/C polymorphism and Alzheimer's disease (Dai et al. 2012). An association of the -174G/C polymorphism with coronary heart disease has been revealed by meta-analysis, which found this polymorphism is associated with a higher risk of the disease in Asian populations (Yin et al. 2012). In addition, the STAT3 locus and IL6ST (gp130) locus are found to associate with ulcerative colitis and rheumatoid arthritis, respectively (Franke et al. 2008) (Stahl et al. 2010). Moreover, the IL-6R gene has been identified as a risk locus for asthma (Ferreira et al. 2011). Recent meta-analyses including data of more than a hundred thousand volunteers suggested that the minor allele of IL-6R in humans, namely Asp358Ala, whose frequency is reported to be 39 %, is associated with an increase of circulating sIL-6R levels and reduced risk of coronary heart disease (Hingorani and Casas 2012; Sarwar et al. 2012). Constitutive activation of gp130/STAT3 signaling by somatic mutations has been demonstrated in benign liver tumors such as inflammatory hepatocellular adenomas (IHCS). The Zucman-Rossi lab reported that 60 % of IHCS harbor somatic mutations in gp130, which renders ligand-independent activation of STAT3 (Rebouissou et al. 2009). The group subsequently showed that a subset of IHCS lacking these gp130 mutations has gain-of-function mutations in STAT3, most of which are located in the SH2 domain, which induces STAT3 dimerization (Pilati et al. 2011). Conversely, a dominant-negative version of STAT3 gene mutations has been reported in hyper IgE syndrome, which is a de novo mutation and characterized by high serum IgE

(Minegishi et al. 2007). Combined, that IL-6 and its signal transduction pathway are essential in animal models of multiple autoimmune diseases, and evidence from the genome-wide association studies (GWAS), demonstrate IL-6 signaling is an attractive therapeutic target for many inflammatory diseases.

## 2.4 IL-6 Signaling as a Therapeutic Target

Clinical efficacy of blocking IL-6 signaling has already been demonstrated in humans. The human version of the anti-IL-6R monoclonal antibody (tocilizumab, or Actemra) has been approved in some countries for treating patients with moderate to severe rheumatoid arthritis. Notably, tocilizumab often shows substantial and better efficacy in patients than other disease-modifying anti-rheumatic drugs (DMARDs). In Japan, tocilizumab has also been used for the treatment of juvenile idiopathic arthritis and Castleman's disease.

As effective as tocilizumab is, there is also an effort to develop antibodies or gp130:Fc fusion proteins that can block IL-6 signaling (Jones et al. 2011). Although tocilizumab may be a “silver bullet” against some chronic inflammatory diseases, including rheumatoid arthritis, a nonbiological small compound that inhibits IL-6 signaling or production is also awaited because of its potential orally active properties and cost-effectiveness, just as other small compound drugs. A novel JAK kinase inhibitor, tofacitinib (or CP690,550), from Pfizer is expected to be launched soon for the treatment of moderate to severe active rheumatoid arthritis in some countries including the United States, Japan, and Europe. Clinical trials of this compound are also underway for psoriasis and inflammatory bowel diseases. STAT3 is another attractive target to interfere with IL-6 signaling. Although many efforts have been made to develop STAT3 inhibitors, to date none has reached the clinical drug stage. Recently, a promising orally available STAT3 inhibitor, BP-1-102, was reported (Zhang et al. 2012). The classical JAK/STAT pathway, which is a major signaling cascade of IL-6, is a relatively simple mechanism because only a couple of factors, namely, JAK and STAT, are involved. Although the pathway therefore makes for a simple model in the understanding of the cellular functions of IL-6, it also means that the number of target molecules is limited. We have recently discovered a novel mechanism for IL-6 and chemokine production under inflammatory situations. This mechanism, termed the inflammation amplifier, involves simultaneous activation of two key transcription factors, STAT3 and NF- $\kappa$ B (Ogura et al. 2008). We have already performed functional genome-wide screening to identify the genes that control its activation (Murakami et al. 2013). Remarkably, the number of genes regulating the inflammation amplifier far exceed the number we had expected, and may offer a vast number of targets for the inhibition of IL-6 production. We discuss the inflammation amplifier and its regulation in the next section.

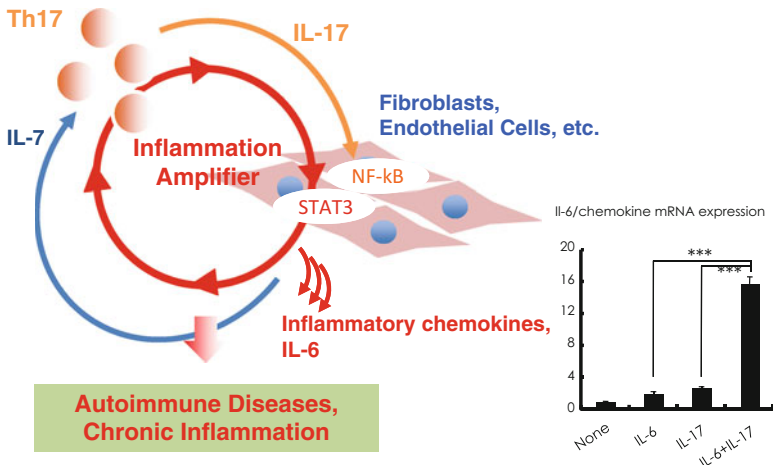
## 2.5 The Inflammation Amplifier

### 2.5.1 *Establishment of an Animal Model to Study the Pathogenesis of Rheumatoid Arthritis*

Although the anti-IL-6R antibody has successfully treated more than 50 % of patients in a clinical setting (Nishimoto et al. 2000), the molecular mechanism responsible remains elusive. One reason is the lack of adequate experimental animal models. Because IL-6 signaling undoubtedly contributes to the pathogenesis of rheumatoid arthritis, we hypothesized that hyperactivation of IL-6 signaling in mice may cause the disease. To prove this hypothesis, we created a knock-in mouse line that has mutated human gp130 via the amino acid substitution Y759F (F759 mice). The tyrosine is an important docking site for the negative feedback regulation by SOCS3 in IL-6 signaling, meaning the mutation is expected to augment the signal transduction (Fig. 2.2a). Indeed, F759 showed hyperactivated STAT3 mediated by IL-6/gp130 signaling in vivo (Ohtani et al. 2000). Furthermore, these F759 mice spontaneously develop an age-dependent autoimmune joint disease in an IL-6-dependent manner (Fig. 2.2b) (Sawa et al. 2006). Also, F759-arthritis is accompanied by an accumulation of memory/activated T cells, and its clinical course is chronic and progressive. The disease symptoms begin as mild swelling and redness in the paws, but eventually lead to decreased joint mobility. Larger joints were affected symmetrically, and all these joints eventually became ankylotic. Radiologic analysis of the affected joints revealed characteristics that resemble advanced human rheumatoid arthritis, as also did histological examination, which showed leukocytes infiltrating the joint space, hyperplasia of the synovium with pannus formation, destruction of the cartilage and bone, and bony ankylosis (Atsumi et al. 2002).

### 2.5.2 *The Discovery of the Inflammation Amplifier*

The F759 arthritis model gives us a chance to investigate the role of IL-6 signaling in autoimmune disease development. We found that in F759 mice the number of memory/activated CD4<sup>+</sup> T cells dramatically increased with age. Additionally, when F759 mice were crossed with CD4-deficient or MHC class II-deficient mice, a significant suppression of disease development was observed in the offspring (Sawa et al. 2006). These observations suggest that excessive IL-6 signaling could have a role in inducing autoimmune arthritis via CD4<sup>+</sup> T-cell activation. Because IL-6 is a multifunctional cytokine and regulates the immune system, we considered whether IL-6 directly activates immune cells including CD4<sup>+</sup> T cells or dendritic cells, but surprisingly found that it in fact inactivates these cells (Atsumi et al. 2009; Kitamura et al. 2005; Park et al. 2004). Subsequent bone marrow chimera experiments demonstrated that wild-type mice transfused with F759 bone marrow did not develop



**Fig. 2.3** The inflammation amplifier. The inflammation amplifier is defined as hyperinduction of IL-6 and chemokines in nonimmune cells that arise from simultaneous activation of STAT3 and NF- $\kappa$ B. IL-7 from nonimmune cells also contributes to enhance the inflammation amplifier by generating Th17 or sustaining Th17 survival. The amplifier is known to be essential for the pathogenesis of F759 arthritis, autoimmune encephalomyelitis (EAE), and chronic graft rejection

arthritis, whereas F759 mice that received wild-type bone marrow did. In spite of the necessity for CD4<sup>+</sup> T cells, it was clearly demonstrated that the gp130 F759 mutation, which causes hyperactivation of IL-6 signaling, is required only in nonimmune cells for F759 arthritis development to occur. Further studies have since shown that excessive IL-6 signaling in nonimmune cells promotes the production of IL-7, which is known to be important for T-cell proliferation and survival, and results in excess homeostatic proliferation of CD4<sup>+</sup> T cells. These results suggest that nonimmune cells actively control the status of immune cells including CD4<sup>+</sup> T cells, and dysregulation of this control can cause autoimmune diseases (Sawa et al. 2006).

Th17 cells have been shown to be involved in many autoimmune disease models and human diseases including rheumatoid arthritis. Consistent with this property, serum IL-17 levels and Th17 cells were both unusually high in aged F759 mice. When F759 mice were bred on an IL-17-deficient background, the arthritis was significantly suppressed. On the other hand, the overexpression of IL-17 in F759 mice accelerated arthritis development. Among the more than 30 kinds of cytokines and chemokines, only serum IL-6 and certain chemokines were upregulated after overexpression of IL-17 in vivo. Moreover, the serum concentration of IL-6 after IL-17 overexpression was significantly higher in F759 mice than in control mice, indicating a positive interaction between IL-17 and IL-6 signaling. In vitro investigation showed that a combination of IL-17 and IL-6 synergistically induces IL-6 and inflammatory chemokines in type I collagen<sup>+</sup> nonimmune cells such as fibroblasts and endothelial cells (see bar graph in Fig. 2.3). This

synergistic effect depends on NF- $\kappa$ B and STAT3. Thus, it was suggested that NF- $\kappa$ B activation by cytokines such as IL-17 stimulates a sufficiently minimal amount of IL-6 in nonimmune cells that in turn synergistically acts with IL-17 to induce more IL-6, which leads to the development of inflammation. In fact, this positive feedback loop of IL-6 signaling, or inflammation amplifier, has been found important for the pathogenesis of autoimmune diseases including F759 arthritis and experimental autoimmune encephalomyelitis (EAE), a disease that resembles multiple sclerosis in animal models (Fig. 2.3) (Ogura et al. 2008). In addition, graft rejection was significantly inhibited in mice with defective CCL2 expression caused by inflammation amplifier activation in the basement cells of tracheal epithelial cells. In this context, CCL2 expression is triggered by traumatic stress, which increases IL-6 and epidermal growth factor, followed by Th1 cell accumulation in the graft (Lee et al. 2012). Thus, the inflammation amplifier can be applied not only to autoimmunity disorders but also to other inflammatory disorders in vivo. These results suggest that the mechanisms behind the clinical efficacy of the anti-IL-6R antibody are likely to be explained by activation of the inflammation amplifier.

### ***2.5.3 Inflammation Amplifier-Regulating Genes as Potential Therapeutic Targets for Human Diseases***

Because the inhibition of IL-6 signaling by anti-IL-6R treatment has been successful in humans, the identification of genes that regulate or are regulated by inflammation amplifier activation may provide therapeutic targets against inflammatory diseases. We conducted a functional genome-wide screening by using a lentivirus library having 65500 shRNAs (16000 ORFs) and DNA microarray experiments to identify those genes related to activation of the inflammation amplifier, finding more than 1,000 (Murakami et al. 2013). When these genes were analyzed by using a public database of human genetic association studies of complex diseases and disorders (Genetic Association Database at NIH; <http://geneticassociationdb.nih.gov/cgi-bin/index.cgi>), a large number were found to be associated with human diseases and disorders. The enriched disease categories went well beyond autoimmune diseases to include metabolic syndromes, such as atherosclerosis and type 2 diabetes, and neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease. These results, then, could be interpreted to show that inflammation, which is induced by the inflammation amplifier, relates to various human diseases and disorders, and that IL-6 inhibitors could have wide-ranging therapeutic effects.



## 2.6 A Four-Step Model for MHC Class II-Associated Autoimmune Diseases

We previously demonstrated that the activation of the inflammation amplifier by IL-17 and IL-6 leads to arthritis in F759 mice. To explain this relationship, we recently proposed a four-step model for MHC class II-associated autoimmune diseases (Murakami and Hirano 2011; Murakami et al. 2011). It has been believed that cognate tissue specific-antigen recognition by autoreactive T cells is a key step for the development of tissue-specific autoimmune diseases, particularly those genetically associated with MHC class II genes such as rheumatoid arthritis (Marrack et al. 2001). Indeed, the development of arthritis in F759 mice is dependent on MHC class II and CD4 (Sawa et al. 2006). Therefore, we investigated the involvement of joint-specific antigens in the development of arthritis in F759 mice. We established F759 mice having a single T-cell receptor (TCR) that recognizes non-joint antigens. F759 mice were crossed with Rag2-deficient mice as well as OT-2 or P25 TCR-transgenic (Tg) mice whose T cells recognize MHC class II-restricted peptides from ovalbumin (OVA) or peptide 25 from *Mycobacterium tuberculosis*. Theoretically, no CD4<sup>+</sup> T cells recognize joint antigens in these offspring. Unexpectedly, CD4<sup>+</sup> T cells bearing a single TCR that recognizes antigens not related to joint tissues induce arthritis in Rag2-deficient mice that have the F759 mutation. We therefore concluded that cognate antigen recognition by effector CD4<sup>+</sup> T cells is not necessary for tissue specificity in F759 mice (Murakami et al. 2011). From this, we hypothesized that disease specificity may be determined by the tissue itself such that local events in the joint may determine and initiate the disease via the inflammation amplifier. If the Th17 cell transfer is done before inducing experimental microbleeding, only the bled leg will develop arthritis. Even Th17 cells derived from TCR transgenic mice induced arthritis in the microbleeding-induced leg of F759 mice. These findings are consistent with the idea that local events determine the disease specificity even if activation of tissue antigen-specific T cells does not occur. We further observed that T cells accumulated in the arthritic joint. This microbleeding-induced accumulation of Th17 cells is dependent on the production of CCL20, a target of the inflammation amplifier, in the joint. Disease induction requires IL-17A produced by T cells, IL-6, and enhanced STAT3 signaling in type I collagen-expressing cells (Murakami and Hirano 2011). Thus, local microbleeding facilitates IL-6- and IL-17-dependent arthritis in the absence of tissue antigen recognition by activated T cells (Murakami and Hirano 2011).

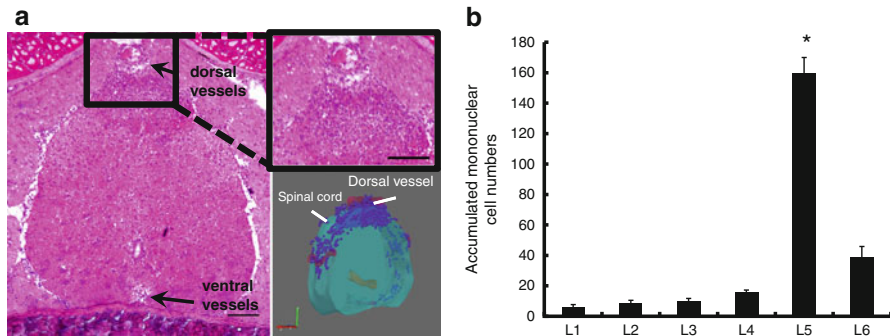
Based on these results, we proposed that certain MHC class-II associated autoimmune diseases such as rheumatoid arthritis arise through a series of at least four steps: (1) T-cell activation regardless of antigen specificity; (2) local events inducing a tissue-specific accumulation of activated T cells; (3) transient activation of the inflammation amplifier, which is triggered by CD4<sup>+</sup> T-cell-derived

cytokines such as IL-17A; and (4) enhanced sensitivity to T-cell-derived cytokines and/or IL-6 in type 1 collagen+ cells in the target tissue just like F759 mutation (Murakami and Hirano 2011; Murakami et al. 2011). After these four steps, chronic activation of the inflammation amplifier followed by development of the autoimmune disease occurs. It is likely that each step interacts with the others, and the degree of the contribution of each to the pathogenesis varies with the disease. Our four-step model provides a plausible explanation why tissue-specific antigens recognized by activated CD4<sup>+</sup> T cells have not been identified in several autoimmune diseases, especially those associated with MHC class II molecules. It is likely that in diseases where tissue antigen-specific T cells play a role, tissue antigen-specific recognition by T cells bypasses the requirement of local events, even though these local events can still affect the accumulation of tissue antigen-specific T cells in the target tissue. Our four-step model, therefore, should be applicable to a wide range of autoimmune and other chronic inflammatory diseases.

## 2.7 A Possible Physiological Role of the Inflammation Amplifier

In the previous sections, we described a pathogenic role for the inflammation amplifier during chronic inflammation. Although aberrant amplification of IL-6 and chemokine production by uncontrolled activation of STAT3 and NF- $\kappa$ B trigger autoimmunity and graft rejection, we recently found that the inflammation amplifier also functions at the steady state. Activation of the inflammation amplifier has been detected in blood vessels adjacent to the central nervous system (CNS). In the course of disease development in EAE, disease-causing CD4<sup>+</sup> T cells infiltrate into the CNS, a well-known immune-privileged tissue that restricts the intrusion of immune cells into the bloodstream by the blood–brain barrier. The blood–brain barrier is a specific blood vessel structure that is mediated by tight junctions and tight liner sheets established by pericytes, astrocytes, and macrophages.

Where and how pathogenic CD4<sup>+</sup> T cells enter the CNS was unclear until recently. Sallusto et al. reported that mice lacking CCR6, a receptor for CCL20, are highly resistant to EAE, and that the choroid plexus, a specialized epithelial structure in the brain, expresses CCL20 constitutively, thereby potentially acting as an attractant for the first wave of CCR6<sup>+</sup> Th17 (Reboldi et al. 2009). In this study, however, the CNS disease was induced by means of complete Freund's adjuvant, which is widely used for active immunization in animals, but at the same time is also an inducer of strong inflammatory responses that potentially affect the pathophysiological status of the whole body including the brain and spinal cord. To reduce background inflammatory responses, particularly at the initiation stage of EAE, we utilized an adoptive transfer model in which Th17 cells obtained from MOG-immunized mice were infused into naïve recipients so that the quiescence

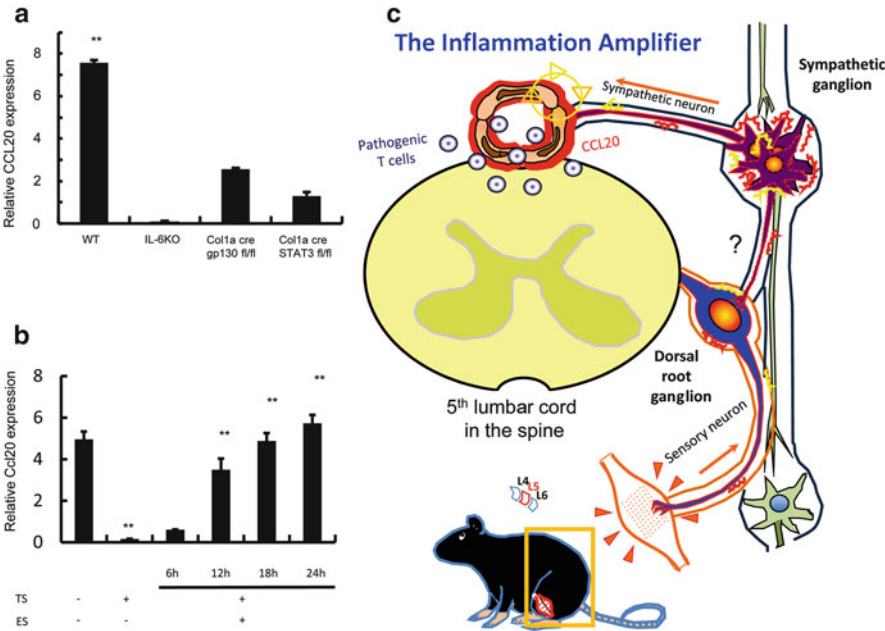


**Fig. 2.4** The fifth lumbar cord is a gateway to the central nervous system (CNS). A cross section of the fifth lumbar (L5) cord (**a**) and actual cell numbers of mononuclear cells accumulated in each lumbar cord segment (**b**) at a preclinical phase of EAE. A magnified image around the dorsal vessel of L5 and a three-dimensional (3D) picture based on ten serial sections of L5 are shown on the right side of (**a**) (top and bottom, respectively)

of the CNS could be preserved. We found that Th17 cells preferentially accumulated in the fifth lumbar (L5) cord rather than the brain or other regions of the spinal cord at the preclinical phase of EAE (Fig. 2.4) (Arima et al. 2012), although we found pathogenic CD4<sup>+</sup> T-cell accumulation in the brain at a later stage after the T-cell transfer. CCL20 mRNA levels were highest in the dorsal vessel of L5 as compared with the other lumbar cords. Interestingly, even in naïve animals, CCL20 as well as many other chemokines were specifically upregulated in the dorsal vessel of L5. Additionally, NF- $\kappa$ B reporter mice showed that NF- $\kappa$ B activity is higher at L5 than L1 or cervical cords. Moreover, the elevated CCL20 levels at L5 were decreased in mice devoid of the inflammation amplifier such as IL-6-deficient mice (Fig. 2.5a). Even under normal conditions, it is known that some immune cells are present in the CNS, suggesting there exists a gateway to enter this restricted area. In this respect, it is tempting to speculate that low-grade activation of the inflammation amplifier at the L5 dorsal vessel in the steady state creates the gateway by inducing some level of chemokines, although direct evidence is needed to link low levels of inflammation amplifier activation and immune homeostasis, including immunological surveillance of the CNS.

## 2.8 Neuroimmune Interactions that Boost Inflammation Amplifier Activation

The answer to why the L5 specifically acts as the gateway comes from an unlikely source. The dorsal root ganglia (DRG) of the sensory neurons from the soleus muscle, which can be activated by a gravitation stimulus, are located beside L5 (Ohira et al. 2004). When mice were tail-suspended so that only the forelimbs could touch the ground and the hind legs were released from gravity stimuli, pathogenic Th17



**Fig. 2.5** Neural stimulation-mediated inflammation amplifier activation creates a gateway into the CNS by chemokine production. **(a)** CCL20 levels under steady state. Note that naïve mice with an inactive inflammation amplifier (*all three columns from right*) showed significantly reduced levels of CCL20, which implicates a physiological role of the inflammation amplifier in the steady state but not in the disease condition (see Fig. 2.3). **(b)** Absence of gravitational stimuli (tail suspension, TS) decreases CCL20 at the L5 dorsal vessel. Electric stimulation (ES) during TS restores the levels in a time-dependent manner. **(c)** Schematic representation of neural stimulation-mediated activation of the inflammation amplifier. Neural signals from gravitational stimuli in soleus muscles reach the L5 dorsal root ganglion. Subsequent activation of sympathetic nerves alters the status of L5 dorsal vessel endothelial cells to enhance the inflammation amplifier, which leads to the production of chemokines including CCL20. Norepinephrine is a mediator between the neural signal and inflammation amplifier activation. A neural network from the soleus muscle-derived sensory neurons to sympathetic neurons that reach L5 is not defined (depicted with a question mark)

cells negligibly accumulated at L5. Instead, these cells utilized a new gateway opened by gravity stimuli to the forelimbs and accumulated at the cervical cords. Consistent with this result, tail suspension significantly inhibited CCL20 expression in L5 dorsal blood vessels and decreased the expression of a neural activation marker, c-Fos, in the L5 DRG. In addition, when the soleus muscles of tail-suspended mice were artificially stimulated by electric pulses, CCL20 expression (Fig. 2.5b), pathogenic Th17 accumulation and c-Fos levels were all restored at L5. These data strongly suggest that neural activation via anti-gravitational responses by the soleus muscles plays a role in the activation of the inflammation amplifier and subsequent expression of chemokines including Th17-attracting CCL20 in L5 dorsal blood vessels before the development of EAE. How do afferent sensory neurons from the soleus muscle

influence the status of the blood vessels at L5? Although a precise neural network remains unidentified, we demonstrated that sympathetic nerves are involved. Blood flow speed at the L5 dorsal vessel became slower when mice were tail-suspended, whereas electronic stimulation of the soleus muscles increased the speed, suggesting a contribution of automatic nerves including sympathetic ones. Importantly, blood flow speeds at other vessels, such as femoral vessels, brain surface vessels, and the portal vein, were not affected by the tail suspension. Furthermore, treatment with the norepinephrine receptor antagonist atenolol significantly suppressed CCL20 expression, NF- $\kappa$ B activation, and pathogenic Th17 accumulation in L5 vessels and abrogated EAE development. Consistent with these *in vivo* results, the addition of norepinephrine to an endothelial cell line culture enhanced inflammation amplifier activation as monitored by IL-6 or CCL20 expression. Thus, neural stimulation of the soleus muscles by gravity causes sympathetic nerve stimulation, which creates a gateway to the CNS at L5 vessels by activating the inflammation amplifier with secreted norepinephrine. MOG-specific, disease-causing Th17 cells exploit this gateway to infiltrate the CNS and induce local inflammation by producing cytokines such as IL-17 and IL-6, which further enhances the activation (Fig. 2.5c). Such neuroimmune interactions have also been reported by Tracey et al., in which they showed that vagus nerve stimulation inhibits proinflammatory cytokine release through the nicotinic acetylcholine receptor  $\alpha 7$  subunit, and identified a subset of T cells that produce acetylcholine and can relay the neural signals (Borovikova et al. 2000); (Wang et al. 2003); (Rosas-Ballina et al. 2011). Therefore, interfering neuroimmune responses may be another promising approach for therapeutic interventions to inflammatory diseases.

## 2.9 Gate Theory

Gravitational stimuli upregulate various chemokines as a result of inflammation amplifier activation at L5 cord vessels via the sensory neurons of soleus muscles extending from the L5 DRG. This neural event creates a local gate for immune cells to enter the CNS. It turns out that stimulating other muscles can create a similar gate into blood vessels where DRG neurons are located. Electronic stimulation of the quadriceps or thigh muscles, which are known to be controlled by L3 DRG neurons, upregulated the expression of the inflammation amplifier target chemokine, CCL20, across L3 cord vessels in mice. Similarly, chemokine levels from the fifth cervical to fifth thoracic cord vessels were elevated by stimulations of epitrochlearis/triceps brachii and upper arm muscles regulated by neurons located at corresponding areas (Arima et al. 2012). In addition, we have found that the location and degree of pathogenic CD4<sup>+</sup> T-cell infiltration into the CNS can be changed in mice under mental stress (unpublished data). These observations led us to propose a gate theory in which the invasion of immune cells into target organs can be modulated by manipulating nerve activity. Although further studies are required to generalize this theory to organs, such a theory has promise for novel therapy against many chronic inflammatory diseases.

## 2.10 Conclusion

In this review, we summarized recent advances in understanding how IL-6 plays a central role in inflammation and various diseases. The success of anti-IL-6R therapy has shown the promise of targeting IL-6 signaling and production for treatment against inflammatory diseases. The inflammation amplifier is one prominent mechanism that produces a large amount of chemokines and IL-6 from nonimmune cells *in vivo* and is therefore a candidate worth considering for future targets. In addition to targeting traditional IL-6 signaling molecules such as JAK and STAT3, finding ways to inhibit the inflammation amplifier and a therapeutic method that can modulate regional neural activity that activates the amplifier may be beneficial to sufferers of chronic inflammatory diseases.

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