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(54) Title: A CRITICAL ROLE OF FEBRILE TEMPERATURE IN REGULATING INTERLEUKIN (IL) -17 PRODUCING CELLS VIA SMAD4

(57) Abstract: Provided is a method of promoting the differentiation or maturation of an IL-17 producing cell, promoting a production of IL-17, improving an immunity of a patient or reducing an immunity of a patient, the method comprises: allowing differentiation or maturation of an IL-17 producing cell under a condition with an environmental temperature higher than a normal body temperature; allowing differentiation or maturation of an IL-17 producing cell lower than a febrile temperature; allowing differentiation or maturation of an IL-17 producing cell in the presence of an inhibitor for SMAD4; allowing differentiation or maturation of an IL-17 producing cell in the presence of an inhibitor for SUMOylated SMAD4; allowing differentiation or maturation of an IL-17 producing cell in the presence of an inhibitor for UBC9; allowing differentiation or maturation of an IL-17 producing cell in the presence of an inhibitor for HSP70; allowing differentiation or maturation of an IL-17 producing cell in the presence of an inhibitor for HSP90; allowing differentiation or maturation of an IL-17 producing cell in the presence of an activator for SMAD4; allowing differentiation or maturation of an IL-17 producing cell in the presence of an activator for SUMOylated SMAD4; allowing differentiation or maturation of an IL-17 producing cell in the presence of an activator for UBC9; allowing differentiation or maturation of an IL-17 producing cell in the presence of an activator for HSP70; allowing differentiation or maturation of an IL-17 producing cell in the presence of an activator for HSP90.



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A CRITICAL ROLE OF FEBRILE TEMPERATURE IN REGULATING INTERLEUKIN (IL)-17 PRODUCING CELLS VIA SMAD4

TECHNICAL FIELD

Embodiments of the present disclosure generally relate to the field of bio-pharmaceutical, more particularly, to a method of promoting or suppressing the differentiation or maturation of IL-17 producing cells, a method of promoting or suppressing IL-17 production, a method of improving or suppressing the immunity of a patient, a method of treating a patient with IL-17 related autoimmune diseases and cancers, an apparatus to promote or inhibit the differentiation or maturation of IL-17 producing cells or IL-17 production or improve or suppress the immunity of a patient, a method of screening a medicament suitable to be used in a treatment or prevention of IL-17 related autoimmune diseases and a method of screening a medicament suitable to be used in enhancing anti-tumor immunity.

BACKGROUND

Fever, a physiological response commonly associated with infections, injuries and neoplasia (Pasikhova et al., 2017), is evolutionarily conserved in both endothermic and ectothermic animals. Febrile range temperatures (1 °C -4 °C above basal core body temperature) are suggested to have a survival advantage in infectious diseases, possibly through inhibiting pathogen growth and boosting protective immune responses (Evans et al., 2015; Hasday et al., 2014; Lin et al., 2019). A key role of fever in immune system is to stimulate innate immune system, such as release of neutrophils in periphery, production of cytokines and nitric oxide from macrophages or dendritic cells, promotion of leukocyte trafficking, and enhancement of their phagocytic, bacteriolytic, cytolytic or antigen presentation functions (Evans et al., 2015; Hasday et al., 2014). Fever is also a shared clinical symptom in many autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, adult onset still's disease, rheumatic fever and inflammatory bowel disease (Limper et al., 2010; Shang et al., 2017). Fever can be observed at both early and active stages of autoimmune diseases, and ~20% of patients with clinical fever of unknown origins are diagnosed later with autoimmune diseases (Limper et al., 2010; Shang et al., 2017), suggest a possible pathogenic role of fever in autoimmune diseases. In addition, fever can be also observed in a number of tumor patients (called as neoplastic fever)(Cornely and Mellingshoff, 2017; Pasikhova et al., 2017), suggesting fever might also regulate anti-tumor or tumor prone immune responses.

Interleukin (IL)-17 plays an important protective role in host defense against fungal and extracellular bacterial infections, as well as in mucosal barrier maintenance. IL-17 producing cells mainly include Th17 cells, a unique CD4⁺ T cell subset identified in 2005 expressing signature cytokines (IL)-17, as well as IL-21, IL-17F and IL-22 (Eming et al., 2017; Stockinger and Omenetti, 2017). In addition, IL-17 can be also produced by a number of other immune cell subsets, including Tc17 cells (a CD8⁺ T cell subset), $\gamma\delta$ T cells, ILC3 cells, NK cells, NKT cells and etc., and these cells share many similar functions and features in various immune responses (Liang et al., 2015; Veldhoen, 2017). However, excessive IL-17 production causes chronic tissue inflammation associated with many human autoimmune diseases (Stockinger and Omenetti, 2017). Up to date, FDA has approved 9 drugs targeting the Th17 cell developmental program in treatment of psoriasis, psoriatic arthritis, ankylosing spondylitis, rheumatoid arthritis and Still's diseases (Fasching et al., 2017). In addition, Th17 cells and IL-17 have been shown to be increased in many cancer

patients or tumor tissues, including melanoma, ovarian cancer, colorectal cancer, lung cancer, ovarian cancer, lung cancer, gastric cancer, etc., and may have a pro-tumor activity or anti-tumor activity, dependent on specific tumor types (Chang, 2019; Qian et al., 2017).

The differentiation of Th17 cells is initiated by interleukin (IL)-6 and transforming growth factor (TGF)- β . IL-6 acts mainly through activating the STAT3 transcription factor (Yang et al., 2007). Downstream signaling of TGF- β involved in Th17 cell differentiation has also been studied. Canonically, TGF- β activates SMAD2 and SMAD3, which form a heterotrimeric complex with SMAD4 and translocate into nucleus to mediate downstream gene expression. SMAD2 is required for Th17 cell differentiation (Malhotra et al., 2010; Martinez et al., 2010), while SMAD3 may play a negative role (Martinez et al., 2009), but also compensate for SMAD2 deficiency (Zhang, 2018). Our earlier report demonstrated a dispensable role of SMAD4 for Th17 cell differentiation *in vitro* (Yang et al., 2008), which was supported by several subsequent studies (Hahn et al., 2011; Zhang et al., 2017). However, a recent publication reported a negative role of SMAD4 in suppressing IL-17 expression when T cells were cultured under IL-6 only conditions, *via* recruiting the transcription repressor SKI (Zhang et al., 2017). In contrast, under complete Th17 cell polarizing conditions, TGF- β causes degradation of SKI to release the inhibitory effect of SMAD4 (Zhang et al., 2017), and together with IL-6 induce robust expression of ROR γ t, the lineage-specific transcriptional factor directly controlling production of IL-17 and IL-17F (Stockinger and Omenetti, 2017), the major effect cytokines in Th17 cells.

Under *in vivo* settings, there is growing evidence that Th17 cells generated in the mucosal tissue, associated with hemostatic barrier regulatory function, are phenotypically distinct from those in the inflamed tissues of autoimmune diseases (Esplugues et al., 2011; Gaublot et al., 2015), and their pathogenicity could be affected by surrounding microenvironments, including cytokines TGF- β 3, IL-1 β and IL-23, salt and microbiota (Kleinewietfeld et al., 2013; Lee et al., 2012; Stockinger and Omenetti, 2017; Wu et al., 2013).

Non-steroid anti-inflammation drugs generally featured with antipyretic properties, including aspirin and rofecoxib, not only reduce inflammation in human patients (Li et al., 2017), but also alleviate experimental autoimmune encephalomyelitis (EAE) model (Mondal et al., 2018; Ni et al., 2007).

SUMMARY

In this study, we examined the role of fever in adaptive immunity and found that febrile temperature significantly enhanced Th17 cell differentiation *in vitro* and *in vivo* and promoted their pro-inflammatory activity and pathogenic function *in vivo* in animal models. Mechanistically, febrile temperature enhanced global amounts of protein SUMOylations, a common response to various stress stimuli (Saitoh and Hinchee, 2000), dependent on HSP70 and HSP90 related heat shock response. We showed that genetic ablation of UBC9, the only E2 SUMOylation conjugating enzyme, or inhibition of HSP70 or HSP90 activity using small molecular inhibitors completely abolished febrile temperature dependent Th17 cell differentiation. Of note, SMAD4, though not required for Th17 cell differentiation under normal temperature, was selectively required for febrile temperature-dependent Th17 cell differentiation *in vitro* and *in vivo*, through SUMOylation at its K113 and K159 residues. Genome-wide RNAseq analysis identified SMAD4 as a critical master transcription factor in controlling febrile Th17 cell differentiation. Genetic ablation of SMAD4, or mutation of the SMAD4 K113/159 SUMOylation sites, abolished the effect of febrile temperature on Th17 cell differentiation *in vitro* and *in vivo*, and alleviated induction of experimental autoimmune encephalomyelitis in mice, a classical Th17-dependent autoimmune disease model. In

addition, febrile temperature could also enhance the differentiation of Tc17 cells (a CD8⁺ T cell subset that expresses IL-17 and shares many similar developmental features to Th17 cells (Liang et al., 2015)) and associated IL-17 expression, likely in a similar mechanism as in Th17 cells. Therefore, our studies reveal a novel mechanism whereby fever promotes IL-17-associated inflammatory responses, including various related autoimmune diseases and pro-tumor or anti-tumor immune response through regulating the differentiation and maturation of Th17 cells, as well as Tc17 cells, or possibly other IL-17 producing cells, including $\gamma\delta$ T, NK, NKT and ILC3 cells

Embodiments of a first broad aspect of the present disclosure provide a method of promoting the differentiation or maturation of an IL-17 producing cell, through modulating environmental temperature. According to the embodiments of present invention, the method comprises: increasing the environmental temperature to enhance the differentiation or maturation of an IL-17 producing cell or IL-17 production *in vitro* or *in vivo*.

It should be noted that the “the environmental temperature” described above is the temperature which IL-17 producing cell can sense during their differentiation or maturation process *in vitro* and *in vivo*.

It should be noted that the “the differentiation or maturation of an IL-17 producing cell” described above indicates the process for naïve T cells or immature T cells or innate immune cells to develop into mature IL-17 producing cells. A naïve T cells includes a naïve CD4⁺ T cell or naïve CD8⁺ T cell. An immature T cell includes a NK T cell or $\gamma\delta$ T cell. An innate immune cell includes an ILC3 cell.

According to the embodiments of present invention, wherein the temperature could be increased up to 39.5°C or above.

According to the embodiments of present invention, wherein an IL-17 producing cell include at least a CD4⁺ T cell (also refer to Th17 cell in the application), CD8⁺ T cell (also refer to Tc17 cells in the application), $\gamma\delta$ T cell, ILC3 cell, NK cell and NK T cell. According to the embodiments of present invention, wherein the *in vivo* environmental temperature is achieved by means of infra-red radiation, hot water, heat-inducing chemical or biological approaches, electronical devices or other physical methods.

Embodiments of a second broad aspect of the present disclosure provide a method of inhibiting the differentiation or maturation of an IL-17 producing cell, through modulating the environmental temperature, in order for reducing IL-17 associated inflammatory response. According to the embodiments of present invention, the method comprises: reduce the environmental temperature to suppress the differentiation or maturation of an IL-17 producing cell *in vitro* or *in vivo*, through using physical, chemical or biological approaches, including anti-pyretic drugs.

Embodiments of a third broad aspect of the present disclosure also provide a method of inhibiting the differentiation or maturation of an IL-17 producing cell, through inhibiting SMAD4. According to the embodiments of present invention, the method comprises: 1) using a chemical or biological inhibitor to suppress the activity of SMAD4 protein; 2) using a chemical or biological reagent to reduce the amount of SMAD4 protein under both *in vitro* or *in vivo* settings.

Embodiments of a fourth broad aspect of the present disclosure also provide a method of inhibiting the differentiation or maturation of an IL-17 producing cell, through inhibiting a SUMOylated SMAD4 protein. According to the embodiments of present invention, the method comprises: 1) using an anti-pyretic drug to reduce the amount of SUMOylated SMAD4 protein; 2) using a HSP70 inhibitor to reduce the amount of SUMOylated SMAD4 under both *in vitro* or *in vivo* settings; 3) using a HSP90 inhibitor to reduce the amount of SUMOylated

SMAD4 chemical or biological reagent to reduce the amount of SMAD4 protein under both in vitro or in vivo settings; 4) using a chemical or biological reagent to inhibit the activity of SUMOylated SMAD4 protein under both in vitro or in vivo settings; or 5) using a chemical or biological reagent to reduce the amount of SUMOylated SMAD4 protein under both in vitro or in vivo settings..

SUMOylated SMAD4 or SMAD4 SUMOylation refers to a SMAD4 protein that is covalently conjugated with one or more small ubiquitin-like modifier (SUMO) moiety. This includes SUMOylation at the K113 or K159 residue of SMAD4.

Embodiments of a fifth broad aspect of the present disclosure also provide a method of inhibiting the differentiation or maturation of an IL-17 producing cell, through inhibiting UBC9-dependent SUMOylation pathway. According to the embodiments of the present invention, the method comprises: 1) using a chemical or biological inhibitor to inhibit the activity of UBC9 protein under both in vitro and in vivo settings; 2) using a chemical or biological reagent to reduce the amount of UBC9 protein under both in vitro and in vivo settings; 3) using a chemical or biological inhibitor to inhibit UBC9 related SUMOylation pathway under both in vitro and in vivo settings.

Embodiments of a sixth broad aspect of the present disclosure also provide a method of inhibiting the differentiation or maturation of an IL-17 producing cell, through inhibiting heat shock response. According to the embodiments of the present invention, the method comprises: 1) using a chemical or biological inhibitor to inhibit the activity of HSP70 protein under both in vitro and in vivo settings; 2) using a chemical or biological reagent to reduce the amount of HSP70 protein under both in vitro and in vivo settings; 3) using a chemical or biological inhibitor to inhibit the activity of HSP90 protein under both in vitro and in vivo settings; 4) using a chemical or biological reagent to reduce the amount of HSP90 protein under both in vitro and in vivo settings.

Embodiments of a seventh broad aspect of the present disclosure also provide a method of promoting the differentiation or maturation of an IL-17 producing cell, through activating SMAD4. According to the embodiments of the present invention, the method comprises: 1) using a chemical or biological reagent to enhance the activity of SMAD4 protein under both in vitro and in vivo settings; 2) using a chemical or biological reagent to increase the amount of SMAD4 protein under both in vitro and in vivo settings.

Embodiments of an eighth broad aspect of the present disclosure also provide a method of promoting the differentiation or maturation of an IL-17 producing cell, through activating SMAD4 SUMOylation. According to the embodiments of the present invention, the method comprises: 1) using a chemical or biological reagent to increase the amount of SUMOylated SMAD4 protein under both in vitro and in vivo settings;

Embodiments of a ninth broad aspect of the present disclosure also provide a method of promoting the differentiation or maturation of an IL-17 producing cell, through activating UBC9-dependent SUMOylation pathway. According to the embodiments of the present invention, the method comprises: 1) using a chemical or biological reagent to enhance the activity of UBC9 protein under both in vitro and in vivo settings; 2) using a chemical or biological reagent to increase the amount of UBC9 under both in vitro and in vivo settings; 3) using a chemical or biological reagent to activate UBC9 related SUMOylation pathway under both in vitro and in vivo settings.

Embodiments of a tenth broad aspect of the present disclosure also provide a method of promoting the differentiation or maturation of an IL-17 producing cell, through activating heat shock response. According to the embodiments of the present invention, the method comprises: 1) using a chemical or biological reagent to enhance the activity of HSP70 protein under both in vitro or in vivo settings; 2) using a chemical or biological reagent to increase the

amount of HSP70 under both in vitro and in vivo settings; 3) using a chemical or biological enhance the activity of HSP90 under both in vitro and in vivo settings; 4) using a chemical or biological reagent to increase the amount of HSP90 protein under both in vitro and in vivo settings.

Embodiments of an eleventh broad aspect of the present disclosure provide a method of treating a patient with IL-17 related autoimmune diseases, comprising: administering an anti-pyretic agent to the patient in need of such treatment; administering an inhibitor for SMAD4; administering an inhibitor for SUMOylated SMAD4; administering an inhibitor for HSP70; administering an inhibitor for HSP90; administering an inhibitor for UBC9.

Embodiments of an twelfth broad aspect of the present disclosure provide a method of treating a patient with IL-17 related cancers in which IL-17 has an pro-tumor activity; comprising: administering an anti-pyretic agent to the patient in need of such treatment; administering an inhibitor for SMAD4; administering an inhibitor or for SUMOylated SMAD; administering an inhibitor for HSP70; administering an inhibitor for HSP90; administering an inhibitor for UBC9.

Embodiments of an thirteenth broad aspect of the present disclosure provide a method of treating a patient with IL-17 related cancers in which IL-17 has an anti-tumor activity; comprising: administering an pro-pyretic agent to the patient in need of such treatment; administering an activator for SMAD4; administering an activator for SUMOylated SMAD4; administering an activator for HSP70; administering an activator for HSP90; administering an activator for UBC9.

According to the embodiments of present invention, wherein the IL-17 related autoimmune disease comprising at least one of the following: psoriasis, psoriasis-like arthritis, ankylosing spondylitis, rheumatoid arthritis, adult's still's disease and multiple sclerosis.

According to the embodiments of present invention, wherein the IL-17 related cancers comprising at least one of the following: melanoma, ovarian cancer, colorectal cancer, lung cancer, ovarian cancer, lung cancer, gastric cancer.

According to the embodiments of present invention, wherein the anti-pyretic agent comprising at least one of the following: aspirin and related salicylates, ibuprofen, ketoprofen, metamizole, nabumetone, acetaminophen, phenazone and docosanol.

Embodiments of a fourteenth broad aspect of the present disclosure provide use of an agent in the preparation of a medicament for improving the immunity of a patient, wherein the agent is suitable to allowing the IL-17 producing cell growing under a condition with an in vivo environmental temperature above 37°C.

Embodiments of a fifteenth broad aspect of the present disclosure provide use of an agent in the preparation of a medicament for suppressing the immunity of a patient, wherein the agent is suitable to allowing the IL-17 producing cell growing under a condition with an in vivo environmental temperature below 42°C.

Embodiments of a sixteenth broad aspect of the present disclosure provide an apparatus to modulate the differentiation or maturation of an IL-17 producing cell or IL-17 expression for improving the immunity of a patient, comprising: an unit suitable to allow the IL-17 producing cell growing under a condition with an in vivo environmental temperature from a range of 37°C or below to 39.5°C or above.

Embodiments of a seventeenth broad aspect of the present disclosure provide use of reagent in preparing medicament, wherein the reagent is used for inhibiting SMAD4 protein, wherein the medicament are used for at least one of the following: inhibiting IL-17

producing cells differentiation; inhibiting production of IL-17; treatment or prevention of IL-17 related autoimmune diseases or cancers.

Embodiments of an eighteenth broad aspect of the present disclosure provide use of reagent in preparing medicament, wherein the reagent is used for inhibiting UBC9, wherein the medicament are used for at least one of the following: inhibiting IL-17 producing cells differentiation; inhibiting production of IL-17; treatment or prevention of IL-17 related autoimmune diseases or cancers.

Embodiments of a nineteenth broad aspect of the present disclosure provide use of reagent in preparing medicament, wherein the reagent is used for inhibiting HSP70, wherein the medicament are used for at least one of the following: inhibiting IL-17 producing cells differentiation; inhibiting production of IL-17; treatment or prevention of IL-17 related autoimmune diseases or cancers.

Embodiments of a twentieth broad aspect of the present disclosure provide use of reagent in preparing medicament, wherein the reagent is used for inhibiting HSP90, wherein the medicament are used for at least one of the following: inhibiting IL-17 producing cells differentiation; inhibiting production of IL-17; treatment or prevention of IL-17 related autoimmune diseases or cancers.

According to the embodiments of present invention, wherein the IL-17 producing cells are Th17 cells, Tc17 cells, $\gamma\delta$ T cells, ILC3 cells, NK and NK T cells.

Embodiments of a twenty-first broad aspect of the present disclosure provide a method of screening a medicament suitable to be used in a treatment or prevention of IL-17 related autoimmune diseases, comprising: contacting a candidate agent with IL-17 producing cells, determining SMAD4 SUMOylation level in the IL-17 producing cells before and after the contacting; the decreased SMAD4 SUMOylation in the IL-17 producing cell after the contacting is an indication that the candidate agent is the medicament.

Embodiments of a twenty-second broad aspect of the present disclosure provide a method of screening a medicament suitable to be used in enhancing anti-tumor immunity, comprising: contacting a candidate agent with IL-17 producing cells, determining SMAD4 SUMOylation level in the IL-17 producing cell before and after the contacting; the increased SMAD4 SUMOylation in the IL-17 producing cell after the contacting is an indication that the candidate agent is the medicament.

According to the embodiments of present invention, the present disclosure provides a method of promoting a maturation of an IL-17 producing cell. According to the embodiments of present invention, the method comprises: allowing the IL-17 producing cell growing under a condition with an environmental temperature higher than a body temperature.

According to the embodiments of present invention, present disclosure provides a method of promoting a secretion of IL-17, comprising: allowing the IL-17 producing cell growing under a condition with an environmental temperature higher than a body temperature.

It should be noted that the “temperature” described above is the temperature which the IL-17 producing cell can sense.

According to the embodiments of present invention, wherein the temperature is sufficient to a SUMOylation of SMAD4.

According to the embodiments of present invention, wherein the temperature is higher than normal body temperature, for example, in a range of 37°C to 42°C.

According to the embodiments of present invention, wherein the temperature is 38.5°C or 39.5°C.

According to the embodiments of present invention, wherein IL-17 producing cell is at least one of Th17 and CD8.

According to the embodiments of present invention, the present disclosure provides a method of improving an immunity of a patient, comprising: allowing the IL-17 producing cell growing under a condition with an in vivo environmental temperature higher than a body temperature.

According to the embodiments of present invention, wherein the in vivo environmental temperature is achieved by means of infra-red radiation, hot water, heat-inducing chemical or biological approaches, electronical devices or other physical methods

According to the embodiments of present invention, the present disclosure also provides a method of promoting a maturation of an IL-17 producing cell or promoting secretion of IL-17. According to the embodiments of present invention, the method comprises: making SMAD4 in IL-17 producing cells SUMOylation.

According to the embodiments of present invention, the present disclosure also provides a method of improving an immunity of a patient, comprising: administrating a reagent used for enhancing SMAD4 SUMOylation in the IL-17 producing cells.

According to the embodiments of present invention, the present disclosure provides a method of treating a patient with IL-17 related autoimmune disease, comprising: administrating an anti-febrile agent to the patient in need of such treatment.

According to the embodiments of present invention, comprising lowering a body temperature of the patient sufficient to suppress a SUMOylation of SMAD4.

According to the embodiments of present invention, the present disclosure provides use of an anti-febrile agent in the preparation of a medicament for the treatment of IL-17 related autoimmune disease.

According to the embodiments of present invention, the present disclosure provides use of an agent in the preparation of a medicament or kit for a maturation of an IL-17 producing cell or promoting a secretion of IL-17, wherein the agent is suitable to allow the IL-17 producing cell growing under a condition with an environment temperature higher than a body temperature.

According to the embodiments of present invention, the present disclosure provides use of an agent in the preparation of a medicament for improving an immunity of a patient, wherein the agent is suitable to allowing the IL-17 producing cell growing under a condition with an in vivo environmental temperature higher than a body temperature.

According to the embodiments of present invention, the present disclosure provides an apparatus to promote the maturation of an IL-17 producing cell or promote a secretion of IL-17 or improve an immunity of a patient, comprising: an unit suitable to allow the IL-17 producing cell growing under a condition with an environmental temperature higher than a body temperature.

According to the embodiments of present invention, the present disclosure provides use of reagent in preparing medicament, wherein the reagent is used for inhibiting SMAD4 SUMOylation, wherein the medicament are used for at least one of the following: inhibiting IL-17 producing cells differentiation; inhibiting production of IL-17; treatment or prevention of IL-17 related autoimmune diseases.

According to the embodiments of present invention, wherein inhibiting SMAD4

SUMOylation is performed through inhibiting UBC9-depended SUMOylation pathway.

According to the embodiments of present invention, the present disclosure provides use of reagent in preparing medicament, wherein the reagent is used for enhancing SMAD4 SUMOylation, wherein the medicament is used for at least one of the following: enhancing secretion of IL-17; enhancing IL-17 producing cells differentiation; enhancing anti-tumor immunity.

According to the embodiments of present invention, wherein enhancing SMAD4 SUMOylation is performed through activating UBC9-depended SUMOylation pathway.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other aspects and advantages of embodiments of the present disclosure will become apparent and more readily appreciated from the following descriptions made with reference the accompanying drawings, in which:

Figure 1 shows febrile temperature promoted Th17 cell differentiation & IL-17 expression;

Naïve CD4⁺ T cells were polarized under Th1 (IFN- γ +IL-12+anti-IL-4+IL-2), Th2 (IL-4+anti-IFN- γ +IL-2), Th17 (IL-6+TGF- β 1 or IL-6+IL-1 β +IL-23+ TGF- β 1) or iTreg (TGF- β 1+IL-2) culture condition for 3-4 days at 37°C or 39.5°C, respectively, and the cells were restimulated with phorbol-12-myristate-13-acetate, ionomycin and Golgi-stop for intracellular staining or with α CD3 for mRNA expression analysis. (A) Top Left: intracellular staining of lineage-specific cytokines or transcriptional factors in Th1, Th2 and iTreg cells; Top right: statistic data of iTreg cells polarized under 37°C or 39.5°C (n=4); Bottom Left: intracellular staining of IL-17 and FOXP3 in Th17 cells polarized under 37°C, 39.5°C or 38.5°C; Bottom right: statistic data of iTreg cells polarized under 37°C or 39.5°C (n=10). (B) Realtime PCR data of mRNA expression in Th17 cells after 3 days' culture under 37°C or 39.5°C. The statistics were performed by Student t-test. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

Figure 2 shows febrile temperature promoted in vitro Th17 cell differentiation & IL-17 production via heat shock response;

A. Time-course mRNA expression of Hsp40, Hsp60, Hsp70, Hsp90aa, Hsp90ab and Hsp110h genes in Th17 cells cultured at 37°C or 39.5°C as determined by realtime PCR assay (student's t test, ** $P < 0.01$; *** $P < 0.001$). B. The protein level of HSF1 and HSF2 in Th17 cells cultured at 37°C or 39.5°C as determined by western blot. The integrated densities (X 105) were measured with Image J software and listed in the figure. The results shown here represents one of two independent results. C. Th17 cells were cultured in the presence or absence of 0.2 μ M HSP90 inhibitor (NMS-E973) or 10 μ M HSP70 inhibitor (VER 155008) under Th17 culture condition (IL-6+TGF- β 1) at 37°C or 39.5°C, respectively. Intracellular staining of IL-17A and FOXP3 in Th17 cells after 3 days' culture.

Figure 3 shows febrile temperature promoted in vivo Th17 cell differentiation & IL-17 production via heat shock response

Naïve OT-II cells were intravenously transferred into *Tcrbd*^{-/-} mice ($\sim 2 \times 10^5$ cells/mouse), followed by OVA+CFA immunization. The mice were treated by oral gavage with aspirin (2 mg/kg body weight, dissolved in 0.5% methyl cellulose solution, n=7) and control solution (n=7) twice a day or ibuprofen (50 mg/kg body weight, dissolved in 0.5% methyl cellulose, n=5) and control (n=5) daily throughout the experiment. The transferred OT-II T cells (CD4⁺CD3⁺) were isolated from the draining lymph nodes at different time points as indicated and analyzed for heat shock-related gene

expression. (A) Relative mRNA expression of *Hsf1*, *Hsf2*, *Hsp60*, *Hsp90* and *Hsp110h* (For each time points, 2-3 mice were sacrificed and the OT-II cells were isolated and pooled together for realtime PCR analysis. The results shown here represents one of the three independent results). (B) Left: Intracellular staining of IL-17 and IFN- γ in the donor OT-II T cells after aspirin or ibuprofen treatment for 7 days; Right: statistic data of IL-17A expression in the donor OT-II T cells after aspirin or ibuprofen treatment. The statistics were performed by Student's t-test. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

Figure 4 shows febrile temperature increased the proinflammatory or pathogenicity of Th17 cells;

Th17 cells induced at 37°C or 39.5°C with IL-6 and TGF- β 1 were collected and used for whole genome transcriptome analysis. (A) Volcano plots of differential gene expression pattern between Th17 cells induced at 39.5°C and 37°C. (B) Overlap of febrile temperature upregulated genes (≥ 1.5 fold increase) with those upregulated by TGF- β 3 + IL-6 (T36) or IL-1 β + IL-6 + IL-23(B623) versus TGF- β 1 + IL-6 (T16). (C) GSEA analysis of RNAseq data obtained from febrile Th17 cells *versus* IL-23 induced Th17 cells, referred to as Th17 (23), or Th17 cells derived in the central nerve system (CNS) of EAE model or homeostatic lamina propria tissues (LP). (D) Left: intracellular staining data of Th17 cells induced using the APCs-OT-II co-culture system at 37°C and 39.5°C, respectively; Middle: intracellular staining data of neutrophils (CD11b⁺Ly6G⁺) infiltrated in the bronchoalveolar lavage fluid (BALF) and lung tissue; Right: statistic data of the percentage of infiltrated neutrophils.

Figure 5 shows febrile temperature promoted Th17 cell differentiation via enhancing UBC9 and SMAD4 SUMOylation;

(A) WT (*Smad4*^{fl/fl}) and *Smad4*^{-/-} (*Smad4*^{fl/fl}CD4^{Cre}) naïve T cells were polarized with complete Th17 polarizing conditions (IL-6 + TGF- β 1 or IL-6 + IL-1 β + IL-23 + TGF- β 1) or IL-6 culture conditions (IL-6 + anti-TGF- β or IL-6 + IL-1 β + IL-23 + anti-TGF- β) at 37°C or 39.5°C, and analyzed by flow cytometry. The results shown here represent one of the two independent experiments. (B) Immuno blot of total cellular SUMO2-conjugated proteins and SUMOylated SMAD4 (immunoprecipitated with α SMAD4 and then blotted by α SUMO2) in Th17 cells induced at 37°C and 39.5°C for 24 hours in the presence of IL-6 and TGF- β 1. β -Actin was blotted as a loading control. (C) Immuno blot of total cellular SUMO2-conjugated proteins and SUMOylated SMAD4 (immunoprecipitated with α SMAD4 and then blotted by α SUMO2) in T cells polarized under Th17 cell condition (IL-6+TGF- β 1) cultured with or without HSP70 inhibitor (10 μ M) or HSP90 inhibitor (0.2 μ M) at 37°C and 39.5°C for 24 hours. β -Actin was also blotted as control. (D) Intracellular staining of IL-17A and FOXP3 in WT (*Ubc9*^{fl/fl}) and Ubc9-deficient (*Ubc9*^{fl/fl}ERT2^{Cre}) Th17 cells induced with IL-6 and TGF- β 1 at 37°C and 39.5°C in the presence of tamoxifen.

Figure 6 shows SMAD4 SUMOylation sites (K113 and K159) were required for febrile Th17 cell differentiation and associated IL-17 production;

(A) Naïve CD4⁺ T cells were polarized with complete Th17 cell condition (IL-6+TGF- β 1), IL-6 only condition (α TGF- β +IL-6) or TGF- β 1 only condition at 37°C and 39.5°C for 24 hrs. The cells were collected, spun down to a cytospin microscope slide, fixed and stained with α SMAD4 followed by staining with Alexa Fluor 488 conjugated secondary antibody. The cellular distribution of SMAD4 was visualized using a confocal fluorescence microscopy. Shown here

represents the merged photos of SMAD4 (green) and DAPI (blue, indicated for nuclear location) staining. Right: Statistic data of SMAD4 nuclear translocation ratio, which was determined by manually counting the percentage of cells containing higher SMAD4 staining intensity in the nucleus *versus* cytoplasm in 3 representative fields revealed by Image-Pro Plus software. The results shown here represent one of the two independent experiments. (B) *Smad4*^{-/-} naïve T cells were activated under neutral condition at 37°C and infected with retrovirus harboring WT *Smad4* or the K113R/159R mutant *Smad4* gene, and then polarized under Th17 cell culture condition (IL-6+TGF-β1) at 37°C or 39.5°C for reconstituting febrile Th17 cell differentiation. Intracellular staining of IL-17 data were gated on GFP⁺ cells infected with retroviruses. (C) Immunofluorescence staining data of SMAD4 (red) in Th17 polarizing cultures (24 hours post retrovirus infection) as shown in (B). Left: immunofluorescence staining data (merged photo, and GFP signal represents retrovirally infected cells and blue DAPI staining represents nuclear location). Right: quantification data of SMAD4 nuclear translocation ratio

Figure 7 shows SMAD4-deficiency abolished febrile Th17 cell differentiation in vivo and reduced experimental autoimmune encephalomyelitis (EAE);

(A) Adoptive T cell transfer experiment: WT 2d2⁺ (CD45.1⁺CD45.2⁺*Smad4*^{fl/+}) and *Smad4*^{-/-} 2d2⁺ (CD45.2⁺*Smad4*^{fl/fl}CD4^{Cre}) naïve T cells were mixed together at 1:1 ratio and transferred into *Tcrbd*^{-/-} mice followed by MOG₃₅₋₅₅ immunization, and the donor cells were isolated from draining lymph nodes and analyzed 7 days later (n=10). When indicated, the mice were treated with aspirin or control solution (0.5% methyl cellulose) by oral gavage at a dose of 2 mg/kg body weight twice a day for 7 days after immunization. Left: intracellular staining of the CD45.1 & CD45.2 congenic markers, IL-17 and IFN-γ in donor cells; Middle: statistic data of the left staining data; Right: statistic data of IL-17 and IFN-γ expression in the WT 2d2 and *Smad4*^{-/-} 2d2 mixed T cell co-transfer experiment followed by aspirin or control treatment. The statistic data shown here represent a combination of 3 independent experiments. (B) Clinical EAE scores in WT (n=8) and *Smad4*^{-/-} (n=7) mice after second MOG₃₅₋₅₅ immunization, and the difference in disease scores were analyzed by Two-way ANOVA analysis (*Smad4* genetic factor***; time factor***: *P* < 0.001). (C) Left: intracellular staining IL-17 and IFN-γ in CD4⁺ T cells infiltrated in the central nerve system of EAE mice; Right: statistic data of IL-17⁺, IFN-γ⁺ and FOXP3⁺ T cells in percentages in the CNS as determined by Student's t test (* *P* < 0.05; ** *P* < 0.01). (D)/(E) *Smad4*^{ΔCD4} (*Smad4*^{fl/fl}*Cd4*^{Cre}) 2D2⁺ T cells were first retrovirally infected with wild-type *Smad4* or the K113/159R-mutant *Smad4*, and then adoptively transferred into *Rag1*^{-/-} mice (n=6-7 for each group) followed by MOG₃₅₋₅₅ immunization to induce EAE disease. The CNS-infiltrating T cells were then isolated from the central nervous system and analyzed for IL-17A and IFN-γ expression. (D) Clinical EAE scores in *Rag1*^{-/-} mice receiving *Smad4*-WT and *Smad4*-K113/159R transduced T cells followed second MOG₃₅₋₅₅ immunization. The difference in disease scores were analyzed by two-way ANOVA analysis (*Smad4* genetic factor***; time factor***: *P* < 0.001). (E) Left: Intracellular staining of IL-17A and IFN-γ in T cells infiltrated in the central nervous system; Right: statistic data of IL-17⁺ % and IFN-γ⁺ % T cells in the CNS as determined by Student's t test (* *P* < 0.05).

Figure 8 shows SMAD4 served as the master transcription factor in regulating febrile Th17 cell differentiation.

WT (*Smad4*^{fl/fl}) and *Smad4*^{ΔCD4} (*Smad4*^{fl/fl}*Cd4*^{Cre}) Th17 cells induced at 37°C or 39.5°C with IL-6 and TGF-β1 for 3 days were collected and used for whole genome transcriptome analysis. (A) Heat map of genome-wide differentially expressed genes in WT and *Smad4*^{ΔCD4} Th17 cells induced at 37°C and 39.5°C. (B) Overlap of *Smad4* upregulated or downregulated genes *versus* the genes induced (left) or repressed (right) at 39.5°C, respectively. (C) Heat map of 42 genes of

febrile temperature induced genes enriched in the “Th17 cell differentiation” and “cytokine-cytokine interaction” pathways (≥ 1.5 fold upregulation)

Figure 9 shows febrile temperature promoted Tc17 cell differentiation & IL-17 expression.

Naïve CD8⁺ T cells obtained from C57BL/6 mice were cultured with IL-6 and TGF- β (similar to Th17 polarizing condition) and cultured under 37°C and 39°C for 4 days, and the cells were collected and restimulated with phorbol-12-myristate-13-acetate, ionomycin and Golgi-stop for intracellular staining of IL-17 and FOXP3.

DETAILED DESCRIPTION

Fever, an evolutionarily conserved physiological response to infection, is also commonly associated with many autoimmune diseases, but its role in T cell differentiation and autoimmunity remains largely unclear. T helper 17 (Th17) cells are critical in host defense and autoinflammatory diseases, with distinct phenotypes and pathogenicity. Here, we show that febrile temperature, selectively regulated Th17 cell differentiation *in vitro* in enhancing interleukin-17 (IL-17), IL-17F and IL-22 expression. Th17 cells generated under febrile temperature (38.5-39.5°C), compared to those under 37°C, showed enhanced pathogenic gene expression, with increased pro-inflammatory activities *in vivo*. Mechanistically, febrile temperature promoted SUMOylation of SMAD4 transcription factor to facilitate its nuclear localization; Smad4 deficiency selectively abrogated the effects of febrile temperature on Th17 cell differentiation both *in vitro*, and ameliorated an autoimmune disease model. Our results thus demonstrate an unexpected role of fever in shaping adaptive immune responses, with implications in autoimmune diseases.

Reference will be made in detail to embodiments of the present disclosure. The embodiments described herein with reference to drawings are explanatory, illustrative, and used to generally understand the present disclosure. The embodiments shall not be construed to limit the present disclosure. The same or similar elements and the elements having same or similar functions are denoted by like reference numerals throughout the descriptions.

Materials and Methods

Primers, inhibitors and antibodies: The primers used for cloning and realtime PCR were listed in Supplementary Table 1 or below. The inhibitors used in this study including: HSP90 inhibitor NMS-E973 (Selleckchem, Cat#S7282), HSP70 inhibitor VER155008 (Selleckchem, Cat#S7751) and TGF- β RI inhibitor SB431542 (Selleckchem, Cat# S1067). The anti-pyretic drugs used in this include: Aspirin (Selleckchem, Cat# S3017), Ibuprofen (Selleckchem, Cat# S1638) and Methyl cellulose (Sigma, Cat# M0262) for its 0.5% solution. The antibodies used in this study include: anti-SUMO2 (Invitrogen, Cat# 519100), anti-SMAD4 for western blotting (Santa Cruz, Cat# sc-7966), anti-HSF1 for western blotting (cell signaling technology, Cat# 4356s), anti-HSF2 for western blotting (Santa Cruz, Cat# sc-13517), anti-SMAD4 for ChIPseq and immunoprecipitation (Abcam, Cat# ab40759), anti-TGF- β (R&D, Cat#MAB1835) for cell culture, as well as antibodies against CD45.1 (eBioscience, Cat#25-0453-81), CD45.2 (eBioscience, Cat# 56-0454), CD11b (eBioscience, Cat# 25-0112-81), CD11c (eBioscience, Cat#48-0114-82), F4/80 (Biolegend, Cat# 123110), Singlec-F (BD, Cat# 562680), Gr-1 (eBioscience, Cat# 45-5931), IL-4 (BD, Cat# 554435), IL-13 (eBioscience, Cat# 12-7133-81), IFN- γ (BD, Cat# 557724),

IL-17A (BD, Cat# 559502) and FOXP3 (eBioscience, Cat# 48-5773-82) for flow cytometry.

Mice. The *Smad4*^{fl/fl} mice were previously described (Chu et al., 2004), and were crossed with *Cd4Cre* (Lee et al., 2001) to generated conditional *Smad4*^{ΔCD4} mice. The *Tcrbd*^{-/-}, *CreERT2*, *CD45.1*, *OT-II* TCR and 2D2 TCR transgenic mice were purchased from Jackson Laboratories. The 2D2 mice and CD45.1 were crossed with *Smad4*^{fl/fl}*Cd4*^{Cre} when indicated. The *Ubc9*^{fl/fl} mice were previously described (Demarque et al., 2011), and were crossed with *CreERT2* mice for preparing inducible *Ubc9* knockout T cells. All the mice were housed in the SPF animal facility at Tsinghua University. All the animal experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee.

Plasmid construction and retroviral transduction. The WT *Smad4* gene (gene access ID: 17128) was PCR amplified using the primers below: CACGCGTTACTCCAGAAATTGGAGAGTTGGAT (forward) and CGGAATTCTCAGTCTAAAGGCTGTGGGTC (reverse), cloned into the pRVKM retroviral vector, and then used for constructing the *Smad4*-K113/159R mutant plasmid by site-direct mutagenesis. The *Smad4* or control plasmids were transfected together with pCL-ECO into 293T cells for preparing retrovirus. Naïve CD4⁺ T cells were activated with plate-bound anti-CD3 plus anti-CD28 for 24 hours under neutral condition, and were infected with virus harboring the WT and *Smad4* mutant genes by spinning. The infected T cells were washed and changed to Th17 polarizing condition for additional two days cultured at 37°C or 39.5°C.

In vitro T cells differentiation and flow cytometry. CD4⁺ T cells were isolated using MACS mouse CD4⁺ T cell isolation kit (Miltenyi) and CD4⁺CD25⁻CD44^{low}CD62L^{high} naïve CD4⁺ T cells were sorted by FACS Aria III cell sorter (BD). Naïve CD4⁺ T cells were cultured in RPMI 1640 medium (Gibco) supplemented with 100 U/mL of penicillin, 100 µg/mL of streptomycin, 0.05 mM of β-mercaptoethanol and 10% fetal bovin serum (Gibco) and differentiated in 48-well plates coated with 2 µg/mL αCD3 (BioXcell) and 2 µg/mL αCD28 (BioXcell) in the presence of different cytokine cocktails: Th1: IL-12 (15 ng/mL), IL-2 (25 U/mL) and αIL-4 (10 µg/mL); Th2: IL-4(20 ng/mL), IL-2 (25 U/mL), αIFN-γ (10 µg/mL); non-pathogenic Th17: IL-6 (15 ng/mL), TGF-β1 (2 ng/mL); pathogenic Th17: IL-6 (15 ng/mL), TGF-β1 (2 ng/mL), IL-23 (25 ng/mL), IL-1β (10 ng/mL); iTreg: TGF-β1 (2 ng/ml), IL-2 (25 U/ml).

For flow cytometry, the cells were re-suspended in 1xPBS for staining with fixable live/dead cell dye (eBioscience, Cat# 65-0866), followed by various surface markers as indicated, and then fixed using the eBioscience Fix/Perm or BD Fix/Perm buffer kit for intracellular staining of FOXP3, IFN-γ, IL-4, IL-13 or IL-17A as indicated, and finally analyzed with the LSR Fortessa cell analyzer (BD) and FlowJo software. For cytokine staining, the cells were first re-stimulated for 5 hours in the presence of phorbol-12-myristate-13-acetate (50 ng/mL), ionomycin (500 ng/mL) and Golgi-stop (2 µM, BD Biosciences, Cat#554724) before staining. The cells obtained in *in vivo* models were blocked by αCD16/CD32 before staining, and the neutrophils were gated as Gr1⁺CD11b⁺ population

Acute lung inflammation model. Naïve CD4⁺ T cells were sorted from OT II mice and co-cultured with antigen presenting cells (APCs) (1:2 ratio) in the presence of OVA peptide (3 µg/ml) under neutral condition (αIFN-γ + αIL-4) for two days at 37°C, and the culture system was then changed to Th17 polarizing condition for another 3 days at 37°C or 39.5°C. One million OVA-specific Th17 cells were then transferred to CD45.1 congenic recipients followed by intra-nasal inhalation of OVA peptide (25 µg/mouse). The mice were then sacrificed and the

BALF and lung tissue were then collected for further analysis according to previous studies.

Adoptive T cell transfer assay and EAE model. Naïve T cells isolated from $CD45.1^+ CD45.2^+ 2d2^+ Smad4^{fl/+}$ ($CD45.1^+ CD45.2^+$ WT) and $CD45.2^+ 2d2^+ Smad4^{fl/fl} CD4^{Cre}$ ($CD45.2^+ Smad4^{\Delta CD4}$) mice were mixed at 1:1 ratio and transferred into the $Tcrbd^{-/-}$ recipient mice (1 million/mouse), followed by subcutaneous immunization at the tail base with MOG₃₅₋₅₅ peptides emulsified in complete Freund's adjuvant (CFA). When indicated, the mice were treated by gavage with aspirin at 2 mg/kg body weight or ibuprofen in 0.5% methylcellulose twice a day throughout the experiment. The mice were sacrificed 7 days later and the draining lymph nodes were then collected for further analysis.

The active EAE was induced by subcutaneous immunization at the tail base with 150 µg/mice MOG₃₅₋₅₅ peptides emulsified in 100 µl of complete Freund's adjuvant (CFA, 5 mg/ml) on day 1 and day 7, followed by i.p. injection of 500 µg/mice pertussis toxin dissolved in 1xPBS on day 2 and day 8. The disease was scored based on the following standards: 0, no clinical sign of disease; 1, loss of tail tonicity; 2, wobbly gait; 3, complete hind limb paralysis; 4, complete hind and fore limb paralysis; 5, moribund or dead. The central nerve system tissues from EAE mice were then isolated and analyzed as previously described (Wang et al., 2012).

Cytospin and immunofluorescence staining. Naïve T cells were polarized under Th17 culture condition for 24 hours and then re-suspended in culture medium at 1 million/mL. ~100 µL of each cell suspensions were added to a slide chamber, and spun down onto the slide using a cytocentrifuge (800 rpm/5 min). The cells were fixed on slide with 4% PFA, permeabilized with 0.01% TrionX-100, blocked with goat serum and stained with αSMAD4 antibody (Santa Cruz, Cat# sc-7966) overnight. The slides were washed and then incubated with goat anti-mouse IgG (Biolegend, Cat# 405319) or APC conjugated goat anti-mouse IgG (Biolegend, Cat# 405308) secondary antibody for 2 hours at room temperature, and finally mounted with mounting medium containing DAPI. The images were obtained by LSM780 fluorescence microscope (Zeiss). The translocation ratio was measured using Image-Pro Plus 6.0 software and calculated based on the relative intensity of SMAD4 staining in the nucleus or cytoplasm.

SUMOylation assay. Total $CD4^+$ T cells, enriched by the MACS mouse $CD4^+$ T cell isolation kit (Miltenyi), were cultured under Th17 polarizing condition at 37°C or 39.5°C for 24 hours, and then harvested and lysed by 1% SDS containing 20 mM NEM to preserve SUMOylation. The cell lysates (containing 50 mM DTT) were denatured at boiling temperature for 10 min followed by sonication to reduce viscosity. After 10-fold dilution using RIPA buffer, the immunoprecipitation was performed using αSMAD4 (Abcam, Cat# ab40759) and Dynabeads Protein A (Life Technologies, Cat# 10002D) to enrich the targeted protein, and SUMOylated bands were detected by western blotting with αSUMO2 antibody (Invitrogen, Cat#519100).

ChIPseq. The ChIP assay was performed using Active Motif's ChIP assay kit (53035) according to manufacturer's instructions with slight modifications (Jiang et al., 2018). Briefly, Th17 cells were harvested and then cross-linked with 1% paraformaldehyde for 10 min and stopped with 125 mM glycine for 5 min at room temperature. The cells were lysed and digested with shearing enzyme followed by 10 cycles' sonication. The cell lysate was then used for immunoprecipitation with antibodies αSMAD4 (Abcam, Cat# ab40759) or control IgG (Abcam, Cat# ab46540) followed by Dynabeads Protein A (Life Technologies, Cat# 10002D) pulldown. The precipitated DNA was then washed, eluted, de-crosslinked and purified for realtime PCR analysis or for deep sequencing carried by BGI Genomics. The sequence data were deposited in

the GEO database under the accession codes: GSE125263. The primers used for ChIP-QPCR are listed in supplementary table.

Clean reads after filtering were aligned to the reference sequence mm10 genome by using bowtie2 (Langmead and Salzberg, 2012). PCR duplicates were removed using picard MarkDuplicates. The uniquely mapped reads were used to call peak with MACS2 (Zhang Y, 2008) using a p value cutoff 0.01. ChIPseeker was used for peak annotation (Yu et al., 2015). Deeptools was used to generate coverage track file (bigWig) which can be visualized in IGV

RNAseq. Th17 cells were collected after 3 days culture and the total RNA was extracted with Trizol (Life Technologies) according to manufacturer's instructions, and the RNA-seq library was constructed and sequenced with BGI500 platform by BGI Genomics. Low quality reads and adaptor sequences were removed by Trim Galore v0.4.4. The clean reads were mapped to the Mus musculus genome (version mm10) by bowtie2 with default parameter. The unique mapping reads were summarized by featureCounts (from Subread package). Differentially expressed genes were identified by at least 1.5 fold change and FDR adjusted p-value 0.01 (Wang et al., 2010). The pathway analysis was performed with ClusterProfiler (R package) (Yu et al., 2012). The sequence data were deposited in the GEO database under the accession codes: GSE125264.

For comparison, febrile temperature induced genes were compared with previously reported pathogenic Th17 cells induced by TGF- β 3 (GSE39820), or generated in the EAE model versus homeostatic state, by overlay or GSEA. The pathogenic and non-pathogenic gene-sets used for comparison was determined by differential expressed genes between TGF- β 3 versus TGF- β 1 induced Th17 cells, or Th17 cells induced with IL-23 versus without IL-23 (GSE23505), or Th17 cells induced in the EAE model versus homeostatic state.

Realtime PCR. T cells derived from the adoptive T cell transfer models will be sorted based on CD4⁺CD3⁺ surface markers and used for mRNA preparation. T cells collected in *in vitro* cultures will be first restimulated with plate bound α CD3 for 4 hours to stimulate cytokine gene expressions before harvesting. The total RNA from these cells was extracted by TRIzol (Invitrogen) according to manufacturer's instruction. The cDNA was synthesized by reverse transcription using M-MLV Reverse Transcriptase (Promega) according to the manufacturer's instructions and used for realtime PCR assay, performed in 1x Hieff qPCR SYBR Green Master Mix (Yeasen) together with 0.2 μ M forward and reverse primers. The mRNA amounts of indicated genes were normalized against that of β -Actin, and the ChIP-QPCR data were normalized to input.

Statistical analysis. All our *in vitro* and *in vivo* data were repeated at least 2-5 times with consistent results. When indicated, the statistical significance was shown as mean $\pm$ SD and generally determined by Student's t test, or Two-way Anova analysis when indicated. (* represents $P < 0.05$; ** represents $P < 0.01$; *** represents $P < 0.001$).

Results

Febrile temperature selectively promotes Th17 cell differentiation *in vitro* via heat shock responses.

To understand the role of fever in T cell response and related autoimmunity, naïve CD4⁺ T cells were cultured *in vitro* under Th1, Th2, Th17 and T regulatory (Treg) cell-polarizing conditions at 37°C or 39.5°C for 3-4 days. Febrile temperature did not affect Th1, Th2 or iTreg cell differentiation, but selectively and robustly enhanced Th17 cell differentiation as determined

by intracellular staining of IL-17A (~2 fold increase) under both sub-optimal (IL-6 plus TGF- β 1) and optimal conditions (IL-6, TGF- β 1, IL-1 β plus IL-23) (Figure 1A). It is noticed that a mild temperature increase (38.5°C) caused a similar degree of enhancement of Th17 cell differentiation, further confirmed a general phenomenon of temperature-sensitive Th17 cell differentiation (Figure 1A). At the mRNA level, febrile temperature significantly enhanced the expression of key Th17 cell cytokine genes including *Il17a*, *Il17f* and *Il22*, as well as cytokine receptor genes *Il1r1* and *Il1r2*, but greatly reduced the expression of anti-inflammatory cytokine *Il10* (Figures 1A-B). However, the mRNA amounts for *Rorc* and *Rora* were not significantly affected (Figure 1B).

Heat shock responses are characterized by activation and induction of heat shock factors and heat shock proteins (Singh and Hasday, 2013). Consistently, expression of heat shock proteins, including *Hsp40*, *Hsp60*, *Hsp70*, *Hsp90* and *Hsp110h*, and the master heat shock factors HSF1 and HSF2 were rapidly induced in Th17 cells cultured at 39.5°C at mRNA or protein levels, respectively (Figure 2A and 2B). Heat shock protein inhibitors, such as NMS-E973 for HSP90 or VER155008 for HSP70, inhibited febrile temperature-enhanced Th17 cell differentiation (Figure 2C). Additionally, shRNA silencing *Hsp70* mRNA expression abolished febrile temperature-associated Th17 cell differentiation, though its overexpression had no effect on Th17 cell induction at normal or febrile temperature (Figures 2D and 2E), suggesting that *Hsp70* upregulation is necessary but not sufficient to potentiate Th17 cell differentiation at febrile temperatures. In multiple experiments, treatment with HSP70 inhibitor slightly but consistently reduced Th17 cell differentiation under 37°C, suggesting a possible minor role for HSP-dependent stress response in normal Th17 cell differentiation.

Febrile temperature regulates Th17 cell differentiation *in vivo*

To investigate the *in vivo* effect of fever, naïve OT-II T cells were adoptively transferred into *Tcrbd*^{-/-} mice, followed by OVA+CFA immunization. As expected, fever was readily induced as in the WT C57BL/6 mice post immunization, and was associated with increased expression of heat shock response related genes in the donor OT-II cells, including *Hsf1*, *Hsf2*, *Hsp60*, *Hsp90* and *Hsp110* (Figure 3A), as well as IL-17 expression (Figure 3B). Treatment with antipyretic drugs, such as aspirin or ibuprofen, not only reduced fever and fever-related gene expression (Figure 3A), but also decreased Th17 cell differentiation in the recipient mice (Figure 3B). These data strongly suggest a T-cell intrinsic effect of fever in regulating *in vivo* Th17 cell differentiation.

Febrile temperature increases the pathogenicity of Th17 cells

To further understand the effect of febrile temperature, an RNA-sequencing assay was performed with Th17 cells generated at both 37°C and 39.5°C. Overall, 1083 genes were upregulated in Th17 cells generated at 39.5°C ($P < 0.01$, fold change ≥ 1.5), compared to those at 37°C (Figure 4A). Pathway analysis revealed that the top listed pathways included genes involved in cytokine-cytokine receptor interaction and Th17 cell differentiation, such as *Il17*, *Il17f*, *Il22* as well as *Il1r1*, *Il1r2* and *Il23r*, which are critical for Th17 cell differentiation or effect function (Stockinger and Omenetti, 2017) (Figures 4A). Moreover, Th1-related transcription factors *Tbx21* and *Stat4*, necessary for Th17-cell mediated autoimmune diseases (Bettelli et al., 2004; Chitnis et al., 2001), were also upregulated by febrile temperature (Figure 4A). In addition, differentiation at 39.5°C led to upregulation of transcription factors *Nr4a2* and *Nfatc1*, both of which directly

regulate IL-17 expression and are important in EAE induction (Doi et al., 2008; Reppert et al., 2015; Zhu et al., 2017), as well as *Cd24a*, a positive regulator for Th17 cells and related autoimmunity (Bai et al., 2000; Zhu et al., 2017) (Figure 4A).

Febrile temperature also repressed 392 genes in Th17 cells (Figure 4A), enriched mostly with biosynthetic and metabolic pathways, including in fatty acids, sugar and carbon backbone metabolism or biosynthesis, possibly due to heat-induced stress response. The most highly repressed genes included *Gpr83*, encoding a Treg cell surface marker involved in suppressive activity (Hansen et al., 2006; Sugimoto et al., 2006), and *Cd62l* (sell), a naïve T cell marker for T cell homing to peripheral lymphoid tissues (Wedepohl et al., 2012) (Figure 4A).

Th17 cells induced by IL-6 plus TGF- β 1 are relatively non-pathogenic, and those generated in the presence of IL-23, or IL-6 in combination with TGF- β 3 or IL-1 and IL-23 are more pathogenic (Ghoreschi et al., 2010; Lee et al., 2012). Among the 99 genes upregulated over 1.5 folds in pathogenic (TGF- β 3 + IL-6) *versus* non-pathogenic (TGF- β 1 + IL-6) Th17 cells (Lee et al., 2012), 30 of them were upregulated in Th17 cells induced at febrile temperatures (Figure 4B), including 22 genes also upregulated in Th17 cells induced by IL-1 β , IL-6 plus IL-23 (Lee et al., 2012), including *Ccl3*, *Cxcl3*, *Tnfsf11*, *Tbx21* and *Stat4* (Figure 4B). GSEA (gene set enrichment analysis) showed that Th17 cells cultured at febrile temperature were more similar to the ones induced by IL-6, IL-1 and IL-23 than those induced by IL-6 plus TGF- β 1 (Ghoreschi et al., 2010) (Figure 4C). In addition, they also exhibited gene expression patterns strongly correlated with those in pathogenic Th17 cells derived from inflamed CNS in EAE, but not with those from non-pathogenic, gut-associated Th17 cells (Gaublomme et al., 2015) (Figure 4C). These data together indicate that Th17 cells generated under febrile temperature exhibit strong correlation with pathogenic Th17 cells in the literatures, supporting that Th17 cells generated *in vivo* may be under the influence of febrile temperature in the draining lymph nodes.

To validate the above findings, an acute lung inflammation model was performed in CD45.1 mice by adoptive transfer of CD45.2 OT-II Th17 cells that were induced by OVA-peptide and antigen-presenting cells (APCs) *in vitro* at 37°C or 39.5°C. Following intranasal administration of OVA protein, as expected, Th17 cells generated with febrile temperature induced significantly increased neutrophil infiltration in both the lung tissue and bronchoalveolar lavage fluid (BALF) than those generated at 37°C (Figures 4D), supporting a highly pro-inflammatory feature of Th17 cells induced at febrile temperatures.

Febrile temperature promotes Th17 cell differentiation through enhancing SMAD4 SUMOylation and its nuclear localization

To understand the mechanism underneath febrile Th17 cell differentiation, we first focused on STAT3 and SMAD2 & SMAD3, critical downstream transcription mediators of IL-6 and TGF- β signaling, respectively. However, febrile temperature did not affect their phosphorylation activation status, and could still upregulate IL-17 expression in SMAD2-deficient T cells, though both IL-6 and TGF- β were indispensable for Th17 cell differentiation, suggesting alternative mechanism(s) involved.

A recent study reported that SMAD4-deficient T cells can differentiate into Th17 cells under IL-6-only culture condition (Zhang et al., 2017), we therefore tested if febrile temperature could further increase IL-6-induced yet SMAD4-independent Th17 cell program. Consistent with

previous findings (Hahn et al., 2011; Yang et al., 2008; Zhang et al., 2017), SMAD4-deficiency did not affect Th17 cell differentiation induced with complete Th17 polarizing cytokine cocktails (IL-6 + TGF- β 1 or IL-6 + IL-1 β + IL-23 + TGF- β 1) under normal 37°C culture condition, but resulted in increased Th17 cell differentiation when cultured with cytokine cocktails containing IL-6 but lacking TGF- β 1 signaling (IL-6 plus anti-TGF- β 1 or IL-6 + IL-1 β + IL-23 + anti-TGF- β 1), in which anti-TGF- β 1 was used to neutralize endogenous TGF- β 1 in the culture (Figure 4A). Under 39.5°C, *Smad4* ^{Δ CD4} T cells failed in upregulating IL-17 expression under both IL-6 only and complete Th17-polarizing culture conditions (Figure 5A), suggesting a necessary positive role of SMAD4 in controlling Th17 cell differentiation at febrile temperatures.

The nuclear localization and transcription activity of SMAD4 is regulated by SUMOylation at its K113 and K159 residues (Lin et al., 2003), and an important consequence of heat shock response is the rapid increase of cellular amounts of protein SUMOylations (Gareau and Lima, 2010). These prompted us to speculate a role of SUMOylation pathway in Th17 cell differentiation at febrile temperatures. We therefore collected T cells activated and cultured under Th17 polarizing conditions for 24 hours at 37°C or 39.5°C, and then analyzed cellular proteins conjugated to SUMO2, a key SUMO moiety in the SUMOylation pathway. As expected, febrile temperature increased total cellular amounts of SUMOylated proteins in Th17 cells, as determined by increase in SUMO2-containing high molecular weight proteins (Figure 5B). Consistently, the amount of SUMOylated SMAD4 was also increased at 24 hours (Figure 5B), which was detected as early as 4 hours post febrile temperature treatment, indicating a direct role of heat-induced stress response in promoting SMAD4 SUMOylation. To further confirm this, T cells were polarized under Th17 cell culture condition (IL-6 + TGF- β 1) in the presence of HSP70 and HSP90 inhibitors. These inhibitors reduced global cellular amounts of SUMOylated proteins, including SUMOylated SMAD4 (Figure 5C), and also abrogated increased IL-17 expression at 39.5°C (Figure 2C), suggesting a functional role of heat shock response in regulating SMAD4 SUMOylation and Th17 cell differentiation.

To confirm the functional of SUMOylation in Th17 cell differentiation, UBC9, the only E2 conjugating enzyme in the SUMOylation pathway (Gareau and Lima, 2010), was selectively ablated in activated T cells using the CreERT2-mediated inducible deletion strategy. As expected, UBC9 deficiency completely abolished the effect by febrile temperature on Th17 cell differentiation (Figure 5D), suggesting an essential role of SUMOylation for febrile Th17 cell differentiation. It is noticed that UBC9 deficiency also reduced IL-17 expression at 37°C, suggesting a role for SUMOylation in Th17 cells at normal physiological temperature, likely in a SMAD4-independent manner (Figure 5D).

To investigate whether SMAD4 SUMOylation indeed regulates Th17 cell differentiation at febrile temperatures, we first examined the subcellular localization of SMAD4 by immunofluorescence microscopy. Not surprisingly, SMAD4 was found mostly localized in the nuclei of Th17 cells 24 hours post culture at 39.5°C, but barely at 37°C (Figure 6A). This phenomenon was dependent on TGF- β since IL-6 alone could not cause SMAD4 nuclear localization, whereas TGF- β alone culture condition was sufficient to induce SMAD4 nuclear localization at elevated temperature (Figure 6A). In addition, when we overexpressed wild-type (WT) SMAD4 or the SMAD4- K113R/K159R double mutant in *Smad4*^{-/-} T cells (infected cells carry GFP reporter signal derived from the retroviral plasmid). WT but not the mutant *Smad4* restored T cell responsiveness to febrile temperature in IL-17 expression in *Smad4*^{-/-} T cells (Figure 6B). As expected, the mutant SMAD4 protein did not respond to the febrile temperature in

their nuclear localization (Figure 6C).

SMAD4 is indispensable for febrile temperature-mediated Th17 cell differentiation *in vivo* and associated autoimmunity

To further investigate whether Smad4 is involved in regulating fever-dependent Th17 cell differentiation *in vivo*, we mixed naïve CD45.1⁺CD45.2⁺ *Smad4*^{fl/+} (WT) T cells and CD45.2⁺ *Smad4*^{fl/fl} *Cd4*^{Cre} (*Smad4*^{-/-}) T cells both carrying the MOG-specific 2D2 TcR transgene at 1:1 ratio and transferred them into *Tcrbd*^{-/-} mice, followed by MOG+CFA immunization. SMAD4 deficiency did not alter the ratio of T cells in recipient mice, but significantly reduced the expression of IL-17A but not IFN- γ (Figure 7A). Importantly, treatment with antipyretic drug significantly reduced IL-17A expression in WT 2d2⁺ T cells, down to amount similar to *Smad4*^{-/-} 2d2⁺ T cells, but barely affected IL-17A expression in *Smad4*^{-/-} 2d2⁺ T cells (Figure 7A). These data together thus demonstrated an indispensable role of *Smad4* in regulating Th17 response *in vivo*.

To validate the above results, we conducted an active EAE model in *Smad4*^{fl/fl} (WT) and *Smad4*^{fl/fl} $\times$ *Cd4*^{Cre} (*Smad4* ^{Δ CD4}) mice. *Smad4* ^{Δ CD4} mice showed delayed disease onset and greatly reduced disease scores (Figure 7B). IL-17⁺ T cells were reduced in CNS in these mice when compared with WT mice, whereas the IFN- γ ⁺ and FOXP3⁺ T cells were comparable between two groups of mice (Figures 7C). To further examine if SMAD4 regulation of EAE requires its SUMOylation, we infected *Smad4*^{-/-} 2d2⁺ T cells with retroviruses containing either wild type or the K113R/159R mutant *Smad4* under neutral culture condition (anti-IL-4 + anti-IFN- γ), and the infected T cells (GFP⁺ cells) were sorted and introduced into *Rag1*^{-/-} mice, followed by MOG immunization for induction of EAE diseases. Consistent with EAE model performed with WT and *Smad4* ^{Δ CD4} mice, mice receiving the *Smad4*-K113R/159R-transduced 2d2⁺ T cells developed less severe diseases (Figure 7D), with reduced Th17 cells in the central nervous system, compared with those receiving WT *Smad4*-transduced T cells (Figure 7E).

SMAD4 orchestrates febrile temperature associated gene expression at genome-wide level

To examine the SMAD4-downstream regulated genes, RNAseq assays were performed with WT and *Smad4*^{-/-} Th17 cells induced at both 37°C and 39.5°C. DEG2 analysis showed over 5000 genes were differentially expressed ($P < 0.01$, fold change ≥ 1), clustered into 4 groups (Figure 8A). Group 1 and Group 3 represent genes most highly expressed or repressed at 39.5°C, respectively, dependent on *Smad4* (Figure 8A). In contrast, Group 2 and 4 represent genes most highly repressed or expressed at 37°C, respectively, also regulated by *Smad4* (Figure 8A). We then focused on the genes with ≥ 1.5 -fold difference between 37°C and 39.5°C. Among the 1083 genes induced at 39.5°C, 405 of them were dependent on *Smad4* (Figure 8B). Among the 392 genes repressed at 39.5°C, 127 were regulated by *Smad4* (Figure 8B). KEGG analysis showed that Th17 cell differentiation and cytokine-cytokine receptor interaction pathways are among the top listed pathways upregulated by febrile temperature, which include 42 genes and 30 of them showed a strong *Smad4* dependence at 39.5°C, but were largely unaffected by *Smad4* at 37°C, including genes critical for the differentiation and effect function of Th17 cells, such as *Il17a*, *Il17f*, *Il21*, *Il1r1*, *Il23r*, *Tbx21*, *Nfatc1*, etc (Figure 8C). These data further confirm the necessary role of

Smad4 in regulating the pathogenicity of febrile Th17 cells.

Febrile temperature promotes the differentiation of Tc17 cells

Tc17 cells represent a CD8⁺ T cell subset that shared many similar features with Th17 cells. In this study, we isolated naïve CD8⁺ T cells and cultures them under Th17-polarizing condition (IL-6 + TGF-β) for 4 days at 37°C or 39°C. These cells were then restimulated for intracellular staining for cytokine expression. Same as Th17 cells, febrile temperature could also promote Tc17 differentiation as showed by increased IL-17 expression (Figure 9).

In addition, terms such as "first" and "second" are used herein for purposes of description and are not intended to indicate or imply relative importance or significance.

Reference throughout this specification to "an embodiment," "some embodiments," "one embodiment," "another example," "an example," "a specific examples," or "some examples," means that a particular feature, structure, material, or characteristic described in connection with the embodiment or example is included in at least one embodiment or example of the present disclosure. Thus, the appearances of the phrases such as "in some embodiments," "in one embodiment," "in an embodiment," "in another example," "in an example," "in a specific examples," or "in some examples," in various places throughout this specification are not necessarily referring to the same embodiment or example of the present disclosure. Furthermore, the particular features, structures, materials, or characteristics may be combined in any suitable manner in one or more embodiments or examples.

Although explanatory embodiments have been shown and described, it would be appreciated by those skilled in the art that the above embodiments cannot be construed to limit the present disclosure, and changes, alternatives, and modifications can be made in the embodiments without departing from spirit, principles and scope of the present disclosure.

References

- Bai, X.F., Liu, J.Q., Liu, X., Guo, Y., Cox, K., Wen, J., Zheng, P., and Liu, Y. (2000). The heat-stable antigen determines pathogenicity of self-reactive T cells in experimental autoimmune encephalomyelitis. *The Journal of clinical investigation* 105, 1227-1232.
- Bettelli, E., Sullivan, B., Szabo, S.J., Sobel, R.A., Glimcher, L.H., and Kuchroo, V.K. (2004). Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis. *The Journal of experimental medicine* 200, 79-87.
- Chang, S.H. (2019). T helper 17 (Th17) cells and interleukin-17 (IL-17) in cancer. *Archives of pharmacal research* 42, 549-559.
- Chitnis, T., Najafian, N., Benou, C., Salama, A.D., Grusby, M.J., Sayegh, M.H., and Khoury, S.J. (2001). Effect of targeted disruption of STAT4 and STAT6 on the induction of experimental autoimmune encephalomyelitis. *The Journal of clinical investigation* 108, 739-747.
- Chu, G.C., Dunn, N.R., Anderson, D.C., Oxburgh, L., and Robertson, E.J. (2004). Differential requirements for Smad4 in TGFbeta-dependent patterning of the early mouse embryo. *Development* 131, 3501-3512.

- Cornely, O.A., and Mellinghoff, S.C. (2017). [Fever in Cancer Patients]. *Deutsche medizinische Wochenschrift* 142, 961-968.
- Demarque, M.D., Nacerddine, K., Neyret-Kahn, H., Andrieux, A., Danenberg, E., Jouvion, G., Bomme, P., Hamard, G., Romagnolo, B., Terris, B., *et al.* (2011). Sumoylation by Ubc9 regulates the stem cell compartment and structure and function of the intestinal epithelium in mice. *Gastroenterology* 140, 286-296.
- Doi, Y., Oki, S., Ozawa, T., Hohjoh, H., Miyake, S., and Yamamura, T. (2008). Orphan nuclear receptor NR4A2 expressed in T cells from multiple sclerosis mediates production of inflammatory cytokines. *Proceedings of the National Academy of Sciences of the United States of America* 105, 8381-8386.
- Eming, S.A., Wynn, T.A., and Martin, P. (2017). Inflammation and metabolism in tissue repair and regeneration. *Science* 356, 1026-1030.
- Esplugues, E., Huber, S., Gagliani, N., Hauser, A.E., Town, T., Wan, Y.Y., O'Connor, W., Jr., Rongvaux, A., Van Rooijen, N., Haberman, A.M., *et al.* (2011). Control of TH17 cells occurs in the small intestine. *Nature* 475, 514-518.
- Evans, S.S., Repasky, E.A., and Fisher, D.T. (2015). Fever and the thermal regulation of immunity: the immune system feels the heat. *Nature reviews. Immunology* 15, 335-349.
- Fasching, P., Stradner, M., Graninger, W., Dejaco, C., and Fessler, J. (2017). Therapeutic Potential of Targeting the Th17/Treg Axis in Autoimmune Disorders. *Molecules* 22.
- Gareau, J.R., and Lima, C.D. (2010). The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition. *Nature reviews. Molecular cell biology* 11, 861-871.
- Gaublomme, J.T., Yosef, N., Lee, Y., Gertner, R.S., Yang, L.V., Wu, C., Pandolfi, P.P., Mak, T., Satija, R., Shalek, A.K., *et al.* (2015). Single-Cell Genomics Unveils Critical Regulators of Th17 Cell Pathogenicity. *Cell* 163, 1400-1412.
- Ghoreschi, K., Laurence, A., Yang, X.P., Tato, C.M., McGeachy, M.J., Konkel, J.E., Ramos, H.L., Wei, L., Davidson, T.S., Bouladoux, N., *et al.* (2010). Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling. *Nature* 467, 967-971.
- Hahn, J.N., Falck, V.G., and Jirik, F.R. (2011). Smad4 deficiency in T cells leads to the Th17-associated development of premalignant gastroduodenal lesions in mice. *The Journal of clinical investigation* 121, 4030-4042.
- Hansen, W., Loser, K., Westendorf, A.M., Bruder, D., Pfoertner, S., Siewert, C., Huehn, J., Beissert, S., and Buer, J. (2006). G protein-coupled receptor 83 overexpression in naive CD4+CD25- T cells leads to the induction of Foxp3+ regulatory T cells in vivo. *Journal of immunology* 177, 209-215.
- Hasday, J.D., Thompson, C., and Singh, I.S. (2014). Fever, immunity, and molecular adaptations. *Comprehensive Physiology* 4, 109-148.
- Jiang, Y., Liu, Y., Lu, H., Sun, S.C., Jin, W., Wang, X., and Dong, C. (2018). Epigenetic activation during T helper 17 cell differentiation is mediated by Tripartite motif containing 28. *Nature communications* 9, 1424.
- Kleinewietfeld, M., Manzel, A., Titze, J., Kvakana, H., Yosef, N., Linker, R.A., Muller, D.N., and Hafler, D.A. (2013). Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells. *Nature* 496, 518-522.
- Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat Methods* 9, 357-359.
- Lee, P.P., Fitzpatrick, D.R., Beard, C., Jessup, H.K., Lehar, S., Makar, K.W., Perez-Melgosa, M., Sweetser, M.T., Schlissel, M.S., Nguyen, S., *et al.* (2001). A critical role for Dnmt1 and DNA

- methylation in T cell development, function, and survival. *Immunity* 15, 763-774.
- Lee, Y., Awasthi, A., Yosef, N., Quintana, F.J., Xiao, S., Peters, A., Wu, C., Kleinewietfeld, M., Kunder, S., Hafler, D.A., *et al.* (2012). Induction and molecular signature of pathogenic TH17 cells. *Nature immunology* 13, 991-999.
- Li, P., Zheng, Y., and Chen, X. (2017). Drugs for Autoimmune Inflammatory Diseases: From Small Molecule Compounds to Anti-TNF Biologics. *Frontiers in pharmacology* 8, 460.
- Liang, Y., Pan, H.F., and Ye, D.Q. (2015). Tc17 Cells in Immunity and Systemic Autoimmunity. *International reviews of immunology* 34, 318-331.
- Limper, M., de Kruif, M.D., Duits, A.J., Brandjes, D.P., and van Gorp, E.C. (2010). The diagnostic role of procalcitonin and other biomarkers in discriminating infectious from non-infectious fever. *The Journal of infection* 60, 409-416.
- Lin, C., Zhang, Y., Zhang, K., Zheng, Y., Lu, L., Chang, H., Yang, H., Yang, Y., Wan, Y., Wang, S., *et al.* (2019). Fever Promotes T Lymphocyte Trafficking via a Thermal Sensory Pathway Involving Heat Shock Protein 90 and alpha4 Integrins. *Immunity* 50, 137-151 e136.
- Lin, X., Liang, M., Liang, Y.Y., Brunicardi, F.C., and Feng, X.H. (2003). SUMO-1/Ubc9 promotes nuclear accumulation and metabolic stability of tumor suppressor Smad4. *J Biol Chem* 278, 31043-31048.
- Malhotra, N., Robertson, E., and Kang, J. (2010). SMAD2 is essential for TGF beta-mediated Th17 cell generation. *The Journal of biological chemistry* 285, 29044-29048.
- Martinez, G.J., Zhang, Z., Chung, Y., Reynolds, J.M., Lin, X., Jetten, A.M., Feng, X.H., and Dong, C. (2009). Smad3 differentially regulates the induction of regulatory and inflammatory T cell differentiation. *The Journal of biological chemistry* 284, 35283-35286.
- Martinez, G.J., Zhang, Z., Reynolds, J.M., Tanaka, S., Chung, Y., Liu, T., Robertson, E., Lin, X., Feng, X.H., and Dong, C. (2010). Smad2 positively regulates the generation of Th17 cells. *The Journal of biological chemistry* 285, 29039-29043.
- Mondal, S., Jana, M., Dasarathi, S., Roy, A., and Pahan, K. (2018). Aspirin ameliorates experimental autoimmune encephalomyelitis through interleukin-11-mediated protection of regulatory T cells. *Science signaling* 11.
- Ni, J., Shu, Y.Y., Zhu, Y.N., Fu, Y.F., Tang, W., Zhong, X.G., Wang, H., Yang, Y.F., Ren, J., Wang, M.W., and Zuo, J.P. (2007). COX-2 inhibitors ameliorate experimental autoimmune encephalomyelitis through modulating IFN-gamma and IL-10 production by inhibiting T-bet expression. *Journal of neuroimmunology* 186, 94-103.
- Pasikhova, Y., Ludlow, S., and Baluch, A. (2017). Fever in Patients With Cancer. *Cancer control : journal of the Moffitt Cancer Center* 24, 193-197.
- Qian, X., Chen, H., Wu, X., Hu, L., Huang, Q., and Jin, Y. (2017). Interleukin-17 acts as double-edged sword in anti-tumor immunity and tumorigenesis. *Cytokine* 89, 34-44.
- Reppert, S., Zinser, E., Holzinger, C., Sandrock, L., Koch, S., and Finotto, S. (2015). NFATc1 deficiency in T cells protects mice from experimental autoimmune encephalomyelitis. *European journal of immunology* 45, 1426-1440.
- Shang, J., Yan, L., Du, L., Liang, L., Zhou, Q., Liang, T., Bai, L., and Tang, H. (2017). Recent trends in the distribution of causative diseases of fever of unknown origin. *Wiener klinische Wochenschrift* 129, 201-207.
- Singh, I.S., and Hasday, J.D. (2013). Fever, hyperthermia and the heat shock response. *Int J Hyperthermia* 29, 423-435.
- Stockinger, B., and Omenetti, S. (2017). The dichotomous nature of T helper 17 cells. *Nature reviews. Immunology* 17, 535-544.

- Sugimoto, N., Oida, T., Hirota, K., Nakamura, K., Nomura, T., Uchiyama, T., and Sakaguchi, S. (2006). Foxp3-dependent and -independent molecules specific for CD25⁺CD4⁺ natural regulatory T cells revealed by DNA microarray analysis. *International immunology* 18, 1197-1209.
- Veldhoen, M. (2017). Interleukin 17 is a chief orchestrator of immunity. *Nature immunology* 18, 612-621.
- Wang, L., Feng, Z., Wang, X., Wang, X., and Zhang, X. (2010). DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. *Bioinformatics* 26, 136-138.
- Wang, X., Zhang, Y., Yang, X.O., Nurieva, R.I., Chang, S.H., Ojeda, S.S., Kang, H.S., Schluns, K.S., Gui, J., Jetten, A.M., and Dong, C. (2012). Transcription of Il17 and Il17f is controlled by conserved noncoding sequence 2. *Immunity* 36, 23-31.
- Wedepohl, S., Beceren-Braun, F., Riese, S., Buscher, K., Enders, S., Bernhard, G., Kilian, K., Blanchard, V., Dernedde, J., and Tauber, R. (2012). L-selectin--a dynamic regulator of leukocyte migration. *European journal of cell biology* 91, 257-264.
- Wu, C., Yosef, N., Thalhamer, T., Zhu, C., Xiao, S., Kishi, Y., Regev, A., and Kuchroo, V.K. (2013). Induction of pathogenic TH17 cells by inducible salt-sensing kinase SGK1. *Nature* 496, 513-517.
- Yang, X.O., Nurieva, R., Martinez, G.J., Kang, H.S., Chung, Y., Pappu, B.P., Shah, B., Chang, S.H., Schluns, K.S., Watowich, S.S., *et al.* (2008). Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity* 29, 44-56.
- Yang, X.O., Panopoulos, A.D., Nurieva, R., Chang, S.H., Wang, D., Watowich, S.S., and Dong, C. (2007). STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *The Journal of biological chemistry* 282, 9358-9363.
- Yu, G., Wang, L.G., Han, Y., and He, Q.Y. (2012). clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* 16, 284-287.
- Yu, G., Wang, L.G., and He, Q.Y. (2015). ChIPseeker: an R/Bioconductor package for ChIP peak annotation, comparison and visualization. *Bioinformatics* 31, 2382-2383.
- Zhang, S. (2018). The role of transforming growth factor beta in T helper 17 differentiation. *Immunology* 155, 24-35.
- Zhang, S., Takaku, M., Zou, L., Gu, A.D., Chou, W.C., Zhang, G., Wu, B., Kong, Q., Thomas, S.Y., Serody, J.S., *et al.* (2017). Reversing SKI-SMAD4-mediated suppression is essential for TH17 cell differentiation. *Nature* 551, 105-109.
- Zhang Y, L.T., Meyer C A, *et al.* (2008). Model-based Analysis of ChIP-Seq (MACS). *Genome biology* 9, R173.
- Zhu, Q., Wu, X., and Wang, X. (2017). Differential distribution of tumor-associated macrophages and Treg/Th17 cells in the progression of malignant and benign epithelial ovarian tumors. *Oncology letters* 13, 159-166.

Claims

1. A method of inhibiting the differentiation or maturation of an IL-17 producing cell or IL-17 producing, comprising:

- 1) using a chemical or biological inhibitor to suppress the activity of SMAD4 protein;
- 2) using a chemical or biological reagent to reduce the amount of SMAD4 protein .

2. A method of inhibiting the differentiation or maturation of an IL-17 producing cell or IL-17 producing, comprising:

- 1) using an anti-pyretic drug to reduce the amount of SUMOylated SMAD4 protein;
- 2) using a HSP70 inhibitor to reduce the amount of SUMOylated SMAD4;
- 3) using a HSP90 inhibitor to reduce the amount of SUMOylated SMAD4 chemical or biological reagent to reduce the amount of SMAD4 protein;
- 4) using a chemical or biological reagent to inhibit the activity of SUMOylated SMAD4 protein; or
- 5) using a chemical or biological reagent to reduce the amount of SUMOylated SMAD4 protein.

3. A method of promoting the differentiation or maturation of an IL-17 producing cell, comprising:

- 1) using a chemical or biological reagent to enhance the activity of SMAD4 protein;
- 2) using a chemical or biological reagent to increase the amount of SMAD4 protein.

4. A method of promoting the differentiation or maturation of an IL-17 producing cell, comprising:

using a chemical or biological reagent to increase the amount of SUMOylated SMAD4 protein.

5. Method of any one claim of 1~4, wherein IL-17 producing cells include at least Th17, Tc17, $\gamma\delta$ T cells, ILC3 cells, NK and NK T cells.

6. A method of treating a patient with IL-17 related autoimmune diseases, comprising:

administering an anti-pyretic agent to the patient in need of such treatment;

administering an inhibitor for SMAD4;

administering an inhibitor for SUMOylated SMAD;

administering an inhibitor for HSP70;

administering an inhibitor for HSP90; or

administering an inhibitor for UBC9.

7. The method of claim 6, wherein IL-17 related autoimmune diseases comprising at least one of the following: psoriasis, psoriasis-like arthritis, ankylosing spondylitis, rheumatoid arthritis, adult still's disease and multiple sclerosis.

8. A method of treating a patient with IL-17 related cancers in which IL-17 plays an

pro-tumor activity, comprising:

- administering an anti-pyretic agent to the patient in need of such treatment;
- administering an inhibitor for SMAD4;
- administering an inhibitor or for SUMOylated SMAD;
- administering an inhibitor for HSP70;
- administering an inhibitor for HSP90; or
- administering an inhibitor for UBC9.

9. A method of treating a patient with IL-17 related cancers in which IL-17 has an anti-tumor activity, comprising:

- administering a pro-pyretic agent to the patient in need of such treatment;
- administering an activator for SMAD4;
- administering an activator for SUMOylated SMAD4;
- administering an activator for HSP70;
- administering an activator for HSP90; or
- administering an activator for UBC9.

10. Method of claim 8 or 9, wherein the IL-17 related cancers comprising at least one of the following: melanoma, ovarian cancer, colorectal cancer, lung cancer, ovarian cancer, lung cancer, gastric cancer.

11. Method of claim 8, wherein the anti-pyretic agent comprising at least one of the following: aspirin and related salicylates, ibuprofen, ketoprofen, metamizole, nabumetone, acetaminophen, phenazone and docosanol.

12. A method of screening a medicament suitable to be used in a treatment or prevention of IL-17 related autoimmune diseases, comprising:

- contacting a candidate agent with an IL-17 producing cell,
- determining SMAD4 SUMOylation level in the IL-17 producing cell before and after the contacting;
- the decreased SMAD4 SUMOylation in the IL-17 producing cell after the contacting is an indication that the candidate agent is the medicament.

13. A method of screening a medicament suitable to be used in enhancing anti-tumor immunity, comprising:

- contacting a candidate agent with an IL-17 producing cell,
- determining SMAD4 SUMOylation level in the IL-17 producing cell before and after the contacting;
- the increased SMAD4 SUMOylation in the IL-17 producing cell after the contacting is an indication that the candidate agent is the medicament.

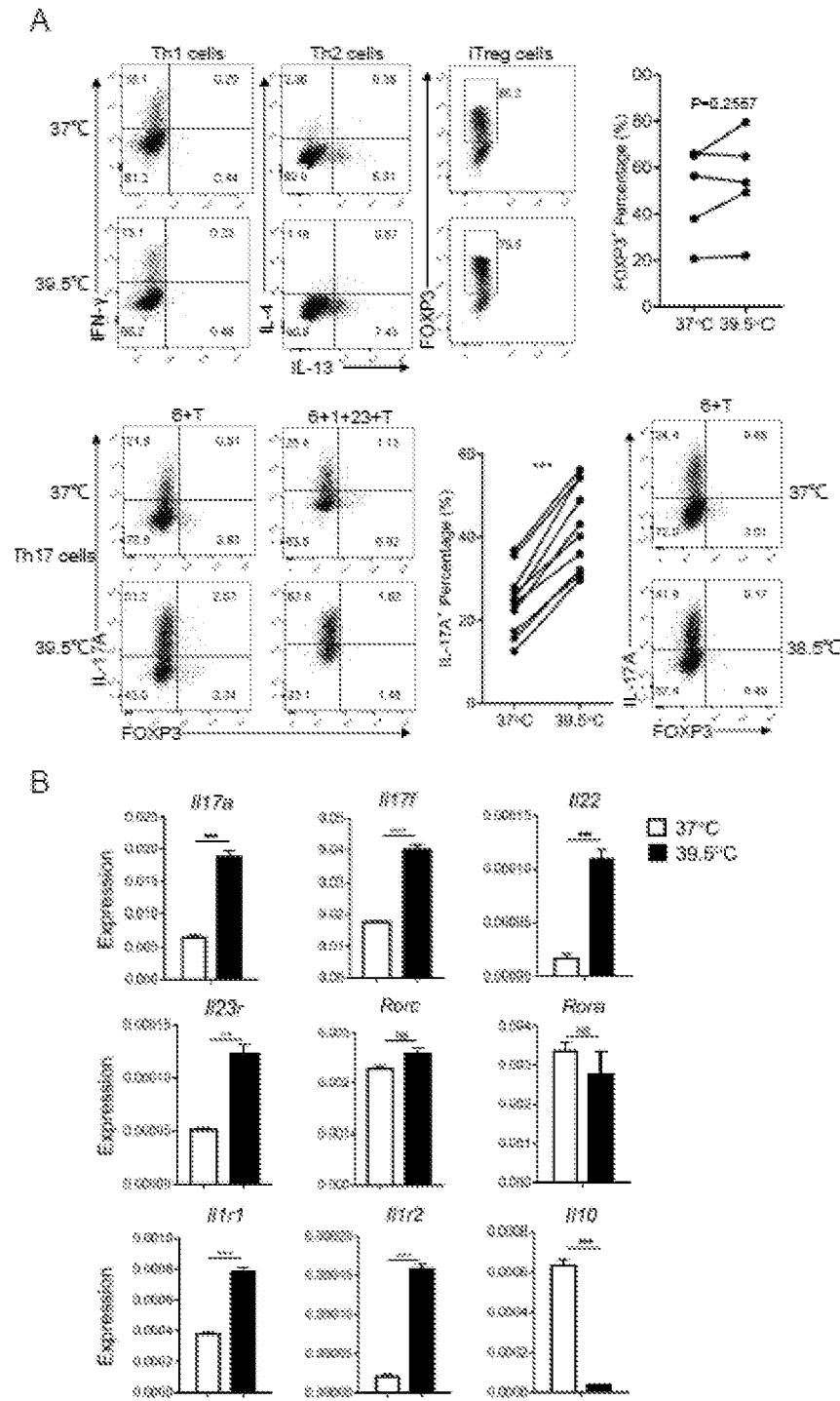


Figure 1

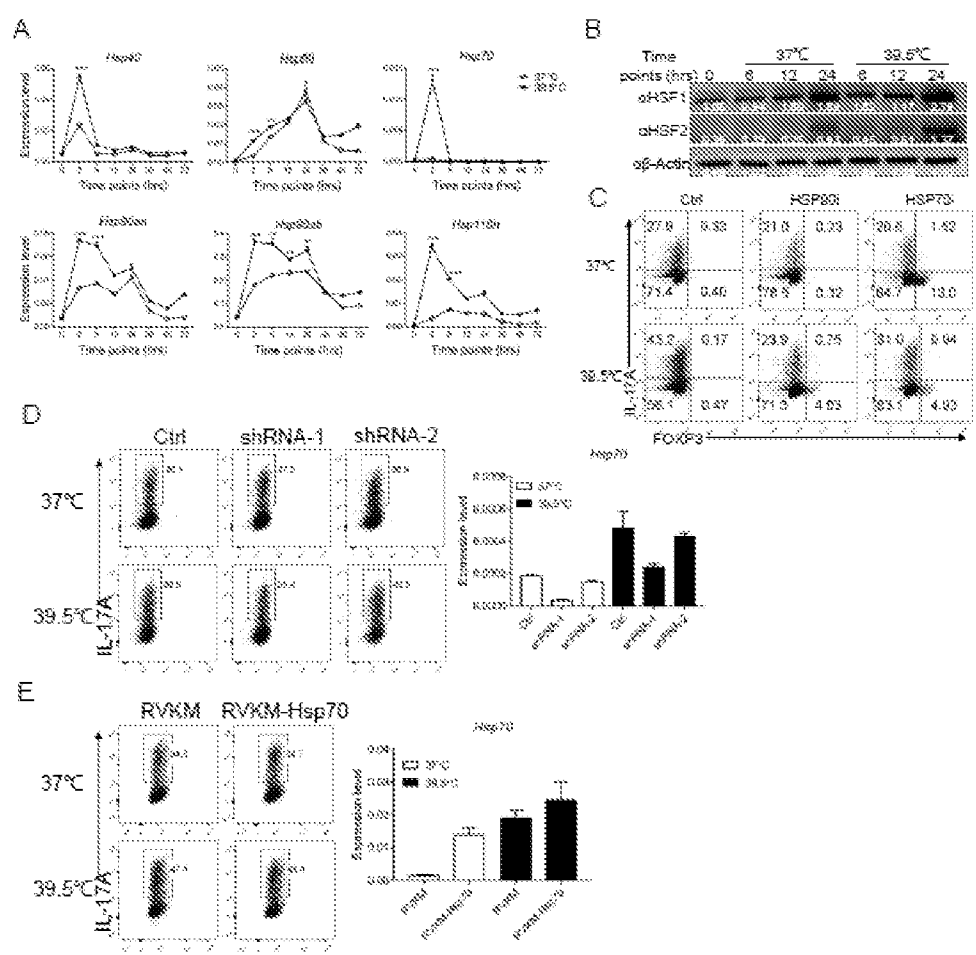


Figure 2

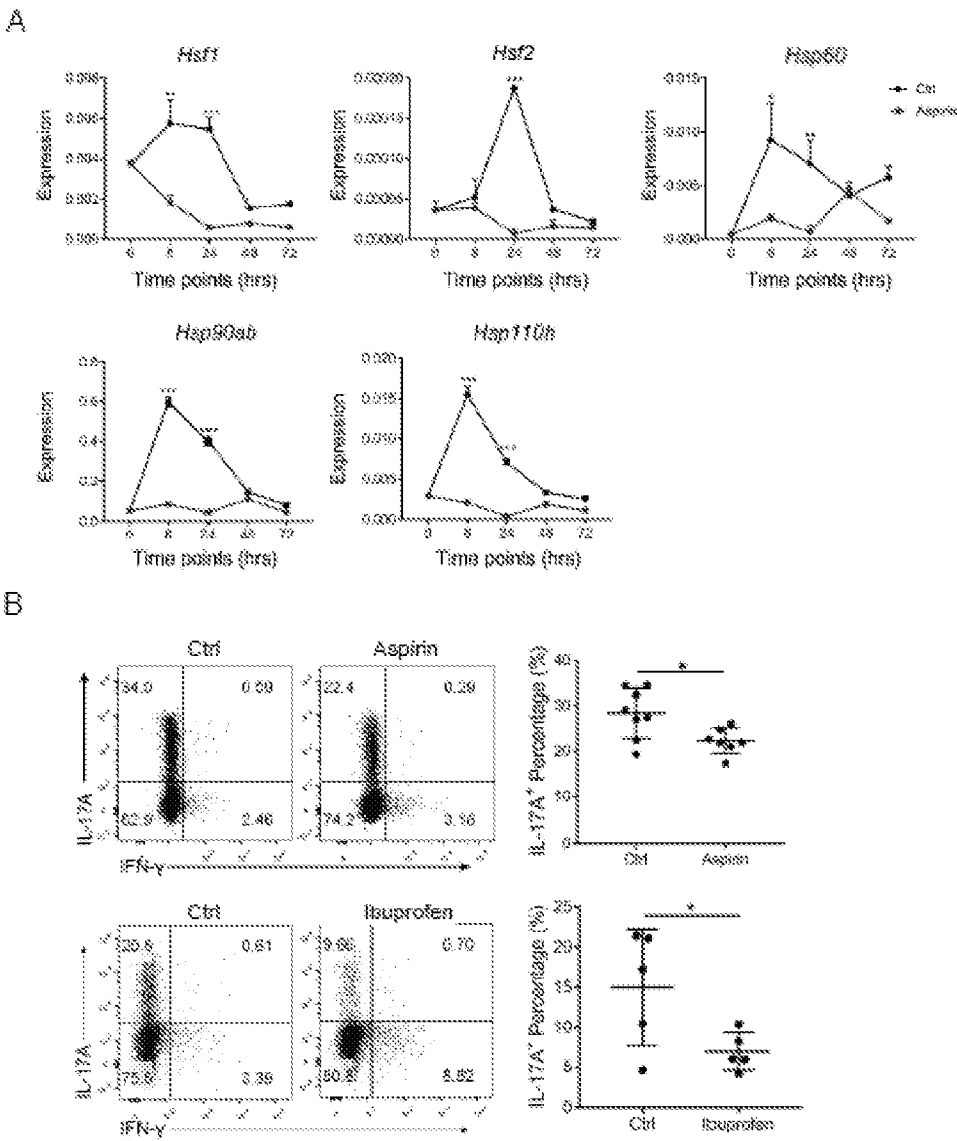


Figure 3

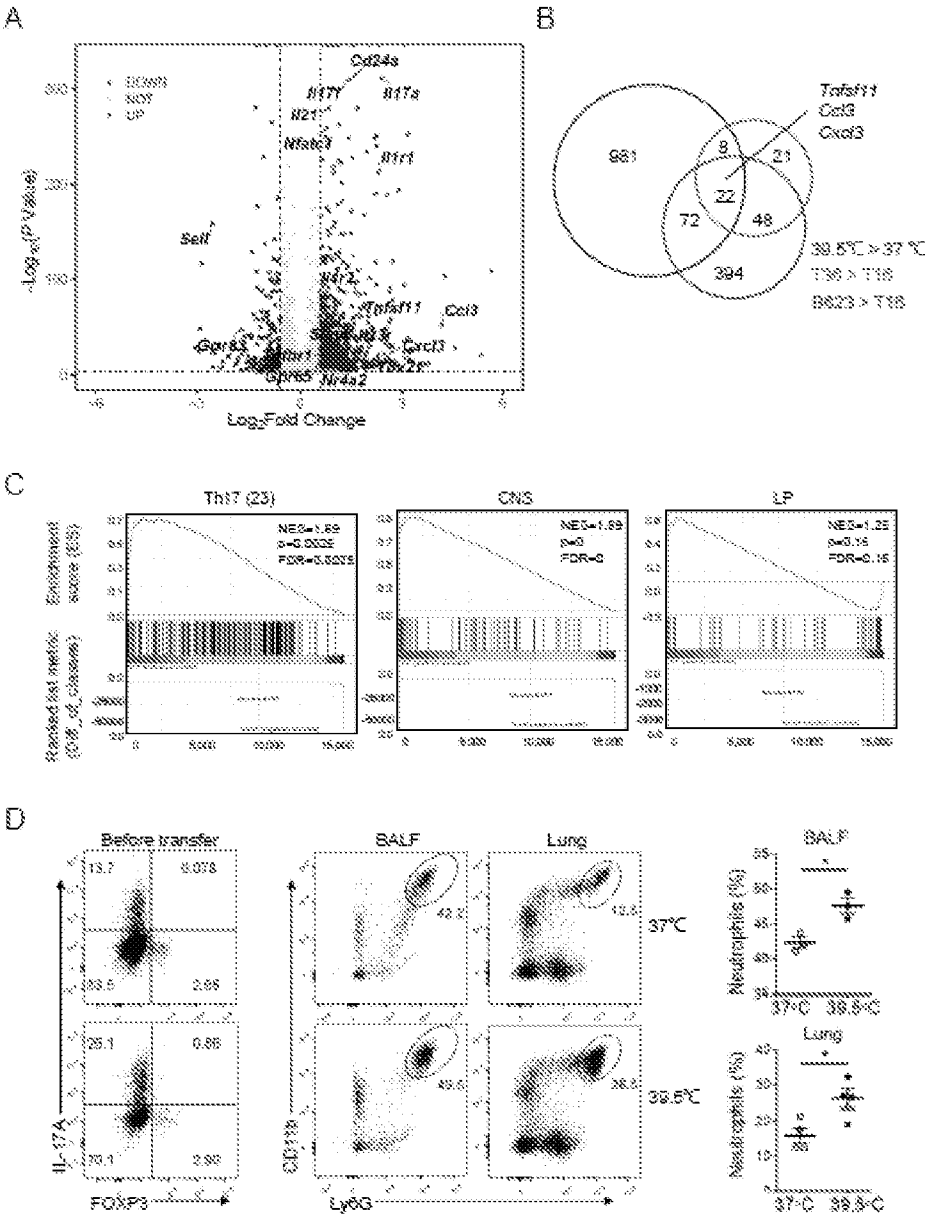


Figure 4

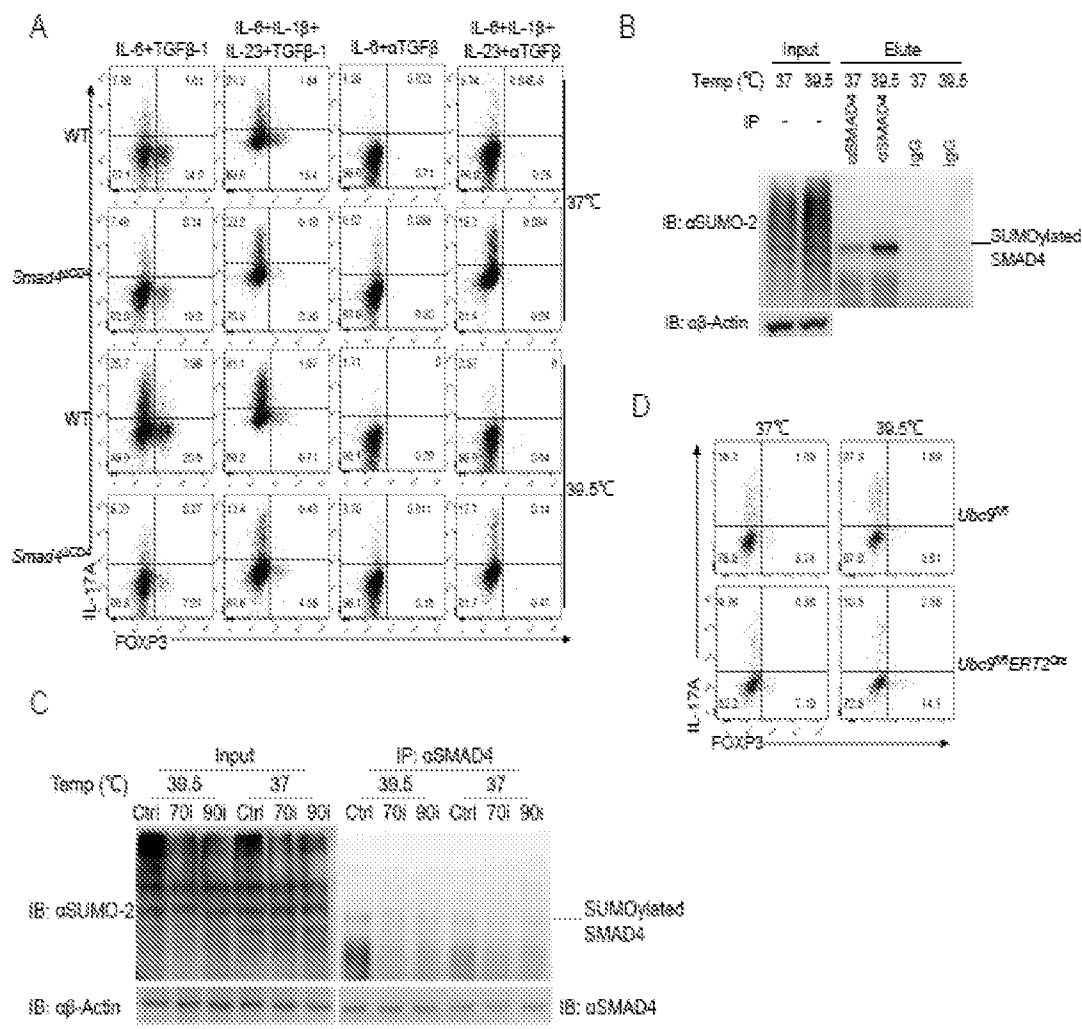


Figure 5

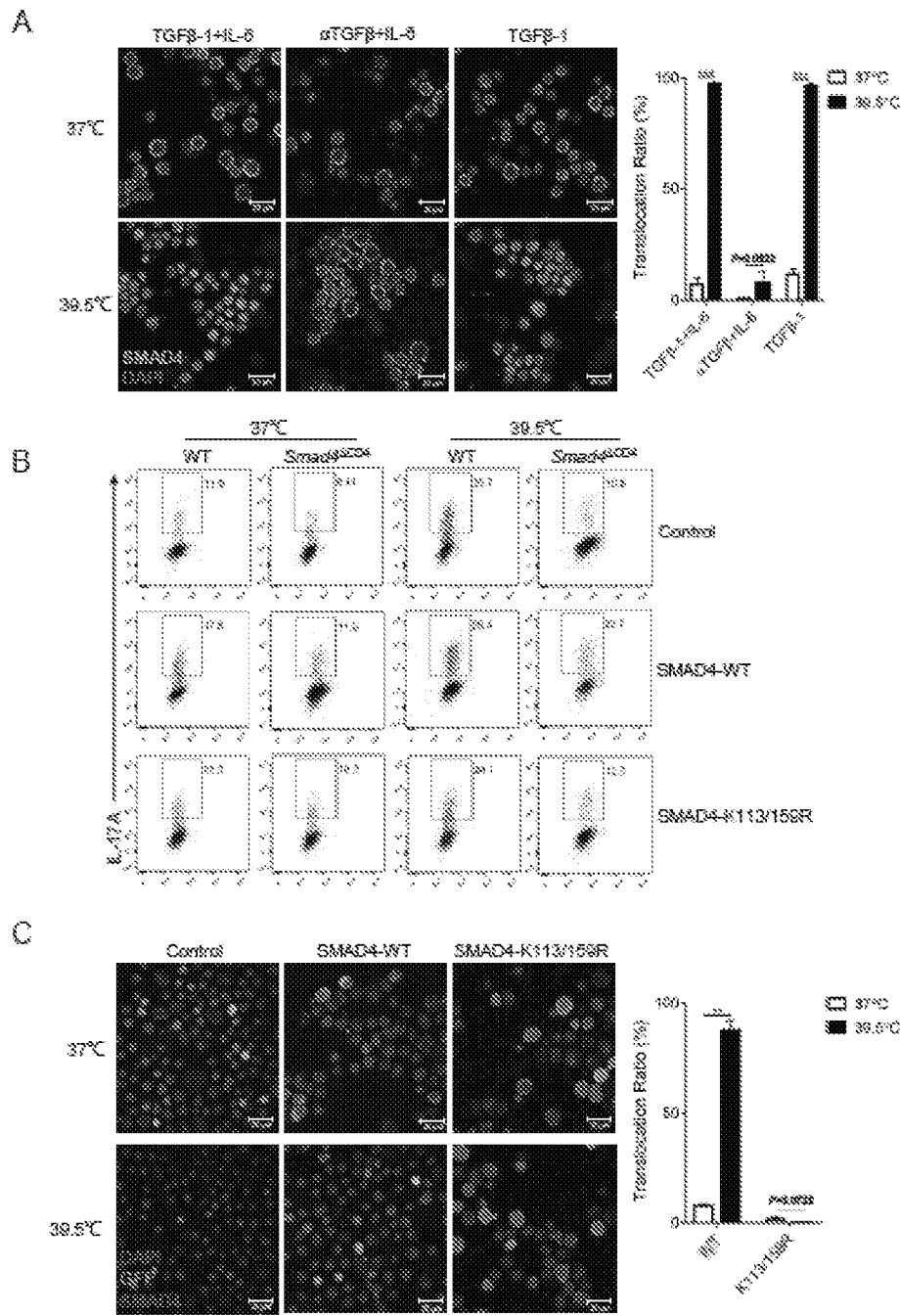


Figure 6

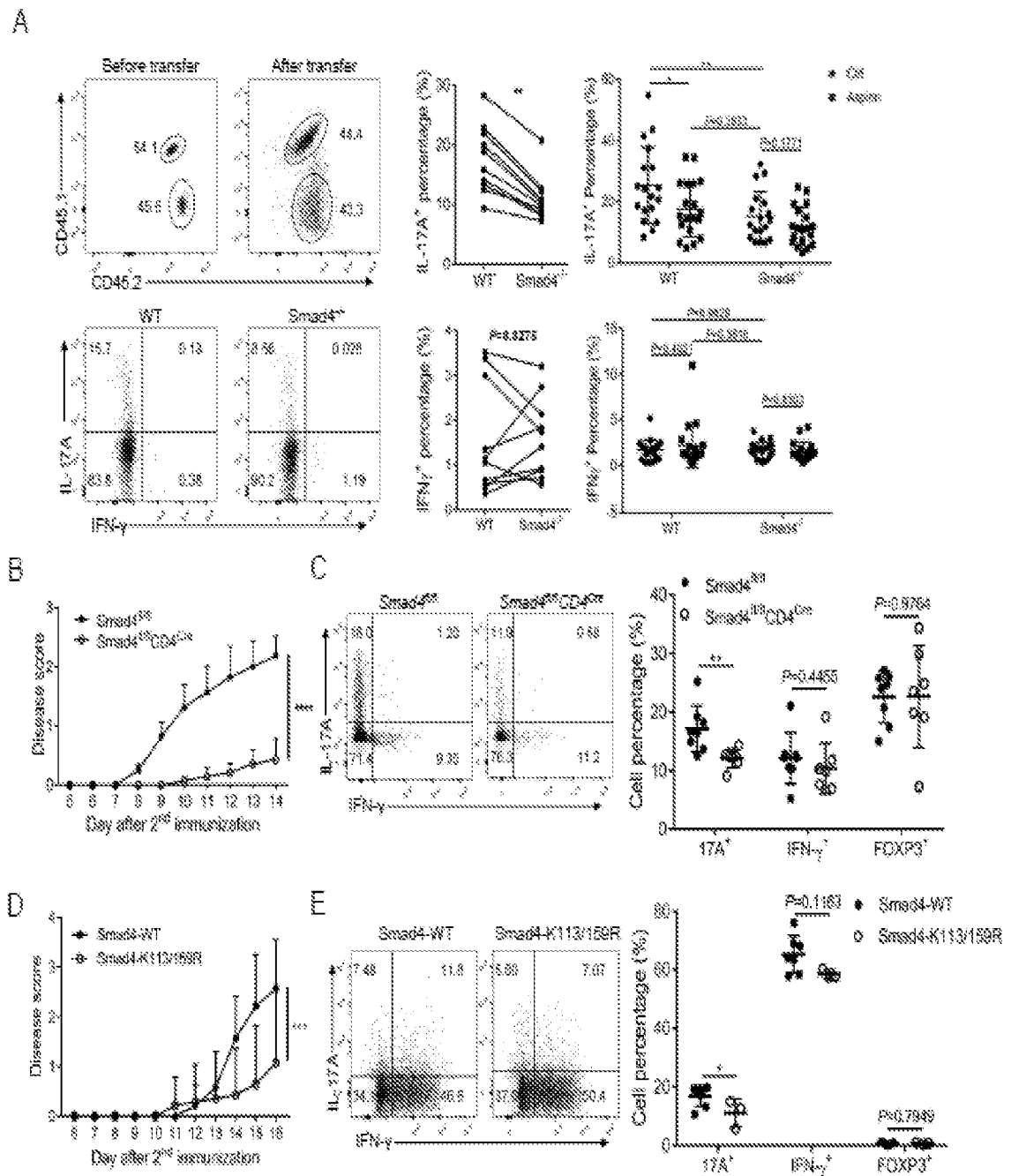


Figure 7

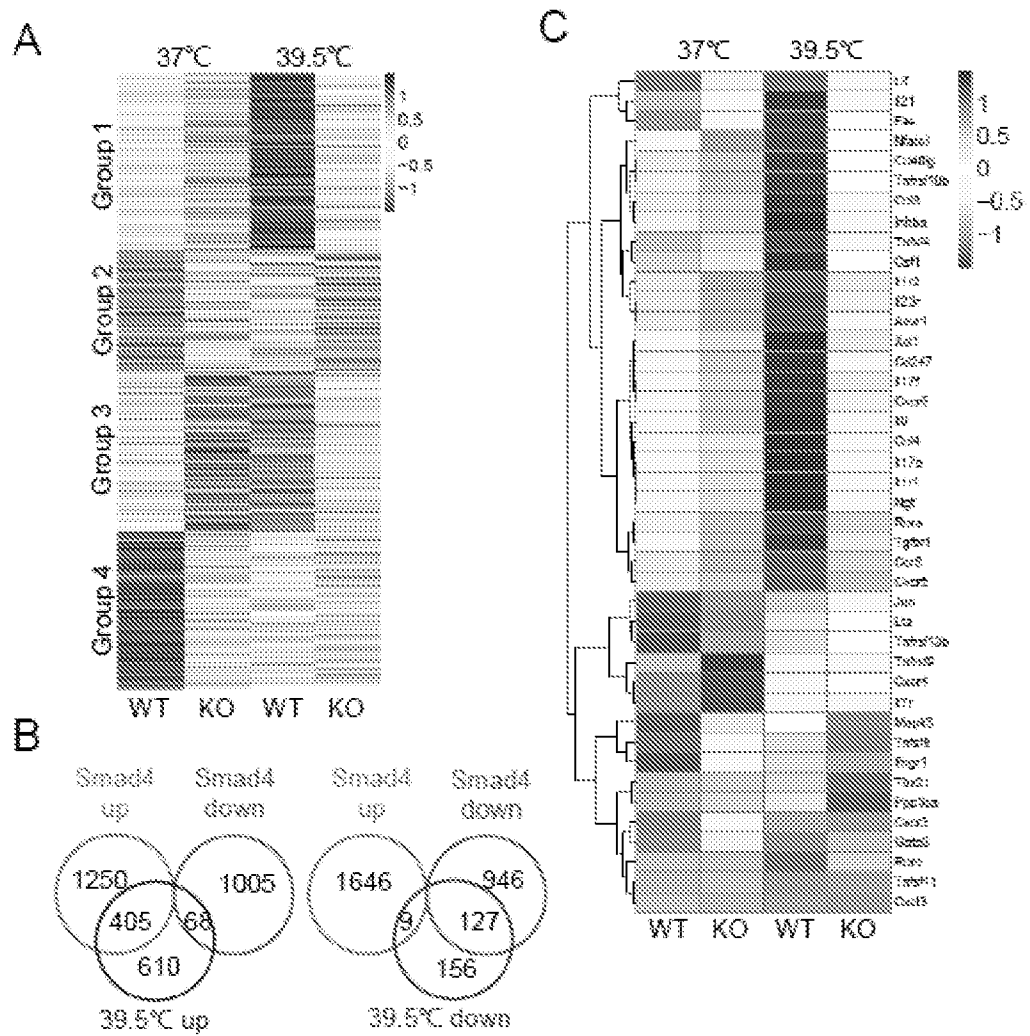


Figure 8

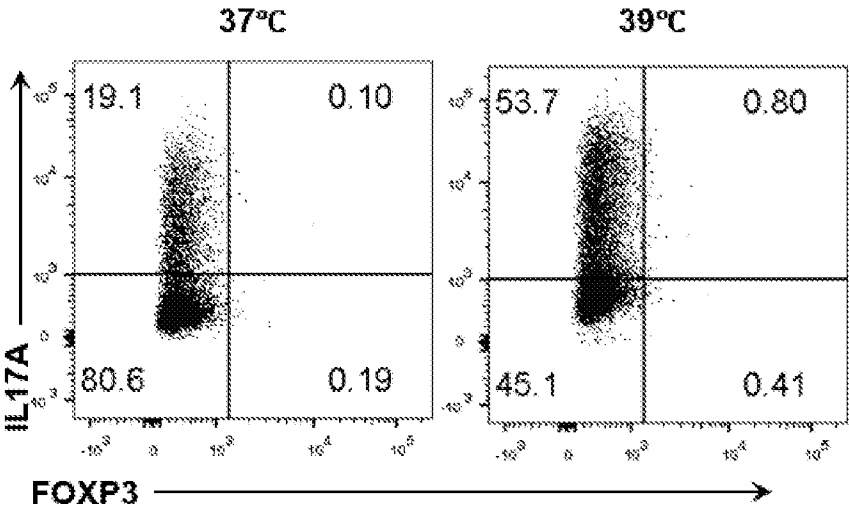


Figure 9

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2020/073271

A. CLASSIFICATION OF SUBJECT MATTER

C12N 5/0783(2010.01)i; A61K 45/06(2006.01)i; A61P 37/02(2006.01)i; A61P 17/06(2006.01)i; A61P 19/02(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N; A61K; A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CNABS, VEN, WOTXT, USTXT, EPTXT, DWPI, CNKI, PubMed, ISI Web of Knowledge; TSINGHUA UNIVERSITY, WANG Xiaohu, DONG Chen, NI Lu, differentiation, maturation, IL-17, inhibitor, SMAD4, SUMO +, Th17, Tc17, $\gamma\delta$ T cells, ILC3 cells, NK, HSP70, HSP90, UBC9, psoriasis, psoriasis-like arthritis, ankylosing spondylitis, rheumatoid arthritis, adult still's disease, multiple sclerosis, aspirin, ibuprofen, ketoprofen, metamizole, nabumetone, acetaminophen, phenazone, docosanol

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHANG, S. et al. "Reversing SKI-SMAD4-mediated suppression is essential for Th17 cell differentiation" <i>NATURE</i> , Vol. 551, 02 November 2017 (2017-11-02), pages 105-109	1
Y	ZHANG, S. et al. "Reversing SKI-SMAD4-mediated suppression is essential for Th17 cell differentiation" <i>NATURE</i> , Vol. 551, 02 November 2017 (2017-11-02), pages 105-107	2-11
X	WO 2008106131 A2 (SCHERING CORP.) 04 September 2008 (2008-09-04) description paragraph [0141], claim 36	6-11
Y	WO 2008106131 A2 (SCHERING CORP.) 04 September 2008 (2008-09-04) description paragraph [0141], claim 36	6-13
X	WO 2012059598 A2 (NOVARTIS AG) 10 May 2012 (2012-05-10) description page 73 paragraph 2	6-7



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
 "E" earlier application or patent but published on or after the international filing date
 "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
 "O" document referring to an oral disclosure, use, exhibition or other means
 "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
 "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
 "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
 "&" document member of the same patent family

Date of the actual completion of the international search

14 September 2020

Date of mailing of the international search report

23 October 2020

Name and mailing address of the ISA/CN

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CHEN, Ying

Telephone No. 86-(010)-53961975

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2020/073271

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 3111954 A1 (NOVARTIS AG) 04 January 2017 (2017-01-04) description paragraph [0240]	6-7
X	US 2007027203 A1 (CHEN, R.H. et al.) 01 February 2007 (2007-02-01) claims 1-8	6-7
Y	LU, G.L. et al. "Research progress on signal transduction of TGF- β pathway mediated by ubiquitination of SMAD4" <i>Journal of Urology for Clinicians (Electronic Version)</i> , Vol. 08, No. 02, 31 December 2016 (2016-12-31), pages 40-44	2-13
Y	YU, J. et al. "The effects of acute heat stress on gene expression of several HSPs and cytokines in lung tissue of mice" <i>Chinese Journal of Veterinary Medicine</i> , Vol. 51, No. 10, 31 December 2015 (2015-12-31), pages 20-23	6-11
A	US 2013011413 A1 (THE UNIVERSITY OF TOKYO) 10 January 2013 (2013-01-10) the whole document	1-13
A	PANG, N.N. et al. "A study of the interaction between Th17 cells and Smads" <i>Journal of Pathogen Biology</i> , Vol. 06, No. 10, 31 October 2011 (2011-10-31), pages 783-785	1-13
A	XIAO, Z. et al. "Emodin inhibits pancreatic cancer cells by upregulating expression of Smad4" <i>Basic & Clinical Medicine</i> , Vol. 36, No. 09, 30 September 2016 (2016-09-30), pages 1242-1245	1-13

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: **6-11**
because they relate to subject matter not required to be searched by this Authority, namely:

[1] The subject matter of claims 6-11 relates to a method of treating a patient with IL-17 related autoimmune diseases or cancers, and therefore does not warrant an international search according to the criteria set out in PCT Rule 39.1(iv). An international search is still carried out on the basis of the use of agents for the manufacturing of a medicament for the treatment of IL-17 related autoimmune diseases or cancers.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2020/073271

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
WO	2008106131	A2	04 September 2008	CA	2679400	A1	04 September 2008
				EP	2056838	B1	25 September 2013
				JP	2010520197	A	10 June 2010
				US	2013287775	A1	31 October 2013
				US	2010135998	A1	03 June 2010
				WO	2008106131	A3	11 December 2008
				JP	5337055	B2	06 November 2013
				CN	103933573	A	23 July 2014
				US	2012156699	A1	21 June 2012
				AU	2008219681	A1	04 September 2008
				BR	PI0807987	A2	24 June 2014
				CN	101668531	A	10 March 2010
				EP	2056838	A2	13 May 2009
				JP	2012251002	A	20 December 2012
				ZA	200905962	B	26 May 2010
				CN	101668531	B	07 May 2014
				NZ	579297	A	30 March 2012
				ZA	200905962	A	26 May 2010
				EP	2425838	A2	07 March 2012
				MX	2009009167	A	04 September 2009
				EP	2417974	A1	15 February 2012
				NZ	597915	A	30 August 2013
				EP	2425838	A3	02 May 2012
WO	2012059598	A2	10 May 2012	US	2018008706	A1	11 January 2018
				KR	20180019247	A	23 February 2018
				RU	2582937	C2	27 April 2016
				ES	2733712	T3	02 December 2019
				JP	2016065094	A	28 April 2016
				MX	362591	B	25 January 2019
				US	2013209480	A1	15 August 2013
				EP	3542820	A1	25 September 2019
				US	2019307880	A1	10 October 2019
				EP	2635303	A2	11 September 2013
				AU	2011325134	A1	04 April 2013
				IL	225309	D0	27 June 2013
				TW	201726170	A	01 August 2017
				MX	2013005060	A	28 June 2013
				US	9744234	B2	29 August 2017
				KR	20140008305	A	21 January 2014
				TW	201517917	A	16 May 2015
				PL	3111954	T3	31 October 2019
				TW	I618543	B	21 March 2018
				TW	201306864	A	16 February 2013
				KR	20150020734	A	26 February 2015
				JP	2014503482	A	13 February 2014
				CL	2013001213	A1	28 March 2014
				SG	189138	A1	31 May 2013
				KR	20160067984	A	14 June 2016
				WO	2012059598	A3	27 September 2012
				MA	34647	B1	02 November 2013

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2020/073271

Patent document cited in search report				Publication date (day/month/year)		Patent family member(s)		Publication date (day/month/year)					
				IL	262559	D0	31 December 2018						
				EP	3111954	B1	03 April 2019						
				LT	3111954	T	10 July 2019						
				JP	2019142912	A	29 August 2019						
				TW	I604851	B	11 November 2017						
				DK	3111954	T3	15 July 2019						
				JP	6049843	B2	21 December 2016						
				SI	3111954	T1	30 August 2019						
				JP	2017002064	A	05 January 2017						
				HR	P20191162	T1	04 October 2019						
				KR	20180129991	A	05 December 2018						
				JP	6250109	B2	20 December 2017						
				RU	2013125775	A	10 December 2014						
				HU	E044038	T2	30 September 2019						
				CN	103189074	A	03 July 2013						
				CN	104800844	A	29 July 2015						
				US	10363307	B2	30 July 2019						
				PT	3111954	T	10 July 2019						
				TR	201909531	T4	22 July 2019						
				AU	2011325134	B2	23 October 2014						
				JP	6515168	B2	15 May 2019						
				JP	2018065826	A	26 April 2018						
				EP	3111954	A1	04 January 2017	US	2018008706	A1	11 January 2018		
								KR	20180019247	A	23 February 2018		
				RU	2582937	C2	27 April 2016						
				ES	2733712	T3	02 December 2019						
				JP	2016065094	A	28 April 2016						
				MX	362591	B	25 January 2019						
				US	2013209480	A1	15 August 2013						
				EP	3542820	A1	25 September 2019						
				US	2019307880	A1	10 October 2019						
				EP	2635303	A2	11 September 2013						
				AU	2011325134	A1	04 April 2013						
				IL	225309	D0	27 June 2013						
				TW	201726170	A	01 August 2017						
				MX	2013005060	A	28 June 2013						
				US	9744234	B2	29 August 2017						
				KR	20140008305	A	21 January 2014						
				TW	201517917	A	16 May 2015						
				PL	3111954	T3	31 October 2019						
				TW	I618543	B	21 March 2018						
				TW	201306864	A	16 February 2013						
				KR	20150020734	A	26 February 2015						
				WO	2012059598	A2	10 May 2012						
				JP	2014503482	A	13 February 2014						
				CL	2013001213	A1	28 March 2014						
				SG	189138	A1	31 May 2013						
				KR	20160067984	A	14 June 2016						
				WO	2012059598	A3	27 September 2012						
				MA	34647	B1	02 November 2013						

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2020/073271

Patent document cited in search report	Publication date (day/month/year)	Patent family member(s)	Publication date (day/month/year)
		IL 262559 D0	31 December 2018
		EP 3111954 B1	03 April 2019
		LT 3111954 T	10 July 2019
		JP 2019142912 A	29 August 2019
		TW I604851 B	11 November 2017
		DK 3111954 T3	15 July 2019
		JP 6049843 B2	21 December 2016
		SI 3111954 T1	30 August 2019
		JP 2017002064 A	05 January 2017
		HR P20191162 T1	04 October 2019
		KR 20180129991 A	05 December 2018
		JP 6250109 B2	20 December 2017
		RU 2013125775 A	10 December 2014
		HU E044038 T2	30 September 2019
		CN 103189074 A	03 July 2013
		CN 104800844 A	29 July 2015
		US 10363307 B2	30 July 2019
		PT 3111954 T	10 July 2019
		TR 201909531 T4	22 July 2019
		AU 2011325134 B2	23 October 2014
		JP 6515168 B2	15 May 2019
		JP 2018065826 A	26 April 2018
US 2007027203 A1	01 February 2007	HK 1160393 A1	08 July 2016
		RS 53372 B	31 October 2014
		JP 2007522114 A	09 August 2007
		CA 2547963 C	10 February 2015
		JP 2015071626 A	16 April 2015
		HK 1096603 A1	31 October 2014
		DK 1706112 T3	22 April 2014
		EP 3020400 A1	18 May 2016
		CA 2871459 A1	04 August 2005
		JP 2012176956 A	13 September 2012
		EP 1706112 B1	12 March 2014
		ME 01878 B	20 December 2014
		EP 2351564 A1	03 August 2011
		HK 1220612 A1	12 May 2017
		JP 5969255 B2	17 August 2016
		HR P20140536 T1	04 July 2014
		US 7855223 B2	21 December 2010
		PL 1706112 T3	29 August 2014
		JP 5383977 B2	08 January 2014
		ES 2559183 T3	10 February 2016
		CA 2547963 A1	04 August 2005
		SI EP1706112 T1	31 July 2014
		EP 1706112 A2	04 October 2006
		PT 1706112 E	02 May 2014
		EP 1706112 A4	10 December 2008
		CY 1115289 T1	04 January 2017
		EP 2351564 B1	11 November 2015
		WO 2005069933 A2	04 August 2005

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2020/073271

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
				ES	2459365	T3	09 May 2014
				US	2005154046	A1	14 July 2005
				WO	2005069933	A3	09 September 2005
				SI	1706112	T1	31 July 2014
US	2013011413	A1	10 January 2013	WO	2011096438	A1	11 August 2011
				JP	WO2011096438	A1	10 June 2013