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Tofacitinib treatment of Rheumatoid Arthritis: increases senescent T cell frequency in patients and limits T cell function *in vitro*

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ABSTRACT

Unravelling the immune signatures in rheumatoid arthritis (RA) patients receiving various treatment regimens can aid in comprehending the immune mechanisms' role in treatment efficacy and side effects. Given the critical role of cellular immunity in RA pathogenesis, we sought to identify T cell profiles characterizing RA patients under specific treatments.

We compared 75 immunophenotypic and biochemical variables in healthy donors (HD) and RA patients, including those receiving different treatments as well as treatment-free patients. Additionally, we conducted *in vitro* experiments to evaluate the direct effect of tofacitinib on purified naïve and memory $CD4^+$ and $CD8^+$ T cells.

Multivariate analysis revealed that tofacitinib-treated patients segregated from HD at the expense of T-cell activation, differentiation and effector function related variables. Additionally, tofacitinib led to an accumulation of peripheral senescent memory $CD4^+$ and $CD8^+$ T cells. *In vitro*, tofacitinib impaired the activation, proliferation, and effector molecules expression and triggered senescence pathways in T cell subsets upon TCR-engagement, with the most significant impact on memory $CD8^+$ T cells.

Our findings suggest that tofacitinib may activate immunosenescence pathways while simultaneously inhibiting effector functions in T cells, both effects likely contributing to the high clinical success and reported side effects of this JAK inhibitor in RA.

Keywords

T cells; Tofacitinib; Rheumatoid arthritis; immunosenescence, JAK inhibitor

Abbreviations

RA: Rheumatoid arthritis

HD: healthy donor

uTx: untreated

DMARDs: disease-modifying antirheumatic drugs

JAKi: Janus kinase inhibitors

JAKs: Janus kinases

SASP: senescence-associated secretory phenotype

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease associated with synovial hyperplasia and bone and cartilage destruction [1]. Numerous studies have demonstrated that dysregulation of T cells plays an important role in the pathogenesis of RA [2, 3], where a network of cytokines originating from tissue-infiltrating leukocytes sustain autoimmunity, maintain chronic inflammatory synovitis and drive the destruction of joint tissue by modulating both innate and adaptive immunity [4]. These advances in our understanding of RA pathogenesis have fostered the successful application of new treatments that selectively antagonize inflammatory cytokines. These drugs, generically called biologic disease-modifying antirheumatic drugs (DMARDs), are used as alternatives or complements to conventional synthetic DMARDs like methotrexate. More recently, Janus kinase inhibitors (JAKi), oral small molecules classified as targeted synthetic DMARDs, have emerged as a new milestone in RA treatment [5].

The Janus kinases (JAKs) are cytoplasmic tyrosine kinases associated with membrane cytokine receptors that mediate signaling of multiple cytokines and growth factors, contributing to the pathogenesis of several autoimmune disorders including RA [5]. Key cytokines in RA pathogenesis activate JAK pathways and JAKi are thought to interrupt the cycle of leukocyte recruitment, activation and pro-inflammatory cytokine expression at sites of inflammation [4, 5]. Tofacitinib, one of the first approved JAKi, is currently used to treat adults with moderate to severe active RA, active psoriatic arthritis, moderate to severe ulcerative colitis or ankylosing spondylitis and children with active polyarticular juvenile idiopathic arthritis [5-7].

Although tofacitinib is very effective at achieving disease remission in RA, it is associated with higher Herpes Zoster incidence and tuberculosis reactivation, among other infections [8, 9]. These side effects are a challenge for patients´ clinical management and may lead to temporary or even permanent treatment discontinuation. Therefore, a deeper understanding of the immune mechanisms underlying tofacitinib's biological effects would improve our knowledge of RA pathogenesis, foster the application of tofacitinib to other diseases, and provide critical information for developing complementary treatments to mitigate JAKi side effects. In this regard, while several studies have investigated the effects of tofacitinib on the immune system [10, 11], knowledge about its impact on the activation, differentiation and effector function of T lymphocytes is fragmentary. Differentiation and cytokine signaling in T cells rely on JAK/STAT pathways [12, 13] and, accordingly, tofacitinib has been reported to reduce T cell activation and interferon gamma (IFN-γ) production [14]. Although the reasons for increased infection risk in tofacitinib-treated patients are considered to be multifactorial, changes in T cell-mediated immunity are likely to play a central role. Severe infections (i.e. herpes virus reactivation, *Cryptococcus neoformans* pneumonia, toxoplasmosis retinitis and disseminated tuberculosis) have also emerged in cancer patients treated with JAKi [15, 16], further indicating a link between this class of drugs and impaired T cell function.

Immunosenescence is a common cause T cell dysfunction associated with increased susceptibility to infection [17]. Senescent T cells are terminally differentiated effector cells that share several features in common with senescent somatic cells (e.g., fibroblasts), including impaired proliferation and increased secretion of cytokines as a consequence of

their senescence-associated secretory phenotype (SASP). However, unlike senescent fibroblasts, senescent T cells are prone to cytokine deprivation-induced apoptosis [18]. Downregulation of the costimulatory molecule CD28, alone or together with CD27 loss, is the classical phenotypic hallmark of senescent T cells [19]. Currently, the expression of CD57 and the inhibitory receptor KLRG1 and the upregulation of proteins involved in DNA damage responses are considered to better reflect a functional senescence status in T cells [20-22]. Increased levels of senescent $CD4^+$ and $CD8^+$ T cells have been found in elderly people but also in young patients with chronic infections, cancer or autoimmune disorders [18]. In RA patients, early studies reported increased frequency of senescent $CD4^+$ and $CD8^+$ T cells, defined by lack of CD28 expression and/or by reduction in markers of recent thymic egress [23]. Higher frequencies of senescent $CD28^-CD4^+$ T cells correlated with extra-articular manifestations and joint involvement [24]. Of note, these results were not reproduced when senescent T cells were defined by the loss of CD28, CD27 and telomere length [25], highlighting the need to further investigate T cell senescence in RA.

Here, we report that among 75 biochemical and immunological variables, the accumulation of T cells with a senescent phenotype and reduced serum IL-22 concentration are the most relevant parameters associated with T cell responses in tofacitinib-treated RA patients. Further comprehensive *in vitro* approaches confirmed that tofacitinib effectively restrained activation, proliferation and effector function of T cells and activated pathways associated with cellular senescence. Remarkably, these tofacitinib-induced effects showed different potency in naïve and memory $CD4^+$ and $CD8^+$ T cell subpopulations, with the strongest effect observed in memory $CD8^+$ T cells. Our findings provide new insight into the biological effects

of tofacitinib, helping to explain its tremendous efficacy in treating inflammatory diseases alongside undesired side effects like increased infection susceptibility.

RESULTS

T cell-related parameters segregate tofacitinib-treated RA patients

We have previously conducted supervised analysis of immunological, biochemical and clinical parameters in RA patients, resulting in the identification of particular features in B and T lymphocytes that are associated with disease activity and/or treatment success [26, 27]. Here, we reasoned that a more comprehensive analysis of the 75 biochemical and immunological variables drawn from our past studies (Table S1), using unsupervised methods such as principal component analysis (PCA), may identify immune signatures specific for RA patients undergoing different treatment regimens. As shown in Figure 1A, the two principal components (PC) of the PCA individually explained 45.7 and 23.1% of the total variation within the groups analyzed, and the contributions of each variable to PC1 and PC2 are shown in Table S1. Notably, although age ranges were comparable among all patient groups, several variables related to cellular responses had a predominant weight in the segregation of the tofacitinib-treated RA group, including % $CD27^-$ $CD28^-$, % $KLRG1^+$ and MFI PD1 for $CD4^+$ T cells, %$CD56^-$ $CD57^+$ and MFI BTLA for $CD8^+$ cells, and interleukin-22 (IL-22) concentration (Figure 1A).

As some of these variables are associated with terminal cell differentiation and an immunosenescence phenotype, we performed a supervised analysis on them in order to

directly compare among groups. This approach further confirmed that only tofacitinib-treated RA patients presented a significant increase in the frequency of cells that lost activation marker expression and/or upregulated senescence surface markers within the $CD4^+$ (Figure 1B) and $CD8^+$ (Figure 1C) T cell gates. While it is known that the population of terminally differentiated effector memory cells (TEMRA) is expected to be enriched with senescent cells [28], we observed no significant changes in the frequency of $CD45RA^+$ $CCR7^-$ $CD4^+$ (Figure 1D) and $CD8^+$ (Figure 1E) T cells between different patient groups and HD. Although inhibitory receptor expression, such as PD-1 and BTLA, was increased in $CD4^+$ and $CD8^+$ T cells from tofacitinib-treated patients, respectively, the frequency of T cells co-expressing two inhibitory receptors, such as CD160 and PD-1, was comparable among the studied groups (Figure 1F and 1G). Finally, we found that tofacitinib-treated RA patients had lower serum IL-22 levels, while IL-17 levels were similar to other patient groups and HD (Figure 1H).

Altogether, these results suggest that treatment with tofacitinib showed specific effects associated with the accumulation of senescent T cells and the inhibition of IL-22 production, without impact in the frequency terminally differentiated effector or exhausted subsets or secretion of IL-17.

Tofacitinib-treated RA patients exhibit increased senescent memory $CD4^+$ and $CD8^+$ T cells

Based on these results, we hypothesized that tofacitinib treatment in RA patients could be linked to increased T cell immunosenescence. To test this, we determined the frequency of T cells lacking expression of the CD27 and CD28 costimulatory markers among PBMCs

obtained from age-matched HD and untreated (uTx) or tofacitinib-treated RA patients. We focused on the memory $CD4^+$ and $CD8^+$ T cell compartments because primed T cells are prone to undergo senescence while naïve T cells are not, and variable frequency of naïve T cells may mask tofacitinib-induced effects when the whole T cell population is evaluated (see gating strategy in Figure S1 and representative contour plots in Figure S2A). Compared to HD, tofacitinib-treated patients presented a significantly higher frequency of $CD27^-CD28^-$ cells within the memory $CD4^+$ (Figure 2A) and $CD8^+$ (Figure 2B) T cell subsets. Concordant results were obtained when senescent cells were classified by the currently used phenotype $CD57^+KLRG1^+$ (Figure S2B and Figure 2C and 2D), particularly in the $CD8^+$ T cell subset. Additionally, we conducted an analysis of the correlation between DAS28-ESR (disease activity score-erythrocyte sedimentation rate) and the frequency of senescent markers in PBMCs (Figure S2C and S2D). Our results indicate a potential negative correlation between DAS28 and CD27-CD28- (but not $CD57^+KLRG1^+$) memory CD4 T cells, as suggested by the p value of 0.0515. Additionally, we observed no significant correlations between disease activity and the CD27/CD28 double negative or the CD57/KLRG1 double positive populations in CD8 T cells. These findings suggest that increased disease activity may not be a major contributor to increased T cell senescence in RA patients.

We next used flow cytometry to examine whether cells with a senescent phenotype were able to produce T cell effector cytokines, finding that different proportions of $CD57^+KLRG1^+$ cells within the memory $CD4^+$ (Figure 2E) and $CD8^+$ (Figure 2F) T cells were able to produce IL-2, TNF or IFN-γ upon stimulation with PMA/Ionomycin in all the groups evaluated.

Altogether, these findings associate tofacitinib treatment in RA patients with an accumulation of $CD4^+$ and $CD8^+$ T cells with senescent phenotypes when compared to HD.

Tofacitinib limits T cell activation and effector functions *in vitro*

Given the results described above, we next assessed whether tofacitinib had a direct effect on the activation and function of T cells in particular differentiation states. To this end, we performed *in vitro* functional studies in cultures of naïve and memory $CD4^+$ and $CD8^+$ T cells purified from HD PBMCs and stimulated for 3 days with anti-CD3/anti-CD28 in the presence or absence of tofacitinib. Tofacitinib significantly reduced the frequency of cells expressing the activation marker CD25, the proliferation antigen Ki-67 and the differentiation factor T-bet in all four T cell subsets analyzed (Figure 3A). These effects were dose-dependent (Figure S3A), and a tofacitinib dose of 1 µM was used in subsequent *in vitro* experiments. Notably, tofacitinib treatment had a greater effect on $CD8^+$ and memory cells than on $CD4^+$ and naïve cells, respectively (Figure 3B). Further phenotypic evaluation focusing on KLRG1, an inhibitory molecule previously linked to a pre-senescent phenotype [22, 29], showed that T cell activation with anti-CD3/anti-CD28 in the presence of tofacitinib significantly increased the expression of KLRG1 in memory $CD8^+$ T cells, but not in the other subpopulations evaluated (Figure 3C).

Next, we aimed to establish whether tofacitinib influenced effector cytokine production. We found that the presence of tofacitinib reduced the frequency of IL-2-secreting cells in all the T cell subpopulations evaluated (Figure 3D and Figure S3B). Tofacitinib treatment also reduced the frequency of IFN-γ-secreting memory $CD8^+$ T cells and had no effect on the

frequency of TNF-secreting cells except for a reduction observed in the already low frequency of TNF-producing naïve $CD8^+$ T cells. We also evaluated the effect of tofacitinib on the expression of molecules associated with cytotoxic effector function and observed that, consistent with the data described above, this treatment significantly reduced granzyme B expression in memory $CD4^+$ and $CD8^+$ T cells (Figure 3E). Finally, we assessed the immunomodulatory effects of JAKi on the secretion of IL-22 and IL-17 by sorted T cell subpopulations. Our results revealed that only memory $CD4^+$ T cells released measurable levels of IL-22 and IL-17, and consistent with the data presented in Figure 1H, treatment with tofacitinib significantly reduced IL-22 levels to a greater extent than IL-17 (Figure 3F).

Tofacitinib upregulates immunosenescence mediators in T cell subpopulations

We next aimed to disentangle the molecular pathways underlying these *ex vivo* and *in vitro* observations, focusing on the impact of tofacitinib on the activation of senescence pathways in T cells. First, we studied the phosphorylation of ATM at Ser1981 (p-ATM) and of histone H2AX at Ser139 (γH2AX), which result in the activation of these two proteins that participate in the earliest stage of the cellular response to DNA double-strand break (DSB) formation [30]. Immunofluorescence analysis revealed that the presence of tofacitinib in T cell subset cultures stimulated with anti-CD3/anti-CD28 resulted in higher p-ATM levels (Figure 4A). Quantitative analysis of p-ATM expression confirmed its induction by tofacitinib in all the subsets except for naïve $CD4^+$ T cells (Figure 4B), with a maximal effect size in memory $CD8^+$ T cells (Figure 4C). For our γH2AX analysis, we focused on the non-proliferating (Ki-67^-) cell compartment, as the endogenous induction of γH2AX during mitosis in proliferating cells

[31, 32], might mask any effects of tofacitinib treatment. Concordantly, tofacitinib treatment also significantly increased the fraction of cells that expressed γH2AX among non-proliferating memory $CD4^+$ and $CD8^+$ T cells (Figure 4D and Figure S4), with the highest effect size observed in the latter subpopulation (Figure 4C).

Senescence-associated variables characterize tofacitinib-treated T cell subpopulations *in vitro*

The results of our supervised analysis of the changes in phenotype, effector function and intracellular signaling induced by tofacitinib in activated T cell subpopulations highlighted the impact of this drug on T cell biology. Based on this, we aimed to identify in an unsupervised manner the set of variables that could specifically characterize cells treated with tofacitinib. We performed a PCA of all the previously assessed variables (Figures 2-4) determined in memory T cell subsets activated polyclonally for 3 days in the presence or absence of tofacitinib. The PCA biplot in Figure 5 depicts the 11 variables measured *in vitro* and highlights the segregation of the T cell groups, with PC1 and PC2 accounting for 85% of the total variance. Notably, the vectors of senescence-associated variables such as p-ATM, KLRG1 expression and γH2AX were particularly associated with exposure to tofacitinib in the memory T cell subpopulations, especially the $CD8^+$ T cells.

DISCUSSION

Numerous studies have revealed distinct variants in the cellular and molecular mechanisms underlying pathophysiology and disease activity in RA [33]. In contrast, little research has been conducted into classifying the heterogeneity of immune signatures among RA patients undergoing different treatments. Here, we present a multivariate analysis of previously reported immunophenotypic and biochemical variables evaluated head-to-head in HD and RA patients undergoing different treatment regimens. We found that patients receiving different treatments segregated apart from untreated RA patients and HD, with tofacitinib-treated patients exhibiting the biggest separation from HD. Variables related to the activation, differentiation and effector function of T cells were predominantly involved in the segregation of patients treated with this JAKi. Accordingly, tofacitinib-treated RA patients exhibited an accumulation of memory $CD4^+$ and $CD8^+$ T cells with a senescent, but not an exhausted, phenotype in peripheral blood. *In vitro* studies demonstrated that tofacitinib directly affects T cells by impairing activation, proliferation, and effector molecule expression, while also activating senescence pathways in both naïve and memory $CD4^+$ and $CD8^+$ subsets. Importantly, this drug showed its strongest impact on memory $CD8^+$ T cell functions. Overall, our findings suggest that tofacitinib imprints an immunosenescence-associated cellular signature in RA patients under treatment, potentially contributing to its high clinical success and reported side effects.

Lately, research in the field of senescence has experienced a radical expansion with the identification of roles for this cellular process in a growing list of diseases and physiological functions, where it has both beneficial and detrimental effects [34, 35]. Discrepancies

concerning these functions of senescent cells, likely arising from differences in the strategies used to identify them, underscore the critical need to more precisely characterize these cells. RA patients were previously reported to exhibit an expansion of $CD4^+$ and $CD8^+$ T cells that showed certain attributes of senescence, such as the loss of CD28 expression or reduction in T-cell receptor excision circles (TRECs) [23, 24]. By contrast, but in agreement with previously reported data [25], we observed no differences between HD and uTx RA patients in the frequency of $CD27^-CD28^-$ senescent $CD4^+$ and $CD8^+$ T cells. However, uTx RA patients did present a higher frequency of senescent $CD4^+$, but not $CD8^+$, T cells characterized by CD57 and KLRG1 co-expression. In addition, we describe for the first time that tofacitinib-treated RA patients showed, in comparison with HD, an increased frequency of senescent $CD4^+$ and $CD8^+$ T cells identified as either $CD27^-CD28^-$ or $CD57^+KLRG1^+$.

Despite their state of permanent growth arrest and limited life span [36], senescent cells are metabolically active and are able to acquire functional phenotypes (e.g., SASP) characterized by increased secretion of bioactive molecules thought to mediate senescent cell effects in various physiological and pathological processes [37]. In this regard, we recently demonstrated that breast cancer patients exhibited an accumulation of senescent $CD57^+KLRG1^+$ $CD4^+$ and $CD8^+$ T cells with higher production of effector cytokines and expression of cytolytic molecules compared to non-senescent subsets [22]. Increased production of cytokines by senescent T cells has been shown to be detrimental in physiological processes (e.g., aging) and in diseases such as cancer by fueling a chronic, systemic low-grade inflammation [38]. Similarly, senescent $CD28^-CD4^+$ T cell levels were

found to be particularly increased in RA patients with more severe extra-articular manifestations and joint involvement [24] and were recently associated with bone loss [39]. Our study revealed that senescent $CD4^+$ and $CD8^+$ T cells from tofacitinib-treated patients, despite being more frequent, exhibited a similar capacity for cytokine secretion as those from uTx RA patients or HD. Interestingly, tofacitinib also had a suppressive effect on T cell effector functions, indicating that senescent cells from RA patients treated with this JAK inhibitor would be unable to secrete cytokines effectively or activate a full SASP phenotype *in vivo*. These findings suggest that tofacitinib has a dual effect regarding senescent T cells, both increasing their frequency while modulating their effector function, which may contribute to the clinical efficacy of tofacitinib in RA treatment.

Previous studies evaluated different JAKi and identified an inhibitory profile involving specific cytokine receptor signaling pathways and lymphocyte activation [12, 32, 40].Our data confirms and expands upon these findings by demonstrating that tofacitinib reduced the activation and proliferation of T cells stimulated by TCR-crosslinking in a dose-dependent manner. Moreover, our functional evaluation revealed that tofacitinib significantly reduced the expression of IL-2, IFN-γ, TNF, granzyme B, IL-22 in specific subsets of T cells activated by TCR engagement. Interestingly, while tofacitinib also reduced IL-17 concentration, the effect was markedly lower than that seen with IL-22, suggesting a differential effect on these two cytokines. Of note, using isolated T cell subsets offered a superior opportunity compared to analyzing total T cells populations to dissect differential effects in naïve versus memory T cells. We thus provide the first demonstration that

tofacitinib affected all the T cell subsets analyzed in this study, though its effects were stronger in memory cells and particularly in $CD8^+$ T cells. Given that T cells have been reported to play an important role in the pathogenesis of RA [3], tofacitinib's effect of limiting effector cellular responses may be instrumental for its high efficacy in treating active disease. At the same time, the reduction of T lymphocyte activation, proliferation and effector function may underlie the increased susceptibility to infection [41, 42], particularly by Herpes Zoster, that is observed in patients treated with tofacitinib [8, 15, 16]. Some recent studies, for instance, report that PBMCs isolated from HD or RA and cultured in the presence of tofacitinib exhibited reduced type 1 Varicella Zoster Virus (VZV)-specific responses [43, 44]. Accordingly, we found that tofacitinib showed the strongest direct effect on TCR-activated memory $CD8^+$ T cells, an immune cell subset fundamental for the control of viruses such as VZV [45]. Additional research will be required to determine whether memory $CD8^+$ T cell subsets with diverse antigen specificities exhibit different sensitivities to tofacitinib.

Inability to proliferate and produce effector cytokines are hallmark features of T cell dysfunction that can arise from multiple mechanisms, including immunosenescence and exhaustion. While both exhaustion and senescence can lead to impaired T cell function, the underlying causes and molecular pathways differ [46] T cell exhaustion is typically characterized by the upregulation of multiple inhibitory receptors after prolonged antigenic exposure, ultimately leading to immune dysfunction [47]. In contrast, replicative or classical senescence occurs due to telomere shortening, which serves as a potent signal for halting

cell proliferation [48]. Certain stimuli or stressors that induce oxidative stress and/or DNA damage [49] can also trigger stress-induced premature senescence independently of the number of duplications that the cell has accumulated [35, 50]. These stimuli include physical and chemical agents as well as biological mediators such as exposure to UV radiation, RAS (oncogene-induced senescence), hyperoxia, drugs and proteasome inhibition. Interestingly, we found that tofacitinib increased the expression p-ATM and γH2AX, two molecules involved in the earliest stage of cellular response to DNA DSB formation and considered sensitive molecular determinants of cellular senescence [30]. Memory $CD8^+$ T cells activated in the presence of tofacitinib also upregulated KLRG1, a marker previously associated with a pre-senescent status [22, 29]. Altogether, our results suggest that tofacitinib induces senescence, but not exhaustion, pathways in activated memory T cells, particularly $CD8^+$ T cells, by attenuating DNA repair and limiting T cell activation and function. Similar molecular events have been recently reported by Reddig *et al.* [32] using an injury model of T cells that involved irradiation without activation by TCR engagement. In contrast, JAK inhibition alleviated cellular senescence in adipocytes [31], suggesting that different cell types can be differently affected by drugs targeting JAK activity.

Given these data, future studies will be needed to shed light on the potential long-term impact of different JAKi on the functions and senescence of specific immune cell populations in RA and other pathologies. Increasing knowledge in this area may guide the design of complementary treatment approaches to prevent immunosuppressive side effects. In this regard, ongoing pre-clinical and clinical development of more selective JAK1 [51] or JAK2

[52] inhibitors may offer not only higher efficacy but also increased safety in terms of infection reactivation. It has been reported that inactivation of JAK2 does not affect T cell reactivity [12], for instance, and novel therapeutic approaches that target senescent cells (known as senotherapeutics) [38, 53] may show promise for reducing susceptibility to infections without compromising the ability of JAKi to restrain autoinflammatory responses.

Some limitations of this study should be acknowledged, including the relatively small sample size and the lack of consideration of disease duration during the ex vivo phenotypic and functional evaluation of senescent T cells. These factors may have limited our ability to detect additional differences among HD, uTx and tofacitinib-treated groups. However, these limitations may have been compensated, at least in part, by a better characterization of immunosenescence using conventional (e.g., $CD28^{-}CD27^{-}$) and recently defined (e.g., $CD57^{+}KLRG1^{+}$) phenotypic markers together with functional attributes (e.g., production of effector cytokines). Also, these *ex vivo* findings were taken as a proof-of-concept further supported by *in vitro* approaches. These studies allowed us to precisely determine tofacitinib´s effects in the activation, proliferation, effector function and senescence induction in T cells, providing unprecedented evidences about distinctive impacts according to the T cell differentiation status.

In summary, our data indicate that tofacitinib-treated RA patients exhibit an accumulation of T cells with a senescence-associated phenotype, opening new avenues for the generation of complementary therapies that address the immunosuppressive side effects of JAKi

treatment. Importantly, these results may also be relevant for other diseases in which JAKi is recommended.

MATERIALS AND METHODS

Detailed methods and study participant characteristics are provided in the online supplementary materials.

Patients. RA patients, either untreated or undergoing different therapies, and healthy donors (HD) included as controls were recruited from the Rheumatology Service (Hospital Nacional de Clínicas). Demographic information, clinical characteristics and treatment regimens of the study subjects are listed in Table 1.

Specimens and cell separation. Heparinized peripheral blood (PB) was drawn from RA patients and HD. Serum samples were obtained for immunological laboratory determinations. Peripheral blood mononuclear cells (PBMCs) were isolated by a standard density-gradient centrifugation technique using Ficoll-Hypaque (GE Healthcare Bio-Sciences AB) and cryopreserved.

Flow cytometry and cell sorting. Flow cytometry for detection of surface molecules, intracellular cytokines and intranuclear transcription factors was performed according to standard protocols described in the Supplementary Material.

***In vitro* cultures.** Sorted naïve and memory $CD4^+$ and $CD8^+$ T cells ($0.2x10^6$) were stimulated in Costar 96-well flat-bottom plates (Corning) pre-coated overnight with 1 µg/ml anti-CD3 (OKT3, BioLegend) and 0.5 µg/ml anti-CD28 (CD28.2, BioLegend) in the presence or absence

of 1 or 10 µM tofacitinib (Pfizer) in complete RPMI + 20% FBS. After 3 days, surface molecules, intracellular cytokines, intranuclear transcription factors and the proliferation marker Ki-67 were evaluated by flow cytometry as described in the Supplementary Material.

Statistical analyses. Statistical analyses were performed with GraphPad Prism version 8.3 (GraphPad Software). *p*-values <0.05 were considered significant. The D´Agostino-Pearson omnibus normality test was initially performed to determine the distribution of the datasets. We conducted a PCA using InfoStat statistical Software (Facultad de Ciencias Agropecuarias, UNC, Argentina). A k-nearest neighbours imputation based on Euclidean distance was used to handle missing data. The effect size of tofacitinib treatment was calculated using Hedges' g. The statistical tests used are indicated in the figure legends.

Ethics approval. This study was approved by the Institutional Ethics Committee of the Hospital Nacional de Clínicas and performed according to the Declaration of Helsinki on studies with human subjects. All subjects provided written informed consent prior to any study procedure.

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CONFLICT OF INTEREST

The authors declare no commercial or financial conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

Alamino Vanina Alejandra: Investigation, Formal analysis, Writing – Original draft, Writing – Review & Editing. **Onofrio Luisina Inés**: Conceptualization, Investigation, Formal analysis, Writing - Original draft, Writing - Review & Editing. **Acosta Cristina del Valle**: Resources, Writing – Review & Editing **Ferrero Paola Virginia**: Resources, Writing – Review & Editing. **Zacca Estefanía Raquel:** Resources, Writing – Review & Editing. **Cadile Isaac Ignacio**:

Resources, Writing – Review & Editing. **Mussano Eduardo Daniel:** Resources, Writing – Review & Editing**. Onetti Laura**: Resources, Writing – Review & Editing. **Montes Carolina Lucía**: Conceptualization, Formal analysis, Writing - Review & Editing. **Gruppi Adriana**: Conceptualization, Formal analysis, Writing - Review & Editing, Funding acquisition. **Eva Acosta Rodríguez**: Conceptualization, Formal analysis, Writing - Original Draft, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

FIGURE LEGENDS

Figure 1. Variables related to T cell activation and differentiation segregate HD and RA patients under different treatment regimens. A, PCA of 75 variables evaluated in whole blood and serum from healthy donors (HD) and RA patients untreated (uTx) or treated with DMARDs, Anti-TNF or tofacitinib (Tofa). Number of donors and demographic features are described in Table 1. Group means (blue squares), variables (yellow circles) and variables that predominantly segregate Tofa (pink circles) are shown. **B-G** Frequency of the indicated population and normalized mean fluorescence intensity (MFI) of the indicated marker expression within $CD4^+$ T and $CD8^+$ cells in each of the five patient groups. **H,** Serum IL-22 (left) and IL-17 (right) concentrations. B-D shows data from patients of the indicated groups according to (A). Data are expressed as mean ± SEM. *p*-values calculated by one-way ANOVA followed by Newman-Keuls multiple comparison test (B-D). ***p<0.001, **p<0.01, *p<0.05 for Tofa vs. HD, uTx, DMARDs and Anti-TNF. #p<0.05 for Tofa vs. HD.

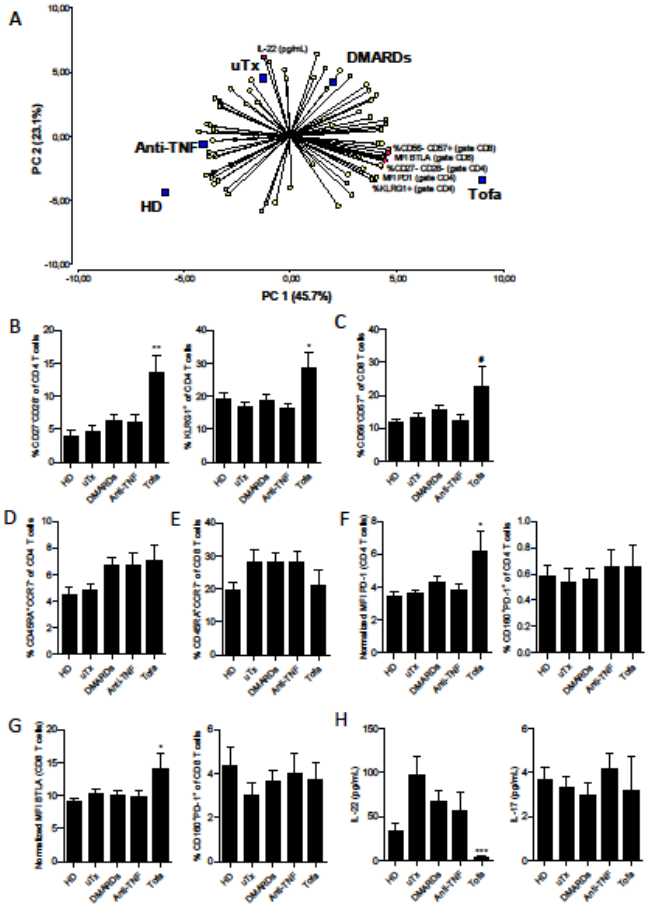

Figure 1

Figure 2. Increased frequency of peripheral memory CD4$^+$ and CD8$^+$ T cells with a senescent phenotype in tofacitinib-treated patients. A-D, Frequency of CD27$^-$CD28$^-$ cells (**A, B**) and CD57$^+$KLRG1$^+$ cells (**C,D**) within memory CD4$^+$ (**A, C**) and CD8$^+$ (**B, D**) T cell gates, evaluated by flow cytometry on PBMCs from healthy donors (HD) and untreated (uTx) and

tofacitinib-treated (Tofa) RA patients. Data are expressed as mean ± SEM. **E-F**, Frequency of cells producing the indicated effector cytokines within senescent ($CD57^+KLRG1^+$) $CD4^+$ (**E**) and $CD8^+$ (**F**) T cells. The horizontal lines depict the mean. Data in A-F are pooled from 7 independent experiments with 2 donors of each group, total sample size N=14/group. *p*-values calculated by one way ANOVA test followed by Newman-Keuls multiple comparison test. ***p<0.001, **p<0.01, *p<0.05.

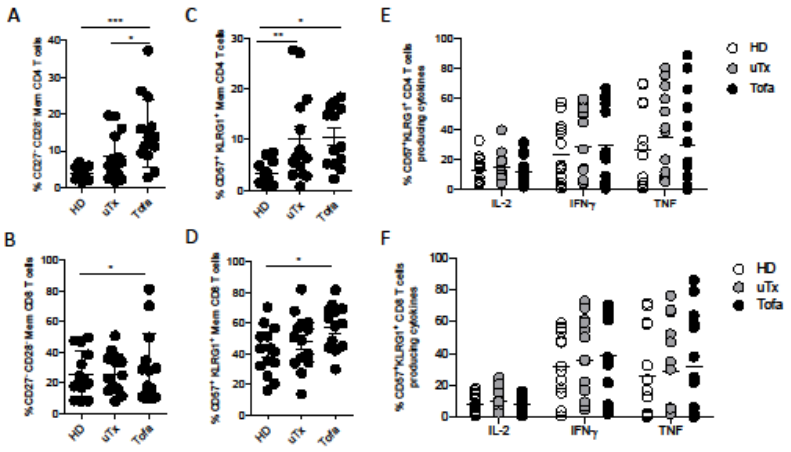

Figure 2

Figure 3. Tofacitinib limits the activation and effector function of T cell subpopulations *in vitro*. A, Frequency of $CD25^+$ (upper), Ki-67^+ (center) and T-bet^+ (lower) cells gated on naïve (N) and memory (Mem) $CD4^+$ and $CD8^+$ T cells after a 3-day stimulation with αCD3/αCD28 in

the presence or absence of 1uM tofacitinib (Tofa). **B**, Representative graph of the magnitudes of the experimental effect sizes (Hedges´ g) calculated from the data shown in (A). **C**, Expression of KLRG1 shown as MFI (arbitrary units) in each treated or untreated T cell subpopulation. **D**, Frequency of cells producing the indicated cytokines among each T cell subpopulation after a 3-day stimulation with αCD3/αCD28 in the presence or absence of 1uM tofacitinib (Tofa) plus 2 hours of PMA/Ionomycin stimulation. **E**, Representative fluorescence microscope image picture (left) and granzyme B expression levels shown as integrated density (in arbitrary units) (right) in the indicated T cell subpopulations. The horizontal lines depict the mean. Data representative of 8 images per sample evaluated in 2 independent experiments, N=2 donors. **F**, Concentration of IL-22 and IL-17 determined by ELISA in supernatants of memory $CD4^+$ T cells, the mean fold decrease is shown. *p*-values calculated by paired Student´s *t*-test (A, C, D, F) and Mann-Whitney test (E). Data in A, B, C, D and F are pooled from 4 independent experiments with 2 donors each, total sample size N=8.

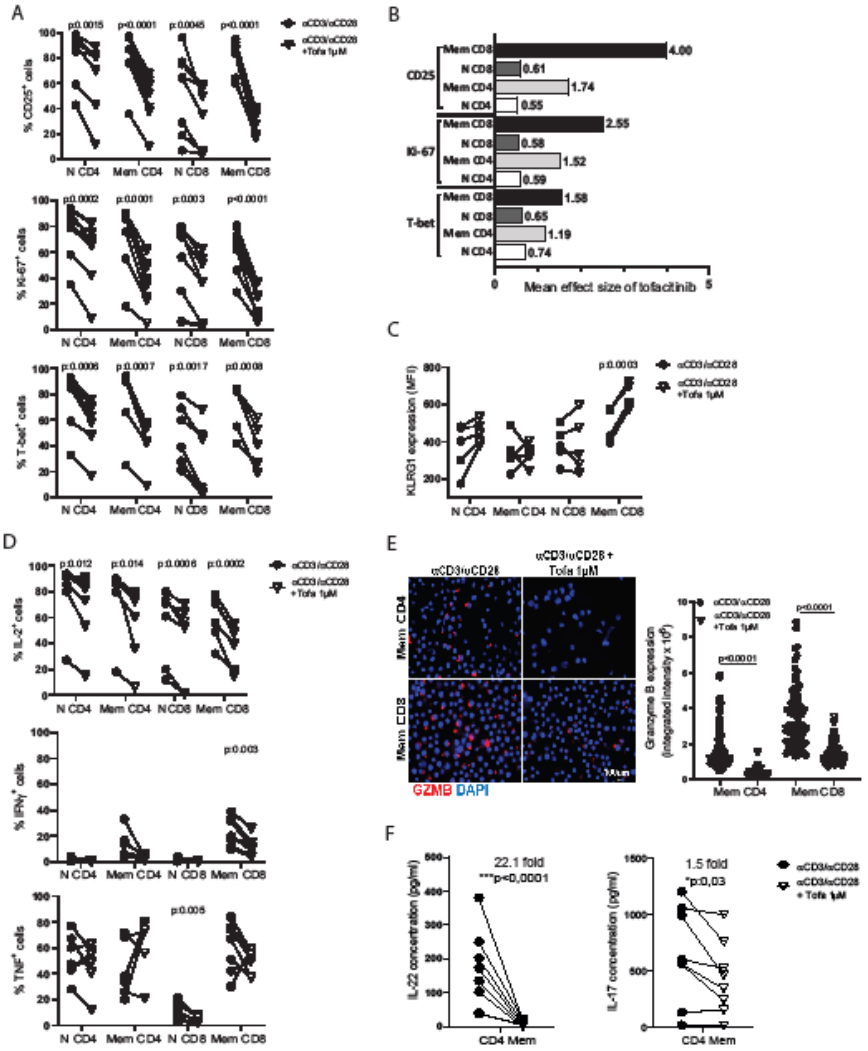

Figure 3

Figure 4. Senescence signaling pathway-associated molecules are activated in tofacitinib-treated memory T cells. **A-B**, Representative immunofluorescence images (**A**) and

accumulated data of the integrated intensity (**B**, in arbitrary units) of p-ATM expression. The horizontal lines depict the mean. **C**, Representative graph of the magnitude of the experimental effect size (Hedges´ g) calculated from the data shown in (B, D). **D**, Frequency of naïve and memory $CD4^+$ and $CD8^+$ T cells that show the $\gamma H2AX^+Ki\text{-}67^-$ phenotype (n: six donors). Data in A are representative images and in B-D are pooled from 3 independent experiments with 2 donors each, total sample size N=6. *p*-values calculated by Mann-Whitney (B) and paired Student´s *t*-test (D).

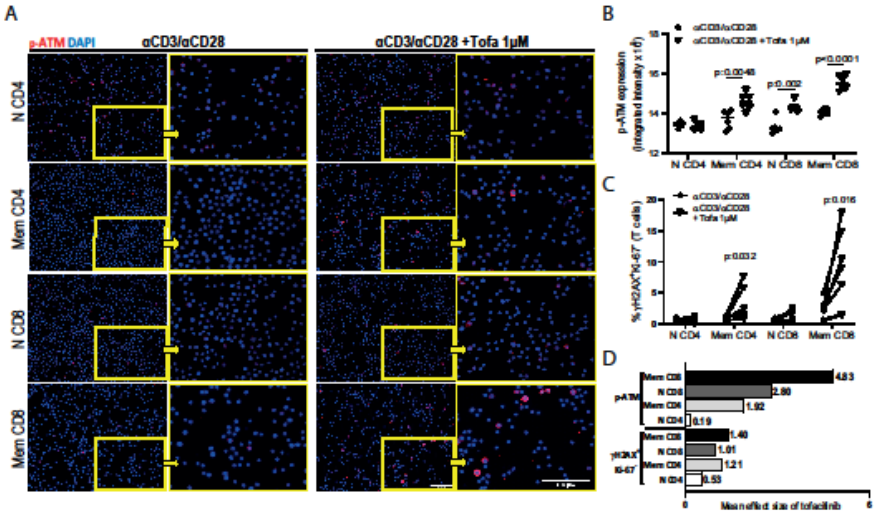

Figure 4

Figure 5. Senescence-related immune variables segregate cells treated with tofacitinib *in vitro*. PCA biplot of variables measured by cytometry, ELISA and microscopy in memory $CD4^+$ and $CD8^+$ T cells from HD activated *in vitro* with anti-CD3/anti-CD8 for 3 days in the presence or absence of tofacitinib. Group means (blue squares) and variables (red circles) are shown. Data collated from 6-8 donors evaluated in 3-4 independent experiments.

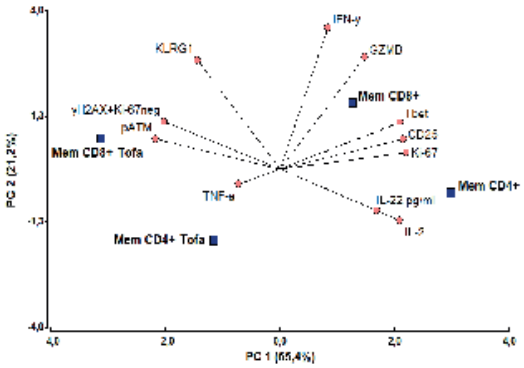

Figure 5

Tables

Table 1. Demographic, biochemical and clinical characteristics of healthy donors and RA patients

	HD	uTx	DMARDs	TNF inhibitors	Tofa
N	36	41	35	23	15
Sex (F/M)	32/4	35/6	25/10	21/2	15/0
Age range (years)	30-80	22-83	22-83	31-75	31-78
ESR (mm) (median±QD)		19±7	12 ± 4	9±3	22±15
CRP (mg/L) (median±QD)		8±4	6±3	6±3	7±2
DAS28-ESR (mean±SD)		4.5±1.3	3.7±1.5 (**p*:0.0101)	4.1±1.5 (*p*:0.2895)	3.8±1.3 (*p*:0.0756)
RF (+/-)		28/13	21/14	17/6	13/2
Anti-CCPs (+/-)		26/15	23/12	16/7	11/4

Anti-CCPs: Anti-cyclic citrullinated proteins, CRP: C-reactive protein, DAS: disease activity score, DMARDs: disease-modifying antirheumatic drugs, ESR: erythrocyte sedimentation rate, HD: healthy donors, QD: quartile deviation, RF: rheumatoid factor, Tofa: tofacitinib, uTx: untreated. *p*-values *vs* uTx was calculated by unpaired Student´s *t*-test.

REFERENCES

1. Smolen, J.S., et al., *Rheumatoid arthritis.* Nat Rev Dis Primers, 2018. **4**: p. 18001.
2. Wehrens, E.J., B.J. Prakken, and F. van Wijk, *T cells out of control--impaired immune regulation in the inflamed joint.* Nat Rev Rheumatol, 2013. **9**(1): p. 34-42.
3. Takeshita, M., et al., *Multi-dimensional analysis identified rheumatoid arthritis-driving pathway in human T cell.* Ann Rheum Dis, 2019. **78**(10): p. 1346-1356.
4. McInnes, I.B. and G. Schett, *Cytokines in the pathogenesis of rheumatoid arthritis.* Nat Rev Immunol, 2007. **7**(6): p. 429-42.
5. Hodge, J.A., et al., *The mechanism of action of tofacitinib - an oral Janus kinase inhibitor for the treatment of rheumatoid arthritis.* Clin Exp Rheumatol, 2016. **34**(2): p. 318-28.
6. Ighani, A., J.R. Georgakopoulos, and J. Yeung, *Tofacitinib for the treatment of psoriasis and psoriatic arthritis.* G Ital Dermatol Venereol, 2020. **155**(4): p. 400-410.
7. Panes, J. and J.P. Gisbert, *Efficacy of tofacitinib treatment in ulcerative colitis.* Gastroenterol Hepatol, 2019. **42**(6): p. 403-412.
8. Redeker, I., et al., *Risk of herpes zoster (shingles) in patients with rheumatoid arthritis under biologic, targeted synthetic and conventional synthetic DMARD treatment: data from the German RABBIT register.* Ann Rheum Dis, 2021.
9. Maiga, M., et al., *Risk of tuberculosis reactivation with tofacitinib (CP-690550).* J Infect Dis, 2012. **205**(11): p. 1705-8.
10. van Vollenhoven, R., et al., *Evaluation of the Short-, Mid-, and Long-Term Effects of Tofacitinib on Lymphocytes in Patients With Rheumatoid Arthritis.* Arthritis Rheumatol, 2019. **71**(5): p. 685-695.
11. Wollenhaupt, J., et al., *Safety and efficacy of tofacitinib for up to 9.5 years in the treatment of rheumatoid arthritis: final results of a global, open-label, long-term extension study.* Arthritis Res Ther, 2019. **21**(1): p. 89.
12. Perner, F., et al., *Specificity of JAK-kinase inhibition determines impact on human and murine T-cell function.* Leukemia, 2016. **30**(4): p. 991-5.
13. Dong, C., *Cytokine Regulation and Function in T Cells.* Annu Rev Immunol, 2021. **39**: p. 51-76.
14. Bedaj, M., et al., *Tofacitinib inhibits CD4 T cell polarisation to Th1 during priming thereby leading to clinical impact in a model of experimental arthritis.* Clin Exp Rheumatol, 2022. **40**(7): p. 1313-1323.
15. Caocci, G., et al., *Reactivation of hepatitis B virus infection following ruxolitinib treatment in a patient with myelofibrosis.* Leukemia, 2014. **28**(1): p. 225-7.
16. Goldberg, R.A., E. Reichel, and L.J. Oshry, *Bilateral toxoplasmosis retinitis associated with ruxolitinib.* N Engl J Med, 2013. **369**(7): p. 681-3.
17. Burton, D.G.A. and A. Stolzing, *Cellular senescence: Immunosurveillance and future immunotherapy.* Ageing Res Rev, 2018. **43**: p. 17-25.
18. Akbar, A.N., S.M. Henson, and A. Lanna, *Senescence of T Lymphocytes: Implications for Enhancing Human Immunity.* Trends Immunol, 2016. **37**(12): p. 866-876.

19. Effros, R.B., et al., *Decline in CD28+ T cells in centenarians and in long-term T cell cultures: a possible cause for both in vivo and in vitro immunosenescence.* Exp Gerontol, 1994. **29**(6): p. 601-9.
20. Sedelnikova, O.A., et al., *Senescing human cells and ageing mice accumulate DNA lesions with unrepairable double-strand breaks.* Nat Cell Biol, 2004. **6**(2): p. 168-70.
21. Ferrara, R., et al., *Circulating T-cell Immunosenescence in Patients with Advanced Non-small Cell Lung Cancer Treated with Single-agent PD-1/PD-L1 Inhibitors or Platinum-based Chemotherapy.* Clin Cancer Res, 2021. **27**(2): p. 492-503.
22. Ramello, M.C., et al., *Polyfunctional KLRG-1(+)CD57(+) Senescent CD4(+) T Cells Infiltrate Tumors and Are Expanded in Peripheral Blood From Breast Cancer Patients.* Front Immunol, 2021. **12**: p. 713132.
23. Koetz, K., et al., *T cell homeostasis in patients with rheumatoid arthritis.* Proc Natl Acad Sci U S A, 2000. **97**(16): p. 9203-8.
24. Pawlik, A., et al., *The expansion of CD4+CD28- T cells in patients with rheumatoid arthritis.* Arthritis Res Ther, 2003. **5**(4): p. R210-3.
25. Petersen, L.E., et al., *Characterization of senescence biomarkers in rheumatoid arthritis: relevance to disease progression.* Clin Rheumatol, 2019. **38**(10): p. 2909-2915.
26. Onofrio, L.I., et al., *Inhibitory Receptor Expression on T Cells as a Marker of Disease Activity and Target to Regulate Effector Cellular Responses in Rheumatoid Arthritis.* Arthritis Rheumatol, 2018. **70**(9): p. 1429-1439.
27. Zacca, E.R., et al., *PD-L1(+) Regulatory B Cells Are Significantly Decreased in Rheumatoid Arthritis Patients and Increase After Successful Treatment.* Front Immunol, 2018. **9**: p. 2241.
28. Salumets, A., et al., *Epigenetic quantification of immunosenescent CD8(+) TEMRA cells in human blood.* Aging Cell, 2022. **21**(5): p. e13607.
29. Voehringer, D., M. Koschella, and H. Pircher, *Lack of proliferative capacity of human effector and memory T cells expressing killer cell lectinlike receptor G1 (KLRG1).* Blood, 2002. **100**(10): p. 3698-702.
30. Bonner, W.M., et al., *GammaH2AX and cancer.* Nat Rev Cancer, 2008. **8**(12): p. 957-67.
31. Xu, M., et al., *JAK inhibition alleviates the cellular senescence-associated secretory phenotype and frailty in old age.* Proc Natl Acad Sci U S A, 2015. **112**(46): p. E6301-10.
32. Reddig, A., et al., *Impact of Different JAK Inhibitors and Methotrexate on Lymphocyte Proliferation and DNA Damage.* J Clin Med, 2021. **10**(7).
33. Zhao, J., et al., *Molecular and Cellular Heterogeneity in Rheumatoid Arthritis: Mechanisms and Clinical Implications.* Front Immunol, 2021. **12**: p. 790122.
34. Palmero, I., V. Gorgoulis, and I. Varela-Nieto, *Editorial: The Role of Cellular Senescence in Health and Disease.* Front Cell Neurosci, 2022. **16**: p. 882417.
35. Munoz-Espin, D. and M. Serrano, *Cellular senescence: from physiology to pathology.* Nat Rev Mol Cell Biol, 2014. **15**(7): p. 482-96.
36. Lian, J., et al., *Immunosenescence: a key player in cancer development.* J Hematol Oncol, 2020. **13**(1): p. 151.
37. Coppe, J.P., et al., *The senescence-associated secretory phenotype: the dark side of tumor suppression.* Annu Rev Pathol, 2010. **5**: p. 99-118.
38. Birch, J. and J. Gil, *Senescence and the SASP: many therapeutic avenues.* Genes Dev, 2020. **34**(23-24): p. 1565-1576.
39. Fessler, J., et al., *Senescent T-Cells Promote Bone Loss in Rheumatoid Arthritis.* Front Immunol, 2018. **9**: p. 95.

40. McInnes, I.B., et al., *Comparison of baricitinib, upadacitinib, and tofacitinib mediated regulation of cytokine signaling in human leukocyte subpopulations.* Arthritis Res Ther, 2019. **21**(1): p. 183.
41. Koszinowski, U.H., M.J. Reddehase, and S. Jonjic, *The role of CD4 and CD8 T cells in viral infections.* Curr Opin Immunol, 1991. **3**(4): p. 471-5.
42. Barry, M. and R.C. Bleackley, *Cytotoxic T lymphocytes: all roads lead to death.* Nat Rev Immunol, 2002. **2**(6): p. 401-9.
43. Almanzar, G., et al., *Tofacitinib modulates the VZV-specific CD4+ T cell immune response in vitro in lymphocytes of patients with rheumatoid arthritis.* Rheumatology (Oxford), 2019. **58**(11): p. 2051-2060.
44. Lee, J.Y., et al., *Effect of Janus kinase inhibitors on T cell responses to herpes zoster in rheumatoid arthritis patients.* Clin Exp Rheumatol, 2022.
45. Weinberg, A. and M.J. Levin, *VZV T cell-mediated immunity.* Curr Top Microbiol Immunol, 2010. **342**: p. 341-57.
46. Zhao, Y., Q. Shao, and G. Peng, *Exhaustion and senescence: two crucial dysfunctional states of T cells in the tumor microenvironment.* Cell Mol Immunol, 2020. **17**(1): p. 27-35.
47. Crespo, J., et al., *T cell anergy, exhaustion, senescence, and stemness in the tumor microenvironment.* Curr Opin Immunol, 2013. **25**(2): p. 214-21.
48. Kim Sh, S.H., P. Kaminker, and J. Campisi, *Telomeres, aging and cancer: in search of a happy ending.* Oncogene, 2002. **21**(4): p. 503-11.
49. Toussaint, O., E.E. Medrano, and T. von Zglinicki, *Cellular and molecular mechanisms of stress-induced premature senescence (SIPS) of human diploid fibroblasts and melanocytes.* Exp Gerontol, 2000. **35**(8): p. 927-45.
50. Kornienko, J.S., et al., *High doses of synthetic antioxidants induce premature senescence in cultivated mesenchymal stem cells.* Sci Rep, 2019. **9**(1): p. 1296.
51. Mascarenhas, J.O., et al., *Primary analysis of a phase II open-label trial of INCB039110, a selective JAK1 inhibitor, in patients with myelofibrosis.* Haematologica, 2017. **102**(2): p. 327-335.
52. Verstovsek, S., et al., *Phase I evaluation of XL019, an oral, potent, and selective JAK2 inhibitor.* Leuk Res, 2014. **38**(3): p. 316-22.
53. Hickson, L.J., et al., *Senolytics decrease senescent cells in humans: Preliminary report from a clinical trial of Dasatinib plus Quercetin in individuals with diabetic kidney disease.* EBioMedicine, 2019. **47**: p. 446-456.

This study identified T cell signatures associated to rheumatoid arthritis patients undergoing various treatments. Tofacitinib-treated patients exhibit an accumulation of senescent T cells. Moreover, tofacitinib impaired T-cell activation, proliferation and effector functions *in vitro*, triggering senescent pathways. These effects may contribute to tofacitinib's clinical success and side effects in rheumatoid arthritis.