Effect of Janus kinase inhibitors on T cell responses to herpes zoster in rheumatoid arthritis patients

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Abstract

Objective

The incidence of herpes zoster (HZ) in rheumatoid arthritis (RA) patients is greater than that in healthy controls (HC), particularly in RA patients treated with Janus kinase inhibitors (JAKi). Here, we examined the effect of JAKi on $CD4^+$/$CD8^+$ T cells, cytokine production, and regulation of transcriptional factors in RA patients and HC.

Methods

Peripheral blood mononuclear cells (PBMCs) obtained from RA patients (n=14) and HCs (n=7) were stimulated with varicella zoster virus lysates and exposed to three JAKi inhibitors (ruxolitinib [JAK1/2 inhibitor]; AG490 [JAK2 inhibitor]; and WHI-P154 [JAK3 inhibitor]) in the presence/absence of methotrexate. The $CD4^+$ and $CD8^+$ T cell populations were measured by flow cytometry. Cytokine levels in culture medium were measured by ELISA. Transcription factor expression was examined by RT-qPCR.

Results

There was a reduction in the $CD4^+IFN\text{-}\gamma^+$, $CD4^+CD69^+IFN\text{-}\gamma^+$, $CD8^+IFN\text{-}\gamma^+$, and $CD8^+CD69^+IFN\text{-}\gamma^+$ populations, and an increase in the $CD4^+CD25^{high}Foxp3^+$ cell population, in PBMCs from RA patients and HCs after exposure to the three JAKi. ELISA revealed a reduction in IFN-γ and granzyme B levels in the presence of JAKi. JAKi reduced expression of mRNA encoding STAT1 and T-bet, but increased that of mRNA encoding STAT5 and Foxp3. Methotrexate plus the highest dose of each JAKi did not affect the Th1, cytotoxic T cell, or Treg populations, the levels of IFN-γ and granzyme B, or expression of transcription factors, significantly.

Conclusion

JAKi reduce the Th1/cytotoxic T cell population and increase the Treg population in both RA patients and HC patients.

Key words

rheumatoid arthritis, herpes zoster, Janus kinase inhibitor, T cells

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Introduction

Rheumatoid arthritis (RA) is an autoimmune-mediated arthritis with a complex pathogenesis involving adaptive and innate immune cells, fibroblast-like synoviocytes, and osteoclasts (1). Among adaptive immune cells, the $CD4^+$ T lymphocyte population comprises several subtypes defined according to expression of cell surface markers (*i.e.* type 1 helper T cells (Th1), Th2, Th17, follicular helper T cells (Tfh), and regulatory T cells (Tregs)); these subsets play different roles in RA pathogenesis (2). Maintaining the balance between Th17 and Tregs is the main aim of RA treatments (3), which include the recently introduced Janus kinase inhibitors (JAKi) (4, 5).

Herpes zoster (HZ) is a painful skin rash, with blisters occurring in localised sensory dermatomes; the cause is reactivation of varicella zoster virus (VZV) in the dorsal root ganglion or cranial sensory nerve. The risk of HZ increases with age (6). The incidence of HZ is higher in RA patients than in the general population (7, 8), and is even more prominent in those treated with JAKi (9). Therefore, the VZV vaccine is recommended to patients with inflammatory arthritis (10). Immune responses against VZV reactivation are both humoral and cell-mediated immunity (CMI). One study showed that CMI, as detected by a skin reaction, is associated with HZ occurrence, whereas the humoral response (*i.e.* VZV-specific antibodies) is not (11). Two *in vitro* studies show that interferon (IFN)-γ suppresses VZV replication (12, 13), and IFN-γ producing T cell responses are important for resolution of VZV infection (12). Also, patients with autoimmune disease who experience VZV reactivation show weaker IFN-γ spot-forming cell responses than patients without VZV reactivation (14), and there is an inverse correlation between VZV reactivation and the number of IFN-γ producing cells (13). Another study reported infiltration of sensory ganglia by $CD4^+$/cytotoxic $CD8^+$ T cells, and up-regulation of major histocompatibility complex class I/II molecules (15), which implies that $CD4^+$/$CD8^+$ T cells play important roles in suppressing VZV replication. Granzyme B, a serine protease secreted by cytotoxic T cells and natural killer cells, induces apoptosis of VZV-infected cells (16). HZ-associated skin lesions in older patients harbor more regulatory T cells (Treg) and fewer IFN-γ producing $CD4^+$ T cells than lesions in younger patients (17), suggesting that increased Treg numbers may negate the anti-viral effects of IFN-γ producing immune cells.

There are four types of JAK, JAK1–3 and tyrosine kinase 2 (TYK2), each of which blocks intracellular signaling via the JAK-STAT pathway (18). Methotrexate (MTX), the most common conventional synthetic disease-modifying anti-rheumatic drug (csDMARD) used to treat RA, also suppresses the JAK-STAT pathway (19). The JAK-STAT pathway is crucial for proliferation and development of T cells (20, 21). JAKi are now used widely to treat various forms of inflammatory arthritis; however, observational studies show a higher risk of HZ in patients treated with JAKi than in those receiving csDMARDs or biologic DMARDs (18). One *in vitro* study demonstrated that tofacitinib suppresses $CD69^+$ $CD3^+$ T cell proliferation under VZV antigen stimulation (22). However, it is unclear whether and how JAKi modulate IFN-γ-secreting $CD4^+$/$CD8^+$ T cells, and/or production of IFN-γ/granzyme B, in RA patients with VZV.

Here, we aimed to examine how JAKi regulate the Th1, $CD8^+$, and Treg populations in PBMCs from RA patients and healthy controls (HC) stimulated with VZV lysates. We also examined expression of transcription factors and cytokines by these cells.

Materials and methods

Patients

RA patients and HC were recruited from a single tertiary Hospital (Konkuk University Medical Center, Seoul, Republic of Korea) from March 2021 to October 2021. The inclusion criteria for RA patients were as follows: 1) fulfill the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for

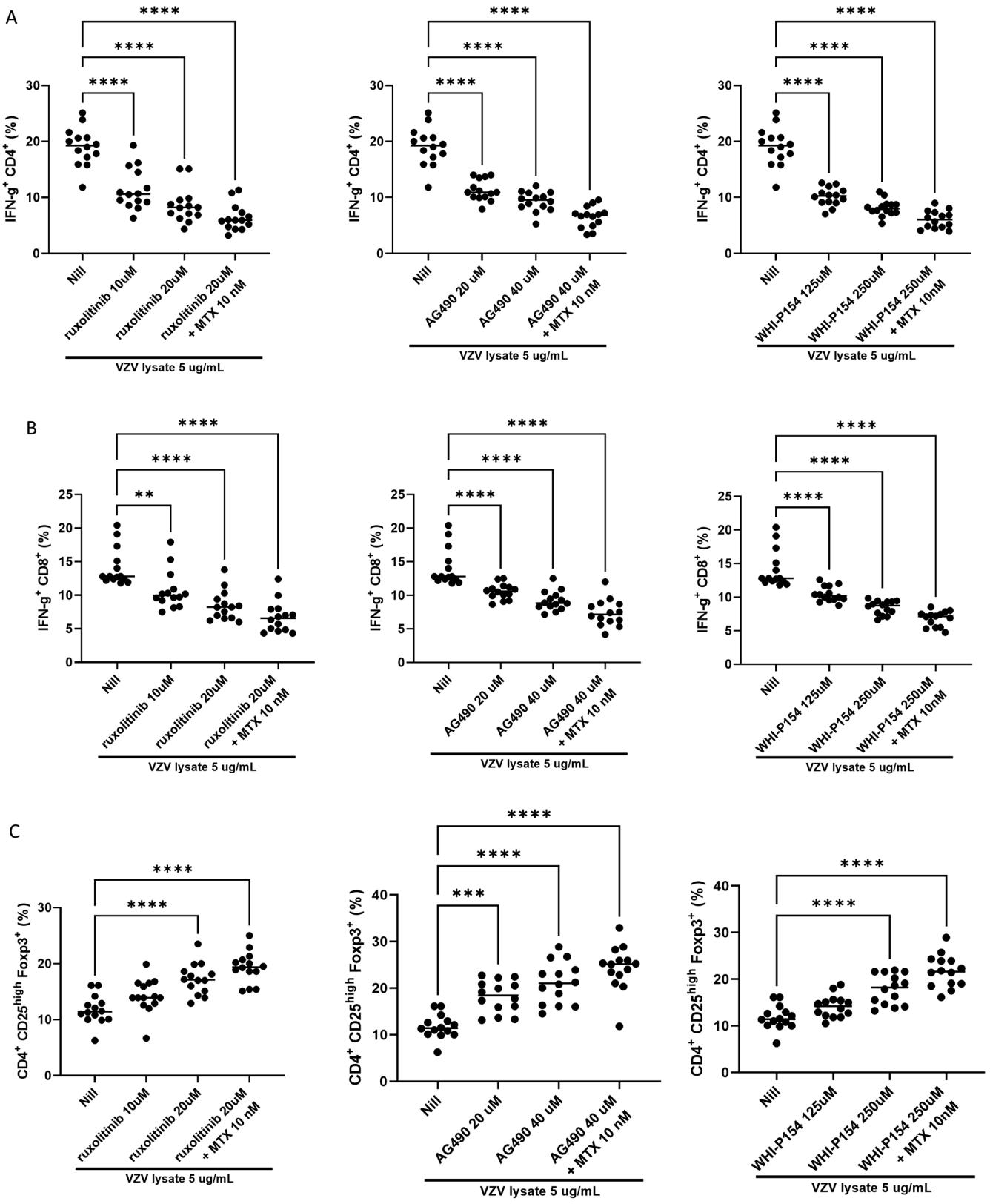

Fig. 1. Regulation of CD4+ and CD8+ T cells by ruxolitinib, AG490, and WHI-P154 in the presence of VZV lysate of RA patients. PBMCs (1×10^6) from RA patients (n=14) were cultured for 72 h in 12-well plates in the presence of VZV lysate (5 μg/mL) with or without various JAKi (ruxolitinib 10 or 20 μM, AG490 20 or 40 μM, and WHI-P154 125 or 250 μM) and methotrexate (10 nM, used at the highest dose of JAKi), and then analysed by flow cytometry. The populations of (**A**) IFN-γ+ CD4+ T cells, (**B**) IFN-γ+ CD8+ T cells, and (**C**) CD4+ $CD25^{high}$ Foxp3+ T cells in the presence of 10 μM ruxolitinib, 20 μM ruxolitinib, or 20 μM ruxolitinib + 10 nM MTX; 20 μM AG490, 40 μM AG490, or 40 μM AG490 + 10 nM MTX; or 125 μM WHI-P154, 250 μM WHI-P154, or 250 μM WHI-P154 + 10 nM MTX were compared with those under the null condition (VZV lysate only).
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

RA (23) and 2) age >19 years. Patients with malignancy, infection, other autoimmune diseases, or inflammatory arthritis were excluded. Peripheral blood was collected from patients and HC. The present study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines, and was approved by the institutional review board of Konkuk University Medical Center (2020-06-031). Before enrolment, all participants provided written informed consent.

Isolation of PBMCs and stimulation with VZV lysate

Peripheral blood was obtained from RA patients (n=14) and HC (n=7). Peripheral blood mononuclear cells (PBMCs) were extracted using the standard Ficoll-Paque density gradient method (GE Healthcare Biosciences, Uppsala, Sweden). Next, a 12-well culture plate (Nunc) was pre-incubated for 1 h at 37°C with an anti-CD3 antibody (1 μg/mL), followed by addition of PBMCs (1×10^6), an anti-CD28 antibody (1 μg/mL; R&D Systems), VZV lysate 5 μg/mL (MBS319900; MyBioSource, San Diego, CA, USA), and various concentrations of JAKi, for 72 h. The following JAKi were used: ruxolitinib (tlrl-rux; 10 or 20 μM; InvivoGen, San Diego, CA, USA), AG490 (tlrl-ag4; 20 or 40 μM; InvivoGen), and WHI-P154 (MBS130580; 125 or 250 μM; MyBioSource). Methotrexate (MTX; 10 nM, M8407; Sigma, St Louis, MA, USA) was also added at the highest JAKi concentration.

Flow cytometry analysis

After 3 days of culture *in vitro*, flow cytometry was performed to measure T cell differentiation. The number of IFN-γ$^+$ cells within the CD4$^+$ and CD8$^+$ T cell and Treg populations was measured. CD69 was used as a marker of activated CD4$^+$ and CD8$^+$ T cells. PBMCs were fixed and immunostained with a PerCP-conjugated anti-CD4 antibody (347324; BD Biosciences, San Jose, CA, USA) and a phycoerythrin (PE)-conjugated anti-CD69 antibody (310906; BioLegend, San Diego, CA, USA), or a PerCP-conjugated anti-CD8 antibody (347314; BD Biosciences), and a PE-conjugated anti-CD69 antibody (310906; BioLegend), and then permeabilised using a Cytofix/Cytoperm Plus kit (BD Biosciences). Next, PBMCs were stained with a FITC-conjugated anti-IFN-γ (554551; BD Biosciences) antibody. A PerCP-conjugated anti-CD4 antibody (347324; BD Biosciences), an allophycocyanin-conjugated anti-CD25 antibody (560987; BD Biosciences), or a PE-conjugated anti-Foxp3 (320207; BioLegend) were used to label Tregs. All cells were detected using a FACSCalibur cytometer (BD Pharmingen, Franklin Lakes, NJ, USA).

ELISA to measure IFN-γ and granzyme B

Briefly, 96-well plates (Eppendorf, Hamburg, Germany) were coated overnight at 4°C with monoclonal antibodies specific for IFN-γ or granzyme B (400 ng/mL; R&D Systems, Minneapolis, MN, USA). After blocking with phosphate-buffered saline (PBS)/1% bovine serum albumin /0.05% Tween 20 for 2 h at room temperature (22–25°C), the test samples were added to the plate and incubated at room temperature for another 2 h. Recombinant IFN-γ and granzyme B (both from R&D Systems) were used as standards. The plates were washed four times with PBS/Tween 20 and then incubated for 2 h at room temperature with biotinylated mouse monoclonal antibodies (300 ng/mL; R&D Systems) specific for IFN-γ and granzyme B. After washing, the streptavidin–alkaline phosphate–horseradish peroxidase conjugate (Sigma, St Louis, MA, USA) was added to the wells for 20 min, followed by another wash. Finally, p-nitrophenyl phosphate (1 mg/mL; Sigma) dissolved in diethanolamine (Sigma) was added to develop the color reaction. The reaction was stopped by addition of 1 M NaOH and the optical density of each well was measured at 405 nm. A standard curve was drawn by plotting the optical density against the log concentration of the recombinant cytokines, and the curve was used to determine the IFN-γ and granzyme B concentrations in the test samples.

RNA preparation and quantification of gene expression levels using real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from cells using an easy-spin™ Total RNA Extraction Kit (Intron Biotechnology, Seongnam, Republic of Korea). RNA samples were then quantified, aliquoted, and stored at -80°C until analysis. Total RNA (500 ng) was reverse-transcribed to cDNA using an AccuPower CycleScript RT PreMix cDNA synthesis kit (Bioneer, Daejeon, Republic of Korea). Next, qPCR was conducted in a total volume of 20 μL comprising 7.2 μL of PCR-grade distilled water, 0.4 μL of forward and reverse primers, and 10 μL of SYBR Green I Master mix (Roche Diagnostics, Mannheim, Germany). The PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 59°C for 15 s, and 72°C for 15 s. All primers were synthesised by Bioneer Corp. (Daejeon, Republic of South Korea). Relative mRNA expression levels were normalised to those of ACTB.

Statistical analysis

Continuous variables are expressed as the mean ± standard error of the mean. Data were compared using one-way analysis of variance with Bonferroni's *post-hoc* multiple comparison test. Statistical significance was set at $p<0.05$. All statistical analyses were performed using Prism 9.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

Suppression of IFN-γ positive helper and cytotoxic T cells and up-regulation of Tregs by JAKi under VZV stimulation

PBMCs were obtained from the 14 RA patients and seven patients with HC. The mean age of the RA and HC patients was 63.0±8.8 and 36.1±4.0 years, respectively. All HC patients were male, whereas only 50% of RA patients were male. All enrolled RA and HC patients were positive for VZV IgG, suggesting that they had either experienced VZV infection or had been vaccinated against VZV. Other characteristics of the recruited RA patients are described

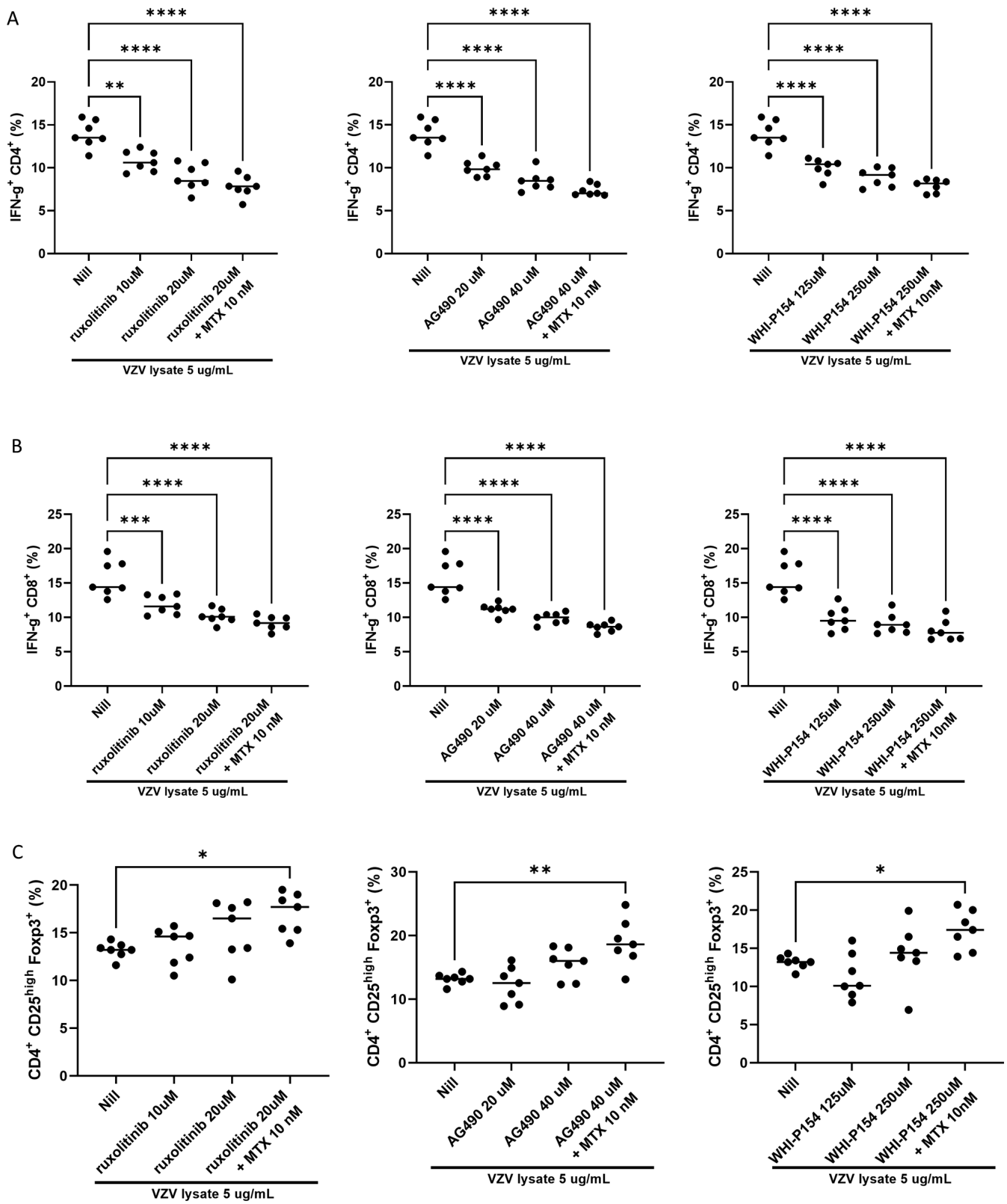

Fig. 2. Regulation of CD4+ and CD8+ T cells by ruxolitinib, AG490, and WHI-P154 in the presence of VZV lysate from healthy controls. PBMCs (1×10^6) from healthy controls (n = 7) were cultured for 72 h in 12-well plates in the presence of VZV lysate (5 μg/mL) with or without various JAKi (ruxolitinib 10 or 20 μM, AG490 20 or 40 μM, and WHI-P154 125 or 250 μM) plus methotrexate (10 nM, used at the highest dose of JAKi), and then analysed by flow cytometry. The populations of (**A**) IFN-γ$^+$ CD4$^+$ T cells, (**B**) IFN-γ$^+$ CD8$^+$ T cells, and (**C**) CD4$^+$ CD25^{high} Foxp3$^+$ T cells in the presence of 10 μM ruxolitinib, 20 μM ruxolitinib, or 20 μM ruxolitinib + 10 nM MTX; 20 μM AG490, 40 μM AG490, or 40 μM AG490 + 10 nM MTX; or 125 μM WHI-P154, 250 μM WHI-P154, or 250 μM WHI-P154 + 10 nM MTX were compared with those under the null condition (VZV lysate only).
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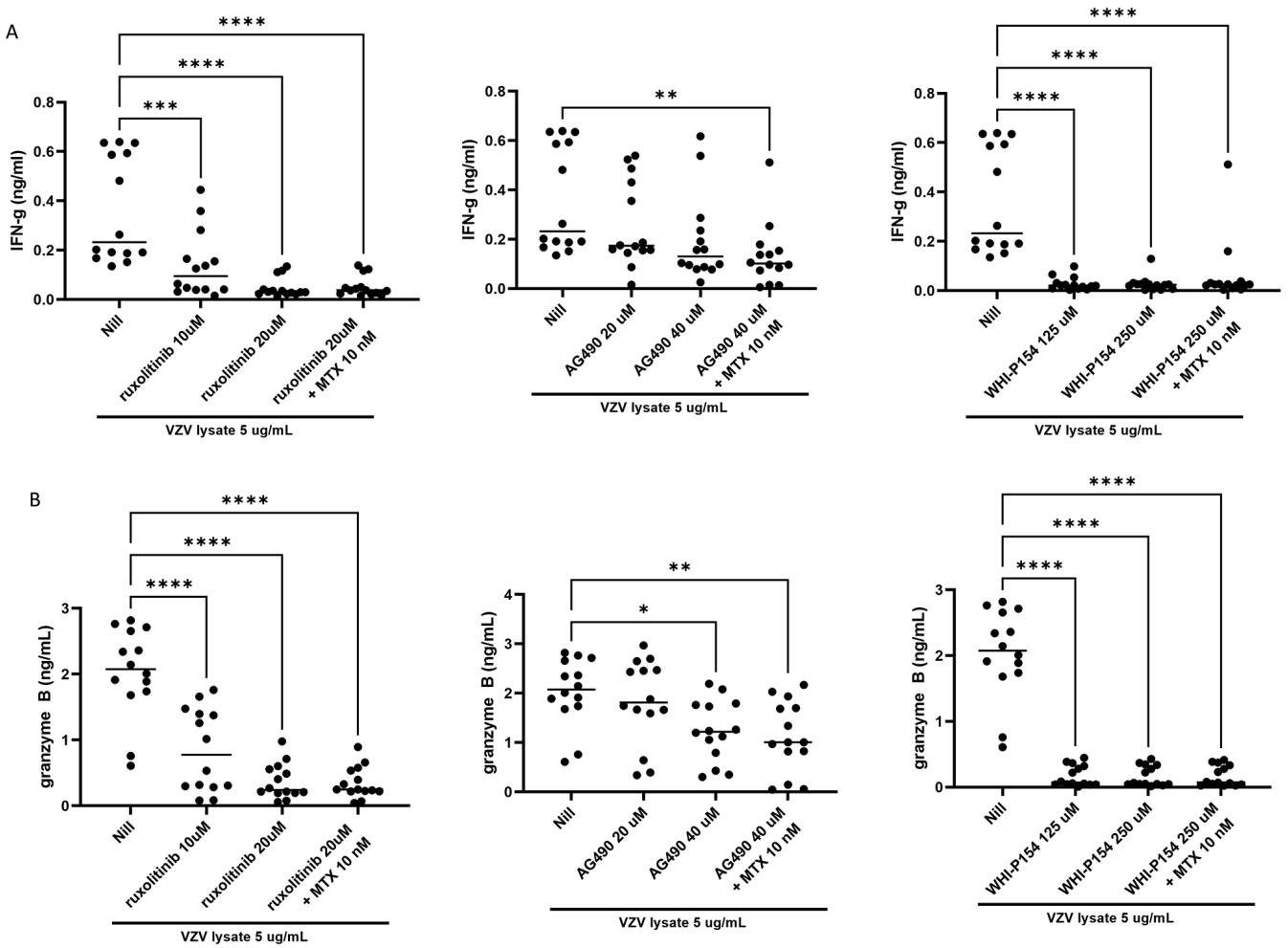

Fig. 3. IFN-γ and granzyme B concentrations in rheumatoid arthritis PBMC culture medium. PBMC culture medium of RA patients (n=14) was obtained after 72 h of *in vitro* culture. Levels of (**A**) IFN-γ and (**B**) granzyme B were measured in the presence of 10 μM ruxolitinib, 20 μM ruxolitinib, or 20 μM ruxolitinib + 10 nM MTX; 20 μM AG490, 40 μM AG490, or 40 μM AG490 + 10 nM MTX; or 125 μM WHI-P154, 250 μM WHI-P154, or 250 μM WHI-P154 + 10 nM MTX and compared with the null condition (VZV lysate only). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

in Supplementary Table S1. The T cell population was quantified by flow cytometry after 3 days of *in vitro* culture in the presence of various JAKi plus VZV lysate. IFN-γ$^+$ CD4$^+$ T cells were suppressed by all three JAKi (ruxolitinib, AG490, and WHI-P154; Fig. 1A). Adding MTX did not decrease IFN-γ$^+$ CD4$^+$ T cell numbers further. In RA patients (n=7), active Th1 cells were defined as IFN-γ$^+$ CD4$^+$ CD69$^+$; these cells were suppressed by all three JAKi in a dose-dependent manner (Suppl. Fig. S1A). In HC patients, the IFN-γ$^+$ CD4$^+$ T cell population was suppressed by JAKi (Fig. 2A). Next, we evaluated cytotoxic T cell regulation by JAKi. The percentage of IFN-γ$^+$ CD8$^+$ T cells in RA patients was reduced by all three JAKi (ruxolitinib, AG490, and WHI-P154; Fig. 1B). In RA patients (n=7), the IFN-γ$^+$ CD8$^+$ CD69$^+$ T cells were suppressed by all three JAKi (Suppl. Fig. S1B). The results for HC patients were similar (Fig. 2B). The CD4$^+$ CD25^{high} Foxp3$^+$ cell population in RA patients increased at the highest dose of ruxolitinib (20 μM) and WHI-P154 (250 μM), and at both doses of AG490, regardless of the presence of MTX (10 nM) (Fig. 1C). Again, the results in HC patients were similar (Fig. 2C).

Cytokine production in the presence of JAKi plus VZV lysate

The concentrations of IFN-γ and granzyme B fell in the presence of VZV plus ruxolitinib or WHI-P154 to a greater extent than in the presence of VZV lysate only (Fig. 3A-B). By contrast, IFN-γ concentrations fell significantly only in the presence of 40 μM AG490 + 10 nM MTX, and the granzyme B concentration decreased in the presence of 40 μM AG490 and 40 μM AG490 + 10 nM MTX (Fig. 3A-B). The findings in HC patients were similar, but the granzyme B concentration did not fall in the presence of AG490 (Fig. 4A-B).

Regulation of transcription factors by JAKi

Next, we measured expression of mRNA encoding *STAT1* and *T-bet*, transcription factors that induce Th1 and cytotoxic T cells. Expression of *STAT1* was down-regulated significantly in the presence of 20 μM ruxolitinib + 10 nM MTX, 40 μM AG490, 40 μM AG490 + 10 nM MTX, 250 μM WHI-P154, and 250 μM WHI-P154 + 10 nM MTX (Fig. 5A). Similarly expression levels of *T-bet* fell in the presence of 20 μM

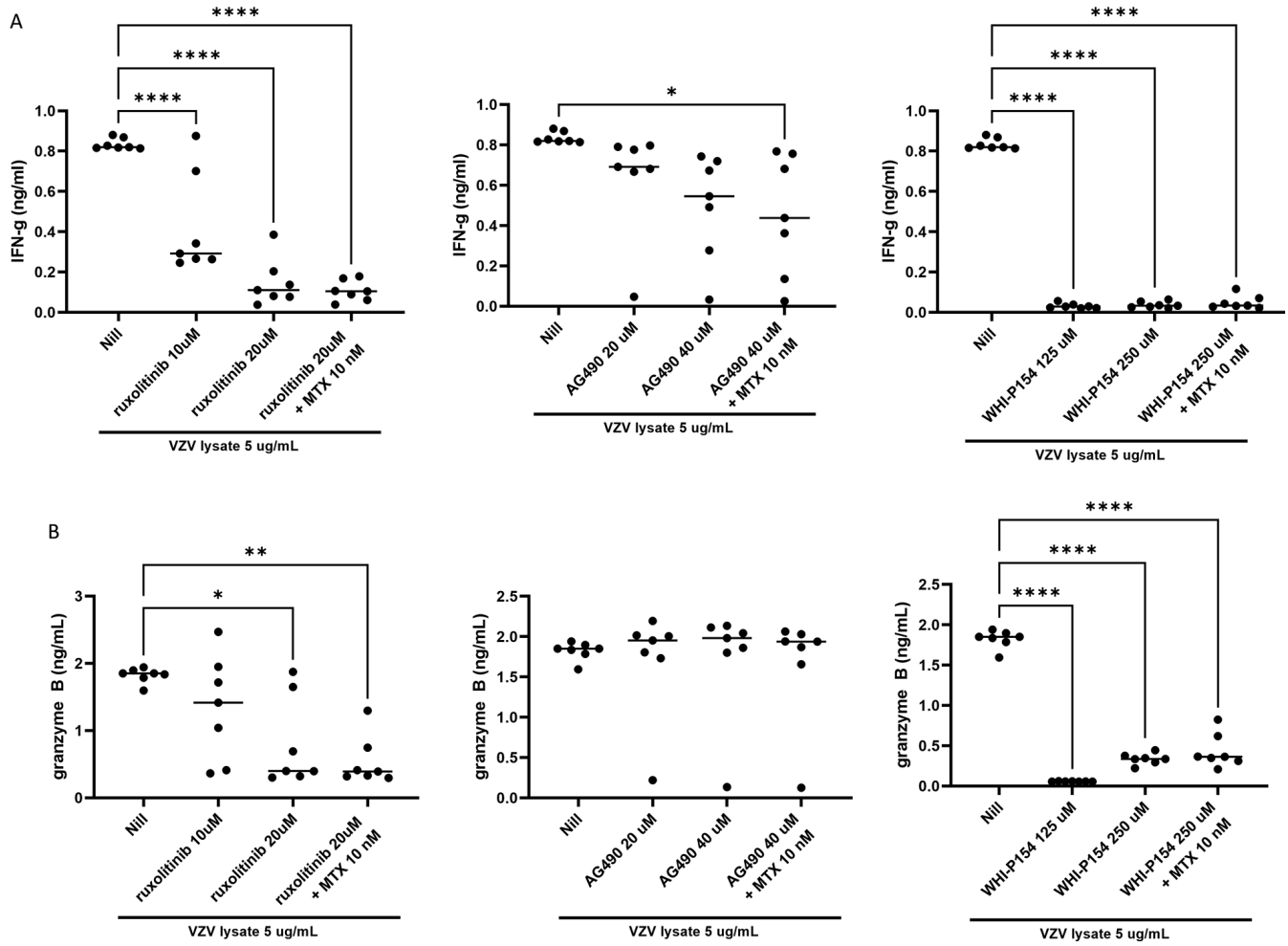

Fig. 4. IFN-γ and granzyme B concentrations in healthy control PBMC culture medium. Medium from healthy control PBMCs (n=7) was obtained after 72 h of *in vitro* culture. Levels of (**A**) IFN-γ and (**B**) granzyme B were measured in the presence of 10 μM ruxolitinib, 20 μM ruxolitinib, or 20 μM ruxolitinib + 10 nM MTX; 20 μM AG490, 40 μM AG490, or 40 μM AG490 + 10 nM MTX; or 125 μM WHI-P154, 250 μM WHI-P154, or 250 μM WHI-P154 + 10 nM MTX, and compared with the null condition (VZV lysate only). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

ruxolitinib, 20 μM ruxolitinib + 10 nM MTX, 40 μM AG490, 40 μM AG490 + 10 nM MTX, 250 μM WHI-P154, and 250 μM WHI-P154 + 10 nM MTX (Fig. 5B). Next, we measured expression of *STAT5* and *Foxp3*, which are transcription factors that induce Tregs. In the presence of 20 μM ruxolitinib, 20 μM ruxolitinib + 10 nM MTX, 20 μM AG490, 40 μM AG490, 40 μM AG490 + 10 nM MTX, 250 μM WHI-P154, or 250 μM WHI-P154 + 10 nM MTX, expression of mRNA encoding *STAT5* increased to a greater extent than in the presence of VZV lysate only (Fig. 5C). Similarly, expression of *Foxp3* increased significantly in the presence of 20 μM ruxolitinib, 20 μM ruxolitinib + 10 nM MTX, 40 μM AG490, 40 μM AG490 + 10 nM MTX, 125 μM WHI-P154, 250 μM WHI-P154, and 250 μM WHI-P154 + 10 nM MTX (Fig. 5D). Down-regulation of *STAT1*/*T-bet* and up-regulation of *STAT5*/*Foxp3* was also observed in cells from HC (Fig. 6A-D).

Discussion

In the present study, we demonstrated that JAKi suppress differentiation of IFN-γ$^+$ helper and cytotoxic T cells, the amounts of effector cytokines secreted by these cells (IFN-γ and granzyme B), and expression levels of their related transcription factors (*STAT1*, and *T-bet*), in the presence of VZV lysate. By contrast, Tregs and their transcription factors (*STAT5* and *Foxp3*) were up-regulated by JAKi. These results were observed in both RA patients and HC.

HZ is caused by reactivation and replication of VZV, which lies latent in the dorsal root ganglion or cranial nerves (24). The initial anti-viral immunologic response is induced by innate immune cells in the affected skin; however, viral clearance and limiting the severity of HZ is the responsibility of adaptive immune cells, especially T cells (24). Among CD4$^+$ T cells, Th1 cells express IFN-γ and exert immunity against intracellular pathogens, including viruses (2). Cytotoxic T cells induce apoptosis of virus-infected cells (25). IFN-γ is produced by various innate and adaptive immune cells, including CD4$^+$ and CD8$^+$ T cells (26). Autocrine production of IFN-γ by CD8$^+$ T cell increases the motility and cytotoxic function of CD8$^+$ T cells (27). Granzyme B, produced by CD8$^+$ T cells, induces apoptosis of virus-infected cells (28). The number of IFN-γ producing immune cells correlates inversely with VZV re-

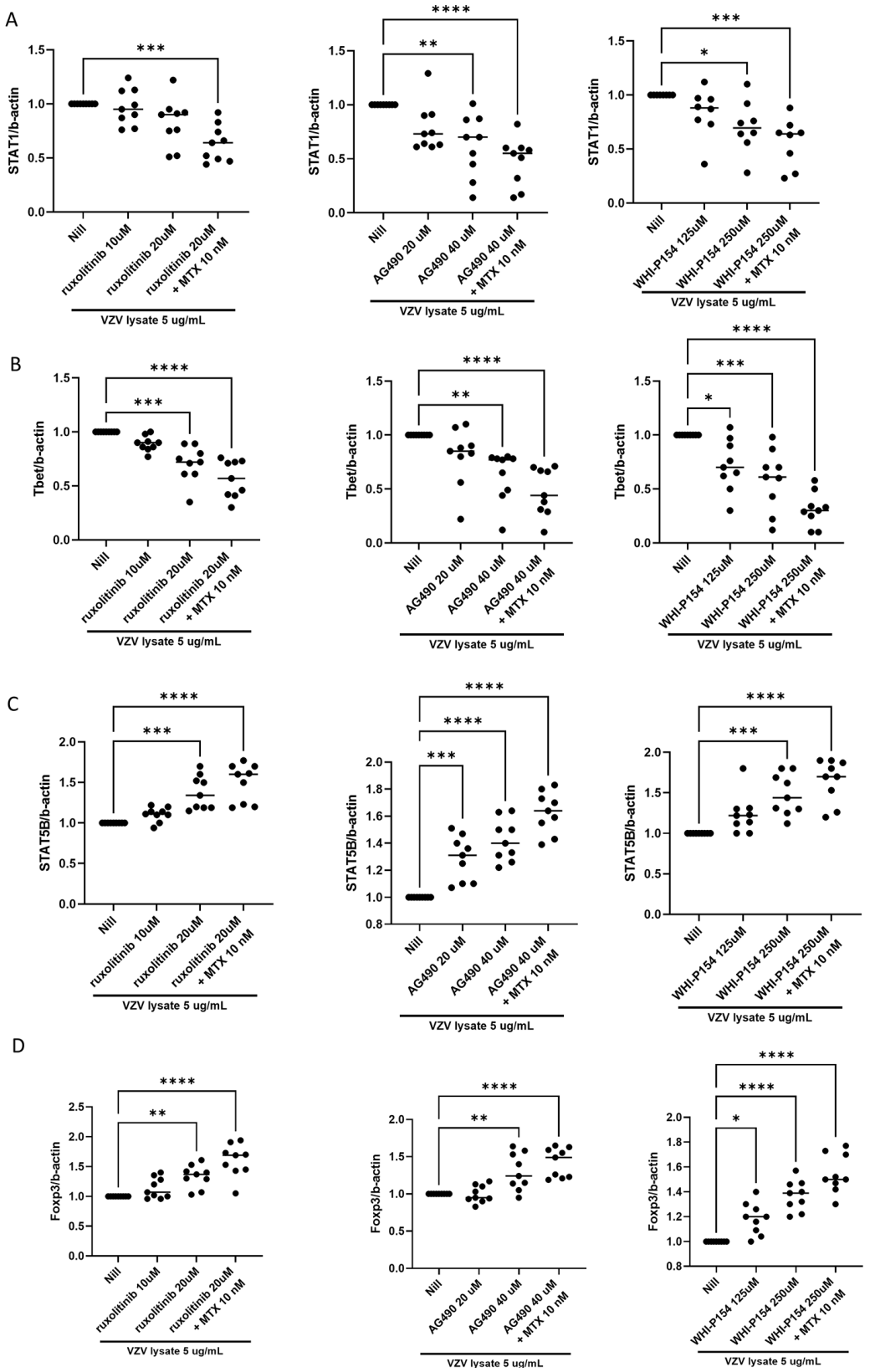

Fig. 5. Expression of genes encoding transcription factors in Th1, cytotoxic T, and Treg cells from rheumatoid arthritis patients. Expression of mRNA encoding *STAT1*, *T-bet*, *STAT5*, and *Foxp3* was quantified by RT-qPCR after 72 h of *in vitro* stimulation by VZV lysate in the presence/absence of JAKi and MTX. (**A**) *STAT1*, (**B**) *T-bet*, (**C**) *STAT5*, and (**D**) *Foxp3* expression in the presence of 10 μM ruxolitinib, 20 μM ruxolitinib, or 20 μM ruxolitinib + 10 nM MTX; 20 μM AG490, 40 μM AG490, or 40 μM AG490 + 10 nM MTX; or 125 μM WHI-P154, 250 μM WHI-P154, or 250 μM WHI-P154 + 10 nM MTX. Expression was normalised to that of *ACTB* and reported as relative expression units. The relative expression level of each JAKi with or without MTX was compared with that under the null condition (VZV lysate only). **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001.

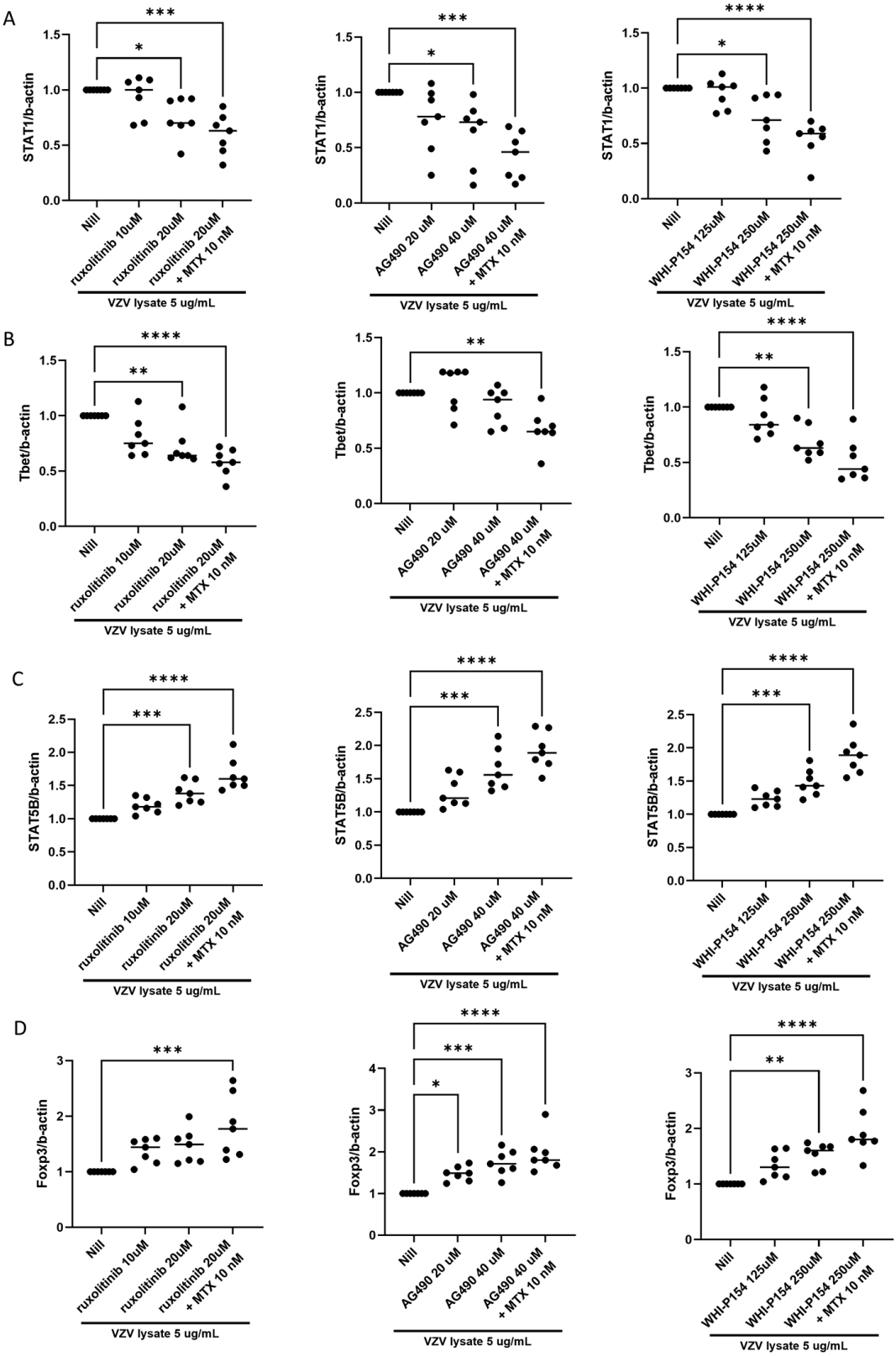

Fig. 6. Expression of genes encoding transcription factors in Th1, cytotoxic T, and Treg cells from healthy controls. Expression of mRNA encoding *STAT1*, *T-bet*, *STAT5*, and *Foxp3* was quantified by RT-qPCR after 72 h of *in vitro* stimulation by VZV lysate in the presence/absence of JAKi and MTX. (**A**) *STAT1*, (**B**) *T-bet*, (**C**) *STAT5*, and (**D**) *Foxp3* expression levels in the presence of 10 μM ruxolitinib, 20 μM ruxolitinib, or 20 μM ruxolitinib + 10 nM MTX; 20 μM AG490, 40 μM AG490, or 40 μM AG490 + 10 nM MTX; or 125 μM WHI-P154, 250 μM WHI-P154, or 250 μM WHI-P154 + 10 nM MTX. Expression was normalised to that of *ACTB* and reported as relative expression units. The relative expression level of each JAKi with or without MTX was compared with that under the null condition (VZV lysate only).
**p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001.

activation, suggesting the importance of IFN-γ in immunity against VZV (13). In the present study, we show that JAKi inhibit differentiation of IFN-γ$^+$ CD4$^+$ and CD8$^+$ T cells within the PBMC population of RA patients in the presence of VZV antigens. Active IFN-γ$^+$ CD4$^+$ CD69$^+$ and IFN-γ$^+$ CD8$^+$ CD69$^+$ T cell numbers were also suppressed by JAKi. Furthermore, production of anti-viral cytokines IFN-γ and granzyme B was suppressed significantly by JAKi in the presence of VZV lysate *in vitro*. Tregs usually induce self-tolerance and protect against autoimmunity; however, in the presence of virus, Tregs can limit host anti-viral immune responses (29). The incidence of HZ increases with age (30), which mirrors the significant increase in cutaneous Treg cells in older patients with HZ relative to that in younger patients; by contrast, the proliferative capacity of CD4$^+$ and CD8$^+$ T cells in young and older patients is similar (17). The increased Treg population in HZ-affected skin may play an important role in VZV reactivation. In the present study, we found that *in vitro* stimulation of RA PBMCs with VZV lysate in the presence of various JAKi increased CD4$^+$ CD25^{high} Foxp3$^+$ T cell differentiation. This reciprocal regulation of IFN-γ$^+$ T cells and regulatory T cells could explain the increased risk of HZ in JAKi users.

JAKs are coupled with transmembrane receptors; thus JAKi modulate intracellular signaling through the JAK-STAT pathway (18). STAT1 and T-bet are the main transcriptional factors of Th1 and CD8$^+$ T cells, whereas Treg differentiation is controlled by transcription factors STAT5 and Foxp3 (31, 32). We used JAKi with different maximal inhibitory concentrations (IC_{50}): 1) ruxolitinib (IC_{50} 3.3 nM for JAK1 and 2.8 nM for JAK2); 2) AG490 (IC_{50} 0.1 μM for JAK2); and 3) WHI-P154 (IC_{50} 1.8 μM for JAK3). All three suppressed *STAT1*/*T-bet* expression and increased expression of *STAT5*/*Foxp3*.

MTX is the first-line treatment for RA patients (5), and in many RA patients co-administration of MTX with JAKi is common. We hypothesised that co-administration of MTX plus JAKi may further modulate T cell immunity because MTX suppresses phosphorylation of STAT3/5 and JAK2 (19). However, addition of MTX to the highest concentration of each JAKi had no addition effect on IFN-γ$^+$ CD4$^+$/CD8$^+$ T cells or Tregs, nor on production of anti-viral cytokines (IFN-γ and granzyme B). Until now, it was unclear whether MTX increases the risk of HZ in JAKi users. The present data suggest not, although further experimental and epidemiologic studies are required to confirm this.

The present study has several limitations. First, all of the results were obtained from *in vitro* studies; we did not examine T-CMI in HZ skin directly. Although systemic immune responses and cutaneous local immune responses to VZV reactivation may differ, our results show that JAKi modulate VZV-specific CD4 and CD8 T cell responses. Second, we did not assess the function or anti-viral capacity of memory T and B cells or innate immune cells against VZV. JAKi modulated IFN-γ$^+$ CD4$^+$/CD8$^+$ T cell/Treg cells and anti-viral cytokines *in vitro*, but similar results were observed in RA and HC PBMCs. Therefore, the present study cannot point to specific immune mechanisms that explain the increased risk of HZ in RA patients taking JAKi. Finally, the HC group only included males, whereas female sex dominance is a characteristic of RA epidemiology.

In conclusion, JAKi suppress the differentiation of IFN-γ$^+$ CD4$^+$ and CD8$^+$ T cells, as well as production of anti-viral cytokines by PBMCs, from RA patients in the presence of VZV lysates. By contrast, Treg cells were up-regulated by JAKi. These data may explain, at least in part, the increased risk of HZ in RA patients using JAKi.

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