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The Gate Theory Explains Regional Neural Regulation of Activated T cells Entering the Central Nervous System

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Abstract

Type 17 helper T (Th17) cells are a subset of activated CD4^{+} T cells that produce interleukin (IL)-17 and contribute to the pathogenesis of autoimmune diseases via inflammation induction. Th17 differentiation is induced by T cell receptor engagement in the presence of several cytokines including IL-1β, IL-23, TGF-β and IL-6. IL-6 is often elevated during inflammation and chronic inflammatory diseases such as autoimmune disorders. We have shown that a combination of IL-17 and IL-6 synergistically induces the production of target molecules including various chemokines and IL-6 itself in non-immune cells such as fibroblasts and endothelial cells. We named this phenomenon the "inflammation amplifier" and determined it essential for the induction of chronic inflammatory diseases. Moreover, our results showed that the inflammation amplifier describes simultaneous activation of NF-κB and STAT3, with the major signal being NF-κB, and STAT3 acting as a costimulatory signal that enhances the expression of NF-κB targets. Thus, the inflammation amplifier can be viewed as a NF-κB loop in non-immune cells that establishes the inflammation status via local chemokine expression. It was recently shown that activation of the inflammation amplifier in blood vessel endothelium is enhanced by regional neural stimuli and results in local upregulation of chemokines and subsequent immune cell infiltration and pathogenic CD4^{+} T cells. Thus, a gate for immune cells from the blood to the site of inflammation, including regions like the central nervous system (CNS), can be opened or closed by regional neuronal stimulations across our entire body. We name this phenomenon the gate theory. In this review article, we summarize our recent data, discuss the physiology of the inflammation amplifier, and gate theory in various inflammatory diseases.

Synergistic Effects of IL-17 and IL-6 on Inflammatory Chemokines: The Inflammation Amplifier

IL-6 is a classical proinflammatory cytokine first cloned in 1986 and IL-6 blockade has shown tremendous effects in the treatment of rheumatoid arthritis [1,2]. In fact, many inflammatory diseases beyond rheumatoid arthritis, including multiple sclerosis and type-1 diabetes have been genetically associated with IL-6 [3,4]. Its effects were traced to the activation of Th17 cells, a subset of activated CD4^{+} T cells [5,6]. We then have investigated the relationship between IL-6 and Th17 cells in autoimmune diseases using mouse models. IL-6 deficient mice are resistant to many inflammatory diseases, in particular to Th17-mediated ones such as collagen-induced arthritis and experimental autoimmune encephalomyelitis [7-10]. Additionally, *in vivo* experiments found that serum levels of IL-6 significantly increase after forced expression of IL-17 [9,11,12], suggesting positive feedback of IL-6 in the presence of IL-17 signaling. Further *in vitro* studies revealed that a combination of IL-17 and IL-6 stimulation induces much larger amounts of IL-6 and inflammatory chemokine production in type I collagen+ non-immune cells such as fibroblasts and endothelial cells, than stimulation by either cytokine alone. This combination effect is not additive but synergistic [9] (Figure 1A). We have also shown that the synergistic effect of IL-17 and IL-6 is dependent on two transcriptional factors: NF-κB and STAT3. Thus, it is possible that IL-17 from Th17 cells induces excess chemokines via an IL-6 positive-feedback loop in non-immune cells (Figure 1B). We named this phenomenon the "inflammation amplifier" and showed it to be important for the pathogenesis of the rheumatoid arthritis model, F759-arthritis, the multiple sclerosis mouse model, experimental autoimmune encephalomyelitis (EAE), and in a mouse model of chronic rejection of lung transplantation [9,13]. We further hypothesized that IL-6 acts as a fuel for the inflammation amplifier and that chemokines

are functional molecules for the development of inflammation. In addition, an immunohistological study using human clinical samples revealed evidence that activation of the inflammation amplifier occurs in the affected area of bronchiolitis obliterans, a common complication via chronic inflammation after lung transplantation [14]. Additionally, genes that regulate the inflammation amplifier or are targeted by it have been functionally screened and identified. These genes were reported to have significantly high levels of genetic associations with a broad range of human diseases and disorders including autoimmune diseases, metabolic syndromes, and other inflammatory diseases [15]. Thus, the inflammation amplifier is involved not only in autoimmunity, but also other inflammatory diseases, with IL-17 being an important factor in driving the inflammation amplifier by activating NF-κB in non-immune cells [16,17].
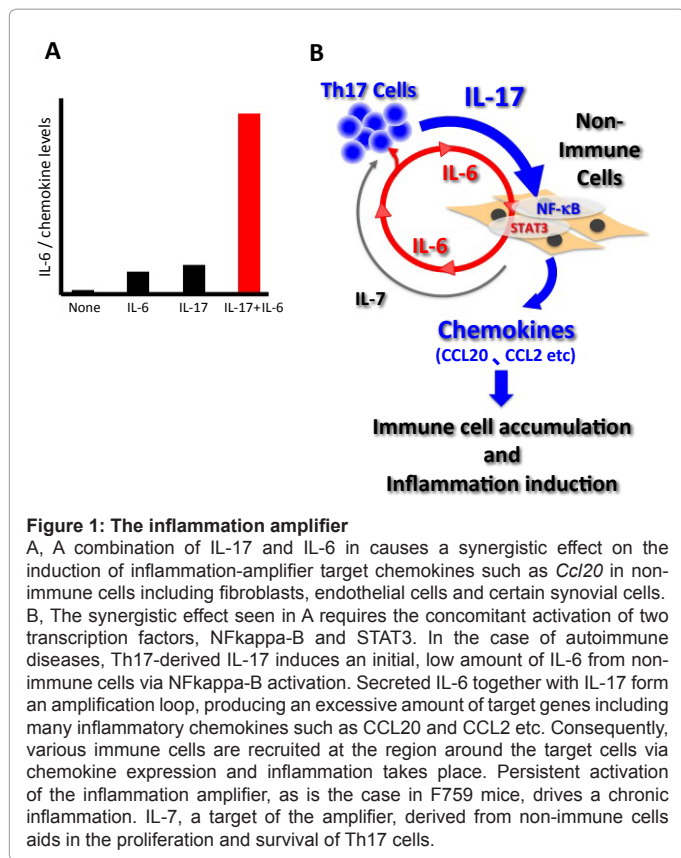
A Th17-dependent Model of Multiple Sclerosis

Inflammatory cytokines such as IL-1, IL-6, IL-17 and TNFα are known to play key roles in the pathogenesis of multiple sclerosis and its animal model, EAE [18]. We have reported the inflammatory amplifier is also an intrinsic part of the molecular mechanism responsible [9,19].

Figure 1: The inflammation amplifier

A, A combination of IL-17 and IL-6 in causes a synergistic effect on the induction of inflammation-amplifier target chemokines such as *Ccl20* in non-immune cells including fibroblasts, endothelial cells and certain synovial cells. B, The synergistic effect seen in A requires the concomitant activation of two transcription factors, NFkappa-B and STAT3. In the case of autoimmune diseases, Th17-derived IL-17 induces an initial, low amount of IL-6 from non-immune cells via NFkappa-B activation. Secreted IL-6 together with IL-17 form an amplification loop, producing an excessive amount of target genes including many inflammatory chemokines such as CCL20 and CCL2 etc. Consequently, various immune cells are recruited at the region around the target cells via chemokine expression and inflammation takes place. Persistent activation of the inflammation amplifier, as is the case in F759 mice, drives a chronic inflammation. IL-7, a target of the amplifier, derived from non-immune cells aids in the proliferation and survival of Th17 cells.

Using EAE as our model, we extended this work to examine how autoreactive CD4+ T cells enter the central nervous system (CNS), considering that the blood-brain barrier (BBB) tightly regulates such transport. We established a Th17-cell dependent adaptive transfer model of EAE, because we thought that more common models by using autopeptide-immunizations in the presence of complete Freund's adjuvant risk modulating the CNS basal state. Th17 cells obtained from myelin oligodendrocyte glycoprotein (MOG)-immunized mice were re-stimulated *in vitro* with IL-23 and infused intravenously in naïve C57BL/6 recipients so that quiescence of the CNS was preserved. Mice showed initial signs of EAE 6 to 7 days after the pathogenic CD4+ T cell transfer. During this period, transferred Th17 cells proliferated, and the chemokine receptor CCR6 increased its expression in secondary lymphoid organs [19]. A recent report by Flügel et al. also suggested that pathogenic Th17 cells might temporally reside in the lung until they have full pathogenicity to attack the CNS [20]. On day 5 after the MOG-specific Th17 transfer, when the day 5 is still in a preclinical stage, we prepared whole-mount sections of adult mice using the CM3600XP (Leica, Germany), a macrotome device, and examined the accumulation of transferred T cells throughout the body by the automatic microscope BZ-9000 (KEYENCE, Japan) after staining with hematoxylin/eosin. Interestingly, we found that transferred Th17 cells preferentially accumulated in the fifth lumbar (L5) cord rather than the brain or other regions of the spinal cords [19]. This observation fits well with a clinical manifestation of typical EAE in which the tail tip is first affected, because L5 dorsal root ganglions (DRG) are connected to sensory neurons in various lower body muscles. On the other hand, we did not observe any significant accumulation of Th17 cells in the lung (data not shown), which disagrees with the findings by Flügel et al. This contradiction may be due to differences in animal species,

donor T cell preparation, and/or time points of sampling. The L5 entry of pathogenic Th17 cells was shown to depend on IL-17 production, as IL-17-deficient Th17 cells obtained from MOC-immunized IL-17-deficient mice did not invade the L5, while IFNγ-deficient Th17 cells did. The antigen specificity of Th17 cells is another critical determinant for CNS invasion, as CNS-irrelevant, ovalbumin-specific Th17 cells did not efficiently traverse the BBB (our unpublished data and [21]). Although how and where this antigen presentation takes place is currently unknown, we propose that cognate TCR stimulation around the L5 region promotes IL-17 secretion from the transferred pathogenic Th17 cells. The IL-17 loosens the BBB by downregulating tight junction molecules [22], allowing the Th17 invasion in the L5 cord. Even if so, this explanation does not explain why MOG-specific Th17 cells are preferentially recruited to L5.

The cervical, thoracic and lumbar cords of mouse can be divided into 7, 13 and 6 segments, respectively [23,24]. Sensory and motor neurons extend from each spinal segment to respective body regions and transmit sensations to the brain or control movements of the respective body parts. MOG-specific Th17 cells are selectively recruited to dorsal blood vessels in the L5 spinal cord at the early stages of EAE [19]. It has been reported that *Ccl20* is a chemokine for Th17 cell migration and is upregulated via NF-κB activation [25-27]. Additionally, we found that NF-κB activity is increased in L5 dorsal vessels compared to vessels in the other spinal segments. Mice lacking IL-6 signal transducer (gp130) or STAT3 in blood vessel endothelial cells do not show this accumulation of MOG-specific Th17 cells at the L5 cord, suggesting the inflammation amplifier in L5 dorsal vessels produces *Ccl20*. Intriguingly, along with the diseased state, a low-grade activation of the inflammation amplifier, namely elevated levels of *Ccl20* and many other chemokines, is evident at L5 vessels even in the healthy state, regardless of pathogenic Th17 cell transfer [19]. This finding led us to hypothesize that some L5 neurons may be constantly activated. It is known that sensory neurons located in DRG at the L5 region are connected to the soleus muscles, which are the muscles primarily responsible for coping with gravity [28]. We speculated that constant stimulation of the soleus muscles by gravity might activate the inflammation amplifier via their sensory nerves. Indeed, when mice were tail suspended so that the hind legs were released from the gravity stimuli, MOG-specific Th17 cells no longer accumulated at the L5 region. Instead, these Th17 cells accumulated at cervical cords as if a new "gate" was opened by gravity stimuli to the forearm muscles. Consistently, the tail suspension suppressed *Ccl20* expression in the L5 dorsal blood vessels and decreased the expression

Figure 2: Effects of regional neural activation on the invasion of Th17 cells into the CNS

Tail suspension (TS) reduces *Ccl20* expression in L5 dorsal vessel. Electric stimulations (ES) to the soleus muscles of TS mice restore the chemokine expression in a time-dependent manner.
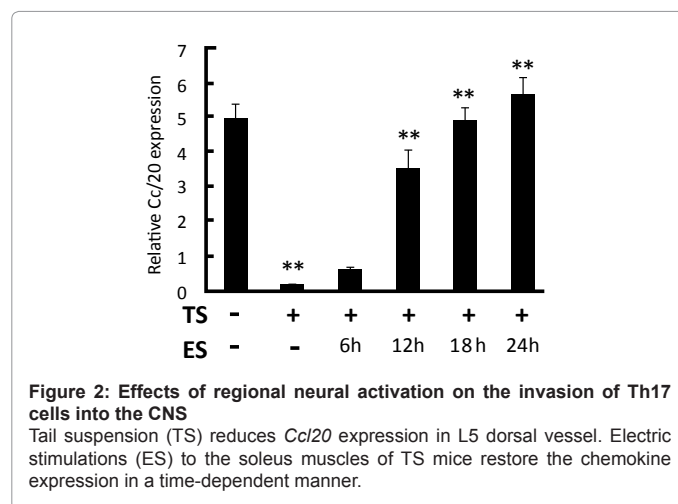
of the neural activation marker, *c-fos*, in L5 DRG. Moreover, electric stimulations to the soleus muscles of tail-suspended mice restored *Ccl20* expression, Th17 accumulation and *c-fos* levels at the L5 cord [19] (Figure 2). These results strongly suggest that neural inputs from soleus muscles in response to gravity play a role in activating the inflammation amplifier and lead to the expression of various chemokines including Th17-attracting *CCL20* in L5 dorsal blood vessels.

What mechanisms do afferent sensory neurons from the soleus muscle use to regulate the status of blood vessels at L5? Although a precise neural network remains elusive, we have shown sympathetic nerves to be involved. Blood flow speeds at L5 dorsal vessels become slower when mice are tail suspended, while electronic stimulation of the soleus muscles increases the flow speed suggesting that autonomic nerves including sympathetic ones are involved in the response. On the other hand, blood flow speeds in blood vessels other than the L5 region, such as femoral vessels, brain surface vessels and the portal vein, are not affected by tail suspension. Furthermore, treatment with atenolol, an α1 adrenergic receptor antagonist, or prazosin, a β1 adrenergic receptor antagonist, significantly inhibits *Ccl20* mRNA expression, NF-κB activation and MOG-reactive Th17 accumulation at L5 vessels and also suppresses clinical signs of EAE [19]. It is also known that the main adrenergic receptors associated with blood vessels are α1 and β2 ones. We hypothesize that the β2 receptor might play a role just like the β1 receptor, because both β1 and β2 receptors activate NFκB pathway [29-31], which is the main pathway of the inflammation amplifier activation. Therefore, it is important to check whether the β2 receptor might play a role by using the inhibitors in our system.

Consistent with these *in vivo* results, the addition of norepinephrine, which is a neurotransmitter from sympathetic neurons, to a culture of endothelial cell lines, enhances the inflammation amplifier based on
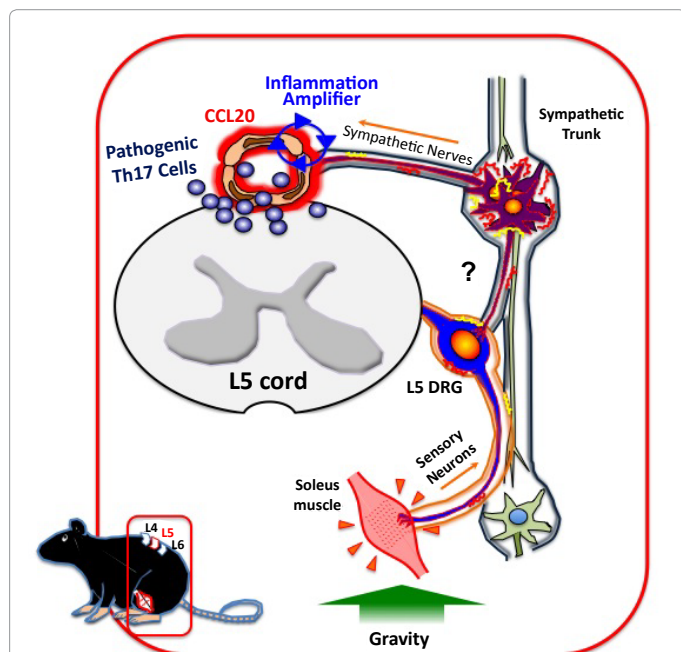

Figure 4: The gate theory
Electric pulses to the upper arm, thigh, and soleus muscles induce activation of the inflammation amplifier: chemokine upregulation, opening a gate in the dorsal vessels of the fifth cervical to fifth thoracic (C5-T5), third lumbar (L3), and fifth lumbar (L5) cords, respectively. This effect offers possible targets for the manipulation of immune cell migration *in vivo*.

IL-6 and *Ccl20* expressions. Thus, anti-gravity responses of the soleus muscles lead to sympathetic nerve stimulation, creating a gateway for immune cells to pass through the CNS via L5 dorsal vessels [19]. However, we do not comprehensively describe the link between the sensory activation and the L5 vascular changes, which makes it unclear if the locally released neurotransmitters directly affect the BBB integrity or the Th17 activity. Based on these findings, we proposed that MOG-reactive, disease-causing Th17 cells make use of the L5 gateway to infiltrate the CNS and induce local inflammation by producing cytokines like IL-17, which further induces chemokines via the inflammation amplifier in parenchymal non-immune cells and results in chronic inflammation in the CNS (Figure 3).

The Gate Theory

Thigh muscles including the quadriceps are known to be regulated by L3 DRG neurons. Interestingly, electronic stimulation of these muscles led to an increased expression of *Ccl20* in L3 cord vessels in mice. In a similar fashion, chemokine levels in the fifth cervical to fifth thoracic cord vessels were upregulated by stimulations of epitrochlearis/triceps brachii and upper arm muscles, which are controlled by neurons located at the corresponding areas [19] (Figure 4). Based on these results, we propose "the gate theory", which describes blood vessels that act as gates depending on the regional neural stimulations for immune cell infiltration into organs. Investigations whether the gate theory can be more generally applied to tissues beyond the CNS are currently undertaken. If true, manipulating the gate should have significant clinical benefits, as closing it in normal cells would dampen autoimmune inflammation in a target organ without any systemic immune suppression, while opening it in tumors could enhance cancer immunotherapy. The precise molecular mechanisms for gating still need to be studied, however, before this potential can be realized.

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Figure 3: The gateway for autoreactive T cells into the CNS
Continuous stimulation of the soleus muscles by gravity leads to the constant activation of L5 DRG sensory neurons, followed by the activation of sympathetic neurons at the L5 region via unidentified circuits. Norepinephrine, a neurotransmitter from sympathetic neurons, augments the inflammation amplifier at the dorsal vessels of L5, causing an elevation of CCL20 and subsequent Th17 infiltration into the CNS.
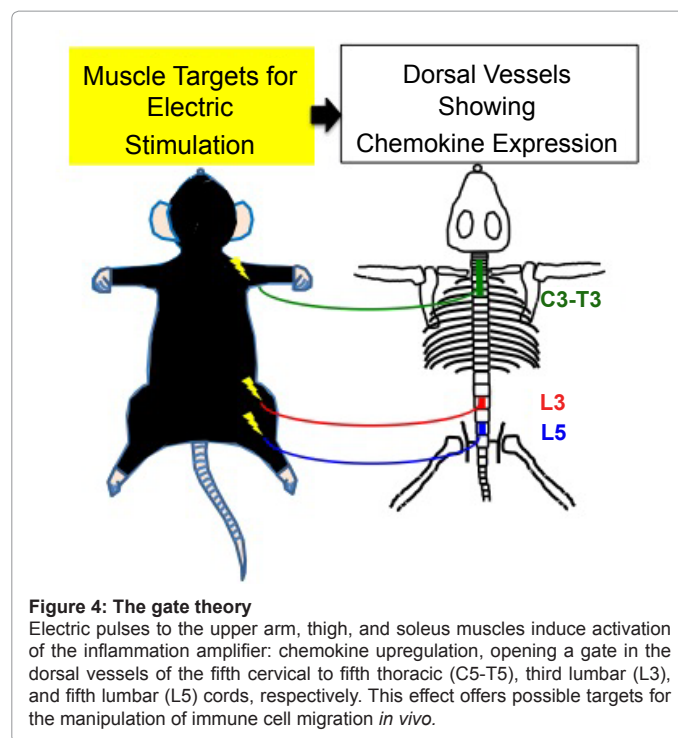
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